Thesis Ref No ----------

MORPHOMETRICAL, PATHOLOGICAL AND BACTERIOLOGICAL STUDY OF MAMMARY GLAND OF COWS WITH SUBCLINICAL AND CLINICAL MASTITIS IN SELECTED FARMS AND ABATTOIRS IN CENTRAL ETHIOPIA

MSc Thesis

By

Mandefrot Meaza Zeleke

Addis Ababa University, College of Veterinary Medicine and Agriculture,

Department of Pathology and Parasitology

MSc Program in Veterinary Pathology

June, 2016

Bishoftu, Ethiopia

MORPHOMETRICAL, PATHOLOGICAL AND BACTERIOLOGICAL STUDY OF MAMMARY GLAND OF COWS WITH SUBCLINICAL AND CLINICAL MASTITIS IN SELECTED FARMS AND ABATTOIRS IN CENTRAL ETHIOPIA

A thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfilment of the requirements for the degree of Master of Science in Veterinary Pathology

By

Mandefrot Meaza Zeleke

June, 2016

Bishoftu, Ethiopia

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Veterinary Pathology and Parasitology

MORPHOMETRICAL, PATHOLOGICAL AND BACTERIOLOGICAL STUDY OF MAMMARY GLAND OF COWS WITH SUBCLINICAL AND CLINICAL MASTITIS IN SELECTED FARMS AND ABATTOIRS IN CENTRAL ETHIOPIA

Submitted by: Mandefrot Meaza ---------- ----------

Name of Student Signature Date

Approved for submittal to thesis assessment committee

1. Dr. Tilaye Demissie (DVM, MSc. Asst. Prof) ---------- ----------

Major Advisor Signature Date

2. Dr. Getinet Abie (DVM, MSc, Asst. Prof.) ---------- ----------

Co-Advisor Signature Date

3. Dr. Yakob Hailu (DVM, MSc., PhD, Assc. Prof) ---------- ----------

Department chairperson Signature Date

Note that: this page must be detached from the dissertation for final submittal of corrected thesis after defence.

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Veterinary Pathology and parasitology

As member of the Examining Board of the final MSc open defence, we certify that we have read and evaluated the thesis prepared by: Mandefrot Meaza titled **morphometrical, pathological and bacteriological study of mammary gland of cows with subclinical and clinical mastitis in selected farms and abattoirs in central Ethiopia** and recommend that it be accepted as fulfilling the thesis requirement for the degree of master of science in Veterinary Pathology

	Signature	Date
Dr. Hagos Ashenafi Chairman	----------	----------
Dr. Dinaol Belila External Examiner	----------	----------
Dr. Bulto Giro Internal Examiner	----------	----------

Final approval and acceptance of the dissertation is contingent upon the submission of its corrected copy to the CGC through the concerned departmental graduate committee.
I hereby certify that I have read the revised version of this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

	Signature	Date
1. Dr. Tilaye Demissie	----------	----------
Major advisor	Signature	Date
2. Dr. Getinet Abie	----------	----------
Co- advisor	Signature	Date
3. Dr. Yakob Hailu	----------	----------
Department chairperson	Signature	Date

TABLE OF CONTENTS

STATEMENT OF AUTHOR

First, I declare that this thesis is my *bonafide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfilment of the requirements for an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is 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.

Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however permission must be obtained from the author.

Name: Mandefrot Meaza Signature: _____

College of Veterinary Medicine and Agriculture, Bishoftu

Date of Submission: _____

ACKNOWLEDGMENTS

First and for most, I would like to thank almighty God, the provider.

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 am grateful to recognize Dr. Tilaye Demissie, my advisor and the thematic research leader, for his friendly intellectual support since the beginning of the MSc. program. Moreover, I want to extend my gratitude to my co-advisor Dr. Getinet Abie.

My heartfelt thanks go to Mr. Muluken Tekle, Mr. Yosef Cherinet, Mr. Getachew Galera, Mr. Debela Taweya Ms. Azeb and Mr. Misgana Tefera because of their laboratory technical support. I am also indebted to acknowledge Dr. Getachew Terefe, Dr. Mehari Teklu, Dr. Olga and Dr. Teshale Sori for their professional, technical and material support.

