RISK FACTORS OF SUB-CLINICAL MASTITIS, ANTIBIOGRAM AND GENOTYPIC ANALYSIS OF *STAPHYLOCOCCUS* SPP AND ENTEROBACTERIA RESISTANT BACTERIA ISOLATED FROM HUMANS AND LACTATING DAIRY COWS FROM SMALL-HOLDER FARMS IN GATUNDU SUB-COUNTY, KENYA

FELIX ODHIAMBO OTENGA

MASTER OF SCIENCE

(Molecular Biology and Bioinformatics)

JOMO KENYATTA UNIVERSITY

OF

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Risk Factors of Sub-Clinical Mastitis, Antibiogram and Genotypic Analysis of *Staphylococcus* Spp. And Enterobacteria Resistant Bacteria Isolated from Humans and Lactating Dairy Cows from Small-Holder Farms in Gatundu Sub-County, Kenya

Felix Odhiambo Otenga

A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Science in Molecular Biology and Bioinformatics of the Jomo Kenyatta University of Agriculture and Technology

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DECLARATION

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

Signature…….....Date……..

Felix Odhiambo Otenga

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

Signature…….....Date……..

Dr. Daniel Muthui Kiboi, PhD

JKUAT, Kenya

Signature…….....Date……..

Dr. John Kagira, PhD

JKUAT, Kenya

Signature…….....Date……..

Dr. Maina Ngotho, PhD

JKUAT, Kenya

DEDICATION

This work is dedicated to my parents; the late Mr. Michael Ochieng Otenga and Mrs. Rose Achieng Ochieng, and my siblings Steve, Sharon, and Michelle for their financial and moral support.

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ABBREVIATIONS AND ACRONYMS

AMR	Antimicrobial resistance
AR	Antibiotic resistance
CLSI	Clinical and Laboratory Standard Institute
DNA	Deoxyribonucleic Acid
ESBLs	Extended spectrum beta lactamases
FAO	Food Agriculture Organization
JKUAT	Jomo Kenyatta University of Agriculture and Technology
MEGA	Molecular evolutionary genetic analysis
MRSA	Methicillin resistant *Staphylococcus aureus*
NAHMS	National animal health monitoring system
NDDB	National dairy development board
PBPs	Penicillin binding proteins
PCR	Polymerase chain reaction
PMF	Proton motive force
RNA	Ribonucleic acid
SCM	Sub-clinical mastitis
UTI	Urinary tract infection

WHO World Health Organization

ABSTRACT

The emergence and subsequent widespread antimicrobial resistance significantly impact global health. The aim of the study was to determine risk factors of sub-clinical mastitis, antibiogram and genotypic analysis of *Staphylococcus* spp. and Enterobacteria against antibiotic resistant bacteria isolated from Humans and lactating dairy cows from small-holder farms in Gatundu Sub-County, Kenya. The cross-sectional field and laboratory study involved the collection of milk samples from one hundred and sixty-four lactating dairy cows, and skin (neck region) swabs from one hundred and twenty human respondents from same household. The milk samples were subjected to California Mastitis Test (CMT) and thereafter cultured and bacteria identified based on growth morphology, color on the media, biochemical tests and use of API 20E Kit. Further, the antimicrobial susceptibility testing was carried out using Kirby Bauer disk diffusion method against 11 antibiotics such as; gentamycin, clindamycin, tetracycline, chloramphenicol, amoyclav, ampicillin, sulphamethoxazole-trimethoprime, oxacillin, vancomycin, cefoxitin and ciprofloxacin. DNA was extracted from the isolated *Staphylococci* spp. and *Enterobacteria* spp. Polymerase chain reaction (PCR) amplification was used to determine isolates positive for the resistant genes, mecA and ESBLs (blaTEM, blaSHV, blaCTX, blaKPC). The amplicons were resolved in a 1.5% gel. The purified PCR samples were sequenced using a 3730xl DNA Analyzer sequencer and based on BigDye™ Terminator v3.1 Cycle Sequencing Kit. The phylogenetic tree was constructed using MEGA11. The evolutionary distances were calculated using the Maximum Likelihood method. The robustness of the tree was assessed with 1,000 bootstrap replicates. From the 164 lactating dairy cows, the prevalence of sub-clinical mastitis based on CMT was 39.6%. The bacteria isolated from the milk samples were Coagulase Negative *Staphylococci* (CoNS) (50.3%), *S. aureus* (31.8%) *Pantoea* spp. (1.9%), *Enterobacter cloacae* (1.3%), *Citrobacter koseri* (0.6%), *Klebsiella oxytoca* (0.6%) and *Serratia* spp (0.6%). The bacteria isolated from humans included. *S. aureus* (49.4%), CoNS (16.9%), *Pantoea* spp. (13%), *Serratia* spp. (13%), *Bukholderia cepacian* (3%), *Enterobacter* spp. (3%), *Yersinia enterocolitica* (1.3%) and *Pasturella aerogenes* (1.3%). Antimicrobial susceptibility testing showed CoNS (100%) and *S. aureus* (86.8%) were mostly resistant to gentamycin but highly susceptible to sulphamethoxazole-trimethoprim (95.5%). Similarly, age had higher burden antimicrobial resistance bacteria among respondents of >50 years ($p = 0.011$, OD=1.745) as well as antibiotic usage ($p = 0.025$, OD = 0.204). The study showed that the cows with previous history of sub-clinical mastitis had a higher prevalence of mastitis ($p = 0.026$, OD = 2.503) as compared to those without such a history. The findings in the current study showed lack of the mecA gene among the 50 *Staphylococc*i spp. isolates screened. However, bla_{TEM} was found in 17 isolates, (41.5%). Phylogenetic analysis showed a close relationship between the Staphylococci and enterobacteria isolates from human and dairy cows. The study recommends further research on differentiating the coagulase negative *Staphylococci* spp. (CoNS) into species level. There is need for involvement of One health approach in control and genomic surveillance in the occurrence of drug-resistant pathogens in the study area.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

The emergence and spread of antimicrobial resistance (AMR) to the available antibiotics is a public health challenge across the globe (Adelowo *et al*., 2014). In human, the spread of AMR has resulted in approximately more than 700,000 deaths on a global scale. It is projected that if there are no significant measures taken to sustain and monitor, surveillance and stewardship measures then, AMR will cost on average ten million lives and about US$100 trillion of economic loss annually by 2050 (WHO, 2016),(Tadesse *et al*., 2017). In human medicine, the resistant bacterial pathogens have led to increased hospitalization increasing the cost of treatment and concomitant derailed economy of the affected country ((Lyon & Skurray, 1987). The predisposing factors to antimicrobial resistance are the excess and indiscriminate use of antibiotics in the human and veterinary field. This has led to selective pressure to the bacteria (Schmidt *et al*., 2017). Further, the increased population across the globe has increased the connectedness of people enabling the microbes to spread rapidly across the human and animal population. Other risk factors cited for the emergence of AMR include increased and over the counter prescription, the occurrence of long therapy on low doses, and termination of medication before completing the therapy dose. (Kumar *et al.,* 2013) Further, usage of antimicrobials in livestock without prescription or professional consultation has led to an increased rise in resistant bacterial pathogens which can transverse between hosts (Rayamajhi *et al*., 2015).

Resistant bacterial pathogens such as *S. aureus* has gained insensitivity to antimicrobials through several mechanisms (Bitrus *et al*., 2018; Cho *et al*., 2018; Rayamajhi *et al*., 2015). These include modification of the antimicrobial target site in the bacteria, production of bacterial enzymes as beta-lactamase to destroy the key component of the antimicrobials such as the lactam ring in beta-lactam antibiotics, modification of the metabolic pathway used by the bacteria, and utilization of alternate pathways for its survival and usage of

efflux mechanisms. Finally, AMR can occur through modification of the membrane receptors of the antimicrobials, therefore, denying entry of antibiotics into the cell for the drug targets (Adeniyi *et al.*,2019; Cesur & Demiröz, 2013; Ebimieowei & Ibemologi, 2016; Kumar & Singh, 2013; Leopold *et al*., 2014).

Staphylococcus aureus is an opportunistic pathogen which is a known pathogen of diseases in both human and livestock. It is a major cause of bacteremia, which is associated with higher morbidity and mortality when compared with bacteremia from other bacterial pathogens (Leopold *et al*., 2014). The burden of *S. aureus* bacteremia, such as methicillin-resistant *S. aureus* bacteremia, considering cost as well as the resource has increased in recent years. In human medicine, the incidence of *S. aureus* has remarkably increased because of the increased frequency of invasive procedures, and increased numbers of immuno-compromised patients (Oliveira *et al*., 2001). This changing epidemiology of *S. aureus* bacteremia, in combination with the inherent virulence of the pathogen, is driving an urgent need for improved strategies and better antibiotics to prevent and treat infections associated with *S. aureus* and their complications 9; (Haag *et al*., 2019; Nizet & Bradley, 2011; D. Oliveira *et al*., 2018; Tong *et al*., 2015). Methicillin-resistant *Staphylococcus aureus* is due to the mecA gene which encodes an alternative penicillin-binding protein, PBP2a (or PBP2'), which has a low affinity for β-lactam antibiotics, which is housed in a large mobile genetic element called staphylococcal cassette chromosome mec (SCC*mec*) (Anjum *et al*., 2019; Oliveira *et al*., 2001).

In human medicine, *E. coli* is a leading cause of bloodstream and urinary tract infection. Systemic infections include bacteremia, nosocomial pneumonia, cholecystitis, cholangitis, peritonitis, cellulitis, osteomyelitis, infectious arthritis, and neonatal meningitis (Adamu *et al*., 2015). A wide range of antimicrobials is used against *Escherichia coli* such as beta-lactam, fluoroquinolones, aminoglycosides, and sulphamethoxazole-trimethoprim for hospital-associated and community-associated infections. In recent years, there has been the emergence of *E. coli* producing beta-lactamases which inactivate beta-lactam antibiotics through hydrolysis. ESBLs are the

predominant enzymes that confer resistance against beta-lactam antibiotics in CTX-M producing *E. coli* which are recognized to cause UTI, bacteremia, and intra-abdominal infections. Resistant strains of *E. coli* increase the cost of treatment by slowing the initiation of therapy and increasing the period of hospitalization (Croxen *et al*., 2013).

Surveillance of the antibacterial resistance is key in tracking and monitoring the extent and widespread. Several molecular technologies have been used in the past for determining the change in resistant strains and the upsurge of variant strains which confers virulence and multidrug resistance. Gene sequencing among others, such molecular typing, represents an essential step in tracking and understanding the spread of antimicrobial resistance.

1.2 Statement of the Problem

The emergence of AMR in the bacterial pathogen is a pressing public health concern. There is widespread indiscriminate use of antibiotics in both humans and livestock. This has led to the increased burden of AMR with the most prominent bacteria being *S. aureus* (Adelowo *et al*., 2014; Anjum *et al*., 2019; Ngaywa *et al*., 2019). Worldwide, AMR in *S. aureus* and *Enterobacteria* is mainly associated with emergence of MRSA and ESBLs, respectively. In most developing countries, like Kenya the human-livestock interface is characterized by close interaction through animal husbandry, as well as consumption of livestock products. This can lead to the exchange of zoonotic pathogenic strains such as *S. aureus* (Maina *et al*., 2020; Oliveira *et al*., 2018; Pekana & Green, 2018; Sharma *et al*., 2018). Today approximately over 150 million households around the globe are engaged in milk production. In East Africa, Kenya is the leading producer of milk, producing an estimated 3.2 billion litres per year by approximately 600,000 smallholder farmers (FAO, 2011).

Many studies have shown that subclinical mastitis (SCM) is more important economically than clinical mastitis (Mdegela *et al*., 2009). This is because SCM is more difficult to detect making it persists longer in the herds and eventually causing more production

losses. Several studies have been carried out in Kenya to determine the prevalence and susceptibility profiles in mastitis causing pathogens (Mbindyo *et al*., 2020). However, Gatundu Sub-County has not received much attention, yet dairy farming is the major economic activity in the region. Further, genomic surveillance is the essential practice in the detection of infectious diseases and gene sequencing has proved an efficient tool in surveillance (Carriço *et al*., 2013; Mason *et al*., 2018; Camp *et al*., 2020).

The current study exploited both phenotypic and molecular test to determine genetics determinants for epidemiological surveillance (Hendriksen *et al.,* 2019). Little is known regarding the use of molecular test to identify most bacteria as *Staphylococci* spp and *Enterobacteria* spp. in most developing countries (Pekana & Green, 2018) and Kenya is no exception. The current study investigated the prevalence, risk factors associated with occurrence of sub-clinical mastitis in the Gatundu sub-county, Kenya. Further the antibiogram of *Staphylococcus aureus* and *Enterobacteriacea*e isolated from lactating dairy cows and human was investigated.

1.3 Justification

The study was undertaken to document the existence of AMR in the sedentary farming setup, as well as to characterize it using molecular methods. Identification of antibiotic sensitivity patterns is needed not only to treat and control mastitis effectively but also to support public health concerns about the judicious use of antibiotics in developed and developing countries. In Gatundu Sub-county and other neighboring regions, there is an increasing demand for milk and milk products to cater for the fast-growing peri urban human population in Nairobi and surrounding counties. In order to assist the farmers and extension agents in the area, it was important to investigate the epidemiology of mastitis and occurrence of antibiotic resistance in the high potential areas such as Gatundu Sub-County. This study focused on livestock-human interface during routine handling and safety of livestock products as the avenue for exposure. The results of this study should inform the Ministry of Livestock on how the unchecked antimicrobial use, unrestricted access and indiscriminate use of antibiotics results to resistant bacterial strains which have

a possibility of being transmitted to human as well as causing bacterial infections in livestock. The results and data of the study should inform the government ministry for human and livestock health on the need for control and surveillance of AMR on other regions the country.

1.4 Hypotheses

There are no risk factors associated with occurrence of bovine mastitis, beta-lactam, and phylogenetic relationship between antibiotic resistance in *Staphylococci* spp. and *Enterobacteria* isolated from human and lactating dairy cows kept by small-holder farmers.

1.5 Objectives

1.5.1 General Objective

To determine the risk factors of Sub-clinical mastitis, antibiogram and genotypic analysis of *Staphylococcus* spp. and Enterobacteria resistant bacteria isolated from Humans and lactating dairy cows small-holder farms in Gatundu Sub-County, Kenya

1.5.2 Specific Objectives

i. To determine the prevalence and aetiology, risk factors of bovine mastitis in small-holder farms in Gatundu Sub-County, Kenya.
ii. To determine the susceptibility of *Staphylococci* spp. and Enterobacteria isolated from both human and dairy cows in small-holder farms Gatundu Sub-County to commonly used antibiotics.
iii. To determine the risk factors associated with occurrence of bovine mastitis, AMR beta-lactam resistance in *Staphylococci* spp. and *Enterobacteria* isolated from human and lactating dairy cows kept by small-holder farmers in Gatundu Sub-County, Kenya.

iv. To determine the antibiotic resistant genes in *Staphylococci* spp. and *Enterobacteria* spp. isolated from human and lactating dairy cows kept by small-holder farmers in Gatundu Sub-County.

v. To determine the phylogenetic relationship of resistant *Staphylococci* spp. and *Enterobacteria* spp. isolated in both human and lactating dairy cows in small-holder farms in Gatundu Sub-County, Kenya.

1.6 Scope and Limitation of the Study

The study focused on the transmission and occurrence of bacteria at the livestock-human interface of the antibiotic-resistant *Staphylococci* spp. and *Enterobacteria* spp in both human and dairy cows in small-holder farms Gatundu Sub-County, Kenya. For molecular study, the study only focused on the *Staphylococci* spp. and *Enterobacteria* spp. which are of both human and livestock importance. The study was limited to milk from dairy cows and skin (neck region) swabs collected from human respondents as they have been shown to have high burden of antimicrobial resistant bacteria in studies done elsewhere.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Staphylococcus* spp. and Enterobacteria Occurrence in Humans and Livestock

2.2 Staphylococcus spp

Staphylococci are ubiquitous, versatile, and highly adaptive pathogens that colonize the skin and mucous membrane of the anterior nares, gastrointestinal tracts, perineum, the genitourinary tracts, and pharynx. Staphylococcal (Haag *et al.,* 2019) bloodstream infections are among the most prevalent and difficult to treat. It causes a wide range of infections in both humans and animals hence having concern about public health due to its ability to acquire resistant and virulence genes as well as the zoonotic capability (Ghalehnoo *et al*., 2018). *Staphylococcus aureus* is the predominant strain of the genus staphylococci causing a wide range of infections as a skin abscess, food poisoning, bacteremia, necrotic pneumonia in children, and endocarditis. In livestock, it causes mastitis in cow, botryomycosis in horses, dermatitis in dogs, septicemia, and arthritis in poultry (Haag *et al.,* 2019; Nizet & Bradley, 2011; Oliveira *et al*., 2018).

2.2.1 Classification of *Staphylococcus* spp

Staphylococcus aureus is a gram-positive non-motile, non-spore-forming facultative anaerobe that is biochemically catalase and coagulase positive. It occurs as an irregularly grape-like cluster and sometimes singly or in pairs, typical colonies are smooth raised yellow to golden yellow color and hemolytic on blood agar containing 5% sheep or horse blood (Tong *et al*., 2015). The genus staphylococci can be biochemically grouped into coagulase-positive and coagulase-negative staphylococci which cause both human and animal diseases. *Staphylococcus aureus* is the potent and pathogenic member of coagulase-positive Staphylococci. Other coagulases positive staphylococcus includes *Staphylococcus intermedius, Staphylococcus hyicus, Staphylococcus pseudintermedius, Staphylococcus lutrae, Staphylococcus schleiferi subspecies coagulants*, and

Staphylococcus delphini which are majorly isolated in animals (Ghalehnoo *et al*., 2018; Haag *et al*., 2019b; Nizet & Bradley, 2011b; Tong *et al*., 2015).

