ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

BOVINE MASTITIS IN DAIRY FARMS IN AND AROUND AKAKI KALITY SUB CITY AND SULULTA TOWN: EMPHASIS ON ISOLATION AND IDENTIFICATION OF PATHOGENIC STAPHYLOCOCCUS AND ANTIMICROBIAL SUSCEPTIBILITY TEST OF *STAPHYLOCOCCUS AUREUS*

MVSc THESIS

BY

GETAHUN TESFAYE

JUNE 2019

BISHOFTU, ETHIOPIA

BOVINE MASTITIS IN DAIRY FARMS IN AND AROUND AKAKI KALITY SUB CITY AND SULULTA TOWN: EMPHASIS ON ISOLATION AND IDENTIFICATION OF PATHOGENIC STAPHYLOCOCCUS AND ANTIMICROBIAL SUSCEPTIBILITY TEST OF *STAPHYLOCOCCUS AUREUS*

A Thesis submitted to the College of Veterinary Medicine, Addis Ababa University in partial fulfillment of the requirements for the Degree of Masters of Veterinary Science in Veterinary Microbiology

By Getahun Tesfaye

Department Of Microbiology, Immunology and Veterinary Public Health

MVSc in Veterinary Microbiology

June, 2019

Bishoftu, Ethiopia

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Immunology and Veterinary Public Health

BOVINE MASTITIS IN DAIRY FARMS IN AND AROUND AKAKI KALITY SUB CITY AND SULULTA TOWN: EMPHASIS ON ISOLATION AND IDENTIFICATION OF PATHOGENIC STAPHYLOCOCCUS AND ANTIMICROBIAL SUSCEPTIBILITY TEST OF *STAPHYLOCOCCUS AUREUS*

Submitted by:

Getahun Tesfaye T/Haimanot _____ _____

Name of Student Signature Date

Approved for submittal to dissertation assessment committee

1. HikaWaktole (BSc, MSc, Asst. Prof.)_____ _____

Major advisor Signature Date

1. Dr. Tilaye Demissie (DVM, MSc, PhD fellow, Assoc. Prof.) _____ _____

Co-advisor Signature Date

1. Dr. Getachew Terefe (DVM, PhD, Assoc. Prof.) _____ _____

Co-advisor Signature Date

2. Dr. Gezahegne Mamo (DVM, MSc, PhD, Assoc. Prof. _____ _____

Department chairperson Signature Date

Addis Ababa University College of Veterinary Medicine and Agriculture

Department of Microbiology, Immunology and Veterinary Public Health,

MVSc program in Masters of Science in Veterinary Microbiology

As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by: Getahun Tesfaye entitled "bovine mastitis in dair y farms in and around akaki kality sub city and sululta town: emphasis on isolation and identifica tion of pathogenic staphylococcus and antimicrobial susceptibility test of *staphylococcus aureus* " and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Veterinary Science in Veterinary Microbiology.

1. Fufa Abuna (Assoc. Prof.)	_____	_____
Chairman	Signature	Date
2. Gobena Ameni (Prof.)	_____	_____
External Examiner	Signature	Date
3. Ashwani Kumar (Prof.)	_____	_____
Internal Examiner	Signature	Date
4. Hika Waktole (Assist. Prof.)	_____	_____
Advisor	Signature	Date
5. Tilaye Demissie (Assoc. Prof.)	_____	_____
Co-advisor	Signature	Date
6. Getachew Terefe (Assoc. Prof.)	_____	_____
Co-advisor	Signature	Date
7. Dr. Gezahegne Mamo (Assoc. Prof.)	_____	_____
Department chairperson	Signature	Date

TABLE OF CONTENTS

AUTHOR DECLARATION

This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture. It is my original work and that all sources of material used for this thesis have been duly acknowledged. The thesis will be deposited at the University/College library to be made available to borrowers under rules of the Library.

I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

Name: Getahun Tesfaye Signature_____

College of Veterinary Medicine and Agriculture, Bishoftu

Date of Submission: June 20, 2019

ACKNOWLEDGMENTS

I am grateful to acknowledge Addis Ababa University Research and Technology transfer for funding this research through thematic research project entitled as “Reproductive Health Management and Dairy Technology RD/LT-038/15”.

I gratefully acknowledge my major advisor: Mr. HikaWaktole Addis Ababa University College of Veterinary Medicine and Agriculture for his supervision, comment, suggestion and critical correction of this paper to the final version.

I wish to express my sincere gratitude my co-advisors: Dr. Tilaye Demissie for allowing and facilitating to do my research through thematic research project and for their encouragement, supervision, comment and suggestion and critical correction of this paper to the final version. Moreover, I want to extend my gratitude to my co-advisor Dr. Getachew Terefe.

LIST OF ABBREVIATIONS

BC	Bacteriological Culture
CMT	California Mastitis Test
PCR	Polymerase Chain Reaction
SCC	Somatic Cell Counts
SPP	Species
CSA	Central Stastical Agency
TSB	Tryptose Soya Broth
CNS	Coagulase Negative *Staphylococcus Aureus*
CPS	Coagulase positive *Staphylococcus Aureus*
PAB	Purple Agar Base
O-F	Oxidation Fermentation
MSA	Mannitol Salt Agar

LIST OF TABLES

LIST OF FIGURES

LIST OF ANNEXES

ABSTRACT

A cross sectional study was conducted from November 2017 to May 2018 in and around Akaki Kality Sub-city of Addis Ababa and Sululta Town with major emphasis of isolation and identification of pathogenic Staphylococcus species and determination of antimicrobial susceptibility pattern of *Staphylococcus aureus* isolates. Besides the prevalence of clinical and sub-clinical mastitis and associated risk factors were investigated. A total of 768 lactating cows were examined by physical examinations of udder and by California mastitis test. Milk from clinical and sub clinical cases were cultured to isolate pathogenic *Staphylococcus* species. Antimicrobial susceptibility pattern of *Staphylococcus aureus* isolates was done by Kirby-Bauer disk diffusion method using eight antimicrobials.The overall prevalence of mastitis was 58.5%, out of which 10.8% was clinical and 47.7% subclinical mastitis.When the prevalence of study sites were considered separately it was 64.6% in Akaki Kality Sub-city, and 52.3% in Sululta Town of which clinical and sub-clinical mastitis were respectively 13.3% and 8.3% in Akaki Kality Sub-city and 51.3% and 44.0% in Sululta Town. In both study sites, univariable logistic regression analysis indicated bovine mastitis was more likely to occur with increasing age of cow, presence of teat lesion, poor housing hygiene and udder/teats hygiene. Based on primary and secondary biochemical characterizations and pathogenicity tests, pathogenic *staphylococci* were isolated in 25.4% at Akaki Kality Sub-city of which 18.5% was *S. aureus*, 4.03% S. *intermidious* and 2.8% *S. hyicus*. at Sululta, the proportions of pathogenic *staphylococci* were 20.9% of which *S. aureus* was 16.9%, *S. intermedious* was 2.5% and *S. hyicus* was 1.5%. A total of 68 *Staphylococcus aureus* isolates (34 of each study area) were assessed for antimicrobial susceptibility. *Staphylococcus aureus* from both study sites were resistant to ampicillin and penicillin. However, susceptibility to gentamicin was (91.2% and 85.3%), oxacillin (85.3 and 82.35%), cefoxitin (67.6% and 85.3%), erythromycin (70.6% and 94.11%), and vancomycin (82.35% and 91.2%) in Akaki Kality and Sululta Town, respectively. It could be concluded that prevalence of bovine mastitis in general, and isolation of pathogenic *Staphylococcus* species in particular was high in the study sites. The *Staphylococcus aureus* that were found to be totally resistant to ampicillin, and penicillin might be due to repeatedly use of these drugs.

Keywords: *Akaki Kality, Antimicrobial susceptibility, Bovine Mastitis, Sululta, Pathogenic Staphylococcus*

1. INTRODUCTION

Mastitis is an inflammation of the mammary gland caused by a variety of microorganisms, mostly bacterial that gain access to the interior of the udder through the teat canal. It is characterized by physical, chemical and bacteriological changes in the milk, and pathological changes in the mammary glandular tissue (Quinn *et al*., 2002).

Mastitis is caused by a wide variety of bacteria, and can be classified as contagious and environmental mastitis. Contagious pathogens are those for which udders of infected cows serve as the major reservoir. They spread from cow to cow, primarily during milking, and tend to result in chronic subclinical infections with flare-ups of clinical episodes. Contagiou s pathogens include: *Staphylococcus aureus* (*S. aureus*), *Streptococcus agalactiae* (*S. agala ctie*) *Mycoplasma spp.* and *Corynebacterium bovis* (*C. bovis*) (Radostits *et al*., 2007). On th e other hand, environmental mastitis can be defined broadly as those intramammary infections caused by pathogens whose primary reservoir is the environment in which the co w lives (Bradley *et al*., 2002). Environmental pathogens include *Escherichia coli, Klebsiell a spp, Streptococcus dysgalactiae* and *Streptococcus uberis* and the majority of infections caused by these pathogens are clinical and of short duration (Quinn *et al*., 2002).

Mastitis occurs mainly in two main forms clinical and subclinical mastitis forms. Clinical mastitis is easily visible and diagnosed by characteristic signs on affected udder/quarter, associated changes in milk composition and by systemic signs on the animal (Gebremichael *et al*., 2013; Duguma *et al*., 2014; Zenebe *et al*., 2014). However, the subclinical mastitis is difficult to be diagnosed visually but characterized mainly by reduction of milk production and alteration of milk constitutes (Gebremichael *et al*., 2013).

Mastitis is generally considered the most costly disease of dairy cows. It reduces the quality and quantity of milk and manufactured milk products and increase somatic cell count (Ababe *et al*., 2016). Its financial loss is a result of permanent loss of production due to

secretor tissue being replaced by fibrous tissue, discarded milk following antibiotic therapy, early culling of cows, veterinary costs, drug costs, increase labor, death of per acute cases, replacement costs and high leukocyte count that lead to a loss of income (Sharif and Muhammed, 2009).

Staphylococcus species are common causes of bovine mastitis in dairy herds. Pathogenic strains are usually coagulase-positive (Gorden *et al*., 2008) and have been found to cause disease in wide range of hosts including human worldwide. Especially the pathogenic strain of *S. aureus* is the most important pathogen among *staphylococci* species related to subclinical intramammary infections in dairy cows leading to severe economic losses in dairy industry worldwide (Godden *et al.*, 2002). Likewise these groups of bacteria are also known for wide transmission to human through contaminated milk of mastitic cows. Increased resistance of *Staphylococcus* species to several antimicrobial agents has been reported (Gentilini *et al*., 2000).

The wide nature of *Staphylococcus* species involvement in bovine mastitis, their zoonotic importance and development of resistance to many antibiotics warrants attention for proper therapy and as well monitoring of the spread of resistant strains throughout the populations. ß-lactam antibiotics are the most frequently used in intramammary infusion therapy. Bacterial resistance mechanisms to this class of antibiotics include production of ß-lactamase and low-affinity penicillin-binding protein 2a (PBP 2a) determined by the presence of the chromosomal gene *mec*A. The latter, designated for Methicillin resistance, precludes therapy with any of the currently available ß-lactam antibiotics, and may predict resistance to several classes of antibiotics (Moon *et al*., 2007).The emergence of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in dairy farms is significant and costly public health concern.

The prevalence of *Staphylococcus* of bovine mastitis in Ethiopia has been studied and found to be 35.8% in Adama (Mekonen and Tesfaye, 2010), 77.1% in Holeta (Mekibib *et al*., 2010), 29.2% in Borena (Bedane *et al*., 2012) and 28.7% in Addis Ababa (Zeryhun *et al*., 2013).But, there was no report from the present study site. Also no study was conducted

to isolate and identify pathogenic *Staphylococcus* species from mastitis and to determine its antimicrobial susceptibility profile.

The control of mastitis in Ethiopia is largely accomplished with the aid of antibiotic; and this indiscriminate uses of drugs may have potential effect on the development of resistant bacteria. This condition would be more dangerous when the zoonotic pathogen like *Staphylococcus* may develop drug resistance. Regular scrutinizing of the degree of the problem of mastitis in dairy cows, assessments of associated risk factors in relation to the type of mastitis, isolation and identification of pathogenic *Staphylococcus aureus* from bovine mastitis and testing for antimicrobial susceptibility would benefit the dairy industry and protect the population if incorporated into mastitis control and prevention program.

Based on the aforementioned background and justification the following objectives were designed:

- To estimate the prevalence of pathogenic *Staphylococcus* species from mastitis cases,
- To estimate the prevalence of *Staphylococcus aureus* from mastitis cases
- To estimate antimicrobial sensitivity profile of *Staphylococcus aureus* isolates, and
- To estimate the prevalence of bovine mastitis investigate factors that may affect its prevalence.

2. LITERATURE REVIEW

2.1. Major Mastitis Causing Bacterial Pathogens

The major pathogens can be further subdivided into contagious and environmental infectious agents (Radostits *et al*., 1994). Contagious pathogens are those for which udders of infected cows serve as the major reservoir. They spread from cow to cow, primarily during milking, and tend to result in chronic sub-clinical infections with flare-ups of clinical episodes. Contagious pathogens include: *S. aureus, S. agalactiae, Mycoplasma spp*. and *C. bovis* (Radostits *et al*., 2007).

Environmental mastitis can be defined broadly as those intramammary infections caused by pathogens whose primary reservoir is the environment in which the cow lives (Bradley *et al*., 2002). Environmental pathogens include *E. coli, Klebsiella spp., S. dysgalactiae* and *S. uberis* and the majority of infections caused by these pathogens are clinical and of short duration (Quinn *et al*., 2002).

2.1.1. S. aureus

One of the most common types of mastitis is caused by Gram positive bacteria and a major cause of economic losses to dairy industry. Often, it is subclinical, where there is neither abnormal milk nor detectable change in the udder, but somatic cell count has increased. Some cows may flare-up with clinical mastitis, especially after calving. The bacteria persist in mammary glands, teat canals, and teat lesions of infected cows and are considered contagious (Tollersrud *et al*., 2000). The infection is spread at milking time, when *S. aureus* contaminated milk from infected cows comes into contact with teats of uninfected cows, and the bacteria penetrate the teat canal. Once established, *S. aureus* usually does not respond to antibiotic treatment, and infected cows eventually must be segregated or culled from the herd. In some herds with somatic cell counts (SCC) below 200,000, dairy

managers have not been able to eradicate *S. aureus*, even when they practiced standard milking time hygiene techniques (Quinn *et al*., 2002).

It causes per acute, acute, and chronic mastitis. The chronic subclinical form is the predominant form. It produces many enzymes/toxins (catalase, coagulase), it is highly invasive (produces hyaluronidase which allows it to invade tissues), is often can resist phagocytosis (it has Protein A on its surface), it resists the immune system (produces teichuronic acid), it is a facultative intracellular pathogen (alive inside phagocytic cells). *S. aureus* can survive to a limited degree in the environment.(Ahmed *et al*.,, 2005).