I am pleased to thank organizations namely National Animal Health Diagnostic and Investigation Center (NAHDIC), Addis Ababa abattoirs enterprise and Adama municipality abattoir, for their kind reception and facilitation of sample collection and laboratory work.

I want to thank all my friends who stood by me during the research work principally Kalkidan Mamo, Buzayehu Kefenie, Ms. Blen Merid, Biruk Medhanite, Workineh Legesse, Dr. kibrom Mebrate, Dr. Mishamo Sulayman, Dr. Gizachew H/Gebriel and Mitiku Tadesse.

I want to acknowledge Dr. Banteyegegn Tamirat, my friend and classmate, Dr. Abdulkarim Jemal and Dr. Misrak for their unlimited support during the research work.

Finally, my deepest gratitude goes to the beloved Meaza's family, for expressing true family fellowship and support in every aspect when needed.

DEDICATION

I dedicate this thesis manuscript to the beloved Meaza's family, for their true family fellowship and practical influence in my life.

LIST OF TABLES

LIST OF FIGURES

ANNEXES

LIST OF ABBREVIATIONS

ALP	Alkaline Phosphatase
AST	Aspartate Aminotransferase
BCS	Body Condition Score
CMT	California Mastitis Test
CNS	Coagulase Negative *Staphylococcus*
DNA	Deoxyribonucleic Acid
EC	Electrical Conductivity
H and E	Hematoxylin and Eosin
IMI	Intramammary Infection
LDH	Lactate Dehydrogenase
LF	Left Front
LH	Left Hind
MECs	Mammary epithelia cells
MSCC	Mastitic somatic cell count
NMC	National Mastitis council
RF	Right Front
RH	Right Hind
SCC	Somatic cell counts
SD	Standard deviation
Spp	*Species*
TSI	Triple Sugar Iron

ABSTRACT

The cross sectional study was conducted from November 2015 to May 2016 in Bishoftu, Addis Ababa and Adama areas to evaluate cow's udder morphometrical changes in relation to mastitis, mammary gland lesion characterization and isolation and characterization of aerobic bacteria from lesions. A total of 252 dairy cow from selected dairy farms in Bishofu and 72 udders from Addis Ababa and Adama municipality abattoirs were included in the study. The study revealed that the overall prevalence of mastitis was found to be 44.44% with clinical and sub-clinical mastitis accounting 16.11% and 36.67%, respectively. Morphometrically, cows with longer and thicker teats, greater distance between hind teats, Longer glandular longitudinal length and shorter teat end to floor distance had higher frequency of subclinical mastitis and the difference was statistically significant ($P<0.05$). However, udder and teat end shape had no significant association ($P>0.05$) with subclinical mastitis. Distance between hind teats and front udder height had direct statistically ($p<0.05$) association with somatic cell count. Out of 217 quarter milk samples cultured, 147 (67.74%) were positive for single colony, 46 (21.2%) for mixed bacterial growth and 24 (11.06%) were negative for bacterial growth. Predominant aerobic bacteria isolated were Coagulase Negative *Staphylococci* 66 (34.2%) followed by *S. aureus* 63 (32.8%) and *E.coli* 40 (20.7%). On the other hand, from culture result of 53 mammary gland tissue samples collected from abattoirs, the major isolated bacteria were *E. Coli* (35cases), *streptococci* (32 cases), *S. aureus* (23 cases), Coagulase Negative *Staphylococci* (14 cases),, and *S. hyicus* (11 cases). Tissue sections from abattoir showed glandular epithelial degeneration and necrosis, atrophy of alveoli and the glands, intestinal fibrosis, acute to sub-acute suppurative inflammation with neutrophil infiltration and chronic inflammation with mononuclear cell infiltration. Serum alkaline phosphatase analysis did not show significant difference between mastitic and non mastitic cows. In conclusion, mastitis was one of diseases of dairy cows in the study areas which could affect dairy production. Some udder traits could be risk factors to mastitis and chronic mammary lesions characterized might severely decreased milk production and could be reason for culling of dairy cows. Therefore udder traits might be part of dairy cattle selection and improvement programs with detailed further study on the subject in the country.

.