2.2.3 Morphology and Biochemical Characteristics of *Staphylococcus aureus*

The word staphylococci were derived from two Greek words *staphyle* which means "a bunch of grapes" and *coccus* which means “spherical bacteria” while *aureus* is a Latin word that stands for "gold" and was given to these bacteria because of yellow to a yellowish-white colonial appearance on enriched medium (Chesbrough *et al*., 2002). *S. aureus* is a gram-positive non-motile, non-spore-forming, facultative anaerobe and pathogenic member of the genus staphylococci approximately 1μM in size. It forms golden or yellowish colonies on rich medium and hemolysis on blood agar containing 5% sheep and horse blood due to the production of carotenoids and β-hemolysin, on gram staining it appears as bluish grape-like colonies because cell division occurs at different planes. *S. aureus* is catalase-positive, oxidase-negative, and can also tolerate high salt concentration. The cell wall is made up of peptidoglycan which contains crosslinks of glycine residue that allows sensitivity towards lysostaphin (Nizet & Bradley, 2011; Oliveira *et al*., 2018; Tong *et al*., 2015).

2.2.4 *Staphylococcus aureus* in Livestock and Human

Staphylococcus aureus is a commensal organism on the skin, nose, and mucous membrane of healthy livestock and human. However, the microorganism has gained significant interest as a livestock pathogen, regarding its epidemiology in veterinary medicine, that which can be attributed to its infectious process, specifically LA-MRSA and the recent emergence of various clonal lineages associated with livestock as well as the zoonotic potential observed in sequence type 398 (Tong *et al*., 2015). In livestock, *S. aureus* is the major cause of chronic bovine mastitis where it is harbored in mammary glands and teats. The infection is transmitted during milking from an infected gland to a healthy one where the pathogen penetrates the teat canal (Mbindyo *et al*., 2020). The pathogen exerts pathogenesis through the secretion of toxins which destroy the cell

membrane of the milk-producing tissues especially tissues that lines the teats and gland cisterns forming scar tissue. The bacteria further establish in the milk-secreting cells in the duct system where they form abscess to prevent the detection of the pathogen by the immune system (Nizet & Bradley, 2011). Alternatively, the bacteria hide in neutrophils and various host cells to escape the antibiotic action. The bacteria are as well found in teat lesions, teat skin, muzzle, and nostrils and also spread through teat cup liners, milkers' hands, washcloths, and flies. The intramammary infection which causes poor milk quality, cost of treatment, and discarding milk affects the dairy industry (Haag *et al*., 2019b; Tong *et al*., 2015).

In human medicine, approximately 30% of the human population harbors *S. aureus* leads to several conditions ranging from superficial skin disease to disease to life-threatening infections as bacteremia (Shittu *et al*., 2012). The pathogenesis of the pathogen is mainly attributed to the diverse pattern of virulence pattern which enables invasion leading to colonization of the host tissue, evasion of the immune system mechanism and helps in acquiring nutrient and spread of the pathogen in human tissues (Reveles *et al*., 2016). Diverse virulence factors include the production of enzymes and cytotoxins such as coagulase, hyaluronidase, leukocidin, nucleases, exfoliative toxin, and staphylokinase. Several strains of the pathogen are capable of producing pyrogenic toxin super-antigens such as Staphylococcal enterotoxin and toxic shock syndrome toxin-1. The secretion of these toxins usually results in scalded skin syndrome, food poisoning, and toxic shock syndrome (Bitrus *et al*., 2018b; Reveles *et al*., 2016).

2.3 Bovine Mastitis

Mastitis is an inflammation of the parenchyma of mammary gland produced by infectious agents that infiltrate the udder, multiply, and produce toxins (Girma *et al*., 2022; Shittu *et al*., 2012). The disease is characterized by physical, chemical and bacteriological changes in milk and pathological changes in the glandular tissue that affects both the normal flow and quality of milk (Mbindyo *et al*., 2020).

Although mastitis is considered as a complex and multi factorial disease, bacterial pathogens share the greatest contributions. According to epidemiology, mastitis is classified as contagious or environmental (Cobirka *et al*., 2020). The major causes of contagious mastitis, which could be cow-associated pathogens, includes *Streptococcus agalactiae* and *Staphylococcus aureus* (Abebe *et al*., 2016) while, Streptococcus *dysgalactiae*, *Streptococcus uberis* and *E. coli* are the main causes of environmental mastitis (Mdegela *et al*., 2009; Tezera & Ali, 2021). The most isolated pathogens include *Staphylococcus* spp, *Streptococcus* spp, *E. coli*, and *Pasteurella* spp. (Girma *et al.*, 2022)

Mastitis can also be classified as clinical and subclinical type. Adamu *et al*., 2020; Cobirka *et al*., 2020; Girma *et al*., 2022). Clinical mastitis showing variation in milk (e.g., color change, clots, consistency, and lowered production) and inflammation symptoms in the udder. In contrast, subclinical mastitis (SCM) is a type where no visible inflammation is noted and is asymptomatic (Birhanu *et al*., 2017; Cobirka *et al*., 2020). Sub-clinical mastitis is more prevalent than clinical mastitis and causes the greatest overall losses in most dairy herds worldwide (Ndahetuye *et al*., 2019; Ogola *et al*., 2007). Thus, subclinical mastitis is challenging to diagnose, persists longer in the herd, and is associated with higher losses compared to clinical mastitis (Mbindyo *et al*., 2020).

Most estimates have shown that mastitic cow result in a 30% reduction in productivity per affected quarter and a 15% reduction in productivity per cow/lactation, making the disease one among the most costly diseases of dairy industry worldwide (Cobirka *et al*., 2020; Dzousse *et al*., 2020). In addition, the bacterial contamination of milk from affected cows may render it unsuitable for human consumption due to zoonosis, food poisoning and antibiotic residue in the milk following mastitis (Khasanah *et al*., 2021).

Approximately 60–70% of all antimicrobials administered on dairy farms are for preventing and treating mastitis. Public health is potentially at risk because mastitis may transmit zoonoses and sicknesses associated with food toxins (Cobirka *et al*., 2020).

2.3.1 Prevalence of Clinical and Sub-Clinical Mastitis

Bovine mastitis is a common and costly disease of dairy cattle, affecting their health, production, and reproductive efficiency (Abebe *et al*., 2016). The disease has a significant impact on the dairy industry globally. The prevalence of bovine mastitis varies between regions and countries (Abebe *et al*., 2016; Mekonnen *et al*., 2017a; Vliegher *et al*., 2012). The prevalence of mastitis varies from season to season since the growth and multiplication of organisms depends on specific temperature and humidity (Adamu *et al*., 2020).

According to a study conducted by the Food and Agriculture Organization of the United Nations (FAO, 2016), the estimated prevalence of bovine mastitis worldwide is between 20% and 25%. In high-income countries, the prevalence is estimated to be between 15% and 20%, while in low-income countries, it is estimated to be between 25% and 30% (Gengler *et al.,* 2007). The prevalence of the disease is higher in countries where dairy production is the main source of income, and the dairy industry is not well-developed, resulting in poor management practices and limited access to veterinary care. In Europe, the prevalence of bovine mastitis varies widely between countries. A study conducted in the Netherlands reported a prevalence of mastitis of 16%, while in Italy, the prevalence was reported to be as high as 30%. In the United States, a study conducted by the National Animal Health Monitoring System (NAHMS) reported a prevalence of 20% in dairy herds.

In developing countries, the prevalence of bovine mastitis is higher due to a lack of resources, poor management practices, and limited access to veterinary care. In India, a study conducted by the National Dairy Development Board (Dairy & Board, 2016) reported a mastitis prevalence of 40% to 50% in dairy. Studies have estimated the prevalence of this disease in 30% of Africa countries, with Ethiopia having the highest prevalence Adamu *et al*., 2020). The prevalence of mastitis is estimated to be between 30% and 40% in Africa. In Cameroon, (Dzousse *et al*., 2020) found the overall prevalence to be 34.88%, with clinical mastitis and sub-clinical as, 9.72% and 25.16%, respectively.

In Nigeria, (Shittu *et al*., 2012)found the prevalence of sub-clinical mastitis at cow-level and quarter level to be 85.33% and 43.25%, respectively. A study in Zimbabwe conducted by (Katsande *et al*., 2009) found the overall prevalence to be 21.1% with clinical and sub-clinical mastitis being 4.8% and 16.3% respectively. Studies in Ethiopia have found the prevalence of mastitis ranges from 11.9% to 74.7% (Abebe *et al*., 2016; Girma *et al*., 2022; Tezera & Ali, 2021).

In East Africa, the prevalence of mastitis varies. In Rwanda, (Ndahetuye *et al*., 2019) found prevalence of subclinical mastitis at cow level and quarter level to be 76.2% and 43.1%, respectively. In Uganda the overall prevalence was found to be 86.2% (Abrahmsén & Persson, 2013). Studies in Kenya have shown that prevalence of the disease ranges from 6% to 87.5%. Consequently, a study in Juja, Kiambu county in Kenya, found the prevalence of sub-clinical mastitis at cow level to be 66.7% while at udder level as 43.3%. There is intensive dairy farming in Kiambu county with farmers practicing zero grazing method (Kagira *et al*., 2022; Mbindyo *et al*., 2020).

2.4 Management of Bacterial Diseases by the Antibiotics

The term antibiotic was derived from the word "antibiosis", which means "against life". Drugs are chemicals which when administered to living organisms produce a biological effect. In early years' antibiotics, were considered to be organic compounds produced by one microorganism which are toxic to other microorganisms by selectively killing or inhibiting the growth of other microorganisms (Adeniyi *et al*., 2019). In contrast, in the modern era, antibiotics include antimicrobial agents produced through synthetic means partly (semi-synthetic) or wholly (synthetic). Some antibiotics can kill bacteria completely and are termed bactericidal while bacteriostatic are those which can only inhibit bacterial growth. Bactericidal are those that completely kill the bacteria while bacteriostatic are those that can only inhibit bacterial growth (Adeniyi *et al*., 2019; Cesur & Demiröz, 2013; Rayamajhi *et al*., 2015).

2.4.1 Classification of Antibiotics

Antibiotics can be classified in various ways, but the most common classification is based on their chemical structures, the spectrum of activity/pharmacokinetics, and mechanism of action.

2.4.1.1 Classification According to the Chemical Structure

Some common classes of antibiotics based on their molecular or chemical structures include Beta-lactams, Tetracyclines, Macrolides, Quinolones, Sulphonamides, Aminoglycosides, Oxazolidinones, and Glycopeptides.

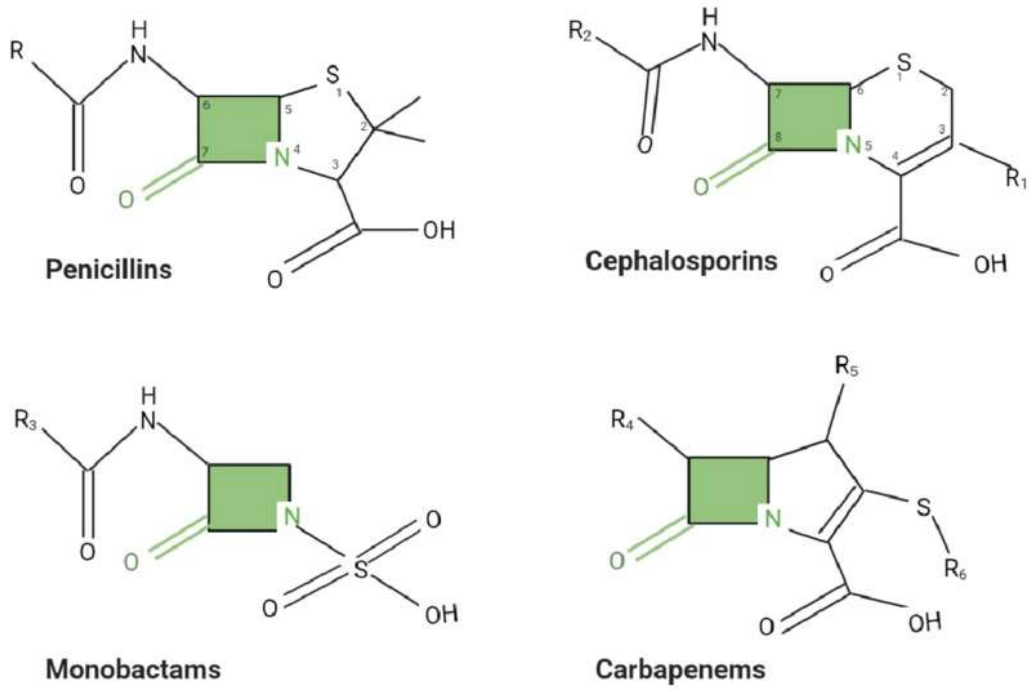


Figure 2.1: Beta-lactam Structure

Source: (Vrancianu *et al* ., 2020)

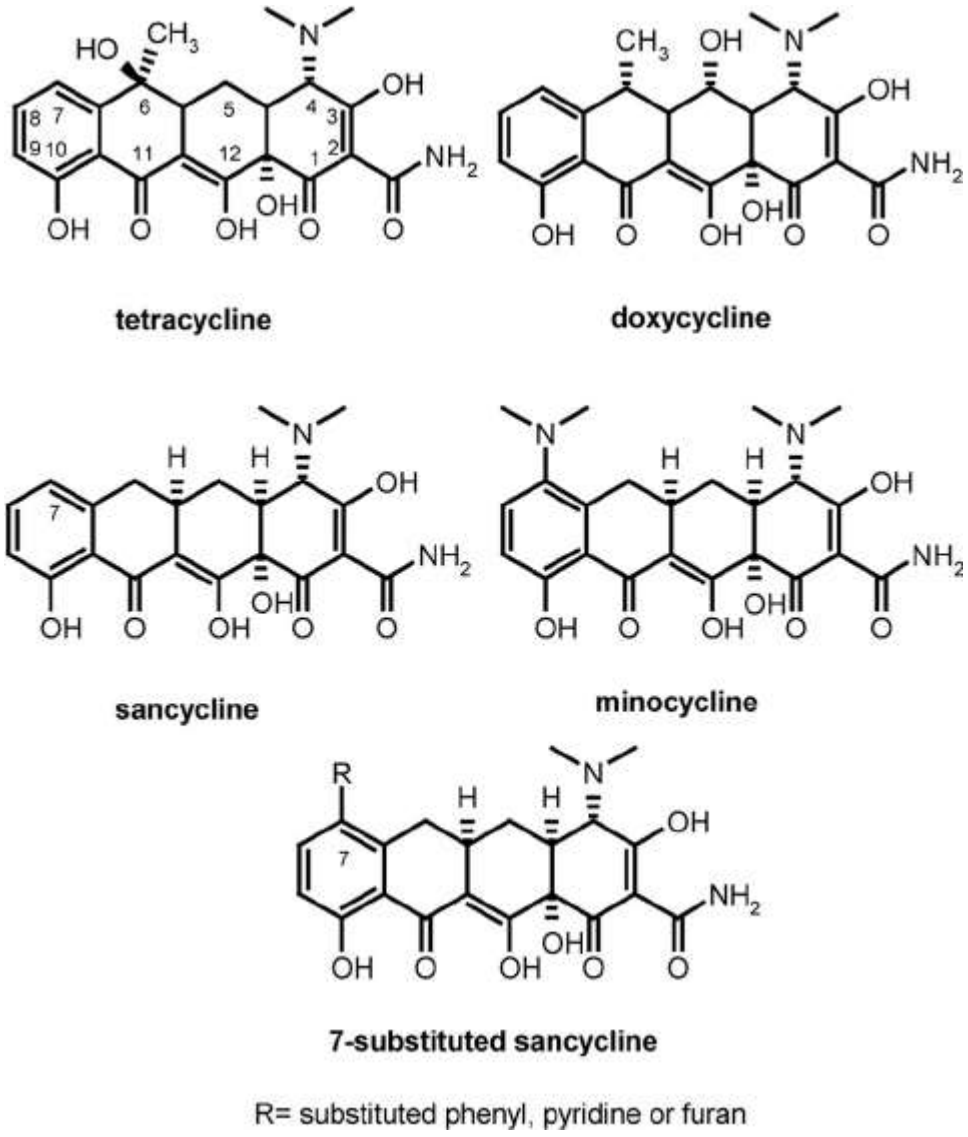


Figure 2.2: Tetracyline Structures

Source: (Draper *et al*., 2013)

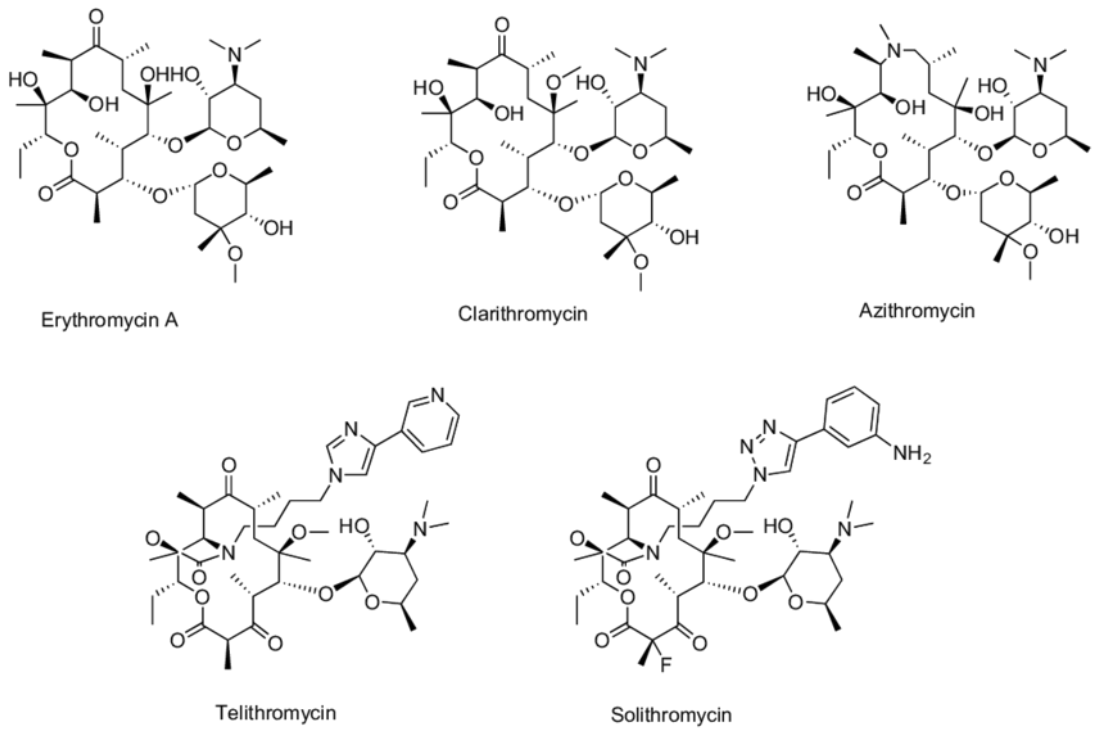


Figure 2.3: Macrolides Structure

Source: (Paljetak *et al*., 2016)