Clinically infected quarters often show moderate swelling and visible signs of chunks of milk, especially in fore stripping. Acute *S. aureus* infections generally develop late in the lactation or just prior to calving. However, the clinical symptoms (udder swelling or hardness, changes in appearance of milk) do not show up until calving or early in the next lactation. It becomes difficult to successfully treat an infection because drugs are not able to penetrate to all infection sites and because the bacteria live inside the white blood cells. *S. aureus* produces an enzyme that inactivates most penicillin-based treatments resulting in ineffective antibiotics (Ahmed *et al*., 2005).

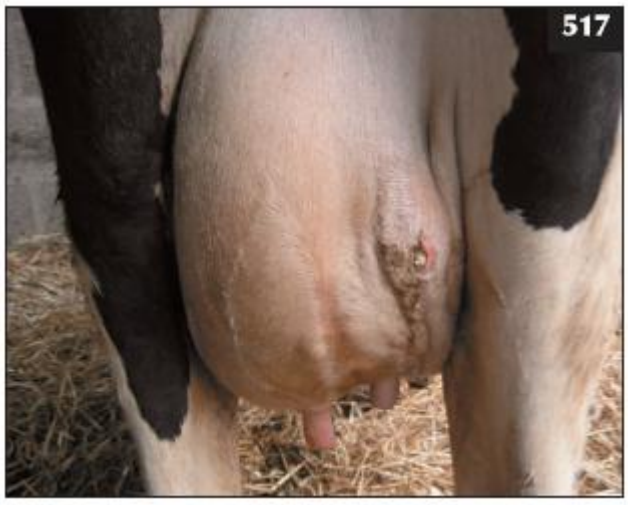


Figure 1: The cow with *S. aureus* infection showing deep abscess formation and a discharging lesion (Source: Scott *et al*., 2011)

2.1.2. Mycoplasma species

Different species of *mycoplasmas* have been shown to cause udder infections in dairy cows with *Mycoplasma bovis* being responsible for most of the mastitis outbreaks. Characteristics of this disease are its highly infectious and contagious nature, complex clinical presentation, and extreme resistance to treatment (Jasper *et al.,* 1987). Mastitis caused by *Mycoplasma* species has highly variable signs ranging from mild clinical cases to complete loss of milk production. The two characteristics of most *Mycoplasma* mastitis infections of individually affected cows are failure to respond to therapy, and spread of the infection from one quarter of the udder to another. Milk transfer during milking is the most common way of *Mycoplasma* mastitis spread between cows (Bayouni *et al*., 1988).

The udder is hard with enlarged mammary lymph nodes. The mammary secretions vary from watery with sandy material present to thick colstrum-like material (Scott *et al.,* 2011).

Figure 2: A watery secretion containing sand-like material from a cow with *mycoplasmal* mastitis (Source: Scott *et al*., 2011)

2.1.3. Streptococcus species

There are three major *streptococci* causing mastitis*; S. agalactiae, S. dysgalactiae* and *S. uberis* (Carrillo-Casas and Miranda-Morales, 2012). *S. agalactiae* causes contagious mastitis, an obligated pathogen of the mammary gland which is transmitted directly among cows during milking (NMC, 1999). *S. agalactiae* infects the gland cistern and ducts of the mammary gland causing irritation, swelling and subclinical mastitis. The infected cow shows mere clinical signs without abnormalities drawn in milk (Hillerton and Berry, 2003).

Streptococcus dysgalactiae is also frequent among the causative agents of clinical and subclinical mastitis. Although *S. dysgalactiae* is conventionally described as a contagious p athogen, it survives well in the environment and thus has some of the properties of an environmental pathogen. It is commonly found on the teat skin (as opposed to the udder), especially if the skin is damaged. It is present in the tonsils and can be transmitted by licking, especially in heifers. It commonly infects dry cows, prepartum heifers and even calves, and is involved in cases of summer mastitis (Rato *et al*., 2011).

S. uberis is widespread in the environment, especially in straw yards, which may contain up to 10^6 bacteria per gram of straw bedding. It is also widespread on the skin of the cow, but relatively rare in faeces when compared with *E. coli*. Outbreaks can occur in cows at pasture, especially in late summer, presumably by transmission from the skin of the cow, via the lying area, to the teat. The clinical signs of *S. uberis* infection vary from subclinical infections to acute severe clinical mastitis with a hard, hot, swollen, painful quarter(s), pyrexia and systemic illness in the cow (Scott *et al*., 2011).

2.1.4. Corynebacterium bovis

This bacterium was thought to be a teat end commensal and thus present as a contaminant in milk samples. However, it has been associated with subclinical mastitis and high SCCs, especially in relation to poor post-milking teat disinfection (Scott *et al*., 2011).

2.1.5. Escherichia coli and Klebsiella pneumoniae

E. coli and *Klebsiella spp.* are Gram-negative bacteria that often cause severe acute clinical mastitis although development of mild and moderate clinical mastitis is also common (Oliveira *et al*., 2013), and subclinical infections can also occur (Bhatt *et al*., 2012). Fecal contamination is the source of infection for *E. coli*. Relaxation of the teat sphincter following milking increases the entrance of the organisms into the teat canal vulnerability to *E. coli* infection. Cows with low somatic cell count are particularly susceptible to infection. The acute form of the disease is characterized by endotoxemia and can be life threatening. Affected animals are severely depressed with drooping ears and sunken eyes. Mammary secretions are watery and contain white flecks (Quinn *et al*., 2002). *K. pneumoni ae* is associated with damp sawdust bedding causing per acute severe coliform mastitis (Scott *et al*., 2011).

2.2. Pathogenesis of Mastitis

Mastitis begins as a results of penetration of pathogenic bacteria pass through the teat duct of a mammary quarter, multiply in the teat and gland cisterns, and progress dorsally to the milk-producing tissues (Akers and Nickerson, 2011). After entry through the teat canal the bacteria enter into the udder tissue, multiply and produce toxins causing inflammation of the udder or the corresponding teat. Due to inflammation, the body releases leucocytes and the quality of the milk gets affected. The milk becomes watery or curdled; sometimes blood streaks may also be present depending on the severity of infection. Infection of the udder usually takes place directly through teat canal. But, organisms may get settled in the mammary tissues via blood. Broadly, two stages have been described viz. invasive stage and infective stage. In invasive stage the organism gets entry from the exterior to the teat canal and milk. Infective stage denotes the stage of bacterial multiplication and their resultant damaging effect on the mammary tissues (Sol *et al*., 2000).

Colonization of the bovine mammary gland by these pathogenic agents result in a series of events which lead to major alterations in the composition of milk secreted from the tissue cells. Initially, elevation in the levels of pathogenic bacteria occur but this is closely followed by marked increase in number of somatic cells. Somatic cells are of two types, sloughed epithelial cells from udder and leukocytes like lymphocytes, neutrophils, monocytes, eosinophils and basophils. During mammary gland inflammation, epithelial cells decrease from 70% to 35%, whereas leukocytes may increase up to 80% or more in milk. High leukocyte count in milk means either the udder is injured or it is infected (Singh *et al*., 2006).

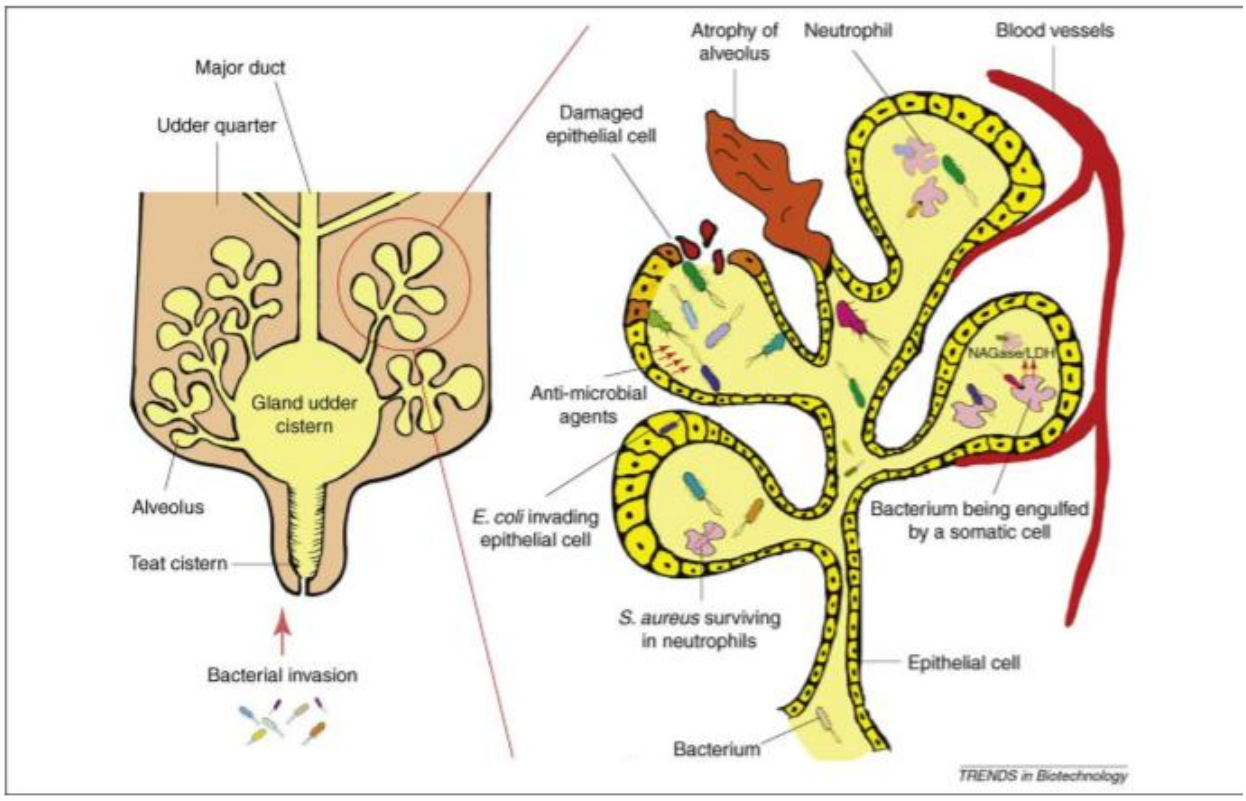


Figure 3: Schematic representation of mastitis development in an infected udder.

(Source: Viguier *et al*., 2009)

2.3. Diagnostic Techniques

Monitoring udder health performance is impossible without reliable and affordable diagnostic procedures. There are wide ranges of diagnostic procedures for mastitis with different principles of actions, where some of them are based on detection of abnormalities of the udder and milk, and inflammatory markers. These procedures include physical and clinical examination of the udder, SCC, CMT, Electrical conductivity test, pH meter, white side test. The other type of diagnosis is more specific and based mainly on isolation and identification of the causative pathogen of mastitis or the immune response such as BC of milk, biochemical tests, and PCR.

Each diagnostic technique has its own advantages and disadvantages and its performance is dependent on many factors some of which are related to the procedures of sample collection, type, preservation and handling in the laboratory, others include the degree of infection and status of infected udder and infected cow, type of causative pathogen and its virulence and finally those related to the experience of the investigator (Mahmmod, 2013).

2.3.1. Clinical Diagnosis

Mastitis may lead to clinical symptoms and, as a consequence, it is often diagnosed directly by visual assessment of udder inflammation or by changes in milk's organoleptic properties (Quinn *et al*., 2002). Milk from healthy, uninfected mammary glands has a white to white-yellow appearance and is free of flakes, clots, or other gross alterations in appearance. Such abnormalities are indicators of milk that is unsuitable for human consumption. The presence of flakes, clots, or other gross alterations in appearance of quarter milk is evidence of clinical mastitis and is by definition, abnormal milk (NMC, 2004; Lechero, 2010). Clinical syndromes are based upon the severity of the inflammatory response. Symptoms include redness, swelling, heat, pain, and loss of function including decreased production,

change in composition, and change in appearance. The clinical syndromes include per acute, acute, sub acute, and chronic (Lechero, 2010).

2.3.2. CMT

The CMT is a cow-side test, so the results are available immediately (milk sample does not have to be sent to a laboratory to obtain the somatic cell count value). For 50 years the CMT has been the only reliable cow-side screening test for subclinical mastitis. Although it does not identify the type of bacteria that cause mastitis, the CMT is useful in identifying quarters that have high SCC. The degree of reaction between a reagent and the DNA of cell nuclei indicates the number of somatic cells in a milk sample, however, the relationship between SCC values and CMT is not precise because of the high degree of variability in SCC values within each CMT score (Argaw, 2016).

The test is very simple, can be performed at milking time, gives instant results and is economical. It is carried out as screening test for sub-clinical mastitis and for selection of samples for culture. A squirt of milk, about 2 ml from each quarter will be placed in each of four shallow cups in the CMT paddle. An equal amount of commercial reagent will be added to each cup. A gentle circular motion will be applied to the mixtures, in horizontal plane for 5 seconds. The reaction will be interpreted based on the thickness of the gel formed by CMT reagent and milk mixture, and the test result will be scored as negative (0), trace (T), + (weak positive), ++ (distinctive positive) and +++ (strong positive) according to Sadashiv and Kaliwal (2014).

Quarters with CMT score of (+) or above is judged as positive. Cows are considered positive when at least one of the quarters becomes positive for CMT and a herd is considered positive, when at least one cow in the herd tested positive with CMT. The CMT gives an indirect estimate of SCC because it is based upon a gelling reaction between the nucleic acid of the cells and a detergent reagent. The CMT is first choice of diagnosis in several investigations because it is more perfect, efficient and reliable than other field and chemical tests for diagnosis of subclinical mastitis (Radostits *et al*., 2007).

Table 1: Interpretations of CMT test

No.	Reaction	Degree of test	Inference (Cells/ml)
1.	No precipitate or colour change	-ve (Negative)	< 200000
2.	Slight precipitate which appears on continuous movement	T (Trace)	150000-500000
3.	A distinct precipitate without gel formation having slight purplish colour	1^+(Weak positive)	400000 – 1500000
4.	Viscosity of milk increased with moderate gel formation	2^+ (positive)	800000 – 5000000
5.	Immediately thick gel formation, sticking at the bottom mostly centralized at mid-center of the cup	3^+ (strong positive)	>5000000
6.	Yellow color of milk (PH around 5.2, which is rarely seen)	Yellow (Acid milk)	Fermentation of lactose
7.	When the mixture is distinctly purple as indicated by a contrasting deep purple colour	+ (Alkaline milk)	Depression of secretory activity

Source: (Singh *et al*., 2006)

2.3.3. pH Determination

The pH of normal milk may vary between 6.5 and 6.8. In mastitis as lactose production decreases and alkaline salts from the blood enter the milk, the milk becomes more alkaline. Thus increasing alkalinity of the milk is characteristics of a progressive mastitis condition. Mastitic milk when drawn from the teat may on rare occasion be acidic and is yellow with Bromocresol purple. The pH should be determined on freshly drawn milk although milk held at refrigerator temperatures for 24 to 28 hours may be used (Coles, 1986).

2.3.4. Electrical Conductivity

Mastitis milk has a higher electrical conductivity than normal milk. This is due to tissue damage and the subsequent increase in sodium and chloride ions in milk**.** The changes in electrical conductivity are one of the earliest manifestations associated with new infections making the early detection and recording of possible mastitis in routine cases. Hand held conductivity meters are also available and may be useful for routine pre milking screening of animals (Savasan, 2002).