Keywords: *Alkaline phosphatase, Bacterial isolation, Cow, Ethiopia, Histopathology, Mastitis, Somatic cell count, and Udder morphometry.*

1. INTRODUCTION

An increase in the global population coupled with the increasing demands for milk as an economic food and as an industrial raw food product has necessitated an increase in production by dairy farms (Javaid *et al.*, 2009). Milk quantity, quality and production efficiency of cows is directly dependent on the udder health (Szencziová *et al.,* 2013). The udder is the most important part of the body of the dairy cow and its morphological and physiological characteristics affect health of cows and play a vital role in sustainable economic milk production (Gulyas and Ivancsics, 2002; Tilki *et al.,* 2005; Tancin *et al.,* 2007). It is also recognized that the udder characteristics are very important in respect to milk production (Shukla *et al.*, 1997).

Mastitis is defined as inflammation of mammary gland parenchyma, which is caused by bacteria and its toxins. Milk secreting tissue and various ducts throughout the mammary gland are damaged due to toxins by the bacteria (Zwald *et al.,* 2004). Despite intensive research and the implementation of various mastitis control strategies over the decades, bovine mastitis has not disappeared and the reduction in the prevalence of mastitis, especially subclinical mastitis, has been minimal (Rajala-Schultz and Grohn, 1999). Mastitis remains unsolved problem because of its complex aetiology **(**Harby *et al*., 1991).

The disease can also occur as a result of chemical, mechanical or thermal injury to udder sac. The udder sac becomes hard, tight and firm. It is the inflammatory condition of the udder irrespective of the cause and characterized by physical, chemical and microbiological changes in the milk and pathological changes in the glandular tissues of the udder. The change in the milk includes change of colour, change of consistency (clot) and presence of abnormally large number of leucocytes (Zwald *et al.,* 2004). In very severe cases, the infection progressed via the ductile system to produce a limited inflammatory reaction but with an extensive involvement of the secretary tissue (Frost and Brooker, 1986).

However, at least 137 biological infectious agents causing bovine mastitis are known to date (Radostits *et al.,* 2000), bacteria are believed to be the major cause of mastitis in cattle and other

species (El-Rashidy *et al*., 1996). The commonest pathogens responsible for mastitis include Staphylococcus species, Streptococcus species and Coliforms (*Staphylococcus aureus, Streptococcus uberis, Streptococcus agalactiae, Streptococcus dysgalactiae,* and *Escherichia coli (E. coli). Staphylococcus aureus(S. aureous)* and *Streptococcus agalactiae* serve as contagious pathogens while *E. coli, Streptococcus uberis,* and *Streptococcus dysgalactiae* act as environmental pathogens (Radostits *et al.,* 2000; Waller *et al.,* 2009).

Characterization of mammary parenchyma indicates the impact of potential milk production on development and secretory cell differentiation (Capuco *et al.,* 1997). Mammary gland infection may result in release harmful toxins in the udder (Yousaf *et al*., 2010) and lesions may vary from increased milk leukocytes counts with no gross changes in milk to increased vascular permeability (Oviedo-Boyso *et al.,* 2007; Ibrahim *et al.,* 2011) or develop fibrosis or severe toxemia (Costa, 1998). Milk leukocytes/somatic cells consist of several types, including neutrophils, macrophages, lymphocytes and a small percentage of epithelial cells (Abera *et al.,* 2010). The activities of the resident and newly recruited leukocytes during the early stages of mastitis play a vital role in the establishment of intra-mammary infection. Marked leukocyte infiltration during mastitis is detrimental to the developing mammary parenchymal tissue (Nickerson, 2009) ultimately leading to milk loss (Piepers *et al.,* 2009).

Somatic cell counts (SCC) in milk are commonly used as indicators of mastitis, on the basis that an increase reflects an immune response to the presence of infection in the mammary gland (Green *et al.,* 2008). Mastitis is associated with high SCC; this is mostly due to increased leukocyte levels, as they are involved in the removal of the infectious agents, and also exfoliated epithelial cells (Bagnicka *et al.,* 2011). Leukocytes are responsible for destroying bacteria, and the enzymes left behind during the phagocytic process significantly reduce the shelf life of milk and milk products (Ingalls, 2009).