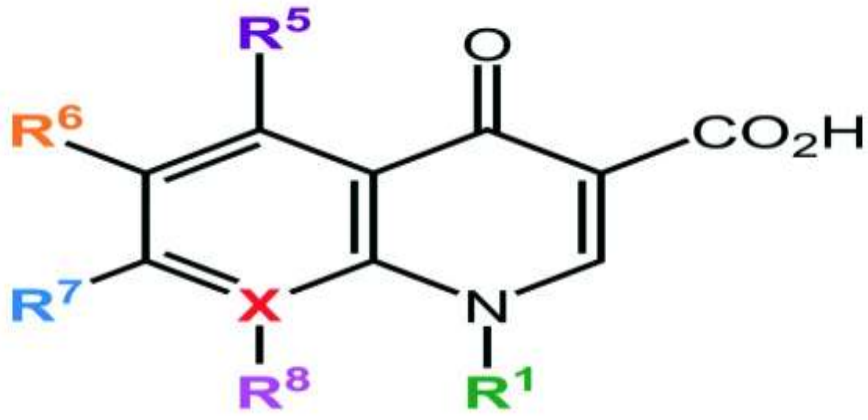


Figure 2.4: Quinolones Structure

Source: (Pham *et al.*, 2019)

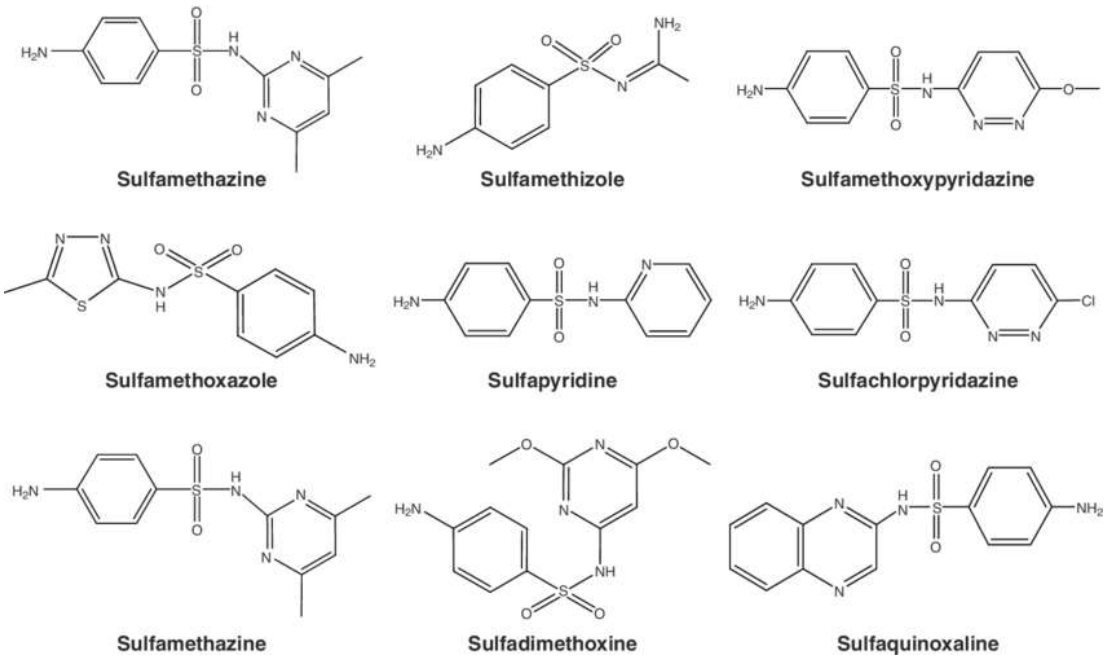


Figure 2.5: Sulphonamides Structure

Source: (Regal *et al*., 2010)

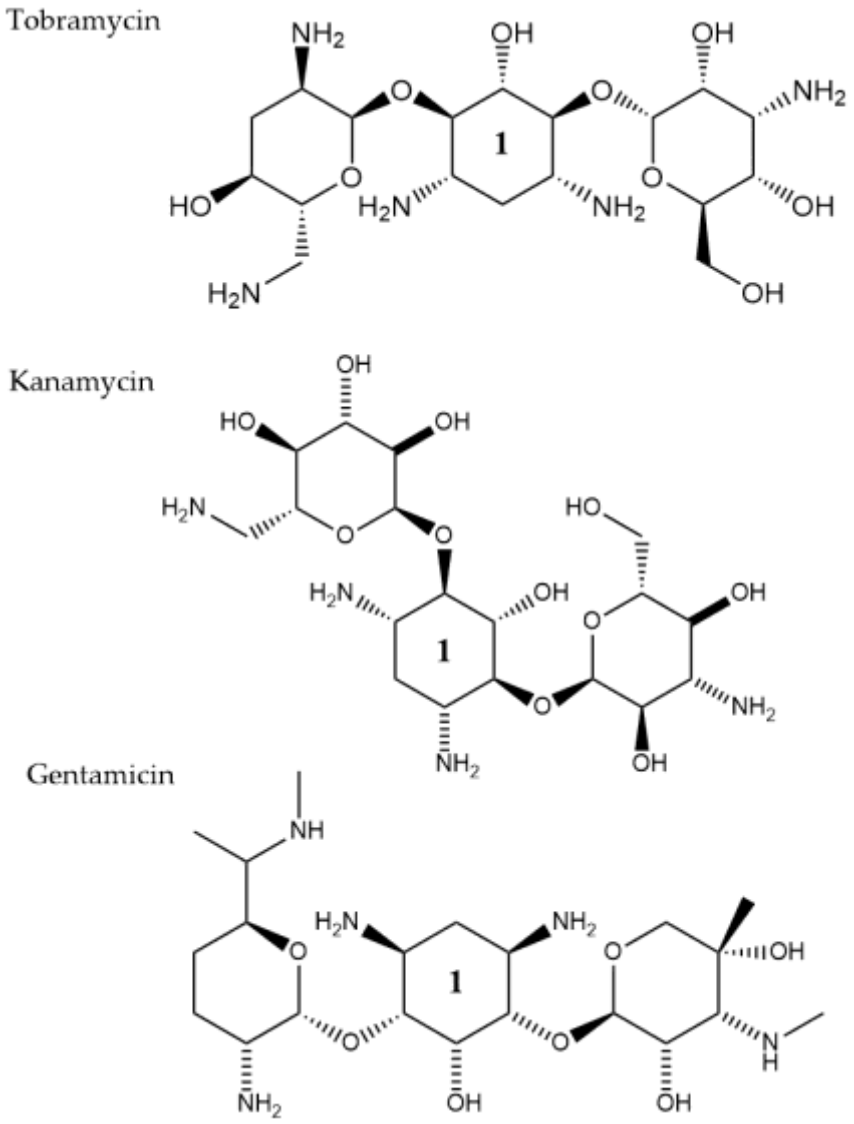


Figure 2.6: Aminoglycosides Structure

Source: (Tsakou *et al*., 2020**)**

2.4.1.2 Classification Based on the Mode of Action.

2.4.1.2.1 Destruction of Nucleic Acid

Antibiotics that destroy bacterial DNA accomplishes this through inhibition of folic acid synthesis (Sulphonamides, Trimethoprim) and inhibition of DNA gyrase

(Fluoroquinolones) as well as through inhibiting RNA synthesis (Rifampin) (Rayamajhi *et al*., 2015).

Folic acid inhibitors are analogs of the substrate for cellular metabolism of bacteria. Therefore, the bacterial enzyme binds to the antibiotic instead of the natural substrate. Sulphonamides mimics tetrahydrofolate, a substrate for folic acid in bacterial cells hence disrupting the synthesis for folic acid which is essential in DNA and amino acid synthesis. Trimethoprim inhibits the dihydrofolate reductase enzyme at a later stage in folic acid synthesis hence synergistic effect with sulphonamide has reduced the mutation rate for resistance against these drugs by the bacteria (Rayamajhi *et al*., 2015).

Bacterial DNA gyrase is an enzyme that nicks the double-stranded DNA, introduces the negative supercoils, and reseals the nicked end. The DNA gyrase is inhibited by fluoroquinolones. DNA gyrase is composed of A and B subunits. The A subunit does the nicking and reseals the nicked the end while the B subunit introduces the negative supercoils. Fluoroquinolones inhibit A subunit therefore interfering with nicking and resealing as well as targeting Topoisomerase IV in Gram-positive bacteria which separate daughter DNA after DNA replication (Fymat *et al*., 2017).

2.4.1.2.2 Disruption of the Cell Membrane

Polymyxins class of antibiotic are responsible for destroying the cell membrane of bacteria but they are specific in every microbial group due to the difference in the types of and nature of lipid in the microbial cell membrane (Cesur & Demiröz, 2013). In the case of Daptomycin which depolarizes the calcium-dependent membrane which leads to the ceasing of the synthesis of the macromolecules and destroying the cell membrane in bacteria (Tadesse *et al*., 2017). Polymyxins bind actively to the lipid moiety of lipopolysaccharide in the bacterial cell destroying the cell membrane (Kumar & Singh, 2013).

2.4.1.2.3 Inhibition of Cell Wall Synthesis

The antibiotics inhibiting cell wall synthesis achieves it through inhibiting peptidoglycan cross-linking (beta-lactam) and inhibiting peptidoglycan synthesis (Vancomycin).

The cell wall is composed of peptidoglycan which surrounds the bacterial cell is made up of long sugar polymers. β-(1-4) –N– acetyl Hexosamine is a cross-linking peptide bond in peptidoglycan and to stay alive the bacteria synthesize peptidoglycan by the activity of Penicillin Binding Proteins (PBPs) which are transpeptidases and transglycosylase (Adeniyi *et al*., 2019).

Beta-lactam target PBPs and also mimics the D-alanyl and D-alanine portion of the peptide chain that binds with PBP, therefore PBPs are not available for the synthesis of new peptidoglycan and this inhibition leads to the lyses the bacterial cell walls and finally killing the cell (Reygaert *et al.,* 2018). Drugs such as Penicillin (penicillin, amoxicillin) and cephalosporin (cephalexin, cefdinir) as well as carbapenems are capable of blocking the cross-linking of peptidoglycan units through inhibition of peptide bond formation which is catalyzed by PBPs. Glycoproteins as vancomycin also prevent the binding of the D-alanyl subunit with PBPs inhibiting cell wall synthesis (Dugassa & Shukuri, 2017).

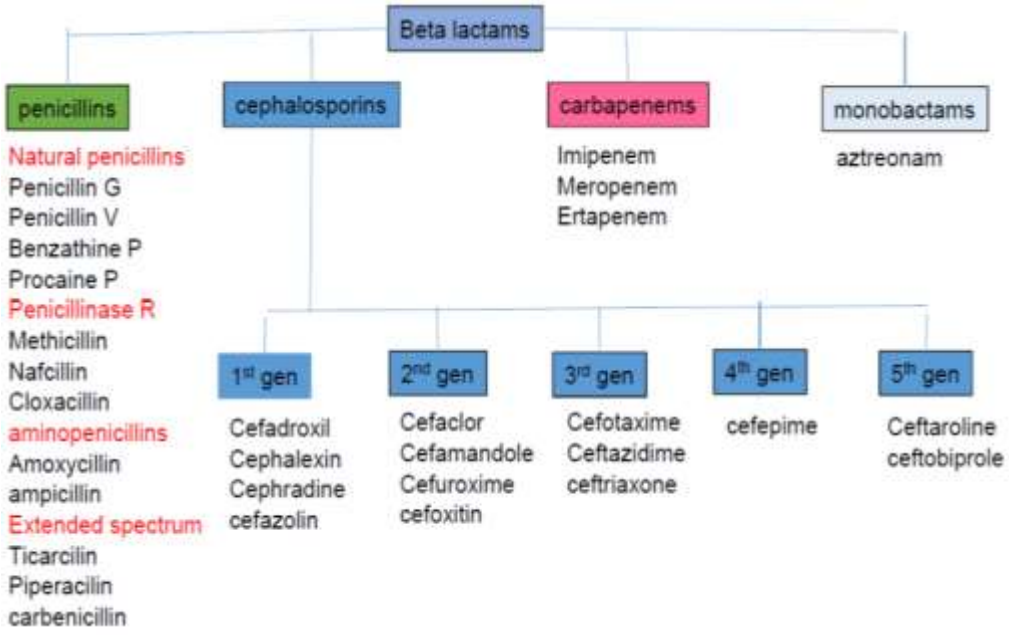


Figure 2.7: The Beta-lactam Family

Source: (Adeniyi *et al*., 2019)

2.4.1.2.4 Inhibition of Protein Synthesis

Antibiotic class inhibiting protein synthesis achieves the inhibition at the 30S ribosomal subunit and 50S ribosomal subunit. During translation, mRNA is synthesized to a protein by the cytoplasmic factors and ribosome. The 30S and 50S ribonucleoprotein comprise the 70S ribosome. Each of them is inhibited by the antimicrobials such as macrolides, tetracycline, aminoglycosides, and chloramphenicol (Adeniyi *et al*., 2019).

30S subunit inhibitor includes aminoglycosides and tetracycline. Aminoglycosides penetrate the bacterium membrane by forming pores and this is possible since it is positively charged hence attaches to the negatively charged outer membrane of the bacteria. To access the ribosome, the antibiotic utilizes proton motive force (PMF) which requires oxygen and this is the reason aminoglycosides are active and effective against aerobic bacteria than to anaerobic bacteria. Aminoglycosides interact with 16S ribosomal RNA through hydrogen bonds near the A site leading to misleading and premature

termination of translation of mRNA. Tetracyclines target the conserved sequences of 16S rRNA of the 30S ribosomal subunit to prevent the binding of transfer RNA (tRNA) to the A site of the ribosome (Wilkinson *et al*., 1976).

Inhibitors of the 50S subunit include chloramphenicol and macrolides, where chloramphenicol targets conserved sequences of the peptidyl transferase cavity of 23S rRNA of the 50S subunit to prevent binding of tRNA to A site of the ribosome inhibiting protein synthesis. On the contrary, macrolides act on the conserved sequences of 23S rRNA of 50S subunit at translocation leading to premature cleavage of the incomplete peptide chains (Adeniyi *et al*., 2019).

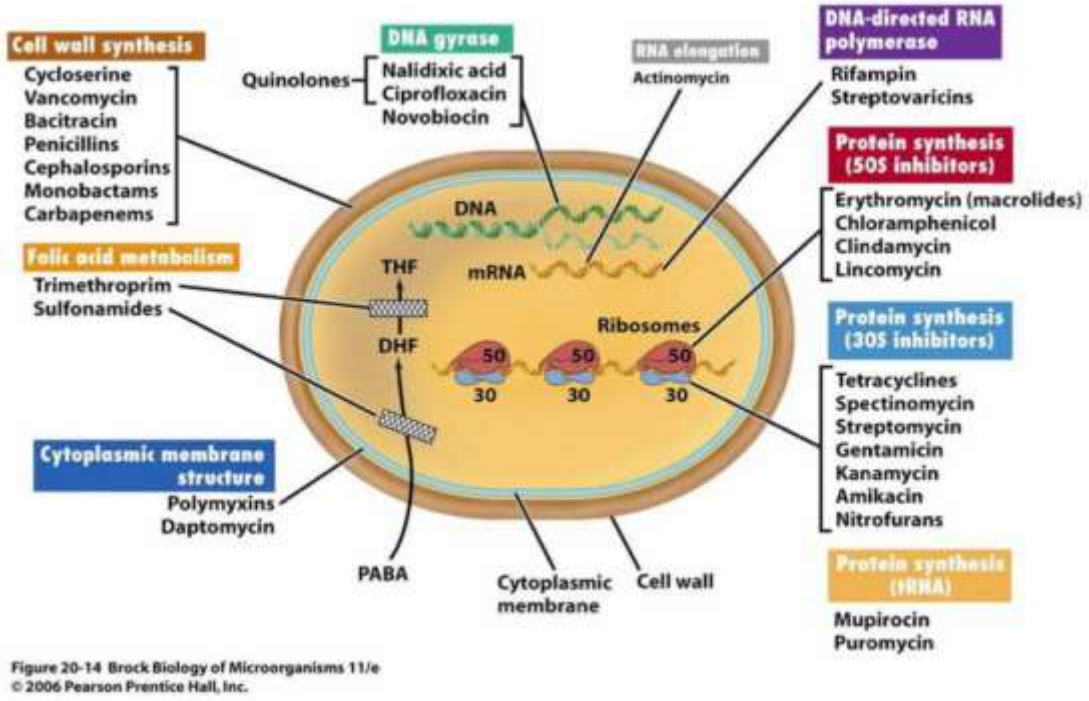


Figure 2.8: Antimicrobial Target Sites

Source: (Ebimieowe et al., 2016).

2.4.1.3 Classification Based on the Spectrum of Activity

This involves how the body responds or relatively how the body interacts with the antibiotics and this is termed as pharmacokinetics. And this interaction involves absorption, distribution, metabolism, and excretion of the drug. This leads to the concentration changes of a drug and this is alluded to body clearance, the volume of distribution, protein binding, or bioavailability (Reygaert *et al.*, 2018).