Conductivity is measured in siemens (S) and is calculated by dividing amphere by voltage. The electrical conductivity of normal milk ranges between 4.0 – 5.5 ms/cm. and the test can be used effectively for detection of sub-clinical mastitis (Singh *et al*., 2006).

2.3.5. Somatic cell count

Once bacteria reach the teat canal and progress to milk producing tissue of the udder, the cow's body reacts by sending larger numbers of white blood cells or somatic cells in to the mammary gland. This local population of somatic cells includes neutrophils, macrophages and lymphocytes which serve as one of the most important defense mechanisms that the cow has to fight infection. The somatic cell count of normal milk is usually less than 200,000 per milliliter. During inflammation, the somatic cell count of the lactating gland increase to millions per milliliter (Nelson and Stephen, 1991).

Somatic cell count is recognized as useful procedure to assess milk quality and udder health (Phillips, 1996). The common somatic cell counting methods are electronic SCC of suspicious milk by using coulter milk cell counter, which counts particles as they flow through an electric field, and the somatic milk cell counter, which stains cells with a fluorescent dye and then counts the number of fluorescing particles. In direct microscopy leukocytes can be counted directly. A known volume of milk amounting 0.01 ml is spread

over a microscopic slide, defatted and stained by methyl blue based stain after counting 50 fields then the number of leucocytes/ml of milk can be calculated (Quinn *et al*., 2002).

SCC in milk is an important indicator of the health status of the udder and remains the most widely used tool to diagnose subclinical mastitis (Nickerson, 2009). Most studies suggest that cows with SCC of less than 200 000 cells/ml are not likely to be infected with major mastitis pathogens, while cows with SCC of 300 000 cells/ml or greater are very likely to be infected (NMC, 1997).

2.3.6. White side test

The principle of this test is based on the increased number of leukocyte in mastitic milk. In this test, 4-5 drops of test milk sample are placed on a clean dry glass slide. To this, add a drop of 4% sodium hydroxide and mix with a glass rod. If the milk is from animal having mastitis, it becomes thickened and flakes appear. While the negative milk sample remain the same (no change after the addition of the test reagent (Chauhan and Agarwal, 2006).

2.3.7. Bacteriological culturing

Bacteriological culturing can be executed at herd, as well as cow and quarter level, each with its own specific goal. Bacteriological culturing is most often used as a diagnostic tool to solve mastitis problems (Lam, *et al*., 2009). The diagnostic test for identifying pathogens in milk is bacterial culture with the presence of bacteria indicated by their growth on an appropriate growth medium following a period of incubation (Hogen *et al*., 1999). Bacterial species are identified based on phenotypic characterstics including colony morphology, serotyping and analysis of enzymatic profiles (Oliver *et al*., 2004).

Bacterial culture is currently regarded as the gold standard for identifying mastitis pathogens (Hogan *et al*., 1999). To effectively use bacteriological culturing as a diagnostic

tool, milk samples have to be collected from the correct cows and quarters at the correct point in time (Lam *et al*., 2009). Proper collection of milk samples is of paramount importance for identification of mastitis pathogens. Aseptic technique is an absolute necessity when collecting milk samples to prevent contamination by organisms found on the cows' skin, udder, and teats; hands of the sampler; and in the barn environment. Contaminated samples result in misdiagnosis, increased work and expense, confusion, and frustration (NMC, 2004).

2.3.8. Biochemical tests

Once the bacteria have been identified to a genus level, further tests can be carried out to identify the species. Pure culture of a single colony type from blood agar will be transferred to nutrient agar slants from which a series of biochemical tests which aid final identification of various pathogens can be done following standard methods (Quinn *et al*., 2002).

2.3.9. Molecular methods

These methods use PCR technology. Testing the presence of a specific bacterial species, a part of the DNA of that pathogen is amplified and subsequently visualized. For a number of mastitis pathogens, PCR-based techniques have been described (Hassan *et al*., 2001). These methods are currently very labour-intensive and it is expensive to do a separate PCR test for every possible mastitis pathogen. For that reason, multiplex PCR tests are of interest in which several pathogens can be tested at the same time (Phuektes *et al*., 2003). Additionally, real-time PCR assays are being developed for detection and quantifying mastitis pathogens in milk. Molecular methods can also be used to differentiate bacterial strains within one bacterial species.

Multiplex PCR: The multiplex PCR assay could be used as an alternative method in routine diagnosis for rapid, sensitive, and specific simultaneous detection of *S. aureus, S. uberis, S. agalactiae, and C. bovis* in milk samples (Phuektes *et al*., 2003).

Real-time PCR: Real-time PCR based assay is an alternative to *in vitro* for detecting bacterial pathogens in milk sample (Taponen *et al*., 2009). Detection of *S. aureus, S. uberis, S. agalactiae, C. bovis,* and *E. coli* from mastitis milk samples using real-time PCR is common. However, the presence of costly instruments and consumables make it difficult to afford particularly in developing countries (Koskinen *et al*., 2009).

2.4. Prevention and Control of Mastitis

The control of mastitis has been successfully achieved through the establishment of effective herd health control programs (Erskine, 2003). Early diagnosis of mastitis with reliable tests facilitates successful treatment and control. The main control principles include: sound husbandry practices and sanitation, post milking teat dip, treatment of mastitis during non-lactating period, and culling of chronically infected animals (Sharif and Muhammed, 2009). Successful control of contagious mastitis pathogens is focused on reducing exposure of teats to pathogens found in milk that originated from infected cows. Control of environmental mastitis can be achieved by reducing the number of bacteria to which teat is exposed, increasing immune resistance of the cow, premilking teat dipping with a germicidal (NAAS, 2013). Animal environment should be as clean and dry as possible. Antimicrobials are routinely used for treatment of dairy cattle affected with clinical and subclinical infections (Aarestrup, 2005).

Proper ventilation and good sanitation at the farm building is necessary to decrease the exposure of pathogens to the mammary gland (Ondiek *et al*., 2013). The milker's hand should be properly washed, dried and cleaned so that chances of spread of disease can be minimized. All milking utensils should also be clean and dry. Dry bedding should be

provided. The dung and urine should be removed immediately as these are constant source of infections at the farm (Sharif and Muhammed, 2009).

The National Mastitis Council of USA and Canada recently expanded the five-point plan to a ten-point plan with 73 sub-points. The ten points are: (a) establishment of goals for udder health; (b) maintenance of a clean, dry and comfortable environment; (c) proper milking procedures; (d) proper maintenance and use of milking equipment; (e) good record keeping; (f) appropriate management of clinical mastitis during lactation; (g) effective dry cow management; (h) maintenance of biosecurity for contagious pathogens and culling of incurable and chronically infected cows; (i) regular monitoring of udder health status; and (j) periodic review of the mastitis control program (NAAS, 2013).

Dry cow treatment, milking technique, post-milking teat dipping and antimicrobial treatment of clinical mastitis are examples of management factors that have a significant effect on the reduction of mastitis cases and bulk tank milk SCC (Lehtolainen *et al*., 2003).

Other general practices to prevent contagious and environmental mastitis include the milking of infected animals last and preventing the animals from lying down after milking. This can be accomplished by feeding them immediately after milking to insure that they are standing for at least 30 minutes. This should allow enough time for the proper closure of the teat orifice (Tomita and Hart, 2001).

3. MATERIALS AND METHODS

3. 1. Study Areas

The study was conducted in selected dairy farms in and around Akaki kaliti sub-city of Addis Ababa administrative Region and Sululta Town of Oromia Region. Akaki Kality is one of the 10 Sub-cities of Addis Ababa. It is the southernmost suburb of the City and borders with the districts of Nifas Silk-Lafto and Bole. It is situated at latitude of 9º3′ North and 38º43′ longitude East. It lies at an altitude of 2500 meters above sea level. It has an average rainfall of 1800 mm per annum. The annual average maximum and minimum temperatures are 26ºC and 11ºC, respectively; with an overall average of 18.7ºC. A highest temperature is recorded in May. The main rainy season extends from June to September (NMSA, 2015).

Sululta is situated between 09º17′84″North and 38º75′79″East latitude and longitude, respectively. It is located at a distance of 23 km Northwest of the capital Addis Ababa. It lies at an altitude of 2505 meters above sea level. The Town takes the lion's share in terms of its milk production potential and contribution to Addis Ababa milk market. The annual average maximum and minimum temperatures are 25ºC and 10ºC, respectively. The annual average maximum and minimum rainfall are1500 and 800mm, respectively(CSA, 2015).

3. 2. Study Design and Animals

A cross sectional study was conducted from November 2017 to May 2018. The study populations are lactating cows of two breeds of cattle (Holstein Friesian (HF) and Cross breed (HF x Local) in selected dairy farms in the study area .

3. 4. Sample Size Determination and Sampling Methods

The sample size was calculated according the formula given by Thrusfield (2007) using 74.7% previous prevalence of clinical and subclinical mastitis (overall) in the same area by Zeryhun *et al*. (2013), 5% absolute precision and 95% confidence level. Based on this 291 cows were expected to be sampled. However, to increase the precision in this study 384 lactating dairy cows were sampled from each study site. Thus, a total of 768 lactating dairy cows were sampled during the study period.

$$n = \frac{1.96^2 pexp\ (1—pexp)}{d^2}$$

Where n= required sample size, Pexp = expected prevalence d = desired absolute precision and 1.96 is multiplier of 95% CI.

Dairy farms were purposively selected based on willingness of the owners to cooperate with the study. Once dairy farms were identified, simple random sampling technique was applied for the selection of individual lactating cows in the farms by applying a lottery system. The herd size included varied from 5 to 40 lactating cows in Akaki Kality and from 12 to 100 lactating cows in Sululta. Thirty five dairy farms were included from Akaki Kality and 26 dairy farms from Sululta based on availability. From each site 384 lactating cows were sampled.

3. 5. Study Methodology

3. 5. 1. Questionnaire survey

Data on potential risk factors for the occurrences of mastitis was collected by using structured questionnaire and by observation. Data were collected on age, breed, and parity, stage of lactation, udder/teats hygiene, and presence of teat lesion. The herd level data include housing hygiene, floor type, washing of milker's hand, udder/teat hygiene, usage of towel and milking of mastitic cows at last.

3. 5. 2. Clinical inspection of the udder and milk

The udder of all selected lactating cows was thoroughly examined for any abnormalities of secretions, abnormalities of size, consistency and temperature. The gland was palpated for any hardening and the teats for evaluation of teat canal potency. Pain reaction upon palpation, changes in the milk (blood tinged milk, watery secretions, clots, pus), and change in consistency of udder was considered as indications of the presence of clinical mastitis.

3. 5. 3. California mastitis test (CMT)

The California mastitis test was carried out as described by Quinn *et al*. (2002). A squirt of milk, about 2 ml from each quarter was placed in each of shallow cups in the CMT paddle. An equal amount of the commercial CMT reagent was added to each cup. A gentle circular motion was applied to the mixtures in a horizontal plane for 15 seconds. The test result was interpreted based on the thickness of gel formed by CMT reagent and milk mixture and scored as 0(negative), T (trace), 1(weak positive), 2(distinct positive) and 3(strong positive). Milk samples for test result of CMT 1, 2 and 3 were classified as evidence of subclinical mastitis (Quinn *et al*., 2002; Radostits *et al*., 2007).

3. 5. 4. Milk sample collection

Milk sample was collected both from clinical and subclinical mastitis separately. The udder of the animal was thoroughly cleaned with water (Chauhan and Agarwal, 2006). The teat orifice was also cleaned using cotton soaked in 70% ethyl alcohol (Quinn *et al*., 2002). After discarding a few streams of milk, by holding the sterile collection bottle nearly horizontal, about 5 ml milk was collected according to the procedures recommended by national mastitis council (NMC, 1990). The milk samples were pooled together from all the 4 quarters in case all are involved. Then the samples were labeled based on temporary ID given to a cow, kept in an ice box and transported to Addis Ababa University College of Veterinary Medicine Microbiology laboratory, Bishoftu.

3. 5. 5. Bacteriological isolation and identification

Milk samples from both clinical and subclinical quarters were bacteriologically examined according to the procedures employed by NMC (1990) and Quinn *et al.* (2002). A loopful of milk sample collected from each infected quarter was inoculated on blood agar base (Oxoid, UK) enriched with 7% defibrinated sheep blood. In case of refrigerated milk samples, as bacteria might be concentrated in the cream layer and held within clumps of fat globules, dispersion of fat and bacteria was accomplished by warming the samples at 25 °C for 15 min before plating on blood agar. The inoculated plates were then incubated aerobically at 37 °C for 24 to 48 hours. When growth was not observed after incubation for 24 to 48 h, the milk sample was reinoculated on an enriched tryptone soya broth (Oxoid, Ha mpshire, England) to amplify the bacterial growth. The plates were examined for growth, morphology and haemolytic characteristics on blood agar. Presumptive colonies were selected and subcultured on nutrient agar (Oxoid, Hampshire, England) and incubated aerobically at 37 °C for 24–48 h to get a pure culture. Final identification of *Staphylococcus* species was done based on Gram reaction (Gram-positive), cellular morphology (coccus) and arrangements of the bacteria, catalase test, O-F glucose test, growth characteristics on Mannitol salt agar, tube coagulase tests (by using rabbit plasma) and purple agar.

Colonies that were identified as *Staphylococcus* by Gram-staining reaction, oxidation fermentation (O-F) test and catalase test were streaked on mannitol salt agar (MSA) plates and incubated at 37^0C and examined after 24-48 hours for growth and change in the colour of the medium. The presence of growth and change of pH in the media (red to yellow colour) were regarded as confirmative identification of *staphylococci*. Phenol red pH indicator detected the acidic metabolic product of mannitol. Fermentation of mannitol by *S. aureus* causes yellow discolouration of the medium. Colonies that develop weak or delayed yellow colour after 24 hours of incubation were taken as *S. intermedius* and colonies that failed to produce any change on the medium were considered as *S. hyicus* and CNS (Quinn *et al*., 2002).

To identify the pathogenic *Staphylococcus* from non -pathogenic coagulase test was used. The tube coagulase test was performed in sterile tubes by adding 0.5 ml of selected isolates of *Staphylococcus* grown on tryptone soya broth (TSB) at 37^0C for 24 hours to 0.5 ml of fresh rabbit plasma. After mixing by gentle rotation, the tubes were incubated at 37^0C along with a negative control tube containing a mixture of 0.5 ml of sterile TSB and 0.5 ml of rabbit plasma. Clotting was evaluated at 30 minutes intervals for the first 4 hours of the test and then after 24 hours incubation. The reaction was considered positive if any degree of clotting from a loose clot to a solid clot that is immovable when the tube is inverted (tilted) was visible within the tube and no degree of clotting would be taken as negative(Quinn *et al*., 2002).

To differentiate among the pathogenic *staphylococci*, that were coagulase-positive purple agar base was used. Purple agar base (PAB) with the addition of 1% maltose was used to differentiate the pathogenic *staphylococci* particularly the coagulase-positive isolates. The suspected culture was inoculated on PAB media plate with 1% of maltose and incubated at 37^oC for 24-48 hours. The identification was based on the fact that *S. aureus* rapidly ferment maltose and the acid metabolic products cause the pH indicator (bromocresol purple) to change the medium and colonies to yellow. *S. intermedius* gives a weak or delayed reaction and *S. hicus* did not ferment maltose but attacks the peptone in the medium producing an alkaline reaction (a deeper purple) around the colonies(Quinn *et al*., 2002).