Position, size, shape and anatomical feature of udder, the dairy cows are susceptible to infection and injuries, which results in mastitis. The morphology of teat, especially apex and streak canal are recognized as parts of the passive defence mechanisms against intramammary infection (Shukla *et al.,* 1997). Anatomical characteristics of dairy cattle are not equal for all breeds, in a

way that the udder and teat morphology could favour an individual performance or a determined breed. Due to these differences, some anatomical measurements of mammary glands have been used in research with dairy herds. In the case of improved breeding, the cow's udder has to undergo rapid changes in relation to size, position and adjustment for rapid removal of large volume of milk and such it is prone to injury and infection (Zwald *et al.*, 2004)

Alkaline phosphatase (ALP) is the enzyme found primarily in the epithelial cell membranes of the bovine mammary gland where the active processes take place (Lenhardt, 1984; Lenhardt *et al.*, 1999). Some studies showed that the ALP test was a reliable parameter for the early diagnosis of subclinical mastitis (Babaei *et al.*, 2007; Guha *et al.*, 2012).

For the dairy industry, mastitis is the most costly common disease, and the economic loss due to mastitis in dairy cattle. Losses are in the form of discarded abnormal milk from clinically infected quarters and as the result of antibiotic therapy, cull cow replacement costs, extra labor to handle mastitic cows, antibiotic and other treatment costs, veterinary services, and most importantly, reduced milk production in subclinically infected cows, which contributes to two-thirds of mastitis losses. In fact, a single quarter infected throughout lactation may reduce milk production of a cow by 10 to 12% (Bramley *et al.*, 1996).

Experience in mastitis control indicates that while the occurrence of inflammation in the udder may not be entirely preventable in all cows within herd, the intensity of clinical attacks may be reduced significantly through selection and better management. Information on selection of genetic characteristics related to individual resistance against mastitis and in establishing management of udder health control is a necessary prerequisite (Guha *et al.*, 2012).

There is need to implement different potential measures to decrease mastitis by selection of animals which are less prone to develop infection, culling of more susceptible animals, potential effects of teat-end size, morphology of teat/udder and association of different lesions (Slettbakk *et al*., 1995; Bhutto *et al*., 2010). So, it is important to investigate the relationship of mastitis with udder and teat gross and histopahological appearance of teat with causative agents.

Therefore, the research was aimed at the following general and specific objectives

General objective:

- ❖ To asses association between cow udder morphometrical trait and mastitis in dairy farms and characterization of pathological lesions of mammary gland of cows slaughtered at abattoirs.

Specific objectives

- ✓ To describe association of udder morphometry with subclinical mastitis
- ✓ To isolate and characterize major aerobic bacteria from mastitis
- ✓ To determine gross and histopathological changes of mammary gland of cows
- ✓ To investigate the changes in serum ALP enzyme as a result of subclinical mastitis.
- ✓ To asses pattern of changes in milk somatic cell count in subclinical mastitis in relation to California mastitis test (CMT) scores

2. LITRATURE REVIEW

2.1. Overview on udder and its health

Udder health has been an important component of dairy farming for the last decades. Udder health disorders were always related to a decreased profitability, and an increase in unexpected culling. More recently, udder health is becoming more important due to strict milk quality regulations. This has further increased the attention of the dairy industry towards udder health. Udder health is affected by a number of interrelating components such as presence and pathogenicity of micro organisms, environment and management, cow factors such as conformation and immunological performance, and treatment and prevention strategies (Poso and Mantysaari, 1996).

In order to understand mastitis pathobiology, the mammary gland has been studied with respect to its anatomy, physiology, and the genomics (Ogorevc *et al.*, 2009). The interior of each quarter is composed of a teat cistern, gland cistern, milk ducts and glandular tissue. The secretary portion known as the glandular tissue contains millions of microscopic sacs called alveoli. Each alveolus is lined with milk- producing epithelial cells and is surrounded by muscle cells which contract and squeeze milk from the alveolus during milking. Blood vessels bring nutrients to each alveolus where the epithelial cells convert them into milk. Milk accumulates in the alveolar spaces, milk ducts and cisterns between milking. It is through the teat duct that the accumulated fluid is removed during milking (Seffner and Pfutzner, 1980).