Tetracycline is administered orally or intravenously and distribution in tissue depends on their lipophilicity with a 20-60% range of plasma protein binding. Orally administered tetracyclines are absorbed in the stomach and proximal small intestine but food reduces their absorption. Tetracyclines have high bioavailability of 75-100% range with 8-25 hours half-life plasma elimination through renal and hepatic route (Ebimieowe *et al*., 2016).

Beta-lactam antibiotics as penicillins are administered orally with absorption in the stomach and proximal small intestine. They undergo minimal metabolism and are excreted through the renal route. Cephalosporins have a varying degree of plasma binding ranging from 6-92%, extensive tissue distribution in lungs, urine, kidney, pleural, synovial, and pericardial fluids. They are mainly eliminated through the renal route with few instances of biliary elimination (Critchley *et al*., 2019).

Macrolides are orally administered and readily absorbed from the gastrointestinal tract. They possess high bioavailability in the spleen, liver, kidney, lungs, pleural and peritoneal fluids. 70% of the administered dose binds to plasma proteins and is inactivated by the metabolic activities of the liver with excretion mainly through the bile (Tenover *et al*., 2006).

Aminoglycosides are administered through the parenteral route with low distribution (<0.3L/Kg) due to poor diffusion in tissues hence highly distributed in blood plasma. They bind weakly to plasma proteins and are mostly eliminated through the kidney with few

instances of bile elimination as well as having plasma elimination half-life of about 2 hours. They are well distributed in fluids as peritoneal, pleural, pericardial, and synovial (Adeniyi *et al*., 2019).

2.5 Antibiotic Resistance

2.5.1 Emergence of Resistance

Antimicrobial resistance is the ability of the microbes to protect themselves against the actions of antimicrobial agents (Adeniyi *et al*, 2019; Wilkinson *et al*., 1976). AMR is a complex phenomenon as a result of the multiple ways of how it is acquired, mechanism of action, and lastly how it spreads. In the middle of the 20th century, there was a revolution in the medical and veterinary field due to the discovery of antibacterial drugs. Bacterial diseases were manageable and this led to increased human and livestock production (Tadesse *et al*., 2017). In livestock the antimicrobials used as therapeutics such as in bovine mastitis, to treat infections, to prevent diseases such as in dry cow therapy, for the disease which could arise due to environmental conditions and exposure to bacteria and also gained enormous use in promoting growth in animals by gaining weight and even enhancing feed efficiency (Reygaert *et al*, 2018). The use of antimicrobial gained popularity and there was a looming danger to the increased indiscriminate use of antibiotics, the reduction of the susceptibility of the bacterial pathogens to the antimicrobials, and the emergence of the resistant strains of bacterial pathogens as MRSA and Extended-spectrum beta-lactamase-producing bacterial pathogens as *E. coli* at the hospitals and the community level. WHO in 2013 warned of the unnecessary and excessive use of antimicrobials as growth promoters because it can lead to greater risk in human health (Bitrus *et al*., 2018; Critchley *et al*., 2019; Fisher & Paterson, 2020; Tadesse *et al*., 2017; Tenover *et al*., 2006). In response, there was the emergence of novel approaches to reduce antibiotic resistance such as herbal medication, ethnoveterinary medicines, bacteriophage therapy, cytokine therapy, mycophage therapy, panchgavya therapies, etc. which are opening new avenues to fight against these superbugs but this is beyond the scope of this review.

Several bacterial pathogens have gained resistance to antimicrobial and genes propelling resistance are generically transferred through various mechanisms as conjugation, transformation, and transduction. The Gram-negative pathogens such as *E. coli* cause a variety of diseases in humans and animals and are resistant to the beta-lactam class of antibiotics by producing up to 1000 different types of β–lactamases. *S. aureus* is recognized as one of the most notorious Gram-positive bacteria. *S. aureus* resides as a nasal commensal in almost 30% of the human population and a major nosocomial infection. Methicillin was the first anti-resistance antibiotic developed against the penicillinases but gained resistance after three years of an introduction leading to the emergence of methicillin-resistant *S. aureus* (MRSA) and progressively it has become multidrug-resistant (Bitrus *et al*., 2018; Critchley *et al*., 2019; Fisher & Paterson, 2020).

The emergence and spread of antimicrobial resistance in bacterial are extensively explored, unfortunately ill-understood. The increased insensitivity of bacterial pathogens to antibiotics makes bacterial infection difficult to treat. This has unfortunately led to increased morbidity and mortality due to bacterial infection across the globe making AMR a serious global health concern. The resistant strain of bacteria such as *S. aureus* (MRSA) and *E. coli* slows down the therapy initiation and this has resulted in increased hospital stay making the cost of treatment and care to escalate. The increased cost of infection has adverse economic constraints in the affected countries across the globe (Reygaert *et al.*, 2018; Lyon & Skurray, 1987).

2.5.2 Mechanisms of Antimicrobial Resistance

There are several proposed mechanisms of resistance and which have extensively been reviewed. They include but not limited to modification of the antibiotic target in the bacterial cell, activation of the efflux mechanism of the efflux pumps, modification of bacterial enzymes to inactivate the antibiotics (beta-lactams, chloramphenicol acyltransferase, and aminoglycoside-modifying enzymes), alteration of membrane permeability denying access of the antimicrobial to the target site and alteration of the

metabolic pathway (Kimera *et al*., 2020; Kumar & Singh, 2013; Rayamajhi *et al*., 2015; WHO, 2016).

2.5.2.1 Enzymatic Degradation Antimicrobial Agents

This involves the degradation of the antibiotic by the bacterial enzyme to protect itself against the action of the antimicrobial agent. This mechanism of antibiotics resistance is the first AMR mechanism to be observed after the discovery of penicillin (Reygaert *et al.,* 2018). The bacterial enzymes destroy the active component of the antibiotic such as observed in antibiotic class as the β–lactam, aminoglycoside, and chloramphenicol (Bitrus *et al*., 2018). This is evident during hydrolytic degradation of the β–lactam ring in penicillin and cephalosporin by the bacterial β–lactamases. Besides Gram-positive and Gram-negative bacteria applies the same mechanism to inactivate aminoglycosides and chloramphenicol through acetylation, adenylation, and phosphorylation (Kimera *et al.,* 2013)

2.5.2.2 Denied Access to the Target Site

A bacterial cell has porins which enable the entry of substances including antibiotic into and out of the cell. And to have a physiologic effect, the antimicrobial has to gain access to the cell through the channels as porins (Kimera *et al*., 2020). Some bacteria to avert the alteration of its function by the antimicrobial agents have changed tact by modifying the cell membrane channels as porins through their number, size and improved selectivity of the substances to enter the cell, this has been exhibited in many Gram-negative bacteria to reduce uptake of aminoglycosides and beta-lactam antibiotic classes. This prevents aminoglycosides and beta-lactams to reach their intended targets, ribosome, and the penicillin-binding proteins (PBPs), respectively (Adeniyi et *et al.,* 2019).

2.5.2.3 Activation of Efflux Mechanism

Bacteria have membrane pumps that help in moving lipophilic or amphipathic molecules across the cell wall. The bacteria possessing the membrane pumps use them to protect

themselves from adverse effects of the action of the antibiotic by pumping. Besides, it helps the bacteria from getting killed by their toxin. The efflux pumps can be very selective to remove from the cell some classes of antibiotics as tetracycline, macrolides, lincosamide, and streptogramins. This results in low intracellular concentrations of antibiotics insufficient to elicit an antibacterial effect (Fymat *et al.,* 2017).

2.5.2.4 Modification of Antimicrobial Target

Modification in target sites enables some bacteria to avoid being recognized by antimicrobial agents. This mechanism is reported in methicillin-resistant *S. aureus* (MRSA) through change or acquisition of different PBPs, in vancomycin-resistant *Enterococcus*, in streptomycin-resistant *Mycobacterium* by modifying 16s rRNA, mutations in RNA polymerase lead to rifampicin resistance in *M. tuberculosis*, and mutations in DNA gyrase lead to resistance for quinolones in many Gram-negative bacteria (Tadedese *et al.,* 2017).

2.5.2.5 Alteration of Metabolic Pathways

Certain antibiotic classes mimic a natural substrate of bacteria for cellular metabolism. Such antibiotics include Sulphonamides and trimethoprim. This, therefore, cause bacterial enzymes to bind to the antibiotic instead of the normal substrate. Specifically, sulphonamides are analogs of tetrahydrofolate which is necessary for the synthesis of folic acid in bacterial cells. Folic acid is vital in the metabolism of nucleic acid and amino acids; therefore, sulphonamides lead to the inhibition of nucleic acids (DNA and RNA) and amino acids synthesis (Reygaert *et al.,* 2018).

2.5.3 Molecular Mechanisms of Antimicrobial Resistance

The genetic mechanisms of antimicrobial resistance are grouped into two types; intrinsic and extrinsic.

2.5.3.1 Intrinsic Resistance

It is the innate ability of bacteria to resist the antimicrobial effect of a particular antibiotic class through its inherent structural or functional characteristics. Such resistance can also be called as "insensitivity" as those microbes have never been susceptible to that particular drug (Fymat *et al.,* 2017). The classical example is the resistance of anaerobes to aminoglycosides and of Gram-negative bacteria to vancomycin. The natural insensitivity can be due to lack of drug targets, the inability of the drug to enter bacterial cell, the expulsion of antimicrobials by chromosomally encoded efflux pump, and innate production of antibiotic inactivating enzymes (Kumar *et al.,* 2013).

2.5.3.2 Acquired Resistance

Acquired resistance involves the ability of bacteria to be resistant to the activity of a specific antimicrobial agent to which it was earlier susceptible. This is largely mediated by mutation or horizontal gene transfer which brings about the various changes in a bacterial genome. Horizontal gene transfer involves transformation, transduction, or conjugation processes (Kimera *et al.,* 2013). This results in a change in the structural and functional characteristics of the bacteria which results in resistance against specific or multiple classes of antibiotics. Various methods of acquired resistance are briefly illustrated as;

2.5.3.2.1 Mutation

A mutation is a result of a spontaneous change in DNA sequence within the gene. A gene is made of sequences of nucleotides that are arranged in codons resulting in amino acid. Therefore, a change in any single nucleotide base pair leads to a concomitant change in the codon responsible for a particular amino acid (s). This consequently changes the affinity of antimicrobials towards the targeted site. In most bacterial pathogen mutations can be as a result of insertions, deletions, and duplication as well as errors of DNA

polymerase during replication resulting in about 0.0033 spontaneous mutation rate for every replication cycle but this largely varies between genes (Fymat *et al.,* 2017).

Mutation occurring at the antibiotic target results in the emergence of multidrug-resistant (MDR) mycobacterial infections. Hydrolysis of lactam ring by β–lactamases is the most common resistance mechanism in penicillin and cephalosporin class of antibiotics. Bacteria acquired the resistance to newer β–lactam antibiotics by a series of point mutations within the lactamase gene (Tadese *et al.,* 2017). Such mutations are common in members of *Enterobacteriaceae*. Penicillin-binding protein (PBP) is responsible for binding with β−lactam antibiotics and inhibition of cell wall synthesis. A mutational change in the *mec*A gene brings an alternative PBP (PBP2a) which in turn leads for methicillin resistance in *S. aureus* (Oliveira *et al*., 2001).

2.5.3.2.2 Horizontal Gene Transfer

Genetic elements such as integrons, transposon, and plasmid carry the antibiotic resistance genes. The genetic elements transfer resistance genes from bacteria to other bacteria that belong to the members of the same species, or different species, or some instance a different genus (Oliveira *et al.,* 2001). Horizontal gene transfer (HGT) is achieved by some mechanisms such as conjugation, transduction, and transformation in a bacterial pathogen (Rayamajhi *et al.,* 2016).

Transformation involves the uptake of short naked DNA fragments and their homologous recombination in naturally competent bacteria. This is commonly observed in the species of streptococci, meningococci and *Acinetobacter.*

Conjugation involves the cell to cell contact via sexual pilli to transfer the piece of DNA. Sex pilli is formed by the responsible genes which are present only in the donor bacteria. Ultimately, the piece of DNA fragments having the resistance genes is transferred from resistant donors to previously susceptible bacteria. Recent studies have demonstrated that

gut microbiota of humans and animals frequently transmit the diverse ranges of antimicrobial resistance genes through conjugation (Tadesse *et al.*, 2017).

Transduction involves the transfer of DNA from one bacterium into another via bacterial viruses called bacteriophage. It is less commonly linked with the transfer of antibiotic resistance genes compared to transformation and conjugation. However, phages are frequently associated with the formation of mobile genetic elements encoding the resistance and virulence genes (Kimera *et al.*, 2013).

2.6 Genomic Surveillance of Antimicrobial Resistance (AMR)

The advancement in rapid and affordable sequencing technologies have revolutionized detection of antimicrobial resistant genes as well as microbial surveillance (Hendriksen *et al.*, 2019). Generation of genomic data has enabled detection of antimicrobial resistance (AMR). I n addition it has enabled tracking of the evolution and spread of AMR bacteria in a hospital and in the community (Boolchandani *et al.*, 2020). The advance sequencing technologies have enabled microbial typing. A study of emerging aminoglycoside-resistant *Campylobacter* in the USA revealed the trend is due to nine different resistance alleles (Arthur &Tsang, 2016). DNA sequence-based surveillance has enabled definition of multidrug resistance (MDR) with greater precision compared to phenotypic assays (Grundmann *et al.*, 2018). They can also be used to reveal co-carriages of various specific genes underlying various MDR patterns. Genomic surveillance of antimicrobial resistance shows cattle and poultry are a moderate source of multi-drug resistance non-typhoidal *Salmonella* in Mexico (Delgado *et al.*, 2021).

2.7 Antimicrobial Resistance (AMR) Burden

Antimicrobial resistance (AMR) constitute one of the enormous threats to public health globally. In human, the spread of AMR has resulted in approximately more than 700,000 deaths on a global scale. It is projected that if there are no significant measures taken to sustain and monitor, surveillance and stewardship measures then, AMR will cost on

average ten million lives and about US$100 trillion of economic loss annually by 2050, (Tadesse *et al*., 2017). A detailed review by Murray *et al.,* and colleagues (2022) found an estimated 4.95 million deaths associated with bacterial AMR in 2019, including 1.27 million deaths attributable to bacterial AMR. At the regional level, death rate attributable to resistance was found to be highest in western Sub-Saharan Africa, at 27.3 deaths per 100000, and lowest in Australasia, at 6.5 deaths per 100000 (Murray *et al*., 2020). The six leading pathogens for deaths associated with resistance (*Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii,* and *Pseudomonas aeruginosa*) were responsible for 929000 deaths attributable to AMR and 3.57 million deaths associated with AMR in 2019. Consequently, methicillin-resistant *S. aureus*, caused more than 100 000 deaths attributable to AMR in 2019 (Murray *et al*., 2020), while six more each caused 50 000–100000 deaths: multidrug-resistant excluding extensively drug-resistant tuberculosis, third-generation cephalosporin-resistant *E. coli*, carbapenem-resistant *A. baumannii*, fluoroquinolone-resistant *E. coli*, carbapenem resistant *K. pneumoniae*, and third-generation cephalosporin-resistant *K. pneumoniae* (Boolchandani *et al.,* 2020).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was carried out in Chania, Mang'u, Ng'enda and Wamwangi administrative wards in the Gatundu Sub- County, Kiambu County, Kenya. The sub-county is located 42Km from Nairobi City and is situated at Longitude of; 36.9050566 and, Latitude of; -1.0130645. The annual rainfall in the area is bimodal and ranges from 500 to 1300mm. The average diurnal temperature is 18.7°C. The elevation from the sea is approximately 1600m. The major economic activities are agriculture and trade. The human population of the sub-county is estimated as 231,978 while the livestock population in Gatundu Sub-county is 31,229 (Kenya National Bureau of Statistics, 2019). Dairy cattle in the area are mainly kept under intensive zero-grazing production system.

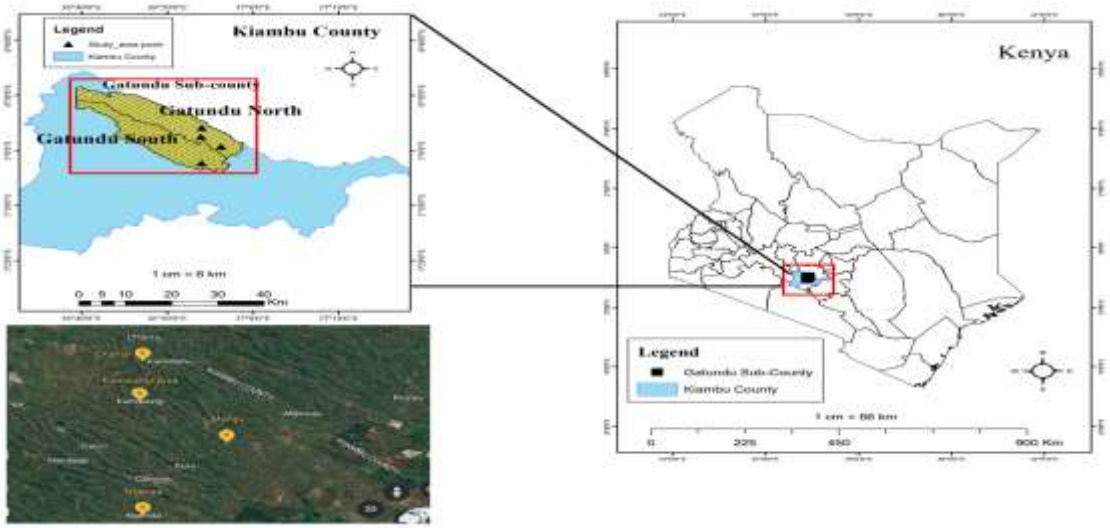


Figure 3.1: Study Sites (ArcGIS v.10.8)

3.2 Study Design and Sampling

Cross-sectional field- and laboratory-based study design was used. Milk samples from lactating dairy cattle and skin swabs (neck region) from humans were collected at the homestead level while the rest of the study was done in the microbiology laboratory at Institute of Primate Research, Kenya.