3. 5. 6. In vitro antimicrobial susceptibility testing of Staphylococcus aureus

The *Staphylococcus aureus* isolates were tested for antimicrobial susceptibility by disc diffusion method (Quinn *et al.,* 2002). A single colony was selected and emulsified in 3 ml sterile normal saline solution in a sterile test tube. The turbidity of the suspension was then adjusted to the density of a barium chloride standard (0.5 McFarland) in order to standardize the size of inoculum. A sterile cotton swab was dipped into the standardized suspension of the bacterial culture, squeezed against the sides of the test tube to remove the excess fluid and swabbed onto the surfaces of Muller-Hinton Agar plate. Then, the following Oxoid antimicrobial susceptibility discs [vancomycin [30 μg], penicillin G [10 IU

], tetracycline [30 μg], ampicillin [10 μg], gentamicin [10 μg], oxacillin [1μg], cefoxitin [30μg], and erythromycin [15μg] were placed onto the agar plate using sterile forceps and pressed gently to ensure the complete contact with the agar surface. The plates were read after 24 hours of incubation at 35°C under aerobic condition. The diameters of the zone of inhibition around the antimicrobial discs were measured to the nearest millimeter using caliper and interpreted as susceptible, intermediate and resistance for each antimicrobial drug tested according to the standard given by (CLSI, 2018 and Quinn *et al.*, 2002) and manufacturer's instructions.

3. 6. Data Management and Analysis

The data collected during the study periods were entered into a Microsoft Excel spreadsheet and analyzed using STATA software 12 (2011). Descriptive statistical analysis such as percentages and frequency distribution were used to describe the nature and the characteristics of the data to describe/bacterial isolates, various risk factor, dependent variables and antimicrobial susceptibility which were expressed as percent/percentile. The Pearson's Chi-square ($\chi 2$) was used to measure the association between the different risk factors and occurrence of bovine mastitis in dairy lactating cows. Also, logistic regression analysis was used to see the association of the potential risk factors with occurrence of bovine mastitis. Odds ratio was calculated to assess the risk levels of categories under each risk factor as the ratio of the odds of diseases occurring among cows exposed to a factor. All the analysis performed at the 95% confidence interval and a P-value <0.05 was considered statistically significant.

3. 7. Ethical Clearance

Before any attempt to collect sample the protocol was approved by Addis Ababa University College of Veterinary Medicine and Agriculture animal research ethical and review committee with reference number VM/ERC/05/10/018, 03/01/2018 (Annex 8).

4. RESULTS

4.1. Overall Prevalence of Mastitis

A total of 768 lactating cows were examined for mastitis and 58.5% (449/768) were positive for mastitis. Furthermore, 10.8% (83/768) and 47.7% (366/768) were categorized into clinical and subclinical mastitis, respectively.

Lactating cows were examined for mastitis at Akaki Kality. Of 384 cows examined in this study site, the prevalence of mastitis was found to be 64.6% (248/384) out of which 13.3% (51/384) and 51.3% (197/384) were categorized into clinical and subclinical forms, respectively.

Lactating cows at Sululta were also examined for mastitis. Out of 384 cows considered in this study site, the prevalence of mastitis was reported to be 52.3% (201/384) and 8.3% (32/384) and 44% (169/384) were classified into clinical and subclinical forms, respectively. The details of clinical, subclinical mastitis and blind teat by quarter level and study sites are indicated in (Table, 2 and 3).

Table 2: Prevalence and distribution of clinical, subclinical mastitis and blind teat by quarters in Akaki Kality

Quarter examined	No with clinical mastitis (%)	No with subclinical mastitis (%)	No with blind teats (%)	Total No (%)
RR	10(7.4%)	66 (17.7%)	22 (25.3%)	98 (25.5%)
RF	19(31.5%)	118 (31.7%)	39 (44.8%)	176 (45.8%)
LR	17(24.0%)	83 (22.3%)	9 (10.3%)	109 (28.4%)
LF	8(7.4%)	105 (28.2%)	17 (19.5%)	130 (33.8%)
Total prevalence	**54(3.7%)**	**372 (25.7%)**	**87 (5.7%)**	**513 (33.4%)**

RR = right rear, RF= right front, LR= left rear, LF= left front

Table 3: Prevalence and distribution of clinical, subclinical mastitis and blind teat by the quarters in Sululta

Quarter examined	No with clinical mastitis (%)	No with subclinical mastitis (%)	No with blind teats (%)	Total No (%)
2RR	12 (34.3%)	96 (29.4%)	23 (31.1%)	131 (34.1%)
RF	5 (14.3%)	79 (24.2%)	8 (10.8%)	92 (23.9%)
LR	3 (8.6%)	66 (20.2%)	17 (23.0%)	86 (22.4%)
LF	15 (42.8%)	85 (26.1%)	26 (35.1%)	126 (32.8%)
Total prevalence	**35 (2.3%)**	**326(21.2%)**	**74 (4.8%)**	**435 (28.3%)**

RR = right rear, RF= right front, LR= left rear, LF= left front

4. 2. Cow level potential Risk Factors at Akaki Kality

Univariate logistic regression analysis to indicate the association of cow level potential risk factors with bovine mastitis in Akaki Kality indicated it was more likely (OR =0.306) to occur in cows that were above 9 years of age with prevalence of 79.6% followed by cows 6-9 years with a prevalence of 72%. Similarly, cows having teat lesion had high prevalence of mastitis (75.6%) than cows with no teat lesion (55.0%). Accordingly, the likelihood of mastitis was 2.5 times more in cows having teat lesion (OR = 2.5) than cows without teat lesion. The details of cow level potential risk factors in development of bovine mastitis are indicated in Table 4.

Table 4: Univariate logistic regression analysis of cow level potential risk factors for the occurrence of mastitis in Akaki Kality Subcity

Risk Factors	Category	Number Examined	Number positive (%)	χ2	P-value	OR	95% CI
Age	3-5	187	102 (54.5)			Ref.	NA
(years)	6-9	143	103 (72)	17.04	**0.001**	0.466	0.292-0.741
	>9	54	43 (79.6)		0.001	0.306	0.149-0.631
Breed							
	HF	315	206 (65.4)	0.51	0.477		0.710-2.077
	Cross	69	42 (60.86)			1.214	
Parity	1-3	207	128(61.8)			Ref.	NA
	4-5	116	76(65.5)	2.25	0.511	0.852	0.530-1.370
	>5	61	44(72.1)		0.143	0.626	0.334-1.170
Lactation	Early 1-4	163	119(73.45)			Ref.	NA
stage	Mid 5-8	142	77(53.84)	11.76	**0.001**	2.283	1.415-3.682
(months)	Late (>8)	79	52(65.8)		0.251	1.404	0.786-2.506
Udder/tea							
t lesion	Yes	46	37 (80.4)				NA
	No	338	211 (62.4)	5.74	**0.020**	2.474	1.156-5.296
	Overall	384	248(64.6)				

Ref.= Reference, NA =not applicable, OR= odd ratio, HF =Holstein Friesian

Multivariate logistic regression analysis showing after removing a variable which were insignificant effect from univariable logistic analysis the final model for multivariable logistic regression analysis to see the association of cow level potential risk factors of bovine mastitis at Akaki Kality indicated that age, lactation stage and udder/teat lesion, which were statistically significant association with the occurrence of bovine mastitis (P<0. 05). Details of the association other cow level risk factors on occurrence of mastitis are indicated in Table 5.

Table 5: Multivariate logistic regression analysisof cow level potential risk factors for the occurrence of mastitis in Akaki Kality

Risk Factors	Category	Number Examined	Number positive (%)	$\chi 2$	P-value	OR	95% CI
Age (years)	3-5	187	102 (54.5)		Ref.	Ref.	NA
	6-9	143	103 (72)		**0.002**	0.468	0.291-0.753
	>9	54	43 (79.6)		**0.003**	0.332	0.159-0.691
Lactation stage (months)	Early 1-4	163	119(73.45)	32.30	Ref.	Ref.	NA
	Mid 5-8	142	77(53.84)		**0.003**	2.283	1.278-3.424
	Late (>8)	79	52(65.8)		0.357	1.404	0.729-2.399
Udder/teat lesion	Yes	46	37 (80.4)		**0.037**	Ref.	NA
	No	338	211 (62.4)			2.287	1.050-4.981

OR= Odd Ratio, CI= Confidence Interval, Ref= Reference, NA= Not applicable

4. 3. Herd level potential risk factors at Akaki Kality

Univariate logistic regression analysis of the association of herd level potential risk factors for the occurrence of mastitis at Akaki Kality indicated that there was statistically significa nt difference ($P< 0.05$) between the occurrences of bovine mastitis and housing of the hygiene as well as udder/teats hygiene($P< 0.05$). The details of herd level potential risk factors are indicated in Table 6.

Table 6: Univariate logistic regression analysis of herd level potential risk factors for the occurrence of mastitis at Akaki Kality Subcity

Risk Factors	Category	Number Examined	Number Positive (%)	χ2	P-value	OR	95% CI
Housing hygiene	Good	143	80 (55.9)				
	Bad	241	168 (69.7)	7.43	**0.007**	0.551	0.359-0.847
Floor type	Concrete	321	205 (63.86)				
	Muddy	63	43 (68.25)	0.44	0.705	0.822	0.461-1.464
Udder/teat hygiene	Good hygiene	298	181 (60.7)				
	Poor hygiene	86	67 (77.9)	8.6	**0.004**	0.438	0.250-0.768
Washing hand before milking	Yes	85	50 (58.8)				
	No	299	198 (66.2)	1.58	0.208	0.728	0.444-1.194
Use towel	Yes	106	67 (63.2)				
	No	278	181 (65.1)	0.12	0.728	0.920	0.578-1.466
Milking Mastitic cow last	Yes	282	178 (63.1)				
	No	102	70(68.6)	0.99	0.320	0.782	0.482-1.268
	Overall	384	248(64.6%)				

OR= Odd Ratio, CI=Confidence Interval

Multivariate logistic regression analysis indicated in table 7 after removing a variable which were insignificant association from univariable logistic regression analysis the final model for multivariable logistic regression analysis to see the association of herd level potential risk factors of bovine mastitis at Akaki Kality indicated that housing hygiene and udder/teat hygiene, which were statistically significant association with the occurrence of bovine masti tis ($P<0.05$).

Table 7: Multivariate logistic regression analysis of herd level potential risk factors for the occurrence of mastitis in Akaki Kality Subcity

Risk Factors	Category	Number Examined	Number Positive (%)	χ2	P-value	OR	95% CI
Housing hygiene	Good	143	80 (55.9)				
	Bad	241	168 (69.7)	7.43	**0.021**	0.616	0.397-0.955
Udder/teat hygiene	Good hygiene	298	181 (60.7)	8.6	**0.015**	0.491	0.277-0.868
	Poor hygiene	86	67 (77.9)				

OR= Odd Ratio, CI= Confidence Interval

4. 4. . Cow level potential Risk Factors at Sululta

Univariate logistic regression analysis to see the association of cow level potential risk factors of bovine mastitis at Sululta indicated that cows greater than 9 years of age had significantly higher prevalence (73.6%) than cows with other age categories (P<0.005), Similarly cows with greater than 5 parities had significantly higher prevalence (75.%) than other categories (P<.0.05). Details of the association other cow level risk factors on occurrence of mastitis are indicated in Table 8.

Table 8: Univariate logistic regression analysis of cow level potential risk factors for the occurrence of mastitis in Sululta

Risk Factors	Category	Number Examined	Number Positive (%)	χ2	P-value	OR	95% CI
Age	3-5	103	33 (32)			Ref.	NA
(years)	6-9	194	104 (53.6)	32.8	**0.000**	0.407	0.247-0.673
	>9	87	64 (73.6)		**0.000**	0.169	0.90-0.318
Breed							
	HF	303	165 (54.4)	2.57		Ref	NA
	Cross	81	36(44.4)		0.110	1.494	0.912-2.447
Parity	1-3	149	69 (46.3)			Ref.	NA
	4-5	186	95 (51.1)	12.8	0.386	0.826	0.536-1.272
	>5	49	37 (75.5)		**0.001**	0.279	0.135-0.578
Lactation	Early (1-	127	62 (48.8)			Ref.	NA
stage	4)			1.28	0.528		
(months)	Mid (5-8)	154	81 (52.6)			0.859	0.537-1.375
	Late (>8)	103	58 (56.3)		0.259	0.740	0.439-1.247
Udder/tea	Yes	81	59 (72.8)			Ref.	NA
t lesion	No	303	142 (46.9)	17.28	**0.000**	3.04	1.773-5.213
	Overall	384	201(52.34)				

Ref.=Reference, NA= not applicable, OR= odd ratio, HF= Holestein-Friesian, CI= Confidence Interval

Multivariate logistic regression analysis shown after removing a variable which were insignificant association from univariable logistic analysis the final model for multivariable logistic regression analysis to see the association of cow level potential risk factors of bovine mastitis at Sululta indicated that age and udder/teat lesion, which were statistically significant association with the occurrence of bovine mastitis ($P<0.05$). Details of the associ ation other cow level risk factors on occurrence of mastitis are indicated in Table 9.

Table 9: Multivariate logistic regression of cow level potential risk factors for the occurren ce of mastitis in Sululta

Risk Factors	Category	Number Examined	Number Positive(%)	χ2	P-value	OR	95% CI
Age (years)	3-5	103	33 (32)	32.8	**0.000**	Ref.	NA
	6-9	194	104 (53.6)			0.308	0.167-0.570
	>9	87	64 (73.6)		**0.000**	0.140	0.059-0.328
Parity	1-3	149	69 (46.3)	12.8	0.062	Ref.	NA
	4-5	186	95 (51.1)			0.1.712	0.972-2.016
	>5	49	37 (75.5)		0.948	0.969	0.378-2.482
Udder/tea t lesion	Yes	81	59 (72.8)	17.3	**0.000**	Ref.	NA
	No	303	142 (46.9)			2.962	1.690-5.192

OR= Odd Ratio, CI= Confidence Interval, Ref= Reference, NA= Not applicable

4. 5. Herd level potential risk factors at Sululta

Univariable logistic regression analysis to know the association of different herd level potential risk factors for the occurrence of bovine mastitis at Sululta indicated that farms with cows having poor udder/teats hygiene and mixed or random milking with mastitic cows with those without mastitis had significantly higher prevalence than the others ($P<0.05$).The details of investigated risk factors and the occurrence of mastitis in dairy farms of Sululta are shown in Table 10.