2.2. Mastitis and intramammary infection (IMI)

The inflammation of the udder is mainly caused by intramammary infection (IMI), where different bacterial species enter the udder quarter via the teat canal. The teat end is the first barrier against invading pathogens. The anatomical and physical characteristics of the teat canal (tightness of closure and keratin lining) inhibit penetration of udder pathogens. Approximately 40% of the keratin lining is removed at each milking (Bitman *et al.,* 1991) and, therefore, it

requires constant regeneration. Consequently, it is important to ensure that there is closure of the teat canal post-milking. After bacteria breach the teat end, they are taken up and destroyed by leukocytes and leukocyte chemotaxis is one of the major factors involved in migration of these cells towards the centre of inflammation. Several experimental infection studies have shown a strong relationship between early leukocyte influx and outcome of infection (Hill, 1981; Kremer *et al.*, 1993; Burvenich *et al.*, 1994).

Mastitis is an inflammation of the mammary gland in response to injury for the purpose of destroying and neutralizing the infectious agents and to prepare the way for healing and return to normal function. Inflammation can be caused by many types of injury including infectious agents and their toxins, physical trauma or chemical irritants (Jones and Bailey, 1998). The inflammatory response increases somatic cell count in milk. Somatic cells are very specific, and are only elevated in the mammary once infection occurred (Timms, 1990).

The IMI response associated with mastitis results in a decrease in milk production and decrease in quality of milk and the manufactured products. Besides increasing somatic cell count in milk mastitis results also in an increase of whey proteins, serum albumin, immunoglobulins, chloride, sodium, pH, free fatty acids the milk. Mastitis also results in a reduction in synthesis of the main components of milk, such as lactose, fat, non-fat solids and casein (NMC, 1996).

The different forms of infectious mastitis occur according to the host response and to the micro-organisms which cause the infection. The occurrence and type of symptomathology are related to the pathogenicity of the micro-organism and to its ability to invade tissues, as well as to the resistance of the mammary gland. These factors determine the severity of the symptoms that can vary from increased cell counts with no macroscopic changes in milk to progressive fibrosis or the occurrence of severe toxaemia (Blood and Radostits, 1991).

2.1.1. Classes of mastitis

It is subdivided into clinical mastitis (inflammation with visual signs of inflammation in the udder or milk) and subclinical mastitis (inflammation without visual signs). Both clinical mastitis

and subclinical mastitis influence milk quality and yield negatively, and mastitis is therefore of major economic concern for the farmer. Clinical mastitis is also of potential concern from an animal welfare perspective (Lundberg, 2015).

Clinical mastitis is defined as an infection of the udder that results in visible changes in the udder quarter and milk (Rodenburg, 1990), may it be acute, sub acute or chronic. The development of clinical mastitis in dairy cows can be detected with high sensitivity and specificity in advance of visible changes in foremilk or udder tissue by determining the electrical conductivity of the foremilk (Milner *et al.*, 1997). Clinical mastitis is characterized by visual clots or discolorations of the milk, often in combination with tender and swollen udder, sometimes in combination with fever, loss of appetite *etc* (Bengtsson *et al.*, 2005).

Subclinical mastitis is characterized by changes in milk composition *e.g.* SCC; leukocytes and epithelial cells), and changes in milk pH and ion concentration, without clinical signs of inflammation (Guidry, 2007). In the healthy lactating mammary gland, the milk SCC is often < 100,000 cells/mL of milk, while the SCC can increase to > 1,000,000 cells/ml of milk during subclinical mastitis. The major factor affecting the SCC at the herd and individual level is the presence of IMI (Radostits, 2007).

2.3. Major pathogens, udder defence mechanism and pathogenesis of mastitis

2.3.1. Major mastitis causing bacterial pathogens and pathogenesis

Mastitis can be cause by many pathogens. It can be classified as either major or minor pathogens. The major pathogens can be further subdivided into contagious and environmental infection agents (Radostits *et al.*, 1994). Infection cause by contagious pathogens is transmitted directly from cow to cow. The most common contagious organisms are *Staphylococcus aureus, Streptococcus agalactiae, Corynebacterium bovis and Mycoplasma* species. Contagious microorganisms are well adapted to survival in the udder and usually establish mild clinical infections of long duration aschronic infections (Erskine, 2001).

The main environmental organisms are gram-negative bacteria. The gram-negative bacteria there are *Escherichia coli, Klebsiella spp., Enterobacter spp., Citrobacter spp., Seratia, Pseudomonas species, Proteus and Actinomyces pyogenes.* The environmental *Streptococci* include *Streptococcus uberis, Streptococcus dysgalactiae, and Streptococcus equinus.* Infections caused by environmental pathogens are frequent and of short duration. These infections usually result in clinical mastitis (Smith and Hogan, 1993).