The sample size for lactating dairy cattle was calculated according to the Fishers formula (Jung *et al*., 2014) formula for infinite populations. An expected prevalence of 73% was assumed based on studies done elsewhere with similar production systems (Okoko *et al*., 2020).

Sample size (n) = $\{[z^2 \times p(1-P)]/e^2\}/1+\{[z2 \times p(1-p)]/e^2N\}$

Where the population size (N) =279,429 (KNBS, 2019), z – score (z)-1.96, margin error (e)-0.05% and standard deviation (p)-0.73. Following the calculation this resulted to a minimum sample size of 304.

Sampling of lactating dairy cattle was based on households keeping dairy cattle. Since the number of dairy cattle in the area were less than 10,000, the adjusted formula, Thrushfield *et al*., (1998), was used to arrive at the more practical sample size. The final minimum sample size was one hundred and fifty cows. The sampling unit of interest was a farm with lactating dairy cows. Since there was no formal list of dairy cow farmers in the study area, a snowballing method was used in identification of farms where the initial farmers were identified with the help of the local government Veterinary Officer. Thereafter, these farmers helped in further identification of other farmers with lactating dairy cows. Using this strategy, a total of one hundred and sixteen farms were identified and visited from where one hundred and sixty four (164) lactating dairy cows were sampled.

3.3 Risk Factors

The risk factors which could be associated with the occurrence of mastitis, antimicrobial resistance and zoonotic transmission were determined through a questionnaire that was administered to the 116 farmers. The specific questions were based on the age, breed, parity, lactation stage, history of mastitis, antibiotic usage. A detailed questionnaire is provided in **Appendix V**.

3.4 Sample Collection

The milk samples for the study were collected from lactating dairy cows and skin swab from around the neck region were obtained from humans at the same homesteads.

3.4.1 Milk Collection

From each cow, the udder was cleaned and dried, the teats were pre-dipped in 70% ethanol for 30 seconds and wiped with a disposable paper towel, and four (4) streams of milk were discarded to minimize the contamination of milk. Three (3) ml of milk from separate teats were milked into a Californian Mastitis Test (CMT) paddle, and an equal amount of a commercial CMT reagent (Immucell RP, USA) was added to the paddle. The CMT paddle was rotated in a circular motion to thoroughly mix the contents. Gel formation was observed within 20 seconds. The results were read on a score of 0-3. A score of 0- trace, 1- negative, while a score of 2 and 3 were considered positive (Quinn *et al*., 2002). Five (5) ml of milk samples were collected from lactating dairy cow into sterile universal bottles. A total of 164 milk samples were collected and transported in cold chain (placed in a cooler box with an ice pack) to the IPR Microbiology Laboratory for bacterial culture, isolation, and identification.

3.4.2 Skin Swab Sampling

The skin swab was applied to the household head who had regular interaction with the dairy animals. Briefly, a sterile swab was wet using normal saline and rubbed on the skin

(neck region) for 15 seconds. The swabs were then aseptically inserted in a cryotube having Stuart transport media, labelled and then placed in a cooler box containing ice packs. The samples were transported to the Microbiology Laboratory at Institute of Primate Research Centre for bacterial culture, isolation, and identification.

3.5 Bacteria Isolation and Identification

The procedures and protocols described by Chesbrough *et al*., (2002) for the culturing and identification of bacteria were adopted in the current study. For identification and isolation of *Staphylococci* spp, the milk sample (100µl) was inoculated into sheep blood agar and mannitol salt agar, streaked and incubated at 37^0C for 24 hours. The identification of the colony was performed using standard bacteriological procedures. The pure isolates of *Staphylococci* spp were stored in a 50% nutrient broth and glycerol at -20^0C until further use (Dabele *et al*., 2021). Isolation of other Enterobacteria species from the milk samples was undertaken using MacConkey agar for 24 hours at 37^0C. Further identification of *Enterobacteriaceae* was done using API 20E kit (Maina *et al*., 2014).

3.6 Antibiotic Susceptibility Test

The isolates of *S. aureus* and coagulase negative *Staphylococcus* spp. were sub-cultured at 37^0C for 24 hours to revive them in nutrient broth where a turbidity standard equivalent to 0.5% McFarland was determined before inoculation on Mueller-Hinton agar. The antibiotic susceptibility test was carried out using the Kirby Bauer disk diffusion method (Hudzicki *et al*., 2012). Susceptibility of eleven (11) antibiotics (Himedia, India) [**Table 3.1**] which are commonly used in the management of mastitis were investigated. Briefly, the disks were gently pressed on the agar, incubated for 24 hours at 37^0C for the examination of zones of inhibition. The results were interpreted according to Clinical and Laboratory Standard Institute, (CLSI, 2019) protocols *E. coli* strain ATCC 25922 and *S. aureus* strain ATCC 25923 were used as quality-control strains (Adelowo *et al*., 2014b). The results were classified as susceptible, intermediate, and resistant.

Table 3.1: Antibiotics Used for Susceptibility Testing in the Current Study

Antibiotic class	Antibiotic	Concentration
Aminoglycoside	Gentamycin	10µg
Macrolide	Erythromycin	15µg
Penicillins	Amoxicillin-clavulanic acid	30µg
	Ampicillin	10µg
	Oxacillin	1µg
Sulfonamides	sulphamethoxazole-trimethoprim	30µg
Cephalosporins (2nd generation)	Cefoxitin	30µg
Quinolones	Ciprofloxacin	5µg
Lincosamide	Clindamycin	2µg
Tetracycline	Tetracycline	30µg
Phenicol	Chloramphenicol	30µg

The antibiotic disks were sourced from HiMedia company a with predetermined concentration.

The occurrence of Multiple Antibiotic Resistance (MAR) index was calculated using the formula:

MAR index = a/b

Where **'a'** is the total number of antibiotics to which a particular bacterium was resistant and **'b'** is the total number of antibiotics against which the bacterium was tested (Amparado *et al*., 2020).

3.7 Genomic DNA Extraction

Bacterial genomic DNA was extracted using Zymo Research (USA) DNA Kits following the manufacturer's protocol. Before DNA extraction, bacterial isolates were harvested following an overnight culture and suspended in 200 µL of genomic lysis buffer for lysis at 55 °C for 30 minutes. Twenty (20) µL of Proteinase K was added to the lysed mixture and then underwent incubation at 55 °C for 30 minutes. The digested mixture was treated with 200 µL of 70% ethanol and further centrifuged to bind the bacterial DNA to silica-

gel-membrane. Inhibitors of PCR were removed following two washing steps. The pure DNA was eluted with elution buffer. The extracted DNA was stored at 4 °C awaiting further analysis. The DNA quantity and purity was assessed spectrophotometrically at 260–280 nm, using a Nano-Drop 1000c spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) (Pekana & Green, 2018).

3.8 DNA Amplification and Resistant Gene Detection

The PCR amplification was conducted following a previously adopted protocol (Taher *et al.,* 2020). DNA amplification for the detection of antibiotic-resistant gene in *Staphylococci* spp. was done using commercial primers of mecA gene (Murakami *et al.,* 1991). The primer sequence which were used for mecA and for *E. coli*, identification of resistant bacteria was performed using primers for ESBLs encoding genes bla $_{CTX-M}$, bla $_{TEM}$, bla $_{SHV}$ bla$_{OXA-1}$ and bla$_{KPC}$ are in **[Table 3.2]** (Pekana & Green, 20. The PCR reaction was carried out using a 12.5 µl PCR mixture containing 2 µl of DNA template, 6.25 µl of the 1X master mix, 1 µl of each reverse and forward primers, and 2 µl of nuclease-free water. The amplification was done using a 96-well thermal cycler. An initial denaturation was done at 95 °C for 3 minutes followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 54 °C for 60 s, and extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. Amplicons were resolved on a 1.5 % agarose gel at 80V for 60 min, and visualization was enhanced through staining with ethidium bromide, and UV-light spectrophotometer was used for visualization and the image was captured using a gel documentation imaging system. A 100kb DNA ladder was used as a molecular size marker. ATCC 25922 *E. coli* strain, EC 6308 and 6307 for ESBL, and NCTC 13552 *S. aureus* strain was used as the positive control. The amplicons were purified using a Zymo Research kit (USA) commercial purification kit (Ali *et al*., 2019; Liu *et al*., 2018; Ngaywa *et al*., 2019).

Table 3.2: Primers Used for DNA Amplification in the Current Study

Primer	Oligonucleotide sequence	Melting temperature (Tm)	Expected amplicon size	Source
mecA	F_AAAATCGATGGTAAAGGTTGGC R_AGTTCTGCAGTACCGGATTTTGC	94ºC	533bp	(Murakami *et al*., 1991)
16S rRNA	F_AGAGTTTGATCCTGGCTCAG R_TACGGYTACCTTGTTACGACTT	56°C	1500bp	This study
bla$_{TEM}$	F_TCGCCGCATACACTATTCTCAGAATGA R_ACGCTCACCGGCTCCAGATTTAT	65°C	445bp	(Monstein *et al.,* 2007)
bla$_{SHV}$	F_ATGCGTTATATTCGCCTGTG R_TGCTTTGTTATTCGGGCCAA	56°C	723bp	(Monstein *et al.*, 2007)
bla$_{CTX-M}$	F_GCCATGAAT AAGCTGATTGC R_CTTTACCCA GCGTCAGATTTT	57°C	193bp	This study
bla$_{OXA}$	F_AATCCGAAT CTTCGCGATACT R_GGTATCTTG AATGTCGATGC	57°C	225 bp	This study
bla$_{KPC}$	F_ATGTCACTGTATCGCCGTCT R_TTACTGCCCGTTGACGCCC	60°C	882bp	(Ribeiro *et al*., 2016)

Table 3.3: PCR Cycling Conditions for Detection of AMR Genes in Staphylococci Spp and Enterobacteria Spp.

Target gene	Primary denaturation	Amplification (35 cycles)			Final extension
		Secondary denaturation	Annealing	Extension	
16S rRNA	95℃, 10 min.	94℃, 30 sec.	56℃, 30 sec.	72℃, 1 min.	72℃, 10 min.
MecA	94℃, 3 min.	94℃, 30 sec.	50℃, 30 sec	68℃, 40 sec.	68℃, 5 min.
Bla_{TEM}	95℃, 4 min.	95℃, 45 sec.	50℃, 60 sec	72℃, 30 sec.	72℃, 7 min.
Bla_{OXA}	95℃, 4 min.	95℃, 45 sec.	54℃, 30 sec	72℃, 30 sec.	72℃, 5 min.
Bla_{CTX-M}	94℃, 3 min.	94℃, 30 sec.	56℃, 30 sec	72℃, 40 sec.	72℃, 5 min.
Bla_{SHV}	94℃, 3 min.	94℃, 30 sec.	56℃, 30 sec	72℃, 1 min.	72℃, 5 min.
Bla_{KPC}	95℃, 4 min.	95℃, 30 sec.	55℃, 30 sec	72℃, 1 min.	72℃, 5 min.

3.9 Sequencing and Analysis

Purified 16S rRNA and bla_{TEM} PCR products (50 ng) were prepared for Sanger sequencing. Sequence editing, end-to-end alignment and generation of consensus was performed on BioEdit program version. Sequence identities were confirmed using the Basic Local Alignment Search Tool (BLAST). Multiple sequence alignment was carried out using the MUSCLE tool. Further, the phylogenetic tree was constructed using MEGA11 (Tamura *et al.*, 2021). The evolutionary distances were calculated using the Maximum Likelihood method where the position containing the gaps and missing data were removed to enable construction of a consensus phylogenetic tree (Ali *et al*., 2019). The robustness of the tree was assessed with 1,000 bootstrap replicates.

3.9.1 Data Analysis

The coded data were entered into an MS Excel spreadsheet (Microsoft, USA) and exported to SPSS v26 (Microsoft, USA) for data analysis. Descriptive statistics were presented as tables. A chi-square test was used to evaluate associations between risk factors and mastitis infection ($p<0.05$). Logistic regression was used to test individual risk factors and their strength of association with a mastitis infection (Kim *et al*., 2017). The

odds ratio was used to determine the strength of associations identified in the logistic regression procedure (Mekonnen *et al*., 2017).

CHAPTER FOUR

RESULTS

4.1 Characteristics of the Respondents in the Current Study

A total of 120 respondents participated in the study and consisted of males (63.3%) and females (37.7%). Most of the respondent were more than 50 years of age (32.5%), while the rest ranged between 30 – 40 years (21.7%) and 40 – 50 years (20.0%). All of them kept dairy cows which were reared in a small-holder zero grazing husbandry system. The respondents were those that interacted closely with livestock through provision of feeds, milking and cleaning of the animal housing units.

Most of the respondents (84.2%) indicated they had suffered various ailments in the past three (3) months before the study. The most common ailment suffered in the last three months included; boils (5.4%), arthritis (1.4%), chest-pain (20.9%), influenza (33.3%), skin rashes (3.4%), stomachache (12.8%), open-wounds (7.4%) and diarrhea (3.4%). The respondents (60.8%) used various drugs for management of the various ailments with the most common drugs being painkillers (40.5%), cough-syrups (13.9%), antibiotics (7.6%), constipation drugs (1.3%) and skin rashes-ointments (1.3%).

4.2 Prevalence of Bacteria Colonizing the Skin of the Respondents

Bacteria were isolated from all (100%) the respondents. A total of eight bacteria species were isolated with the most prevalent being *S. aureus* (49.4%) followed by coagulase-negative Staphylococci (CoNS) sp (16.9%) and *Pantoea* spp. (13.0%). The least isolated bacteria were *Yersinia enterocolitica* (1.3%) and *Pasteurella aerogenes* (1.3%).

Table 4.1: Prevalence of Bacteria Isolated from the 120 Participating Respondents on Small Scale Farms in Gatundu Sub-County, Kenya

Bacterial species	n	%
Staphylococcus aureus	38	49.4
Coagulase negative *Staphylococci*	13	16.9
Pantoea spp.	10	13.0
Serratia spp.	10	13.0
Bukholderia cepacian	2	3.0
Enterobacter spp.	2	3.0
Yersinia enterocolitica	1	1.3
Pasteurella aerogenes	1	1.3

Key: n = number of isolates, %-percentage frequency, CoNS - coagulase negative staphylococci

4.3 Antibiogram for *S. aureus* Isolated From the Skin of Human Respondents

The overall prevalence of antibiotic resistance among *S. aureus* isolates was 37.3%. In descending order, the *S. aureus* were resistant against gentamicin (86.8%), followed by oxacillin (86.8%), vancomycin (73.7%) and ampicillin (60.5%). Most *S. aureus* were sensitive to amoxycillin-clavulanic acid (89.5%), chloramphenicol (73.7%), cefoxitin (65.8%) and ciprofloxacin (65.8%). (Table 4.2)

Table 4.2: Susceptibility Profile of S. Aureus Isolated from Human Having Close Contact with Dairy Cows in Small Scale Farms in Gatundu Sub- County, Kenya

Antibiotic	Antibiogram for *S. aureus* (n=38)					
	Resistant		Intermediate		Susceptible	
	n	%	n	%	n	%
GEN	33	86.8	0	0	5	13.2
DA	11	28.9	20	52.6	7	18.4
E	10	26.3	25	65.8	3	7.9
TE	11	28.9	8	21.1	19	50
C	10	26.3	0	0	28	73.7
AMC	1	2.6	3	7.9	34	89.5
AMP	23	60.5	0	0	15	39.5
MEL	15	39.5	6	15.8	17	44.7
SXT	0	0	9	23.7	29	50
OX	33	86.8	0	0	5	13.2
VA	28	73.7	8	21.1	2	5.3
FOX	13	34.2	0	0	25	65.8
CIP	13	34.2	0	0	25	65.8

Key: GEN-gentamycin, DA-clindamycin, E- erythromycin, TE- tetracycline, C-chloramphenicol, AMC-amoxyclav, AMP-ampicillin, MEL-mecillinum, SXT-sulphamethoxazole-trimethoprim, OX- oxacillin, VA-vancomycin, FOX- cefoxitin, CIP-ciprofloxacin. n – number of isolates, % - percentage frequency

4.4 Antibiogram for CoNS Isolated From Neck Skin Swab of Human Respondents

The overall prevalence of antibiotic resistance CoNS was found to be 63.1 %. Most CoNS were resistant to gentamycin (100%), clindamycin (84.6%), erythromycin (84.6%) and ciprofloxacin 84.6%. The CoNS were most sensitive to sulphamethoxazole-trimethoprim (76.9%) and chloramphenicol (69.2%). (Table 4.3)

Table 4.3: Antibiogram of Coagulase Negative Staphylococci (Cons) Isolated from Skin Swab from Human Respondents having Close Contact with Dairy Cattle in the Small-Scale Farms in Gatundu Sub-County, Kenya

	Antibiogram of CoNS (n = 13)					
Antibiotic	**Resistant**		**Intermediate**		**Susceptible**	
	n	**%**	**n**	**%**	**n**	**%**
Gen	13	100.0	0	0	0	0
DA	11	84.6	2	15.4	0	0
E	11	84.6	0	0	2	15.4
TE	8	61.5	1	7.7	4	30.8
C	4	30.8	0	0	9	69.2
AMP	11	84.6	0	0	2	15.4
OX	8	61.5	0	0	5	38.5
FOX	5	38.5	0	0	8	61.5
CIP	11	84.6	0	0	2	15.4
SXT	0	0	3	23.1	10	76.9

Key: Gen-gentamycin, DA-clindamycin, E- erythromycin, TE- tetracycline, C-chloramphenicol, AMP-ampicillin, SXT- sulphamethoxazole-trimethoprim, OX-oxacillin, FOX- cefoxitin, CIP- ciprofloxacin. n – number of isolates, % - percentage frequency

Table 4.4: The Prevalence of Staphylococci Spp. and Risk-Factors Associated with Human Colonization in Small-Holder Farms in Gatundu Sub-County, Kenya.