Table 10: Univariate logistic regression analysis of herd level potential risk factors for the occurrence of mastitis in Sululta

Risk Factors	Category	Number Examined	Number Positive (%)	χ2	P-value	OR	95% CI
Housing hygiene	Good	222	114 (51.4)				
	Bad	162	87 (53.7)	0.2	0.649	0.909	0.606-1.365
Floor type	Concrete	346	180 (52)				
	Muddy	38	21 (55.3)	0.14	0.704	0.877	0.447-1.721
Udder/teat hygiene	Good hygiene	260	120 (46)				
	Poor hygiene	124	81 (65.3)	12.36	**0.000**	0.455	0.292-0.708
Washing hand before milking	Yes	318	166 (52.2)				
	No	66	35 (53)	0.01	0.902	0.967	0.568-1.645
Use towel	Yes	236	121 (51.3)				
	No	148	80 (54.1)	0.28	0.595	0.894	0.592-1.350
Milking mastitic cow last	Yes	317	157 (49.5)				
	No	67	44 (65.7)	5.77	**0.016**	0.512	0.295-0.889
	Overall	384	201(52.34%)				

OR= Odd Ratio, CI=Confidence Interval

Multivariate logistic regression analysis indicated in table 11 after removing a variable which were insignificant effect from univariable logistic analysis the final model for multivariable logistic regression analysis to see the association of herd level potential risk factors of bovine mastitis at Sululta indicated that udder/teat hygiene and milking mastitic cow last which were statistically significant association with the occurrence of bovine masti tis ($P<0.05$).

Table 11: Multivariate logistic regression of herd level potential risk factors for the occurrence of mastitis in Sululta

Risk Factors	Category	Number Examind	Number Positive (%)	χ2	P-value	OR	95% CI
Udder/teat hygiene	Good hygiene	260	120 (46)	12.36	**0.003**	0.455	0.315-0.783
	Poor hygiene	124	81 (65.3)				
Milking mastitic	Yes	317	157 (49.5)				
	No	67	44 (65.7)	5.77	**0.002**	0.512	0.355-1.113

OR= Odd Ratio, CI= Confidence Interval

4.6. Isolation and Identification pathogenic Staphylococcus

Biochemical characterization of pathogenic *staphylococcus* isolates at Akaki Kality Subcity indicated that 25.4% (63/248) isolates were *staphylococci species*. The majority of them were *S. aureus* (18.5%, 46/248), followed by *S. intermidius* (4.03%, 10/248) and *S. hyicus* (2.8%, 7/248). Of the 201 milk sample collected from mastitis case at Sululta 20.9% (42/201) isolates were identified as *Staphylococci species*. The majority of them were again *S. aureus* (16.9%, 34/201) followed by *S. intermedius* (2.5%, 5/201) and *S. hyicus* (1.5%, 3/201) are indicated in Table 12.

Table 12: Isolation of pathogenic Staphylococcus from bovine mastitis in Akaki Kality Sub-city and Sululta

	Akaki Kality Sub-city			Sululta		
	Number of Isolation (%)	χ2	P-value	Number of Isolation (%)	χ2	P-value
S. aureus	18.5%(46/248)			16.9%(34/201)		
S. intermedius	4.03%(10/248)	41.33	**0.000**	2.5%(5/201)	42.93	**0.000**
S. hyicus	2.8%(7/248)			1.5%(3/201)		
Overall	25.4%(63/248)			20.9%(42/201)		

4.7. Isolation and identification of *Staphylococcus aureus*

Isolation and identification of *Staphylococcus aureus* from 248 milk samples collected from mastitis case cows (51 clinical and 197 sub-clinical) in Akaki Kality sub-city indicated *S. aureus* to be isolated at a rate of 18.5% (46/248) of the cases. When the type of mastitis was separately considered, *S. aureus* was isolated at a rate of 17.6% (9/51) from clinical mastitis and 18.8% (37/197) from sub-clinical mastitis. Of the 201 milk samples collected from mastitis cases (32 clinical and 169 sub-clinical mastitis) in Sululta indicated *S. aureus* to be isolated in 16.9% (34/201) of the cases. When the types of mastitis were considered separately, *S. aureus* was isolated at rate of 21.9% (7/32) from clinical and at 15.4% (26/169) from sub-clinical mastitis.

4. 8. Antimicrobial Susceptibilities of *S. aureus*

Antimicrobial sensitivity test was conducted on all isolates of *S. aureus* from both study areas. The result of antimicrobial sensitivity test indicated total resistance (100%) to ampicillin and pencillin on isolates from both study sites while 76.5% isolates from AkakiKality and 52.9% from Suluta were resistant to tetracycline. The details of antimicrob ials used and their susceptibility profile of the isolates are indicated in Table 13.

Table 13: Antimicrobial susceptibility of *S. aureus* isolates from Akaki Kality and Suluta

Study site	Antimicrobial Agents	Resistance No (%)	Intermediate No (%)	Susceptible No (%)
Akaki Kality	Gentamicin	3(8.8%)	0	31(91.2%)
	Oxacillin	0	5 (14.7%)	29 (85.3%)
	Cefoxitin	11 (32.4%)	0	23(67.6%)
	Ampicillin	34 (100%)	0	0
	Erythromycin	3 (8.8%)	7 (20.6%)	24 (70.6%)
	Tetracycline	26 (76.5%)	6 (17.6%)	2 (5.9%)
	Pencillin	34(100%)	0	0
	Vancomycin	2 (5.88%)	4 (11.76%)	28(82.35%)
Sululta	Gentamicin	0	5 (14.7%)	29 (85.29%)
	Oxacillin	4 (11.76%)	2 (5.88%)	28 (82.35%)
	Cefoxitin	3 (21.42%)	2 (5.88%)	29 (85.29%)
	Ampicillin	34 (100%)	0	0
	Erythromycin	2 (5.88%)	0	32 (94.11%)
	Tetracycline	18 (52.9%)	12 (35.3%)	4 (11.8%)
	Pencillin	34 (100%)	0	0
	Vancomycin	3 (8.8%)	0	31 (91.2%)

5. DISCUSSION

The overall prevalence of mastitis in dairy cows in the present study was found to be 58.8% (449/768) in both study sites. This result was in agreement with the previous observation of Bedane *et al*. (2012), Benta and Habtamu. (2011) and Tassew *et al*. (2016) who reported 59.1%, 56.5% and 56% overall prevalence of mastitis, respectively in different parts of Ethiopia and 56.16% in Oran region of west Algeria (Benhamed *et al*., 2011). However, Biffa *et al*. (2005), Getahun *et al*. (2008) and Kerro and Tareke (2003), reported the prevalence of 34.9%, 33.6% and 40%, respectively which were lower than the present prevalence. Additionally, the present prevalence was lower than that reported by Sori *et al*. (2011), Zeryhun *et al*. (2013), Mekibib *et al*. (2010) and Hailemeskel *et al*. (2014) who reported the overall prevalence of 75.22%, 74.7%, 71% and 88.9%, respectively. Mastitis is a complex disease involving interactions of several factors, mainly of management, environ ment, and factors relating to animal and causative organisms, its prevalence is expected to vary from place to place. And also variations in husbandry practices between different areas might, at least, partly explain the difference in prevalence reported by different authors (Biffa *et al*., 2005; Radostits *et al*., 2007).

The overall prevalence of clinical mastitis in the present study was 10.8% (83/768) and that of sub-clinical mastitis was 47.7% (366/768) in Akaki Kality and Sululta. This prevalence was similar with those reported by Abera *et al*. (2013) and Tassew *et al*. (2016) who reported the prevalence of 10% and 10%, each of them, however, it was higher than those reported by Bitew *et al*. (2010), Moges *et al*. (2011), Benta and Habtamu. (2011) who reported 3.0%, 0.93% and 5.3%, respectively. The 47.7% overall prevalence of sub-clinical mastitis was in agreement with the reports of Mekibib *et al*. (2010) who reported 46.6%, but higher than the those of Sori *et al*. (2011) and Fufa *et al*. (2013) who reported 36.67% and 36.86%, respectively. Tadesse *et al*. (2014) reported 85.4% overall prevalence of sub-clinical mastitis in dairy farms of selected sites in Addis Ababa. In most reports including the present study, clinical mastitis was far lower than subclinical mastitis. This may be

attributed to the difficulty of detecting sub-clinical mastitis by the owners compared to the easily detectable clinical cases which prompt owners seek treatment for their animals (Radostits *et al*., 2007).

Comparison of the current finding by study sites indicated the prevalence of clinical mastiti s was to be 13.3% in Akaki kality and 8.3% in Sululta which were lower than the prevalence of clinical mastitis reported by Mekibib *et al*.(2010) and Workineh *et al*. (2002) who reported prevalence of 22.4% and 21.5%, respectively. The 8.3% prevalence of clinical mastitis from Sululta was similar with the 10% prevalence reported by Abera *et al*. (2013) and Tassew *et al*. (2016) but lower when compared with the reports of Zeryhun *et al*, (2013) and Tilahun and Aylate (2015) who reported 19.6% and 21.2%, respectively. On the other hand, the result of the present finding was higher when compared with the reports of Bitew *et al*. (2010) who reported 3.0%. The prevalence of subclinical mastitis was 51.3% in Akaki Kality and 44.0% in Sululta and these were lower than the prevalence of 85.4% s ubclinical mastitis reported by Tadesse *et al*, (2014) in Addis Ababa. The prevalence of rep orted by the present study was similar with the 48.6% prevalence reported from Holota tow n by Mekibib *et al.* (2010), and with the 49.2% reported by Mulushet *et al*. (2017) in Addis Ababa. However, it was higher than 36.18% report in and around Walaita Sodo, southern Et hiopia by Biniam *et al.* (2017) and 36.7% in Adama town by Abera *et al*. (2010). Mastitis is a complex disease involving interactions of several factors, mainly of management, environ ment and factors relating to animal and causative organisms. Its prevalence is expected to v ary from place to place (Biffa *et al*., 2005).

Quarters' level prevalence of clinical mastitis found to 3.7% in Akaki Kality and 2.3% in Sululta. These finding agreed with Mokonen and Tesfaye (2010) in Adama and Zeryhun *et al*. (2013) in Addis Ababa who reported prevalence of 2.4% and 5.2%, respectively. The quarters level clinical mastitis was lower than Matios *et al*. (2009) in Asela and Mekibib *et al*. (2010) in Holota who reported 14.9% and 10%, respectively. However, the present prevalence was higher than the reports of 0.9% at Sellale small holder dairy farm (Getahun *et al*., 2008) and 1.2% in Addis Ababa (Zeryhun *et al*., 2013).

The present study also revealed that quarters' level subclinical mastitis was 25.7% in Akaki Kality and 21.2% in Sululta. These prevalence were similar with workineh *et al*. (2002), Bitew *et al*. (2010) in Bahir Dar and Belayneh *et al*. (2014) at Akaki district who re ported 21.5%, 25.3% and 20.8%, respectively. The quarters' level subclinical mastitis was greater than the reports of Belayneh *et al*. (2014) at Akaki district who reported 2.7%. However, the current prevalence was lower than Mekibib *et al*. (2010) in Holota and Zeryhun *et al*. (2013) who reported 34.8% and 42.7%, respectively.

The current study among the total quarters' level prevalence of blind teats was 5.7% at Akaki Kality and 4.8% at Sululta. These reports were similar those of with Matios *et al*. (2009) at Asela, Etifu (2012) in Alage state dairy farms and Fufa *et al*. (2013) in Addis Ababa who reported 4.5%, 5.2% and 4.61%, respectively. The quarters wise prevalence differences in clinical and subclinical mastitis observed in the current study and previous studies may be due to the difference in breeds of animals, immune status and management practices. The blind teat which may be an indication of serious mastitis problems in the herd and lack of screening test, treatment of subclinical mastitis, and inadequate follow up of chronic mastitis were considered to be the major reasons for the development of quarter level blindness (Biffa *et al*., 2005).

Risk factors geared study considered age, parity, lactation stage, udder/teat lesion, udder/teat hygiene, housing hygiene and milking mastitic cow last were statistically associa ted with the occurrence of mastitis ($P<0.05$). Tassew *et al*. (2015), Zeryhun *et al*. (2013) an d Biffa *et al*. (2005) indicated that mastitis increases with increasing cows' age. Radostits *e t al*. (2007) explained that older cows have the largest teats and more relaxed sphincter muscles which increase the accessibility to infectious agent in the cows' udder. Similarly, prevalence of mastitis was higher during early lactation (1-4 months) as compared to mid lactation (5-8 months) and late lactation (>8 months). Absence of dry cow therapy regime could possibly be among the major factors contributing to high prevalence at early lactation. During a dry period due to the low bactericidal and bacteriostatic qualities of milk, the pathogens can easily penetrate into the teat canal and multiply; this can be carried over into the post parturient period and ultimately develop into clinical mastitis (Radostits *et al*., 2007).

The prevalence of bovine mastitis in cows with udder/teat lesion was higher (80.4%) than those without udder/teat lesion (62.4%) in the present study. Similarly, Biffa *et al*. (2005) reported high level of bovine mastitis in cows with injured udder/teat. The teat/udder injury could contribute a favorable environment for pathogens to enter and multiply. Cows with p oor udder/teat hygiene had higher prevalence (77.9%) than those with good udder/teat hygi ene (60.7%) and it was in agreement with the finding of Abebe *et al*. (2016). In dairy animals managed under poor hygienic houses, manure and wet bedding materials were not frequently removed which in turn favour high occurrence of bovine mastitis. This is consistent with the findings of earlier works in Ethiopia that implicated poor barn hygiene causes high prevalence of mastitis (Sori *et al*., 2005; Abera *et al*., 2013; Yohannis and Molla, 2013).

The occurrence of mastitis has significant association with parity of the animal. The increased occurrence of mastitis with increased parity in the current study is in agreement with the previous reports of Mulugeta and Wassie (2013), Mekibib *et al*. (2010) and Haftu *e t al*. (2012). This could be due to that fact that primiparous cows have more effective defense mechanism than multiparous cows (Enerike, 2001). The other important factor for the occurrence of mastitis in this study was milking of mastitis cows at the last. Mastitis positive cows should be milked at the end to prevent cross contamination to the healthy animals.

Pathogenic Staphylococcus isolates were *S.aureus* (18.5%), *S. intermedius* (4.03%) and *S. h yicus* (2.8%) in Akaki kality and similarly were *S. aureus* (16.9%), *S. intermedius* (2.5%) and *S. hyicus*(1.5%) in Sululta. These frequencies were lower than that of Kedir *et al*. (2016) in dairy farms of Dire Dawa city who reported 48.4% *S. aureus*, 12.7% *S. intermedius* and 4.5% *S.hyicu*,.Workineh *et al*. (2002),Tadesse *et al*. (2014), Abera *et al*. (2 013) and Bitew *et al*. (2010) reported 39.2%, 24.4%, 42.1% and 20.3%, respectively. How ever, these reports were similar to the reports of Abebe *et al*. (2013) who reported 16.2%. *S. aureus* which is well adapted to survive in the udder and usually establishes mild subclinical infection of long duration from which it is shed through milk serving as source of infection for other healthy cows and transmitted during the milking process (Radostits *et al*., 2007).

S. aureus isolate originated from Akaki kality dairy farms in the present study showed total resistance (100%) to Ampcillin and Pencillin and 76.5% to Tetracycline. On the other hand it was 52.9% to Tetracycline in Sululta. Similarly, isolates of *S. aureus* originated from Sululta showed similar resistance pattern to Ampcillin and Pencillin. This was in agreement with the finding of Abebe *et al*. (2016) and Tassew *et al*. (2016) who reported similar resistance to Pencillin, Ampicillin and Tetracycline. The resistance of *Staphylococcus aureus* to certain antibiotic groups in a specific region might be due to their frequent and long-term use of antibiotics (Sabour *et al*., 2004; Moon *et al*., 2007). Furthermore, many *S. aureus* strains can resist antibiotic therapy by the production of beta-lactamase, an enzyme that inactivates penicillin, and closely related antibiotics. Probably around 50% of mastitis caused by *S. aureus* strains produce beta-lactamase and there is evidence that these strains are more difficult to cure with all antibiotics (Levy and Marshall, 2004); Andrew *et al*., 2004).