2.3.2. Udder defence mechanism

The antibacterial defence of bovine udder comprises of anatomical features of the gland and humoral and cellular defence mechanisms (Colditz and Watson, 1982) that can be separated into two distinct categories: innate immunity and specific immunity. The optimal protection is the collaborated function of the both above mentioned immune mechanism. Innate immunity, also known as nonspecific responsiveness, is the predominant defence during the early stages of infection. Nonspecific responses are present or are activated quickly at the site of infection by numerous stimuli; however, they are not augmented by repeated exposure to the same insult. Nonspecific or innate responses of the mammary gland are mediated by the physical barrier of the teat end, macrophages, neutrophils, natural killer cells, and by certain soluble factors (Rainard and Riollet, 2006).

The specific or acquired mammary immune system recognizes specific determinants of a pathogen. Activation of specific mammary immune defences results in the selective elimination of mastitis-causing pathogens. Recognition of pathogenic factors is mediated by several lymphoid populations, macrophages, and antibody molecules. Because of the “memory” of certain lymphocytes, specific immune responses can be augmented by repeated exposure to a pathogen. Vaccination of dairy cattle against certain pathogens can occur if specific mammary immune mechanisms are effectively activated (Sordillo and Streicher, 2002).

Keratin present within teat duct is one of the most important limiting factors in penetration of bacteria via teat duct which is major entry site for most of mammary pathogens. The infectious agents that are not blocked by keratin and get entry into gland are attacked by humoral and

cellular defences. The humoral defense comprises of different antimicrobial molecules and enzymatic pathways while cellular defense consists of different types of defense cells including lymphocytes, macrophages and neutrophils (Oviedo-Boyso *et al.*, 2007)

2.3.3. Pathogenesis of mastitis

Intramammary infection results once 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 in case of tuberculous mastitis. 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).

Once the organisms breach the teat duct and the cisternal spaces of the udder, adherence of bacteria to tissues lining the interior of the mammary gland may affect their ability to remain inside the gland, especially during lactation when the contents of the udder are periodically flushed during each milking; up to 4 times a day, or more with robotic milking. Interaction of bacteria with milk leukocytes affects the establishment of infection. In milk of uninfected, healthy mammary glands, macrophages are the predominant leukocyte type, and serve as nonspecific sentinels for the detection of invading pathogens (Sol *et al.*, 2000; Akers and Nickerson, 2011).

After detection of bacteria, macrophages release chemoattractants that recruit polymorphonuclear neutrophilic leukocytes from the vasculature into the area of infection. The polymorphonuclear neutrophilic leukocytes extravasate in large numbers, and initially accumulate around alveoli, with the goal of migrating across the alveolar, ductal, and cistrnal

lumina to contact, engulf, and kill the invading pathogens. Inflammation that ensues in response to bacterial presence is initiated by the release of interferons, interleukins, and tumor necrosis factor (TNF-α) (Akers and Nickerson, 2011)

2.4. Correlation of udder conformation with mastitis

The occurrence of mastitis in dairy herds results from a complex interaction between the host, environment and agent. Generally, the most common risk factors for Clinical mastitis in dairy herds can be divided in two groups: individual cow risk factors and risk factors from the environment. Many authors report risk factors for clinical mastitis associated with farm management, hygiene management, the breeding environment, milking technology, feeding, the calving season and preventive health management (Van Dorp *et al.,* 1999). In an individual herd, cow factors are responsible for the differences among cows in contracting clinical mastitis. A great number of individual cow-specific risk factors for clinical mastitis have been identified, including breed, parity, period of lactation, udder and teat morphology, age at first calving, milk leakage, udder edema, milk production, number of milk somatic cells and reproductive disorders (Peeler *et al.,* 2000; Nyman *et al.,* 2007; Valde *et al.,* 2007).