Risk factors		***S. aureus* (n=38)**	**CoNS (n=13)**
Ward	Wamwangi	15	7
	Ng'enda	11	2
	Chania	8	1
	Mang'u	4	3
Gender	Male	20	8
	Female	18	5
Age (years)	<20	4	1
	21 – 30	7	1
	30 – 40	7	3
	40 – 50	11	5
	> 50	9	3
Previous ailments	Yes	22	7
	No	16	6

4.5 Association of Antimicrobial Resistance (AMR) and Risk Factors (Table 4.5)

Most males (59.2%) had AMR isolates compared to females (52.3%). However, the relationship between antimicrobial resistance and gender was statistically insignificant (p = 0.463, OD = -0.121). Most of the respondents having AMR isolates were in the age categories of >50 years old (n=9) and 40 – 50 years old (n=11) (61.5%). The prevalence of AMR isolates was significantly (p = 0.011, OD = 1.745) related to age of the respondents. The proportion of AMR isolates in respondents who had used antibiotics (55.2%) was higher than those who had not (47.3%) ((p = 0.025, OD = 0.204).

The current study also sought to find out on the recovery of the respondents who have protracted use of drugs. Among the respondents, 52.5% did not recover and 47.5% recovered. Further, 65.1% of who had not recovered had AMR resistant isolates while 56.1% of those recovered also had AMR resistant isolates (p = 0.859, OD = 1.105).

Over half (54.1%) of the participants who had visited the healthcare center for treatment had AMR resistant isolates. The relationship between the prevalence of antimicrobial resistance and health facility visit was not statistically significant (p = 0.287, OD = 0.577).

Table 4.5: Association between Risk Factors and Proportion of Antimicrobial Resistance (AMR) Isolates in Respondents from Gatundu Sub-County, Kenya

Risk factor	N	%	p-value	OD
Gender			0.463	-0.121
Male	45/76	59.2		
Female	23/44	52.3		
Age (Years)			0.011	1.745
≤20	9/16	56.3		
21 – 30	7/15	46.7		
30 – 40	10/26	38.5		
40 – 50	18/24	75.0		
>50	30/39	76.9		
Medication usage			0.025	0.204
No	25/53	47.3		
Yes	37/67	55.2		
Recovery			0.859	1.105
No	41/63	65.1		
Yes	32/57	56.1		
Hospital visit			0.287	0.577
No	45/59	76.3		
Yes	33/61	54.1		

Key: n – proportion of respondents with AMR resistant isolate, % - proportion in percent of respondents with AMR resistant isolate at the small-holder farms.

4.6 Occurrence of Multiple Antibiotic Resistance (MAR) Index and Multidrug Resistance Pattern in Human Respondents

The MAR Index for *S. aureus* and CoNS was 0.92 and 0.92, respectively. This shows that for both bacteria, the MAR index was greater than 0.2. Occurrence of multidrug resistance (MDR) was noted amongst the various *S. aureus* and CoNS isolates. For *S. aureus*, the MDR was expressed against the following antibiotics: 66 (14.7%%) isolates expressed MDR to gentamycin and oxacillin, 26 isolates (5.8%) were resistant to cefoxitin and ciprofloxacin, 22 isolates (4.9%) were resistant to clindamycin and tetracycline while 20 isolates (4.4%) were resistant to chloramphenicol and erythromycin. For CoNS, 44

(33.8%) isolates expressed MDR to clindamycin, erythromycin, ampicillin and ciprofloxacin, and 16 (3.6%) isolates were resistant to tetracycline and oxacillin.

Table 4.6: Multiple Resistance Patterns of S. Aureus and CONs Isolated from Human Respondents in Small-Holder Farms in Gatundu Sub-County, Kenya

Bacteria	Antibiotic	No. of isolates	%
Staphylococcus aureus	GEN & OX	66	14.7
	FOX & CIP	26	5.8
	TE & DA	22	4.9
	E & C	20	4.4
CoNS	DA, E, AMP & CIP	44	33.8
	TE, OX	16	3.6

Key: GEN-gentamycin, DA-clindamycin, E- erythromycin, TE- tetracycline, C-chloramphenicol, AMP-ampicillin, OX- oxacillin, FOX- cefoxitin, CIP- ciprofloxacin. n – number of isolates, % - percentage frequency

4.7 Prevalence and Risk Factors of Sub-clinical Bovine Mastitis Based on CMT

The overall prevalence of sub-clinical mastitis at the host level as determined by CMT was 39.6%. The prevalence varied with geographical/administrative Wards, with highest the highest prevalence being recorded in cows from Wamwangi (47.6%) Ward, followed by those from Chania (34.8%), Ng'enda (9.8%) and Mang'u (7.9%) wards. There were no significant differences in prevalence of mastitis in lactating dairy cows originating from different administrative wards.

Table 4.7: Prevalence of Sub-Clinical Mastitis as Categorized Different Administrative Wards in the Gatundu Sub-County

Wards of origin	Prevalence		
	No. of samples	No. positive	%
Wamwangi	78	30	38.5
Chania	57	20	35.1
Ng'enda	16	10	62.5
Mang'u	13	5	38.5
Total	**164**	**65**	**39.6%**

4.8 Farm Based Prevalence and Risk Factors of Sub-Clinical Mastitis Based on CMT

The overall prevalence of sub-clinical mastitis at the farm-level as determined by CMT was 46.6%. The prevalence varied with wards, with the highest prevalence being recorded in small-scale farms from Ng'enda (63.6%) Ward, followed by those from Wamwangi (48.1%), Chania (41.9%), and Mang'u (40.0%) wards (p = 0.061).

Table 4.8: Farm-Based Prevalence of Sub-Clinical Mastitis in different Administrative Wards in the Gatundu Sub-County, Kenya

Ward of origin	Farm-based prevalence		
	No. samples	No. positive	%
Wamwangi	52	25	48.1
Chania	43	18	41.9
Ng'enda	11	7	63.6
Mang'u	10	4	40.0
Total	**116**	**54**	**46.6%**

4.9 Relationship Between Prevalence of Mastitis and Various Risk Factors (Table 4.9)

The highest prevalence of sub-clinical mastitis was found in cows having five (5) parities and above (55.6%) while the lowest prevalence was observed in cows having single parity. However, there was no significant (p=0.323, OD=0.872) relationship between the prevalence of sub-clinical mastitis and the parity of the dairy cows.

Cows with low milk production (2 litres/day or less) had higher (66.7%) cases of mastitis than those producing 4 litres per day. However, the relationship between milk production and prevalence of sub-clinical mastitis was not significant (p=0.502, OD=0.891).

Most cows, (80.5%) had no previous history of clinical mastitis. Those animals having previous history of mastitis had significantly (p=0.026, OD=2.503) higher prevalence (56.3%) of sub-clinical mastitis than those without such a history.

Prevalence of sub-clinical mastitis was higher (37.5%) in cows kept in poor hygiene condition than those kept in good condition. The relationship between prevalence of mastitis and the hygiene condition in the dairy cows-housing was statistically insignificant (p=0.411, OD=1.340).

The main breed of cattle kept by farmers in the study was Friesian (86.0%). The relationship of between prevalence of mastitis and the breed of cattle was statistically (p=-0.407, OD=0.902) insignificant.

Table 4.9: Risk Factors Associated with Sub-Clinical Mastitis in Cows kept by Small Holder Farmers in Gatundu Sub-County, Kenya.

Risk factors	n	%	P-value	OD
Parity			0.323	0.872
>5	5/9	55.6		
1st	21/53	39.6		
2nd	15/39	38.5		
3rd	15/37	40.5		
4th	7/22	31.8		
5th	2/4	50		
Milk volume (Liters)			0.502	0.891
>5	60/149	40.3		
1	0/1	0		
2	2/3	66.7		
3	0/1	0		
4	3/10	30		
History of mastitis			0.026	2.503
No	47/132	35.6		
Yes	18/32	56.3		
Hygiene/sanitation			0.411	1.340
Poor	43/114	37.7		
Good	22/50	44		
Cattle Breed			0.497	0.902
Ayrshire	4/10	40		
Ayrshire - Friesian cross	0/2	0		
Ayrshire-Jersey cross	1/2	50		
Friesian	58/141	41.1		
Guernsey	1/5	20		
Holstein - Friesian cross	1/3	33.3		
Friesian - Indigenous cross	0/1	0		

Key: n – Proportion positive for sub-clinical mastitis, % - Percentage, OD – Odds Ratio

4.10 Bacteria Identified in Milk Samples

In descending order, the bacteria identified were CoNS (50.3%), *S. aureus* (31.8%), *Pantoea* spp. (1.9%) and *Enterobacter cloacae* (1.3%), *Klebsiella oxytoca* (0.6%), *Serratia* spp. (0.6%) and *Citrobacter koseri* (0.6%). (Table 4.10)

Table 4.10: Bacterial Species Identified from the Milk Samples Collected from Dairy Cows kept by Small Holder Farmers in Gatundu Sub – County, Kenya

Bacterial species	**n**	**%**
CoNS	79	50.3
S. aureus	50	31.8
Pantoea spp.	3	1.9
Enterobacter cloacae	2	1.3
Citrobacter koseri	1	0.6
Klebsiella oxytoca	1	0.6
Serratia spp.	1	0.6
Average	**157**	**100**

CoNS – Coagulase negative *Staphylococci*

4.11 Antibiogram of *S. aureus* Isolated From Milk Samples

Staphylococcus aureus isolates from milk exhibited highest resistance to vancomycin (77.3%), mecillinum (54.5%) and gentamycin (50.0%). The bacteria were highly sensitive to cefoxitin (100%), ciprofloxacin (100%) and clindamycin (95.5%). (Table 4.11)

Table 4.11: Antibiogram of S. aureus Isolated from Milk Obtained from Dairy Cows kept in Small-Holder Farms in Gatundu Sub-County, Kenya

Antibiotic	Antibiogram for *S. aureus* (n=22)					
	Resistant		Intermediate		Susceptible	
	n	%	n	%	n	%
Gen/CN	11	50	0	0	11	50
DA	0	0	7	31.8	15	68.2
E	1	4.5	15	68.2	6	27.3
TE	3	13.6	2	9.1	17	77.3
C	1	4.5	0	0	21	95.5
AMC	0	0	2	9.1	20	90.9
AMP	10	45.5	0	0	12	54.5
MEL	12	54.5	2	9.1	8	36.4
SXT	1	4.5	1	4.5	20	90.9
OX	10	45.5	0	0	12	54.5
VA	17	77.3	3	13.6	1	4.5
FOX	0	0	0	0	22	100
CIP	1	4.5	0	0	21	95.5

Key: Gen/CN-gentamycin, DA-clindamycin, E- erythromycin, TE- tetracycline, C-chloramphenicol, AMC-amoyclav, AMP-ampicillin, MEL-mecillinum, SXT-sulphamethoxazole-trimethoprim, OX- oxacillin, VA-vancomycin, FOX- cefoxitin, CIP-ciprofloxacin. n – number of *S. aures* isolated from milk samples, % - percentage frequency

4.12 Antibiogram of Coagulase Negative Staphylococci (CoNS) Isolated From Milk Obtained From Dairy Cows in Small-Holder Farms in Gatundu Sub-County, Kenya

Coagulase negative Staphylococci exhibited resistance to gentamycin (86.7%) and ampicillin (70.0%). The bacteria were sensitive to chloramphenicol (86.7%), sulphamethoxazole-trimethoprim (80.0%), cefoxitin (73.3%), tetracycline (70.0%), ciprofloxacin (63.3%) and oxacillin (63.3%). (Table 4.12)

Table 4.12: Antibiogram of CONs Isolated from Dairy Cows Kept in Small-Holder Farms in Gatundu Sub-County, Kenya

Antibiogram of CoNS (n = 30)						
Antibiotic	**Resistant**		**Intermediate**		**Susceptible**	
	n	**%**	**n**	**%**	**n**	**%**
Gen	26	86.7	2	6.7	2	6.7
DA	9	30.0	8	26.7	13	43.3
E	9	30.0	5	16.7	16	53.3
TE	9	30.0	-	-	21	70.0
C	4	13.3	-	-	26	86.7
AMP	21	70	-	-	9	30.0
OX	11	36.7	-	-	19	63.3
FOX	8	26.7	-	-	22	73.3
CIP	11	36.7	-	-	19	63.3
SXT	3	10.0	3	10	24	80.0

Key: Gen/CN-gentamycin, DA-clindamycin, E- erythromycin, TE- tetracycline, C-chloramphenicol, AMP-ampicillin, SXT- sulphamethoxazole-trimethoprim, OX-oxacillin, FOX- cefoxitin, CIP- ciprofloxacin. n – number of isolates, % - percentage frequency

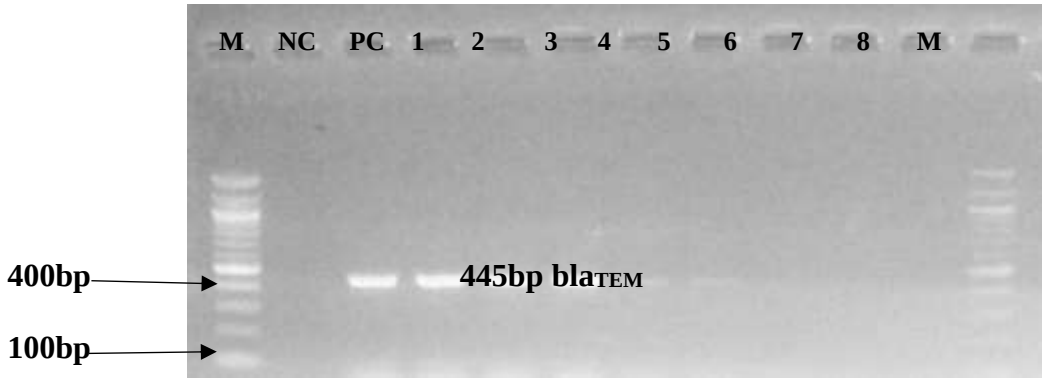
4.13 Occurrence of Multiple Antibiotic Resistance (MAR) Index and the Antibiotics Resistance Pattern in Dairy Cows in Small-Holder Farms in Gatundu Sub-County.

The MAR Index for *S. aureus* and CoNS was 0.77 and 1.00, respectively. This shows that for both bacteria species, the MAR index was greater than 0.2 threshold.

4.14 Molecular Detection of AMR Genes

The *Staphylococci* spp isolates did not harbor mecA gene which is used for the determination of methicillin-resistance. Isolated *Pantoea* spp, *Enterobacter cloacae*, *Citrobacter koseri*, *Klebsiella oxytoca* and *Serratia* spp were screened for the presence of beta-lactam resistant genes such as blaOXA, blaTEM, blaKPC, blaCTX-M and blaSHV.

The screened enterobacteria were only positive for blaTEM gene (17 isolates of 81, 21%). (Figure 4.1)



Legend: M – 100bp DNA Ladder, NC - Negative control, PC – Positive control, 1-3 positive prominent bands, 4-5 positive faint bands, 6-8 Negative (No bands)

Figure 4.1: Gel Image for blaTEM Gene Detected in Pantoea spp and Enterobacter cloacae

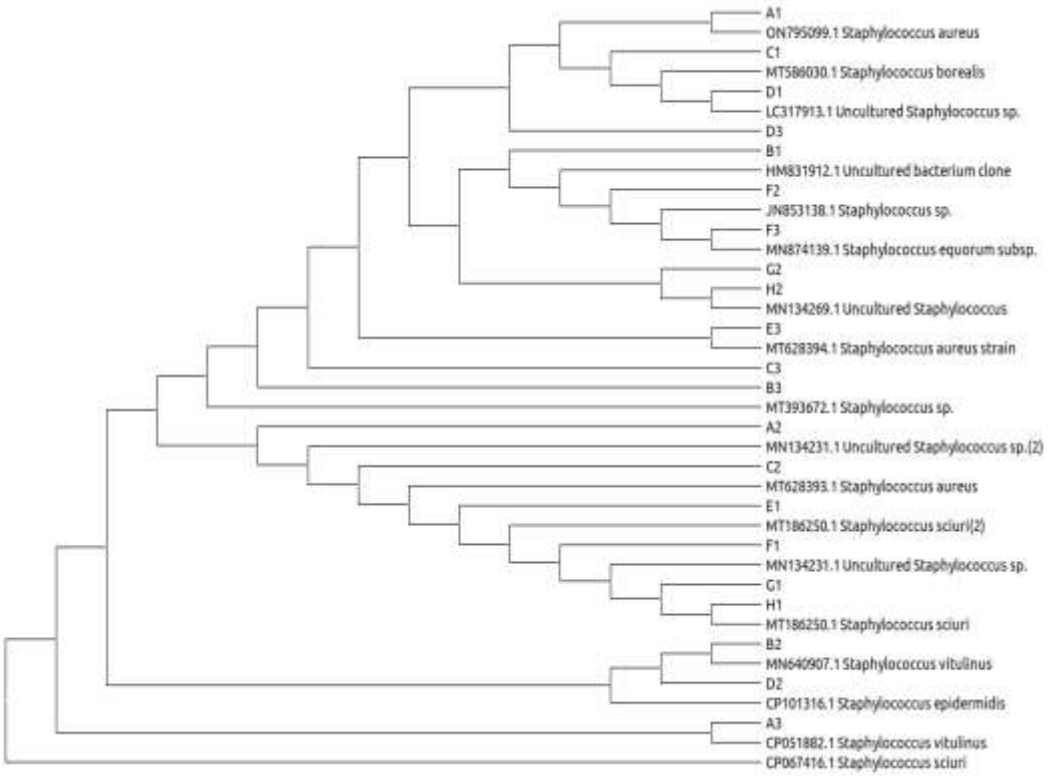
4.15 Evolutionary Analysis by Maximum Likelihood Method

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model for 16S rRNA and blaTEM gene for *Staphylococci* spp. and Enterobacteria (*Pantoea* spp. and *Enterobacter cloacae*), respectively (Table 4.14). The Staphylococci isolates relatives retrieved from NCBI reveals almost similar sources of the isolates with the current study. Most (9/14, 64.3%) of the retrieved from human skin, milk and cow teat skin which agrees with the current study.