S. aureus originated from Akaki kality mastitis case in this study was found to be suscepti ble to Gentamicin (91.2%), Oxacillin (85.3%), Vancomycin (82.35%), Erythromycin (70.6 %) and Cefoxitin (67.6%) in respective orders mentioned. Isolates of *S. aureus* originated from mastitis case from Sululta showed lower susceptiblity to Gentamycin (85.29%) compared to AkakiKality and also Oxacillin (82.35%) showed lower susceptibility on Suluta isolates. However, Cefoxitin (85.3%), and Erythromycin, (94.11%) showed higher susceptibility on Suluta isolates which was in agreement with the report by Tassew *et al*. (2016) who reported susceptibility to Cefoxitin, Erythromycin and Gentamycin. Mekuria *et al*., (2013) also reported susceptibility to oxacillin and vancomycin.

6. CONCLUSIONS AND RECOMMENDATIONS

The results of the present study indicated a relatively high prevalence of bovine mastitis in dairy cows in the study areas. Similarly, the prevalence of subclinical mastitis was higher than clinical mastitis. Higher infection rates were observed in cows with old age, early lactating stage, udder/teats lesion, poor housing hygiene, poor udder/teats hygiene, multiple parity and mixing of milking mastitis cow normal cows. Mastitis caused by pathogenic Sta phylococcus in general and *Staphylococcus aureus* in particular was higher than many previ ous reports in Ethiopia. *Staphylococcus aureus* isolates from both study sites were found to be totally resistant to pencillin, and ampicillin but showed good susceptibility gentamicin, vancomicin, erythromicine, oxacillin and cefoxitin.

Based on this concluding remark the following points can be recommended:

- Regular investigation of mastitis especially the subclinical form should be practiced.
- Good housing and udder/teats hygiene as well as appropriate treatment of cows during dry and lactation period should be practiced.
- Prevention of teat injuries and on time treatment if any to alleviate the problem.
- Awareness should be created to dairy farm owners and dairy workers on the effect of mastitis.
- Mechanisms to control the risk factors associated with the disease should be implemented.
- *Staphylococcus aureus* mastitis control strategy should be initiated and promoted in the study areas.
- Further study involving large area should be conducted on the role of pathogenic Staphylococcus species and susceptibility to microbial so as to undertake measurable control.

7. REFERENCES

Aarestrup, F.M. (2005): Veterinary drug usage and antimicrobial resistance in bacteria of animal origin. *Journal of Basic and Clinical Pharmacology and Toxicology*., 96:27 1-281.

Abebe, M., Daniel, A., Yimtubezinash, W. and Genene, T. (2013): Identification and antimi crobial susceptibility of *Staphylococcus aureus* isolated from milk samples of dairy cows and nasal swabs of farm workers in selected dairy farms around Addis Ababa, Ethiopia. *Africa Journal of Microbiology Research*., **7**(27):3501-3510.

Abera, M., Demie, B., Araga, K.,Regassa, F. and Regassa, A. (2013): Isolation and identific ation of *Staphylococcus aureus* from bovine mastitic milk and their drug resistance patterns in Adama town, Ethiopia. *Africa Journal Dai. Far. and Mil. Prod*., **1**:019-023.

Ahmed, Y., Oleg, K., Gedeon, F., Elimelech, D. H., Natan, G., Arthur, S. and Gabriel, L. (2005): *Staphylococcus aureus Leucocidin*, a Virulence Factor in Bovine Mastitis, *Journal of Dairy Research.*, **72**: 188-194

Akers, M. R. and Nickerson, C. S. (2011): Mastitis and its impact on Structure and Function in the Ruminant Mammary Gland. *Journal Mammary Gland Biology Neoplasia.*,**16**(4): 275-89.

Andrews, A. H., Blowey, R. W., Boyd, H. and Eddy, R. G. (2004): Bovine medicine disease and husbandry of cattle 2nd edition., 186-230.

Argaw, A. (2016): Review on epidemiology of clinical and subclinical mastitis on dairy cows. Food Science Quallity Management., **52**: 56-65.

Bayouni, A., Faruer, T. B., Bushnell, B. and Olivera, A. (1988): Enzootic *mycoplasmal* ma stitis in a large dairy during an eight-year period. *Journal of the American Veterinary Association.*, **192**: 902–905

Bedane, A., Kasim, G., Yohannis, T., Habtamu, T., Asseged, B. and Demelash, B. (2012): Study on prevalence and risk factors of bovine mastitis in Borana pastoral and agro-pastoral settings of Yabello District, Borana Zone, and Southern Ethiopia,

American-Eurasian *Journal of Agricultural and Environmental Science*.,**12**(10): 1274.

Belayneh, R., Belihu, K. and Tesfaye, A. (2014): Microbiological study on bacterial causes of bovine mastitis and its antibiotics susceptibility patterns in East Showa Zone, Akaki District, Ethiopia. *Journal of Veterinary Medicine and Animal Health*., ***6***(4), 116-122.

Benhamed, N., Moulay, M., Aggad, H., Henni, J. E. and Kihal, M. (2011): Prevalence of mastitis infection and identification of causing bacteria in cattle in the oran region West Algeria. *Journal of animal and veterinary Advances*.,**10**(22): 3002-3005.

Benta, D. B. and Habtamu, T. M. (2011): Study on prevalence of mastitis and its associated risk factors in lactating dairy cows in batu and its environs, Ethiopia. Global Veterinaria., **7**(6): 632-637.

Bhatt, V. D., Ahir, V. B., Koringa, P. G., Jakhesara, S. J., Rank, D. N., Nauriyal, D. S., Kunjadia, A. P. and Joshi, C. G. (2012): Milk microbiome signatures of subclinical mastitis affected cattle analyzed by shotgun sequencing. *Journal of Applied Microb iology*.,**112**(4) : 639-650.

Biffa, D., Debela, E. and Beyene, F. (2005): Prevalence and risk factors of mastitis in lactati ngdairy cows in Southern Ethiopia. International *Journal of Applied Research and Veterinary Medicine*., **3**(3): 189–198.

Biniam, T. D., Biruk, T. B., Tesfaye, S. T. and Ashenafi , K. W. (2017): Isolation and Identi fication of Methicilin Resistant *Staphylcoccus aureus* (MRSA) from Bovine Mastitic Milk in and around Wolaita Sodo, Southern Ethiopia., *Journal of Veterinary Science and Research*., **2**(3): 136.

Birhanu, A., Diriba, L. and Iyob, I. (2013): Study of bovine mastitis in Asella government dairy farm of Oromia Regional state, South Eastern Ethiopia. International *Journal of Current Research and Academic Review*.,**1**(2): 134-145.

Bitew, M.,Tafere, A. and Tolosa, T. (2010): Study on bovine mastitis in dairy farms of Bahir Dar and its environs. *Journal of Animal and Veterinary Advances*., **9**(23): 2912-2917.

bovine mastitis and its antibiotics susceptibility patterns in East Showa Zone, Akaki District, Ethiopia. *J. Vet. Med. Anim. Health*, **6**(4): 116-122.

Bradley, A.J. (2002): Bovine mastitis an evolving disease, *The Veterinary Journal*., **164**: 116-128.

Carrillo-Casas., E. M. and Miranda-Morales, R. E. (2012): Bovine mastitis pathogens: prevalence and effects on somatic cell count. In Milk Production-An Up-to-Date Overview of Animal Nutrition, Management and Health. New York: *INTECH*, pp. 359–374.

Chauhan, R. S. and Agarwal, D. K, (2006): Text book of Veterinary Clinical and Laborator y Diagnosis, 2nd ed. India, Japee brothers, pp. 162-164

CLSI (2018): Performance Standards for Antimicrobial Susceptibility Testing. 28th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute., **38**(3): 54-140.

Coles, E. H. (1986): Veterinary clinical pathology. 4th Edition, W.B. Saunders Company, Philadelphia, PP 360-369.

CSA (Central Statistical Agency). (2015): The Federal Democratic Republic of Ethiopia; Central Statistical Authority Federal Democratic Republic of Ethiopia Agricultural samples Enumeration Statistical Abstract, PP 446-539.

Duguma, A., Tolosa, T. and Yohannes, A. (2014): Prevalence of clinical and sub-clinical mastitis on cross breed dairy cows at Holleta Agricultural Research Center, Central Ethiopia. *Journal Veterinary Medicine Animal Health*., **6**:13–17.

Eriskine, R. J. (2001): Intramuscular administration of ceftiofursodiu versus intrammamary

Erskine, R.J. (2003): Antibacterial therapy of clinical mastitis part I. Drug selection. Part II Administration. North America Veterinary Confrence, Proc, pp 13-16.

Etifu, M. (2012): Study on prevalence of mastitis and associated risk factors with isolation and anitimicrobial susceptibility of major pathogs in alage state dairy farm., 47

Fufa, A., Gemechis, F., Bekele, M. and Alemayehu, R. (2013): Bovine Mastitis: Prevalence , Risk Factors and Bacterial Isolation in Small-Holder Dairy Farms in Addis Ababa City, Ethiopia. *Global Veterinarian*.,**10** (6): 647-652.

Gebremichael, L., Deressa, B., Begna, F. and Mekuria, A. (2013): Study on prevalence of bovine mastitis in lactating cows and associated risk factors in and around Areka town, Southern of Ethiopia. *African Journal Microbiology Resistance*.,**7**:5051–5056.

Gentilini, E., Denamiel, G., Llorente, P., Godaly, S., Rebuelto, M. and DeGregorio, O. (200 0): Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Argentina. *Journal of dairy science*.,**83**(6): 1224-1227.

Getahun, K., Kelay, B., Megra, B. and Fikiru, L. (2008): Bovine mastitis and antibiotic resistance pattern in sellale smallholder dairy farms, Ethiopia.Trop.Anim.Health Product., **40**: 216-268.

Godden, S. M., Jansen, J. T., Leslie, K. E., Smart, N. L. and Kelton, D. F. (2002): The effect of sampling time and sample handling on the detection of *Staphylococcus aureus* in milk from quarters with subclinical mastitis. *The Canadian Veterinary Journal*.,**43**(1): 38.

Gordon, J.R.., Lowy, F.D. (2008): Pathogenesis of *methicillin*-resistant *Staphylococcus aureus* infection. Clin. Infect. Dis., **46**: 350–359.

Haftu, R., Taddele, H., Gugsa, G. and Kalayou, S. (2012): Prevalence, bacterial causes, and antimicrobial susceptibility profile of mastitis isolates from cows in large-scale dairy farms of Northern Ethiopia. Tropical animal health and production, **44**(7), 1765-1771.

Hailemeskel, D., Admasu, p. and Alemu, F. (2014): prevalence and identification of bacteri al pathogens causing bovine mastitis from crossbreed of Dairy cows in North showa zone of Ethiopia, Global veterinarian., **3**(2): 189-195.

Hassan, A. A., Khan, I. U. and Abdulmawjood, A. (2001): Evaluation of PCR methods for rapid identification and differentiation of *Streptococcus uberis* and *Streptococcus para uberis. Journal of Clinical Microbiology.*, **39**: 1618-1621.

Hillerton, J. E., and Berry, E. A. (2003): The management and treatment of environmental *streptococcal* mastitis. The Veterinary Clinics of North America Food Animal Practice **19**(1): 157-69.

Hogan, J. S., Gonzalez, R. N., Harmon, R. J., Nickerson, S. C., Oliver, S. P., Pankey, J. W., and Smith, K. L. (1999): Laboratory Handbook on Bovine Mastitis. Revised Edition 1999. National Mastitis Council. *Inc., Madison, WI*. Infusion of penicillin/n ovobiocin for treatment of *Streptococcus agalactiae* mastitis.

Jasper, D.E., Booth by, T. T. and Thomas, C. B. (1987): Pathogenesis of bovine Mycoplas ma mastitis. *Israel Journal of Medical Sciences***., 6**: 625–627

Kedir, J., Disassa, H., Jaleta, H., Zenebe, T. and Kebede, G. (2016): A Study on Bovine Mastitis, Isolation and Identification of *Staphylococcus* Species in Dairy Farms of Dire Dawa City, Eastern Ethiopia.

Kerro, D., and Tareke, F. (2003): Bovine Mstitis in selected areas of Southern Ethiopia. Trop Anim Health Prod.,**35**(3): 197-205.

Koskinen, M. T., Holopainen, J., Pyorala, S., Bredbacka, P., Pitkala, A., Barkema, H. W. and Kelton, D. (2009): Analytical specificity and sensitivity of a real-time polymerase chain reaction assay for identification of bovine mastitis pathogens. *Journal of Dairy Science*., **92**(3): 952-959.

Lam,T., Olde, R., Sampimon, O. and Smith, H. (2009): Mastitis diagnostics and performanc e monitoring: a practical approach. *Irish Veterinary Journal*., **62**:120-128.

Lechero, E. (2010): Questions about milk quality: What is the difference between clinical and subclinicalmastitis.Availableat:http://ansci.illinois.edu/static/ansc438/Mastitis/syndre. html Accessed Dec. **18**, 2015.

Lehtolainen, T., Pohjanvirta., Pyorala, S. and Pelkonen S. (2003): Association between Virulence Factors and Clinical Course of *Escherichia coli* Mastitis. *Acta veterinary Scand.*, **44**: 203-205.

Levy, S. B., & Marshall, B. (2004): Antibacterial resistance worldwide: causes, challenges and responses. Nature medicine., **10**(12), 122.

Mahmmod, Y. (2013): The Future of PCR Technologies in Diagnosis of Bovine Mastitis Pathogens. Advance Dairy Research Medicine. 2nd ed. Toronto: W.B. Saunders Company, Pp. 229-273.

Matios, L., Tadele, T. and Worku, T.(2009): Prevalence and major bacterial causes of bovine mastitis in Asella, South Eastern Ethiopia, Tropical Animal Health and Production., **41**: 1525-1530.

Mekibib, B., Furgassa, M., Abuna, F., Megersa, B., and Regassa, A. (2010): Bovine mastitis: prevalence, risk factors and major pathogens in dairy farms of Holeta town, Central Ethiopia. Vet. Wor., **9**: 397 - 403.

Mekonnen, H. and Tesfaye, A. (2010): Prevalence and etiology of mastitis and related management factors in market oriented smallholder dairy farms in Adama, Ethiopia *Journal of Veterinary Medicine*., **161**(12): 574-579.

Mekuria AS., Daniel A., Yimtubezinash W., Genene, T. (2013): Identification and antimicrobial susceptibility of *Staphylococcus aureus* isolated from milk samples of dairy cows and nasal swabs of farm workers in selected dairy farms around Addis ababa, Ethiopia. *African journal microbial Res*., **7**(27): 3501-3510.