It is well established that a favourable association exists between mastitis resistance and several udder type traits. The literature data are generally similar about the genetic correlation between udder depth, udder attachment to the cow's body, milk production and association of these factors with mastitis incidence (Sorensen *et al.,* 2000; Klein *et al.,* 2005; Ptak *et al.,* 2011). Several studies have identified udder and teat conformation as risk factors for clinical mastitis (Rupp and Boichard, 2003; Bhutto *et al.,* 2010; Singh *et al.,* 2013). According to them, cows with less desirably shaped udders and more udder depth are more susceptible to lesions and contamination by mastitis-causing pathogens which increase the risk of mastitis. However, it should be emphasized that clinical mastitis also influences udder and teat morphological characteristics (Klaas *et al.,* 2004).

Teats and teat tips conformation in female cattle can be classified according to their shape, ranging from undesirable to desirable shapes. Cows with teats and teat tips with undesirable

conformation are more susceptible to injury and infection by pathogens, increasing the risk of mastitis. The udder teats are the first line of defence against IMI. The probability of mastitis occurring varies considerably between different teat shapes, sizes, teat placement and the morphology of the teat tip (Bardakcioglu *et al.*, 2011). In any case, there is no consensus in the literature about the influence of teat morphology on mastitis occurrence (Haghkhah *et al.*, 2011; Singh *et al.*, 2013). Some studies have reported that decreasing teat-end to floor distance is a risk factor for clinical mastitis (Singh *et al.*, 2013). Also, an increasing proportion of teat lesions, with decreasing teat end to floor distance, is a well-documented risk factor for mastitis (Bhutto *et al.*, 2010).

Prospective longitudinal study by Nakov *et al.* (2014) has shown that individual cow factors, along with farm management, are important in influencing the risk of clinical mastitis during lactation, and these factors indicate different susceptibilities to clinical mastitis from animal to animal. The conformation udder traits are strong arguments that can be used to improve udder health. Therefore, it has been suggested that selection of cows with desirable udder and teat morphology might help reduce the incidence of mastitis and improve milk quality. The distance between the front teats of the cow's udder, difference of productivity between udder quarters and the difference of productivity between udder quarters influenced sub clinical mastitis rate of cows (Slyzius *et al.*, 2014). A study done by Porcionat *et al.* (2010) on Gir cows revealed that teat end to floor distance is related to SCC.

2.5. Histopathology and Evaluation of Serum ALP activity of mammary gland

When there is presence of microbial infection in the mammary gland of the dairy cow has been shown to decrease milk yield, but how is this reduction in milk synthesis brought about? Paradoxically, it is believed that the host immune response and toxins produced by mastitis causing bacteria are the causal mechanisms that have deleterious effects on mammary tissue. The mammary tissue response to mastitis in dairy cows as a result of both natural and experimental infections has been quantified using histological and cytological techniques. In general, quarters with natural infections demonstrate reduced ability of secretary tissue to synthesis and secrete

milk; namely, decreased percentages of tissue areas occupied by alveolar epithelium and lumina, and increased interalveolar stromal areas (Akers and Nickerson, 2011).

2.5.1. Histopathology of mammary gland of cows

The histopathological features, due to the direct action of pathogens or due to the indirect action of the inflammatory response, are relevant aspects although there is sparse international literature on this subject (Stabemfeldt and Spencer, 1965; Jubb *et al.,* 1993; Zarkower and Norcross, 1966 ; Benites *et al.,* 2002). The histopathological changes including number of alveoli, alveolar diameter and secretary alveolar cell population significantly decreased in mastitic cattle. These results indicated pathological changes occurring in udder tissue and could be due to severe tissue damage due to different mastitis pathogens (Hussain *et al.,* 2012)

Histological analyses have been widely used since the 1970s and are still being used today for assessing damage to secretory tissue in the bovine mammary gland caused by mastitis pathogens. Benites *et al.* (2002) revealed 96.9% (n=184) of samples showed inflammatory response. According to Zhao and Lacasse (2007) lesions of the breast tissue reduces the number and activity of epithelial cells and therefore contributes to lower milk production with increasing proportions of lymphocytes and macrophages and a decrease cell number of polymorphonucléaires. Chandler (1973) examined mammary parenchymal tissue samples from lactating cows infected naturally with S. aureus and reported a massive polymorphonuclear neutrophils infiltration and necrosis of secretory tissues.