Most (13/20, 65%) of the 16S S*taphylococci* spp. sequence from the current had 100% closeness to their NCBI BLAST relatives. Similarly, most (9/14, 64.3%) of the NCBI sequence retrieved through blast were isolated from skin, milk, cow teat skin and skin. This is consistent with the source of samples from this study, which was milk from lactating dairy cow and skin from human respondent. (Table 4.13)

Table 4.13: Sample Identities and their NCBI Closest Relative for 16S rRNA in Staphylococci sp

S/NO.	Sample ID	NCBI closest relative	Source	% closeness	Accession No.
1.	A1	*Staphylococcus aureus*	-	100	ON795099.1
2.	A2	Uncultured *Staphylococcus aureus*	Skin	100	MN134231.1
3.	A3	*Staphylococci* spp.	-	99.76	MT393672.1
4.	B1	*Bacterium*	Water	95.53	KC734181.1
5.	B2	*Staphylococcus vitulinus*	Endophyte (*Carica papaya* pulp)	100	MN640907.1
6.	B3	*Staphylococcus sciuri*	Water	100	CP067416.1
7.	C1	*Staphylococcus borealis*	Blood culture	100	MT586030.1
8.	C2	*Staphylococcus sciuri*	-	99.75	MH880111.1
9.	C3	*Staphylococcus aureus*	-	100	MT628393.1
10.	D1	Uncultured *Staphylococcus aureus*	Leaves of Awa Bancha tea	96.28	LC317913.1
11.	D2	*Staphylococcus vitulinus*	Ground beef	99.98	CP051882.1
12.	D3	*Staphylococcus epidermidis*	Human skin	100	CP101316.1
13.	E1	*Staphylococcus sciuri*	Milk	100	MT186250.1
14.	E3	*Staphylococcus aureus*	Cow	99.74	MT628394.1
15.	F1	Uncultured *Staphylococcus aureus*	Skin	100	MN134231.1
16.	F2	*Staphylococci* spp.	Cow teat skin	98.72	JN853138.1
17.	F3	*Staphylococcus equorum*	-	100	MN874139.1
18.	G1, G2	*Staphylococcus sciuri*	-	100	MH880111.1
19.	H1	*Staphylococcus sciuri*	Milk	100	MT186250.1
20.	H2	Uncultured *Staphylococci* spp.	Skin	100	MN134269.1



Legend: A1, C1, D1, D3, B1, F2, G2, H2, E3, C3, B3, A2, C2, E1, F1, G1, H1, B2, D2 and A3 are the sample for this study.

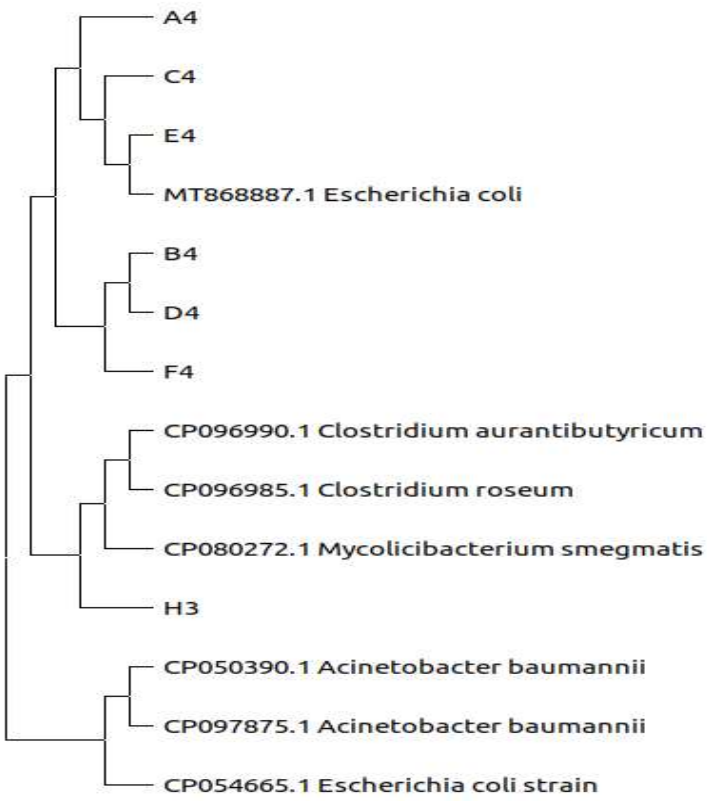
Figure 4.2: 16S rRNA Phylogenetic Tree for Staphylococci sp.

All of the blaTEM sequence from this study have 99.33% to 100% closeness to their NCBI BLAST relative. In contrast, all of the sequences retrieved were isolated from different sources. Most of blaTEM the sequences retrieved from NCBI were from *Acetinobacter bauminnii* (2 out of 7), *Escherichia coli* (2 out of 7), *Clostridium aurantibutyricum* (1out of 7), *Clostridium roseum* (1 out of 7) *and Mycolicibacteruim smegmatis* (1 out of 7). (Table 4.14)

Table 4.14: Sample Identities and their NCBI Closest Relative for bla TEM Gene in Enterobacteria

S/NO.	Sample ID	NCBI closest relative	Source	% closeness	Accession No.
1.	A4	*Clostridium aurantibutyricum*	-	100	CP096990.1
2.	B4	*Acetinobacter bauminnii*	blood	100	CP050390.1
3.	C4	*Clostridium roseum*	-	100	CP096985.1
4.	D4	*Acetinobacter bauminnii*	Hospital ICU	99.71	CP097875.1
5.	E4	*Escherichia coli*	Norville Karst	100	MT868887.1
6.	F4	*Escherichia coli*	-	100	CP054665.1
7.	H3	*Mycolicibacteruim smegmatis*	-	99.33	CP080272.1

The seven samples under study harbored blaTEM gene. Samples A4, C4 and E4 clustered together. They exhibited evolutionary events. Sample A4 was isolated from milk, while C4 and E4 were isolated from skin of human respondents from Ng'enda and Wamwangi administrative wards. Samples; B4, D4, and F4 clustered together. All the three samples were isolated from skin of human respondent within Wamwangi administrative ward but not from the same homestead. Similarly, sample H3 was isolated from skin of human respondent in Ng'enda administrative ward.



Legend: A4, C4, E4, B4, D4, F4 and H3 are sample for this study.

Figure 4.3: Phylogenetic Tree Based on blaTEM Gene for Enterobacteria

CHAPTER FIVE

DISCUSSION

Information about the current magnitude of the burden of bacterial AMR, trends in different parts of the world, and the leading pathogen–drug combinations contributing to bacterial AMR burden is crucial (WHO, 2016). With minimal surveillance, the spread of AMR could make many bacterial pathogens much more lethal in the future than they are today (Murray *et al*., 2022). The current study investigated the occurrence of AMR among human having close contact with dairy cattle in small-scale set-ups in Gatundu Sub-County, Kenya.

The findings of the current study showed the farmers had previous skin infections such as boils, skin rashes and open wounds, which they treated using various drugs. Most could not remember the specific names of the drugs and thus the investigator could not identify the drugs. The presence of these ailments could have led to the high number of coagulase negative *Staphylococci* spp. and *S. aureus* colonizing the skins of the farmers. Other bacteria isolated from the respondents' skins included *Pantoea* spp., *Serratia* spp., *B. cepacia*, *Enterobacter* spp., *Y. enterocolitica* and *P. aerogenes*. These results are consistent with a study conducted in milkmen from Uganda, where the prevalence of *S. aureus* and CoNS was high (71%) (Kateete *et al*., 2013). The present study also agrees to a study conducted in Romania which found *S. aureus* and CoNS being dominant bacteria colonizing the skin, representing, 48% and 64%, respectively (Gizaw *et al*., 2020). *Staphylococci* spp. are skin commensals and very adaptive on mucous membrane.

The present study found high number of respondents having *S. aureus* that were resistant to gentamycin, oxacillin and vancomycin. This could be attributed to high prescription of these antibiotics in the study area. The highest susceptibility of *S. aureus* to antibiotics used in this study was observed in amoxycillin-clavulanic acid, chloramphenicol, ciprofloxacin and cefoxitin. The result from the current study are consistent with a study by others (Kateete *et al*., 2013), which found susceptibility to oxacillin and cefoxitin

(36%). However, further studies are needed to determine the causes of the high burden of resistance which was observed.

This study observed a high proportion of CoNS from human respondents were resistant to gentamycin, clindamycin, erythromycin, ampicillin and ciprofloxacin. A similar study conducted in Egypt (Gizaw *et al*., 2020b) found intensity of antimicrobial resistance by CoNS varied according to the site of isolation. The prevalence of resistant CoNS from the nasal and hands of butchers were 20.0%, 13.5%, respectively. The percentage of CoNS milker's hand was 4.0%. In the Egyptian study, CoNS had lower resistance to gentamycin (3.6%), ciprofloxacin (3.6%), sulphamethoxazole-trimethoprim (17.9%), chloramphenicol (25.2%) and cefoxitin (60.7%). The observed prevalences could be related to selection pressure due to specific antibiotics used in a given area.

Overall, the current study found that large proportion of *S. aureus* and CoNS isolated from the human respondents exhibited multidrug resistant trait, which agrees with studies done elsewhere (Beyene *et al*., 2017; Gizaw *et al*., 2020b). The latter study (Kateete *et al*.,(Kateete *et al*., 2013) reported that all *S. aureus* isolates from milkmens' hands were resistant to a combination of cefoxitin, gentamycin, tetracycline, erythromycin and chloramphenicol. Multidrug drug resistant was also reported in other countries like Ethiopia, where MDR of 7 of 9 (77.8%) antimicrobial classes was noted in CoNS. This resistance can be attributed to consistent therapeutic and/or indiscriminate use of these antimicrobials in these study areas.

Sub-clinical mastitis is of global concern, especially where milk and milk products cater for the fast-growing human population (Gitau *et al*., 2014). This creates a need for extensive research on the status of mastitis and mastitis-associated pathogens to improve the existing control measures and guide treatment (Mahlangu *et al*., 2018). The current study investigated the prevalence, risk factors, and antibiogram of *Staphylococcus* sp. isolated from dairy cows kept by small-holder farmers in the Gatundu Sub-County, Kenya.

The prevalence of subclinical mastitis reported in the present study was lower than those reported in other countries (Amer *et al*., 2018; Mbindyo *et al*., 2020; Mekonnen *et al*., 2017), but was close to that reported in Philippines (42.7%) (Salvador *et al*., 2012). However, the prevalence was higher than reported in Ethiopia (Haftu *et al*., 2012), Rwanda (Mpatswenumugabo *et al*., 2017). Several studies in Kenya showed higher prevalence of sub-clinical mastitis, Embu (73.5%,), Kajiado (72.8%) counties (Mbindyo *et al.,* 2020). In Kiambu, Juja Sub-county the prevalence was reported as 66.7% (Kagira *et al.,* 2022). Mastitis has a multifactorial nature, with evidence of interaction between host, agent, and environment (Balemi *et al*., 2021a). Studies have attributed the various differences to poor hygiene, environmental factors, and non-adherent to mastitis control measures such as; standard milking procedures, proper pre-, and post-udder washing, as well as the usage of teat dips after milking (Balemi *et al*., 2021). In the current study, cows with previous history of mastitis had higher prevalence indicating possibilities of relapses or existence of risk factors in such farms which could be exposing cows to mastitis. Thus, efforts should be made for regular screening of sub-clinical mastitis using a CMT kit. In general, the findings of the present study found a higher prevalence of sub-clinical mastitis in dairy cows producing low amount of milk after previously encountering mastitis. This could be due to the fact that mastitis lowers the yield of milk production (Amer *et al*., 2018; Dabele *et al*., 2021).

The most prevalent bacteria isolated in the current study were the *Staphylococci* spp. where both coagulase-negative Staphylococcus (CoNS) and *Staphylococcus aureus* were observed. These findings are consistent with other studies which have found the *Staphylococci* species to be the major causal agent of mastitis in dairy cows ((Mbindyo *et al*., 2020). Staphylococci infections mainly develop as sub-clinical mastitis which more often develops to clinical form if not well treated. Further, this infection causes loss of milk, high cost of treatment, as well as culling of the affected cow. On the other hand, consumption of milk contaminated with *S. aureus* is associated with food poisoning amongst other human infections.

This study also revealed the presence of enterobacteria such as *Citrobacter, Klebsiella, Enterobacter*, and *Serratia* spp. in the milk samples which is in agreement with a study by others (Mahlangu *et al*., 2018), who noted that coliforms are the drivers of environmental mastitis in dairy animals. Poor hygiene in the cow pens, like those witnessed in some farms in Gatundu Sub- County, promotes the presence of these coliforms. This is consistent with other studies in Kenya, done by Mbindyo *et al*. (2020) and Kagira *et al*. (2022)

Most of the *S. aureus* isolated in the present study were resistant to vancomycin, mecillinum, and gentamycin. Further, the present study revealed that most CoNS isolated in the study area were resistant to gentamycin and ampicillin. These could be possibly attributed to the long-term usage and probably the ease of access of these drugs in treating mastitis and other diseases in the area. On a positive note, all the *S. aureus* isolates were susceptible to cefoxitin, ciprofloxacin, and amoxicillin-clavulanic acid. In addition, most of the CoNS isolated in the present study were sensitive to chloramphenicol, sulphamethoxazole trimethoprim, cefoxitin, tetracycline, ciprofloxacin, and oxacillin. These could be due to the rare usage of these bacteria in the treatment of sub-clinical mastitis in the study area. These antibiotics should be used in the treatment of sub-clinical mastitis under the guidelines of the veterinary officer.

Overall, the current study found that large proportion of *S. aureus* and CoNS isolates were exhibited multidrug resistant trait, which agrees with studies done elsewhere (Balemi *et al*., 2021) (Haftu *et al*., 2012). The latter studies reported that all *S. aureus* isolates from cows were resistant to a combination of ampicillin, erythromycin, clindamycin, and chloramphenicol. Multidrug drug resistant was also been reported other countries like France, where Botrel *et al*., (2010), found both *S. aureus* and CoNS which had MDR to gentamycin, tetracycline, cefoxitin, erythromycin and sulphamethoxazole- trimethoprim. This resistance might be due to repeated therapeutic and/or indiscriminate use of these antimicrobials in these study areas.

In the current study, the staphylococcal isolates were screened for methicillin-resistant staphylococcal gene. The screening was targeting mecA gene. The presence of the mecA gene is considered the gold standard of detecting MRSA and other homologues of mecA, such as emergent mecC and mecB. However, the current study did not detect mecA in all the staphylococcal isolates. These findings agree with other studies carried out in Kenya (Mbindyo, 2021), Egypt (Taher *et al.,* 2020), and elsewhere (Virgin *et al.,* 2009, Haran *et al.,* 2012). The current findings are in contrast with a study in Egyptian (Mousa *et al.,* 2021) which reported the presence (73% of the screened staphylococcal isolates) of mecA gene.

The current study also investigated the presence of extended spectrum beta-lactamases (ESBLs) encoding genes. Among the screened ESBLs were bla_{OXA}, bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$ and bla_{KPC}. However, the current study found only bla_{TEM} (17 of 41 isolates, 41.5%) among the ESBLs screened. The current study agrees with study in Egypt (Taher *et al.,* 2020), which reported 43% of bla_{TEM} screened.

In the current study, genotypic and diversity analysis on staphylococcal isolates was done by sequencing of the 16S rRNA gene. This is the first step of molecular characterization for the identity of the bacteria. The evolutionary analysis in the current study revealed the close relationship between the isolates from humans and livestock independently. In the instances of closes evolutionary relationship between the isolates between human and livestock isolates, the isolates were from different farms situated in different administrative wards.

The cattle study also identified several enterobacteria (coliforms) such as *Pantoea* spp, *Serratia* spp, *Bulkholderia cepacian*, *Enterobacter* spp, *Yersinia enterolitica, Pasturella oxytoca* and *Citrobacter koseri*. These finding agrees with several studies done elsewhere (Mbindyo *et al.,* 2020, Kalmus *et al.,* 2011, Pascu *et al.,* 2021, Abdi *et al.,* 2021). The isolates positive for bla_{TEM} among the enterobacteria were sequenced. The BLAST search revealed among the close relatives in the NCBI were *Escherichia coli*. Surprisingly, this bacterium was not isolated when using the normal culturing in the laboratory These

coliforms are the causative agents for environmental mastitis since their presence confirms environmental contamination.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The current study concludes that:

- The overall prevalence of sub-clinical mastitis at the farm-level as determined by CMT was 46.6%. The prevalence varied with wards, with the highest prevalence being recorded in small-scale farms from Ng'enda (63.6%) Ward, followed by those from Wamwangi (48.1%), Chania (41.9%), and Mang'u (40.0%) wards. The present study showed that sub-clinical mastitis caused by CoNS and *S. aureus* is a challenge in cows in the study area.
- Further, high prevalence of antimicrobial resistance was noted in these isolates. In human, the overall prevalence of antibiotic resistance CoNS was found to be 63.1 %. Most CoNS were most resistant to gentamycin (100%) and most sensitive to sulphamethoxazole-trimethoprim (76.9%). The overall prevalence of antibiotic resistance among *S. aureus* isolates was 37.3%. *S. aureus* isolates were most resistant to gentamicin (86.8%) and most sensitive to amoxycillin-clavulanic acid (89.5%). In milk, Coagulase negative Staphylococci exhibited high resistance to gentamycin (86.7%) and most sensitive to chloramphenicol (86.7%). *S. aureus* isolates from milk exhibited highest resistance to vancomycin (77.3%), mecillinum (54.5%) and highly sensitive to cefoxitin (100%), ciprofloxacin (100%) and clindamycin (95.5%).
- Age and drug usage were found to be potential risk factors to prevalence of antibiotic resistant bacterial pathogen to human who frequently and closely handle dairy cows in the study area. Ages 40 – 50, and >50 had high burden of antimicrobial resistant bacteria in the study area. Respondent who has protracted drug usage (55.2%) and this is hypothesized to be reason for the presence of

antimicrobial drug resistant bacteria in the study area. In dairy cows in the study area, history of mastitis is a potential risk factor for colonization for AMR bacteria.