Moges, N., Asfaw, Y. and Belihu, K. (2011): A Cross Sectional Study on the Prevalence of Sub Clinical Mastitis and Associated Risk Factors in and Aronund Gondar, Northern Ethiopia. *International Journal of Animal and Veterinary Advances*., **3**(6): 455-459.

Moon, J. S., Lee, A. R., Kang, H. M., Lee, E. S., Kim, M. N., Paik, Y. H. and Koo, H. C. (2007): Phenotypic and genetic antibiogram of methicillin-resistant *staphylococci* isolated from bovine mastitis in Korea. *Journal of dairy science*.,**90**(3): 1176-1185.

Mulushet, Y., Derso, S., and Nigus, A. (2017): Prevalence Of Bovine Subclinical Mastitis And Associated Risk Factors In Addis Ababa, Central Ethiopia. *Journal Animal Feed Res*., **7**(5): 124-133.

Mulugeta, Y. and Wassie, M. (2013): Prevalence, risk factors and major bacterial causes of bovine mastitis in and around Wolaita Sodo, Southern Ethiopia. *Africa Journal Microbiol. Res*., **7**(48):5400-5405.

NAAS. (2013): Mastitis Management in Dairy Animals. Policy Paper. National Academy of Agricultural Sciences, New Delhi., **61**:12-36.

National Mastitis Council, (1990): Microbiological Procedures for the Diagnosis of Bovine Udder Infection. national mastitis council.3rd edition, Arlington, Virginia, USA.

National Mastitis Council, (NMC). (2004): Guidelines on Normal and Abnormal Raw Milk Based on SCC and signs of clinical mastitis. Available at: https://nmconline.org/docs/abnmilk.pdf

Nelson, W. P. and Stephan, C. N. (1991): Mastitis counter attacks: Babson. Bros. Co. Naperville, PP 18-59.

Nickerson, S.C. (2009): Control of heifer mastitis: Antimicrobial treatment: An overview. Veterinary Microbiology., **134**: 128-135.

NMC – National Mastitis Council (1997): A Look at Physiological and Regulatory SCC Standards in Milk. NMC Newsletter Udder Topics.

NMSA (National Meteorological Service Agency). (2015): Rainfall and Temperature data, Addis Ababa, Ethiopia.

Oliveira, L., Hulland, C. and Ruegg, P. L. (2013): Characterization of clinical mastitis occurring in cows on 50 large dairy herds in wisconsin. *Journal of dairy science*., **96**(12): 7538-7549.

Oliver, S. P., Gonzalez, R. N., Hogan, J. S., Jayarao, B. M., and Owens, W. E. (2004): Microbiological procedures for the diagnosis of bovine udder infection and determination of milk quality. *Verona WI, USA: National Mastitis Council*.

Ondiek, O., Ogore, P., Shakala, E. and Kaburu, G.(2013): Prevalence of bovine mastitis, its therapeutics and control in Tatton Agriculture Park, Egerton University, Njoro District of Kenya. Basic Research *Journal of Agricultural Science* and *Review*., **2**(1):15-20.

Phuektes, P., Browning, G. F., Anderson, G., and Mansell, P. D. (2003): Multiplex polymerase chain reaction as a mastitis screening test for *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae and Streptococcus uberis* in bulk milk samples. *Journal of Dairy Research.*, **70**(2), 149-155.

Quinn, P. J., Leonard, F. C., Markey, B. K., Carter, W. E. and Donnelly, W. J. (2002): Clinical veterinary microbiology. Wolfe, Baltimore, pp. 465-472.

Quinn, P. J., Leonard, F. C., Markey, B. K., Carter, W. E. and Donnelly, W. J. (2002): Clinical veterinary microbiology. Wolfe, Baltimore, PP 327-475.

Quinn, P. J., Leonard, F. C., Markey, B. K., Carter, W. E. and Donnelly, W. J. (2002): Clinical veterinary microbiology. Wolfe, Baltimore., **4:** 65-75.

Radostits, O. M., Leslie, K. E., and Fetrow, J. (1994): Planned animal health and production in swine herds. Herd Health*: Food Animal Production Medicine*, PP 435-526.

Radostits, O. M., Gay, C.C., Hinchcliff, K.W and Constable, P.D. (2007): Veterinary Medic in: A text book of the disease of cattle, horses, sheep, pigs and goats. 10th ed. Elsevier London, pp. 674-762

Rato, M. G., Nerlich, A. and Bergmann, R. (2011): Virulence gene pool detected in bovine Group C *Streptococcus dysgalactiae subsp*. *Dysgalactiae* isolates by use of a Group A *S. pyogenes* virulence microarray. *Journal Clinical Microbiology*., **49**: 2470–2479.

Sabour, P. M., Gill, J. J., Lepp, D., Pacan, J. C., Ahmed, R., Dingwell, R. and Leslie, K. (2004): Molecular typing and distribution of *Staphylococcus aureus* isolates in Eastern Canadian dairy herds. *Journal of clinical microbiology.*, **42**(8): 3449-3455.

Sadashiv, S.O. and Kaliwal, B.(2014): Antibiotic resistance of *Staphylococcus aureus* and Coagulase-Negative *Staphylococci* isolated from Bovine mastitis in the region of north Karnataka, India, World *Journal of Pharmaceutical Research*., **3**(01): 571-586.

Savasan, S. (2002): Clinical examination a rapid detection of gram positive and gram negative bacteria in bovine mastitis. Year book publishers, Inc. London, PP 23-27.

Scott, P.R., Penny, C.D. and Macrae, A.I. (2011): Cattle medicine, PP 218-221

Sharif A. and Muhammed G. (2009): Mastitis control in dairy animals. *Pakistan Veterinary Journal*., **29**(3):145-148.

Singh, A. K., Jaiswal, S., Singh, D.D. and Singh, H. (2006): Bovine mastitis and udder affections. 1st edition, PP 52-53.

Sol, J., Sampimon, O. C., Snoep, J. J. and Schukken, Y. H. (2000): Factors associated with bacteriological cure after dry cow treatment of subclinical *Staphylococcus* mastitis. *Journal of dairy science.*,**77**(1):75-79.

Sori, T., Hussien, J., and Bitew, M. (2011): Prevalence and susceptibility assayof *Staphylococcus aureus* isolated from bovine mastitis in dairy farms of Jimma town, South West Ethiopia. *Journal of Animal and Veterinary Advances*.,**10**(6): 745-749.

Tadesse, T., Mulisa, MK. and Teka, F. (2014): Bovine Mastitis: Prevalence and Isolation of Major Pathogens in Dairy Farms of Selected Sites in Addis Ababa, Ethiopia. *Applied Journal of Hygiene*.,**3** (3): 31-37.

Taponen, S., Salmikivi, L., Simojoki, H., Koskinen, M. T., and Pyorala, S. (2009): Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. *Journal of dairy science*.,**92**(6): 2610-2617.

Tassew, A., Negash, M., Demeke, A., Feleke,A. and Tesfaye, B. (2016): Isolation, identification and drug resistance patterns of methicillin resistant *Staphylococcus aureus* from mastitic cow's milk from selected dairy farms in and around

Kombolcha, Ethiopia. *Journal of Veterinary Medicine and Animal Health*.,**8**(1): 1-10.

Thrusfield, M. (2007): Veterinary epidemiology. 3rd ed. Blackwell Science Ltd, a Blackwell Publishing company, UK. 188-265

Tilahun, A. and Aylate, A. (2015): Prevalence of bovine mastitis in lactating cows and its public health implications in selected commercial dairy farms of Addis Ababa. *Global Journal of Medical Research*., 15(2):

Tollersrud, T., Kenny, K., Reitz, J. R. and Lee, J. C. (2000): Genetic and serologic evaluati ve of capsule production by bovine mammary isolates of *Staphylococcus aureus* an d other Staphylococcus spp. from Europe and the United States. *Journal Clinical M icrobiology*.,**38**:2998-3003.

Tomita, G. and Hart S. (2001): The Mastitis Problem. In Proc. 16th Ann. Goat Field Day, Langston University, Langston; 6-9.

Viguier, C., Arora, S., Gilmartin, N., Welbeck, K. and O'Kennedy, R. (2009): Mastitis dete ction: current trends and future perspectives. *Trends in biotechnology*.,**27**(8):486-493.

Workineh , S., Bayleyegn, M., Mekonnen, H. and Potgieter, L. (2002): prevalence and a etiology of Mastitis in cows from two major Ethiopian dairies. Tropical Animal Health Production., **34**: 19-25.

Yohannis, M., and Molla, W. (2013): Prevalence, risk factors and major bacterial causes of bovine mastitis in and around WolaitaSodo, Southern Ethiopia. *African journal of microbiology research*, **7**(48): 5400-5405.

Zenebe, N., Habtamu, T. and Endale, B. (2014): Study on Bovine mastitis and associated risk factors in Adigrat, Northern Ethiopia. *Africa Journal Microbiology Research*, **8**:327–331.

Zeryehun, T., Aya, T.andBayecha, R. (2013): Study on prevalence, bacterial pathogens and associated risk factors of bovine mastitis in small holder dairy farms in and around Addis Ababa, Ethiopia. *Journal Animal Plant Sci.*, **23**(1): 50-55.

8. ANNEXES

Annex 1. Questionnaire format

Owners Name: _____Address_____

Date of sample Collection_____

1. History of cow:

   Breed_____

   Age_____

   Parity_____

   Stage of lactation_____

   Teat lesion: present _____ Absent_____

   Blindness of teat canal: RR_____ RF _____ LF _____ LR _____

   Sample collection from: RR_____RF _____LF _____LR _____

   CMT score: RR _____ RF _____ LF _____ LR _____

2. Milking practice:

   2.1.Do you wash hand before milking ?

   yes_____ no_____

   2.2.Udder/teats hygiene

   Good hygiene_____ poor hygiene_____

   2.3.Do you use towel

   yes ____ no ____

   2.4.Do you practice milking mastitic cows last

   yes_____ no _____

3. Housing

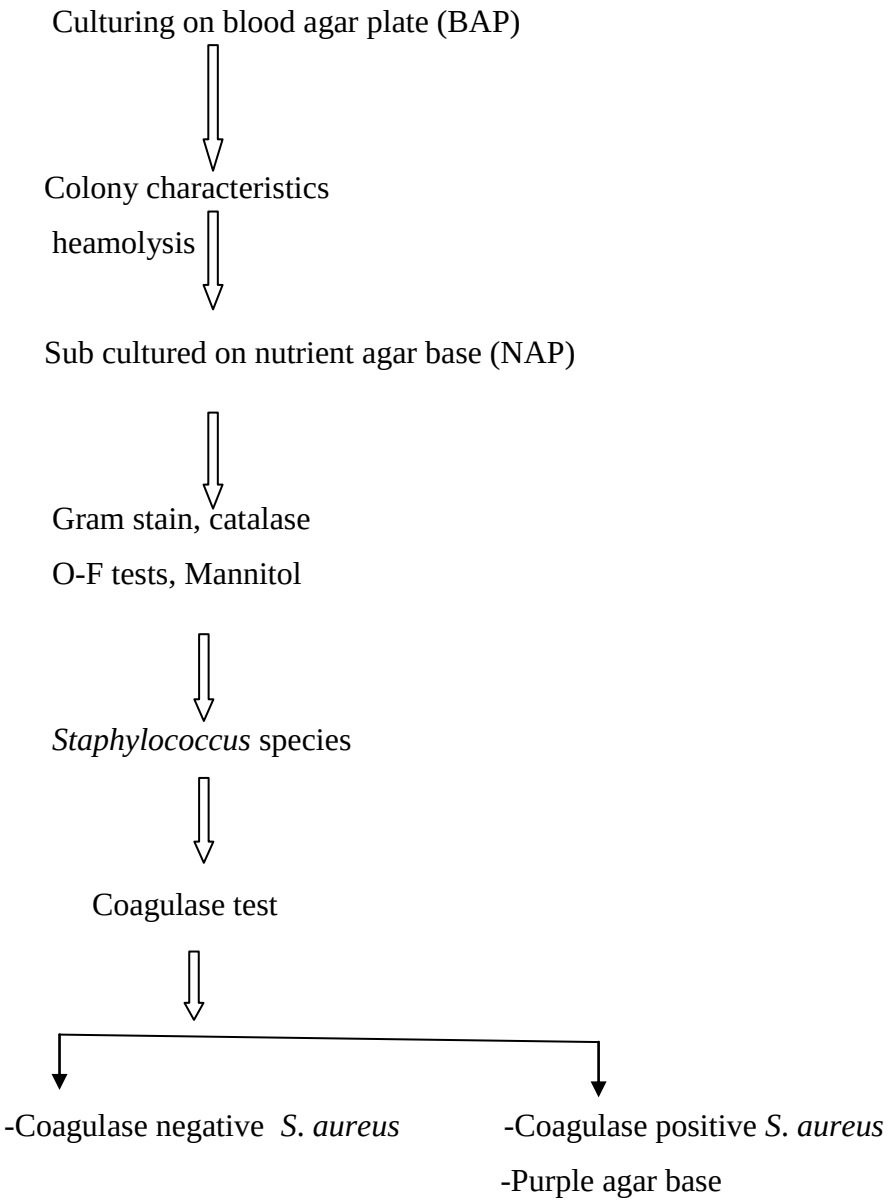
   3.1. Floor type

   concrete _____ muddy _____

   3.2. Housing hygiene

   good _____ bad _____

Annex 2: Flow chart of the laboratory analysis protocol



Annex 3: Biochemical test procedure

Catalase Test (Quinn *et al*., 2000)

Principle: the breakdown of hydrogen peroxide into oxygen and water is mediated by the enzyme catalase.

Procedure: a loopful of the bacterial growth is taken from the top of the colonies avoiding the blood agar medium. The bacterial cells are placed on a clean microscope slide and a drop of 3% H_2O_2 is added. An effervescence of oxygen gas, within a few seconds, indicates a positive reaction.

O-F test (Oxidative and Fermentative test) (Quinn *et al*., 2000)

Procedure: prepare O-F base medium and when the O-F base has cooled to 50^0c add 20ml of sterile glucose solution into 200ml of O-F base, for final concentration of 1% glucose and dispense in to tubes.

Two tubes of the O-F medium are heated in a breaker of boiling water immediately before use to drive off any dissolved oxygen and the tubes are then cooled rapidly under cold running water. Both tubes (sealed tube) to a depth of about 1cm and the tubes are incubated at 37^0c and examined in 24hrs and then daily for up to 14 days.

Mannitol salt agar (Quinn *et al*., 2002)

The colonies that were identified by Gram-staining reaction, O-F glucose, oxidase and catalase test as Staphylococcus were streaked on MSA plates and incubated at 370C and examined after 24-48 hours for growth and change in the colour of the medium. The presence of growth and change of pH in the media (red to yellow colour) were regarded as confirmative identification of Staphylococci. Phenol red pH indicator detected the acidic metabolic product of mannitol. Fermentation of mannitol by S. aureus causes yellow discolouration of the medium. Colonies that develop weak or delayed yellow colour after

24 hours of incubation were taken as S. intermedius and colonies that failed to produce any change on the medium were considered as S. hyicus and CNS.

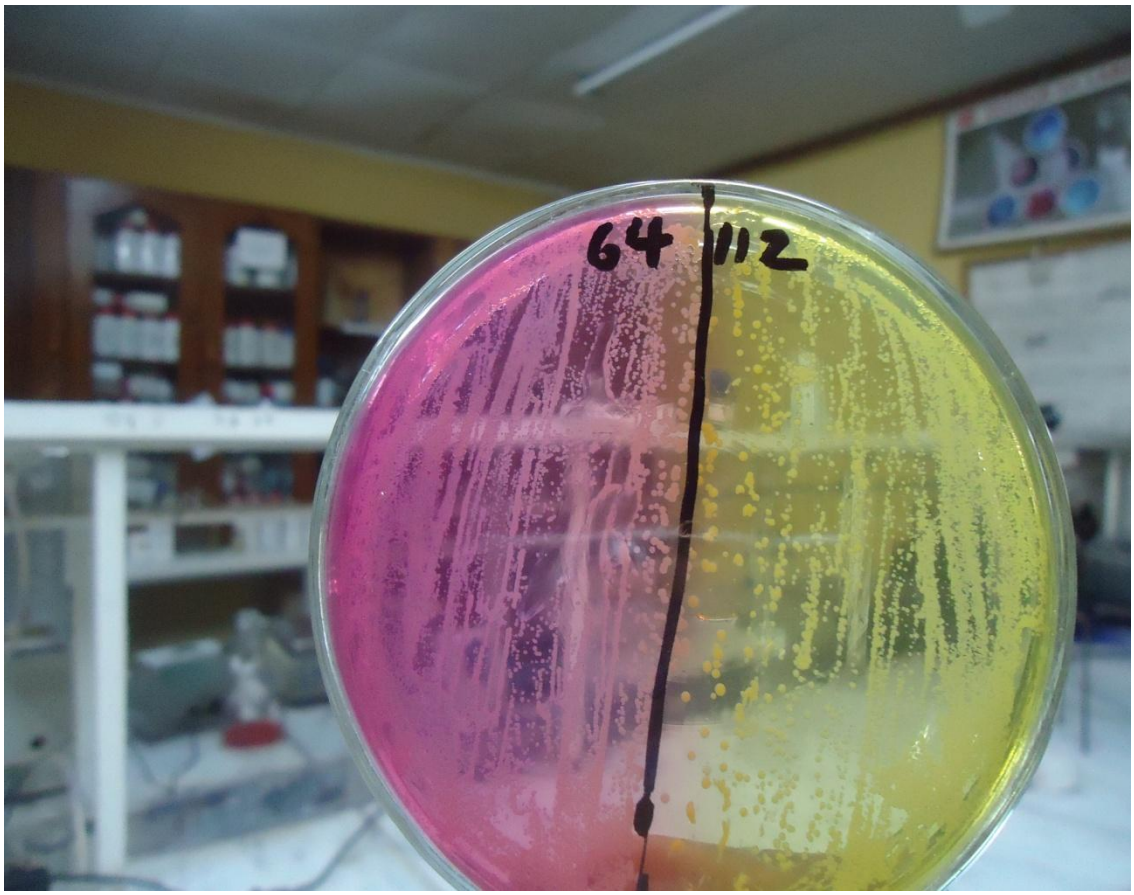


Figure 4: Innoculation of Staphylococcus species on mannitol salt agar

Coagulase test (Quinn et al., 2002)

The tube coagulase test was performed in sterile tubes by adding 0.5 ml of selected isolates of Staphylococcus grown on tryptone soya broth (TSB) at 370C for 24 hours to 0.5 ml of fresh rabbit plasma. After mixing by gentle rotation, the tubes were incubated at 370C along with a negative control tube containing a mixture of 0.5 ml of sterile TSB and 0.5 ml of rabbit plasma. Clotting was evaluated at 30 minutes intervals for the first 4 hours of the test and then after 24 hours incubation. The reaction was considered positive, if any degree

of clotting from a loose clot to a solid clot that is immovable when the tube is inverted (tilted) was visible within the tube and no degree of clotting would be taken as negative.

Annex 4: Antimicrobial susceptibility test

- Preparation of inoculums

Inoculation of distinct colony in to 5ml nutrient broth incubated at 35-37^{0}c for about 8 hours. Then the turbidity is compared with 0.5 Mac Farlandstandard. This standard is prepared by adding 0.5ml of 1 % (11.75g/liter) Bacl2 2H20 to 99.5ml of 1% (0.36N) H_2SO_4.

- Inoculation to Muller- Hinton Agar

Muller-Hinton Agar cooled to 50 ^{o}c and poured into a sterile petridish on level surface to a depth of 4mm. this is equivalent to 60ml in 15cm plate and 25 ml in 10cm plate. Then a sterile cotton swab on a wooden applicator stick is used to transfer the diluted bacterial suspension to a plate; excess fluid must be squeezed out by rotating the swab against the sides of the tube. The plate is seeded uniformly by rubbing the swab against the entire agar surface in three different planes roughly 60 degrees to each others.

- Disc application

Within 15 minutes (time used to dry the inoculums) after the plates are inoculated, antibiotic impregnated discs are applied to the surface of the inoculated plates by hand using a sterile forceps. All discs gently pressed down on to the agar with forceps to ensure complete contact with the agar surface. The disc should no closer than 1.5 cm to the edge of the plate and they should rest 24 mm apart from each other. The large Petridishes accommodate 6 discs in outer ring and three in the center, where as no more than 5 should be placed in small plates (10cm plates). Incubate the plates inverted aerobically for 24 hours at 35^{0}c but not 37^{0}c.

❖ Interpretation

Zone of inhibition is measured in millimeters using a transparent ruler on the under surface of the Petri dish. For measuring purpose, the end is taken as complete inhibition of growth as determined by naked eye. The result is interpreted according to the table presented below.

Table 14: Zone of inhibition interpretation chart for Antimicrobials

Antimicrobial		**In mm (milliliter)**		
Agent	Disc potency	Resistance	Intermediate	Susceptible
Gentamicin	10µg	≤ 12	13-14	≥ 15
Oxacillin	1µg	≤ 17	_	≥ 18
Cefoxitin	30µg	≤ 21	_	≥ 22
Ampicillin	10µg	≤ 16	_	≥ 17
Erythromycin	15µg	≤ 13	14-22	≥ 23
Tetracycline	30µg	≤ 14	15-18	≥ 19
Pencillin	10U	≤ 28	_	≥29
Vancomycin	30µg	≤15	_	≥15

Source: (CLSI, 2018)

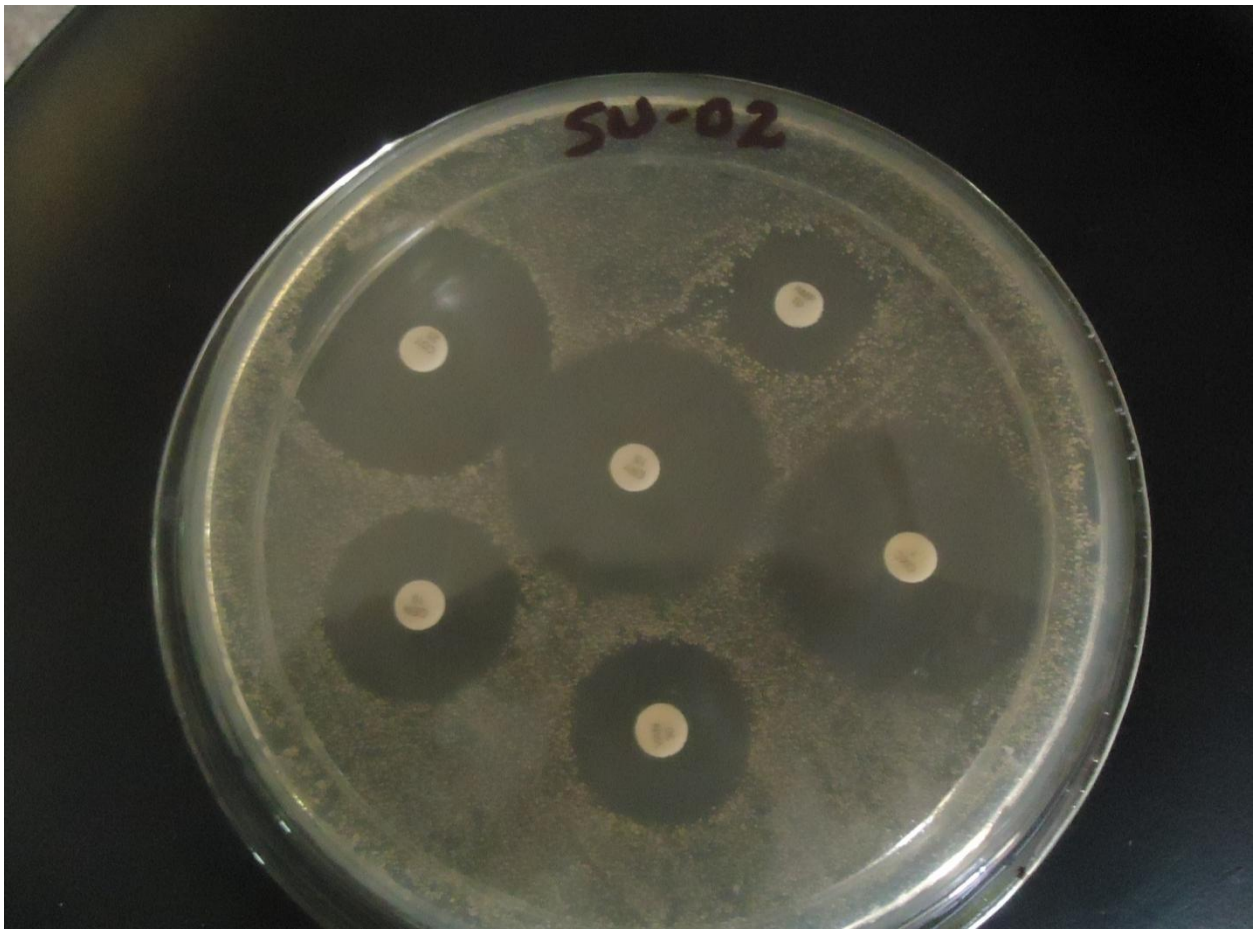
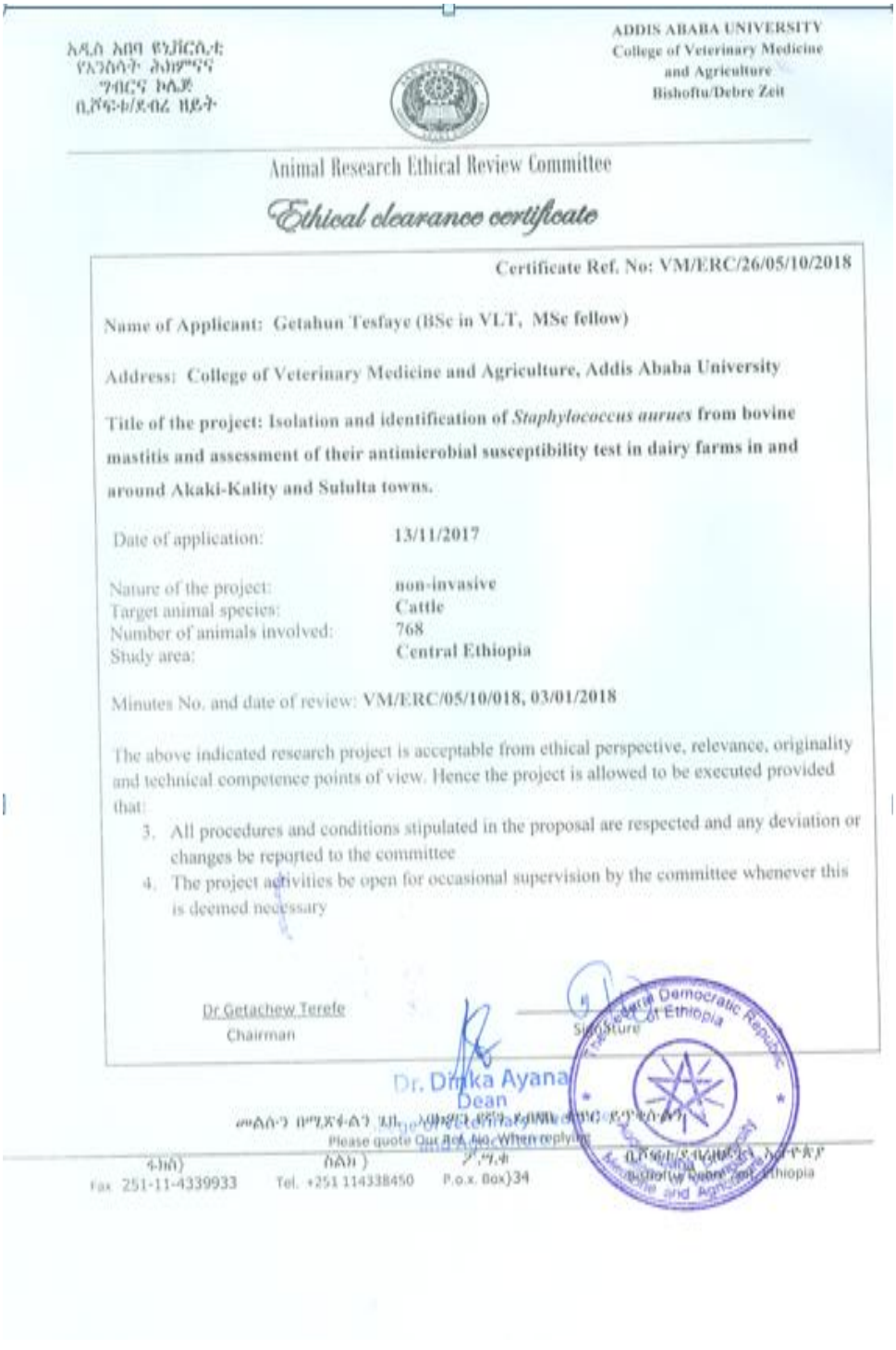


Figure 5: Antimicrobial sensitivity test of *S.aureus*

Annex 5: Ethical clearance



አዲስ አበባ ዩኒቨርሲቲ
የእንስሳት ሕክምናና
ግብርና ኮሌጅ
ቢሾፍቱ/ደብረ ዘይት

ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu/Debre Zeit

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/26/05/10/2018

Name of Applicant: Getahun Tesfaye (BSc in VLT, MSc fellow)

Address: College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: Isolation and identification of *Staphylococcus aurues* from bovine mastitis and assessment of their antimicrobial susceptibility test in dairy farms in and around Akaki-Kality and Sululta towns.

Date of application: 13/11/2017

Nature of the project: non-invasive
Target animal species: Cattle
Number of animals involved: 768
Study area: Central Ethiopia

Minutes No. and date of review: VM/ERC/05/10/018, 03/01/2018

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is allowed to be executed provided that:

3. All procedures and conditions stipulated in the proposal are respected and any deviation or changes be reported to the committee
4. The project activities be open for occasional supervision by the committee whenever this is deemed necessary

Dr Getachew Terefe
Chairman

Signature

Dr. Dinka Ayana
Dean

Please quote Our Ref. No. When replying

ፋክስ) Fax 251-11-4339933
ስልክ) Tel. +251 114338450
ፖ.ሳ.ቁ P.o.x. Box)34