- Current study found no MRSA among the Staphylococci spp. and only bla_{TEM} among the screened ESBLs.
- The current study found close relationship between the Staphylococci and enterobacteria isolates from human and dairy cows.

6.2 Recommendation for Future Study

The study recommends:

1. There is need to differentiate the coagulase negative *Staphylococci* spp. (CoNS) up to species level using molecular methods.
2. The antibiogram of isolated enterobacteria species should be determined to determine the specific antibiotic resistant enterobacteria species circulating in the study area.
3. Exploiting whole-genome and molecular typing to assess the clonal lineages of *Staphylococci* spp. circulating in the study population is encouraged.
4. Creating awareness on the risk factors causing antimicrobial resistance.

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APPENDICES

Appendix I: Informed Consent

Study title: Risk factors of Sub-clinical mastitis, antibiogram and genotypic analysis of *Staphylococcus* spp. and Enterobacteria resistant bacteria isolated from Humans and lactating dairy cows from small-holder farms in Gatundu Sub- County, Kenya.

Principal Investigator: Mr. FELIX ODHIAMBO OTENGA

Purpose of the study

In this study we are interested in knowing the prevalence of antibiotic resistant infections in Gatundu Sub-County and if there are some activities that put one at risk. Overuse and misuse of antibiotic drugs both in humans and cattle cause emergence of resistance. Humans acquire these infections from the cattle directly: animals-human contact, by-product handling or indirectly in the food chain.

Study procedures

If you agree to participate in this study a questionnaire that capture details on your demographic data and further questions assessing if you are exposed to any activities that cause emergence of antibiotic resistant bacteria or put you or your cattle (if any) at risk of being infected with antibacterial resistant infections will be given to you. A nasal swab will be taken from two members of your household. This procedure does not intrude your privacy nor induce any pain except if you have wounds in your nasal cavity. However, depending with the depth you might experience some bleeding for a few minutes but this doesn’t pose any serious health issue. The samples will be taken to IPR laboratories for further analysis. The obtained colonies from your sample may be used to prepare a cryobank for future use.

In case of a positive result, you will be contacted and will be advised on how to get treated for the infection.

Confidentiality

All the information obtained will be strictly confidential and data password protected. Only the Principal investigator and the co-investigators will be able to access the data. Participants in the study will be kept anonymous, being identified only by specific unique numbers assigned by the co-investigators.

Benefits

By choosing to participate in this study, you will not have any direct benefits from it other than that of a free test to know your health status. However, the information obtained from the study will be useful to the country in general by giving information on the antibiotic resistant infection status and can be used in formulation of policies and laying appropriate action plans for management of the zoonosis of antibiotic resistance.

Risks

There is a little discomfort in obtaining the nasal swab samples; however, there are no other foreseeable risks that will arise from participating in the study. You will however take about 45min-1hr from your daily routine to go through the study procedures.

In the case that you are found infected you will be contacted confidentially and told how to be treated.

Voluntariness

Taking part in this study is voluntary. You have the right to choose not to take part in this study.

Questions

If you have any questions about this study now or later, you may contact the FELIX ODHIAMBO OTENGA using the following phone number 0723685369/0740105674 or email; felixotenga1@gmail.com

If you have any questions about your rights as a study participant, you can contact the Chairperson of Institutional Research and Ethical Committee (JKUAT).

Co-investigators:

Professor Naomi Maina [JKUAT] - 0727726785

Dr. John Kagira [JKUAT] - 0726731970

Appendix II: Written Consent

I, the undersigned have understood the above information which has been fully explained to me by the investigator. I have agreed to voluntarily consent to participate. I was given the chance to ask questions and I received satisfactory responses.

Signature of participant …… Date …….

I certify that I have followed the study SOP to obtain consent from the participant. She/he has understood the nature and the purpose of the study and consent to their participation in the study. She/he has been given opportunity to ask questions which have been answered satisfactorily.

Signature of Principal Investigator …… Date …….

Thank you for agreeing to participate in the project.

Appendix III: Formed Consent in Swahili

Mada Ya Utafiti

kuchunguza jinsi bakteria *Staphylococcus aureus* na *Enterobacteria* ambao wasioweza kudhibitiwa na antibiotics kwa binadamu na mifugo na kusababisha upinzani wa antibiotics kwenye kaunti ndogo ya Gatundu, Kenya.

Mtafiti: Felix Odhiambo Otenga

Huu utafiti unanuia kuchunguza hali na kiwango cha uwapo wa bakteria wasioweza kudhibitiwa na antibiotics, madhara na magonjwa unaotokana upinzani wa antibiotics. Kutumia kwa antibiotics kwa jinsi isiyofaa kwenye binadamu na mifugo, huchangia uwepo wa bakteria wasioweza kudhibitiwa na antibiotic na magonjwa yasiyoweza kutibiwa kwa urahisi. Binadamu huambukizwa na hivi bakteria kwa kutangamana na mifugo kwa hali ya moja kwa moja na mifugo amabao wameathiriwa au kwa kutumia mazao (maziwa) kutoka kwa mifugo ambao wameathiriwa.

Taratibu

Utafiti huu ni hiari, ukikubali kushiriki maelezo kukuhusu yatanikiliwa kwa njia ya dodoso, na kuchunguza vile vitendo ambavyo vinachangia uwepo wa bakteria vivisivyoweza kidhibitiwa na antibiotics. Sampuli kutoka kwenye pua wa wawili kweneye familia. Hili tendo linaweza sababisha maumivu iwapo tu mshiriki ana vidonda kwenye pua. Wakati mwingine mshiriki anaweza tokwa na damu kwa muda mfupu isioweza na madhara kiafya. Iwapo patatokea aliye na bakteria visivyoweza kudhibitiwa na antibiotic, atashauriwa jinsi ya kupata matibabu.

Siri: Maelezo na majibu kuhusu mshiriki katiaka huu utafiti yatalindwa kutumia password. Mtafiti mkuu na watafiti wenza ndio pekee ambao wanaidhini ya maelezo na majibu ya mshiriki. Mshiriki kwenye dodoso atabanwa ila atatambuliwa kwa nambari za siri (unique) na watafiti pekee.

Faida

Huu utafiti hauna faida ya moja kwa moja ila matokea wa uwepo wa bakteria wasioweza kudhibitiwa na antibiotics yatapokezwa kwa mshiriki nakisha kushuriwa jinsi ya kupata matibabu. Haya matokeo yatasaidia kushauri kwa njia tofauti ya kuzuia kuchipuka kwa bakteria wasioweza kudhibitiwa na antibiotics na njia sahihi ya kutumia antibiotics.

Madhara ya Kushiriki Kwa Utafiti

Utafiti huu hauna madhara yoyote kiafya ila tu wakati wa kuchukua sampuli, mshiriki anaweza kuwa discomfortable. Kuchukua sampuli huenda ukachukua dakika 30-40. Iwapo mshiriki atapatikana na hivi bakteria visivyoweza kudhibitiwa na antibiotic, atashauriwa njia ya siri (confidential) na kushauriwa jinsi ya kupata matibabu.

Hali ya kujitolea kwa utafiti huu

Utafiti huu ni wa kujitolea kwa hiari. Una ruhusa

ya kutoshiriki ama kukataa kujibu swali lolote lile. Ukibadilisha nia yako ya

kushiriki, una ruhusa ya kujiondoa wakati wowote. Iwapo kuna jambo lisiloeleweka,

ama kuhitaji habari zaidi tutakujuza.

Maswali

Iwapo una swali lolote kuhusiana utafitia, mfikie FELIX ODHIAMBO OTENGA kwa njia ya simu ya rununu kwa nambari 0723-685-369 au kupitia barua pepe: felixotenga1@gmail.com.

Iwapo una swali lolote kuhusiana na haki yako kama mshiriki katiaka huu utafiti, mfikie Chairperson of Institutional Research and Ethical Committee (JKUAT).

Watafiti wenza:

Professor Naomi Maina [JKUAT] - 0727726785

Dr. John Kagira [JKUAT] – 0726731970

Appendix IV: Written Consent in Swahili

Uamuzi wa anayejitolea

Nmekubali na kuamua kushiriki kwenye huu utafiti unaokuchunguza jinsi bakteria *Staphylococcus aureus* na *Enterobacteria* ambao wasioweza kudhibitiwa na antibiotics kwa binadamu na mifugo kwenye kaunti ndogo ya Gatundu, Kenya

Nimesoma ujumbe wote kuhusu utafiti huu, nimeelewa lengo lake na wajibu wangu iwapo nitashirikishwa. Nimeelezwa hatari na faida zo zote zile iwapo zipo na maswali yangu yote yamejibiwa. Nakubali kwa hiari yangu kushiriki katika utafiti huu.

Sahihi yamtafiti_____Tarehe_____

Ninahakikisha kuwa nmefuatilia utaratibu unaopaswa kupata hiari ya ushiriki (consent). Mshiriki ameelewa fika namna, desturi na lengo la huu utafiti na amekubali bila kushurutishwa kushiriki kwenye huu utafiti. Amepewa nafasi kikamilifu wa kuuliza maswali kuhusiana na huu utafiti na amepokea majibu sahihi la kuridhisha kuhusiana na huu utafiti.

Sahihi ya Mtafiti mkuu_____Tarehe_____

Asante kwa kushiriki.

Appendix V: Questionnaire Administered to Household Head

Questionnaire number: ……

GPS : longitude__: latitude__

Date:

Unique identifier: _____

Socio-demographic data

1. Village name

1. Ward name

2. What is your Gender?

   Male ☐

   Female ☐

3. What is your age (in years)?

   ≤20years ☐

   21-30 years ☐

   30-40 yeas ☐

40-50 years ☐

>50 years ☐

4. What is your Marriage status?
   Single ☐

   Married ☐

   Widowed☐

   Divorced ☐

   Separated ☐

5. What religion do you ascribe to?
   Christian ☐

   Muslim ☐

   African Traditional religion ☐

Animal health

10. Do you use teat dips; Yes No

11. What is the local name given to mastitis……...

a) What are the clinical signs you observe to rule your cow has mastitis……?

d) Are any teat abnormalities observed during mastitis infection? ….……

e) Are any milk abnormalities observed during mastitis infection? ….……

f) Do you have a protocol for managing the mastitis? Y N

g) Have you experienced a case of mastitis in your herd in the last one month?

Yes No

12. Was an antibiotic used to treat the animal?

Yes No

a) If yes do you remember the name of the antibiotic used?

Yes No

b) If yes, what is name of the antibiotic that was used in the treatment of the mastitis……

Yes No

14. Did the mastitis reoccur for the case that was treated?

Yes No

19. Is the milk pasteurized before it is consumed? Yes No

20. Are the any preferences for raw milk consumption by this household?

Yes No

If yes, reason? .....

Human section

1. . Which one of the following has you or someone in your family suffered from in the past 3months?

Boils	
rashes	
Chest pain	
Burns	
Flu	
stomachache	
Open wounds	
Diarrhea	
Other (specify)	

2. Are there specific members of your household who often suffer from (boils, rashes, burns, flu, chest pain, stomach-ache, burns, open wounds and diarrhea)?
3. What is your first line treatment option for any of the conditions listed above? ***(Please mark one)***

a) Have you or any of your household members used medicine used in the past 6 months?

Yes	
No	

b.) If yes, what type of medicine? ***(Please list all used)***

4. In-cases of (Boils, rashes, burns, flu, chest pain, stomach-ache, diarrhea) among household members are medicine shared among the infected individuals?

Yes	
No	

5. **10.** How many visits to the Health Centre have been made by you or any household member in the past 3months?

Appendix VI: Questionnaire Administered to Household Head in Swahili

Nambari ya dodoso:

GPRS

Maelezo ya watu:

Tarehe:

Nambari ya wanaishi nyumbani:

_____

_____

Jinsia:

Mwanamke	
mme	

Mahali anapotoka:

_____

_____

Miaka:

_____

_____

Dini

mkristo	

Mkristo wa katholoki	
Muislamu	
Dini ya jadi	
Nyingine	

Huduma za afya

Je! Ni ugonjwa wa hivi karibuni ambao mtu aliye katika familia ameambukizwa kutoka? (Majipu, misuli, kuchoma, mafua, maumivu ya kifua, stomachache, kuhara nk?

Ni dawa gani ya hivi karibuni ya antibiotic inayotumiwa nyumbani

_____

_____

Ni mara ngapi wewe hukaribiana na wanyama

Siku nzima	
Mara tano au Zaidi kwa siku	
Hakuna wakati nakaribiana na wanyama	

Animal health

Mbinu ipi ya ufugaji wa wanyama inayofwatwa na boma hii?

Kukuza sifuri	
paddocking	
Wanyama wa kutembea tembea	
nyingine(eleza zaidi)	

Kumekua na mnyama yeyote amepatwa na ugonjwa kwa mda wa miezi mitatu iliyopita?

Ndio	
La	

Je, kuna dawa za kigeni zilizohifadhiwa kwa wanyama wako?

Dawa izi zilipatakana vipi?

_____

_____

Unatumia mbinu zipi kuzuia ugonjwa kwenye ng'ombe wako?

Appendix VII: Ethical Approval Letter

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY
P.O BOX 62000(00200) NAIROBI, Tel:(067) 58700001-4
(Office of the Deputy Vice Chancellor, Research Production and Extension Division)

JKUAT INSTITUTIONAL ETHICS REVIEW COMMITTEE

REF: JKU/2/4/896B **Date: 10th June 2021**

Felix Odhiambo Otenga
Department of Biochemistry, JKUAT.

Dear Mr. Otenga,

RE: GENOTYPIC ANALYSIS OF *STAPHYLOCOCCUS AUREUS* AND *ESCHERICHIA COLI* RESISTANT BACTERIA ISOLATED FROM HUMANS AND CATTLE IN GATUNDU SUB-COUNTY, KENYA

This is to inform you that JKUAT Institutional Ethics Review Committee has reviewed and approved your amendment to change your research study site from Thika Sub-County to Gatundu Sub-County subject to the terms and conditions of your ethics approval number **JKU/IERC/02316/0062** that was issued on 8th February 2021.

This approval is subject to compliance with the following requirements;

i. Only approved documents including (informed consents, study instruments, MTA) will be used
ii. All changes including (amendments, deviations, and violations) are submitted for review and approval by JKUAT IERC.
iii. Death and life threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to JKUAT IERC within 72 hours of notification
iv. Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study participants and others or affect the integrity of the research must be reported to JKUAT IERC within 72 hours
v. Clearance for export of biological specimens must be obtained from relevant institutions.
vi. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report to support the renewal.
vii. Submission of an executive summary report within 90 days upon completion of the study to JKUAT IERC.

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://oris.nacosti.go.ke and also obtain other clearances needed.

Yours sincerely

Dr Patrick Mburugu
Chair, JKUAT IERC

INSTITUTIONAL ETHICS REVIEW COMMITTEE
10 JUN 2021
APPROVED
P.O. Box 62000-00200 NAIROBI
JKUAT

JKUAT is ISO 9001:2015 and ISO 14001:2015 certified
Setting Trends in Higher Education, Research, Innovation and Entrepreneurship

Appendix VIII: Front Page of the Manuscript

International Journal of Community Medicine and Public Health
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Original Research Article

Prevalence, risk factors and antibiogram of bacteria isolated from skin of human having close contact with dairy cows in small-holder farms in Gatundu Sub-County, Kenya

Felix O. Otenga[1], John M. Kagira[2]*, Kenneth Waititu[3], Daniel M. Kiboi[1], Maina Ngotho[4], Naomi Maina[1]

[1]Department of Biochemistry, [2]Department of Animal Sciences, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya
[3]Department of Animal Science, Institute of Primate Research, Nairobi, Kenya
[4]Department of Clinical Studies, University of Nairobi, Nairobi, Nairobi, Kenya

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***Correspondence:**
Dr. John M. Kagira,
E-mail: jkagira@jkuat.ac.ke

ABSTRACT

Background: Antimicrobial resistance is a global health crisis which needs surveillance especially in people having close contact with animals.
Methods: A total of 120 skin swabs from around the neck region were obtained from human respondents and subjected to bacteriological analyses. Antimicrobial susceptibility test was done using Kirby Bauer disk diffusion method and results were interpreted according to the Clinical and Laboratory Standard Institute guidelines.
Results: The results showed that the skin was colonized by *Staphylococcus aureus* (49.4%), coagulase-negative staphylococci (CoNS) (16.9%), *Pantoea* spp. (13%), *Serratia* spp. (13%), *Bukholderia cepacian* (3%), *Enterobacter* spp. (3%), *Yersinia enterocolitica* (1.3%) and *Pasteurella aerogenes* (1.3%). The CoNS were mostly resistant to gentamycin (100%), clindamycin (84.6%), erythromycin (84.6%) and ciprofloxacin 84.6% and sensitive to sulphamethoxazole-trimethoprim (76.9%). For *S. aureus*, most isolates were resistant to gentamycin (86.8%), oxacillin (86.8%) and vancomycin (73.7%) but susceptible to amoxycillin-clavulanic acid (89.5%) amongst other antibiotics. The multiple antibiotic resistance index for *S. aureus* and CoNS was 0.92 and 0.92, respectively. Respondents aged more than 40 years had higher burden of AMR compared to the other respondents ($p = 0.011$, OD=1.745). Similarly, the AMR burden was higher in respondents who had previous history of using medication compared to those who had not ($p=0.025$, OD=0.204).
Conclusions: The study showed a high prevalence of antibiotic resistance in CoNS and *S. aureus* isolates from skin of people having regular contacts with dairy cows in the study area. Interventions strategies to stem the emergence of AMR should be undertaken.

Keywords: Prevalence, Human, Risk-factors, Antibiotics, Antimicrobial resistance, *Staphylococci* spp., *Enterobacteriaceae*

INTRODUCTION

For decades, antibiotics have played a critical role in the treatment and management of bacteria-associated infections in human and animals. However, indiscriminate use of antibiotics in agriculture, veterinary and medical sectors drive the selection-pressure of bacteria and this has led to emergence of antibiotic-