According Trinidad *et al.* (1990), mammary glands infected with *Staphylococcus aureus,* showing an increase of connective tissue inter-cellular and a reduction in epithelial cells and alveolar lumen. Kheira and Abdellatif (2014) found 34.61% of samples showed inflammatory lesions different from the disappearance of the alveolar lumen, through fibrosis to the complete destruction of the parenchyma. When the infection persists and the channels are blocked, the milk within the alveoli increases the pressure there, the secretary cells lose their ability to synthesize and the cells begin to atrophy. Substances released by white blood cells cause destruction of cellular structures, which are replaced by scar.

Histopathologically, the udder section from healthy cattle showed no pathological lesions and the milk secretion was observed in alveoli. However, the tissue sections from mastitic animals revealed mild, moderate or severe atrophy of alveoli. The cellular exudate in udder tissue was present in the lumen of the alveoli in varying amounts in a number of cases. The acute inflammatory changes were recorded in 56% and chronic inflammation in 44% cattle. Infected mammary parenchymal tissues showed destruction of alveoli and fibrous tissue proliferation. Cellular infiltration mainly was observed in different areas of mammary tissues such as in teat cistern lining, gland cistern and deep parenchyma. Mastitic udder showed significantly lower alveolar epithelial cell population, alveolar luminal diameter and number of alveoli per plate (Hussain *et al.,* 2012).

The study done by Benites *et al.* (2002) evidenced for repair process replaces permanently the glandular tissue by connective tissue and consequently leads to reduction in milk production. In this study, it was observed that 71 (55.5%) of the samples which had positive microbiological results, showed inflammatory response associated to repair and 10 (7.8%) samples showed only repair process. Therefore a total of 81 (63.3%) of these samples showed loss of parenchyma, the area where milk is produced, due to the repair process (fibrosis and/or cystic dilatation).

Some recent reviews on bovine mastitis and its impact on structure and function in the ruminant mammary gland covers the most aspects of direct or indirect damage caused to mammary tissue by mastitis (Zhao and Lacasse, 2007; Akers sand Nickerson, 2011). Mastitis is a multi-etiological disease (El-Metwally and Asfour, 2008) and the S. *aureus* is considered to be an important root cause of acute mastitis (Sharma *et al.,* 2007; Sharma *et al.,* 2010). This bacterium produces a toxin that destroys mammary endothelial cells (MECs) and permanently damages milk-producing tissue (vesicle), resulting in complete loss of milk production due to fibrosis.

In severe and chronic intramammary infections the secretary tissue changed into non-secretory or fibrous tissue (Kumar and Singh, 2011). Hence, during and immediately post mastitis, the infected udder has less of alveolar epithelium, adipose tissue, luminal areas and it has more inter-alveolar connective tissue. Ensuingly, there is reduction in milk secretary activity, resulting in drastically reduced milk production (Nickerson, 2009). It is a well-established fact that milk

production in dairy cattle is a function of number and activity of MEC and these factors can be influenced by diverse environmental and management practices (Singh *et al.,* 2010).

2.5.2. Evaluation of ALP as Indicator of Subclinical Mastitis

A positive diagnosis of mastitis should fulfil two criteria a positive bacteriological test and an inflammatory change. Bacteriological diagnosis has been a standard method of examination for mastitis, but inflammatory reactions in the mammary gland may originate from mechanisms other than infection; bacteriological examination of milk has not been fully reliable for detecting mastitis and it is difficult to avoid contamination during sampling (Babaei *et al.*, 2007). Cell counts have generally been used for the latter purpose. The quantification of cells in milk, or somatic cell count (SCC), is estimated using direct microscopic or electronic cell counters or by an indirect method of estimating SCC using the CMT. The CMT is the most commonly used cow-side test for measurement of SCC in milk. It is well developed for cattle, and is most accurate in this species (Donovan *et al.*, 1992).

The invasion of polymorphonuclear leukocytes and macrophages is one of the essential body defences against clinical and subclinical mastitis. During the inflammatory process, these cells and damaged cells of the udder's epithelial and interstitial cells secrete products that include hydrolytic enzymes. These enzymes can be non-lysosomal enzymes, such as lactate dehydrogenase, or lysosomal enzymes such as β-galactosidase (Oliszewski *et al*., 2002).

The enzymes Lactate dehydrogenase (LDH), alkalinePhosphatase (ALP) and aspartate Aminotransferase (AST) are secreted by the epithelial cells of mastitic mammary gland. In mastitis, muscle and tissues of mammary gland are damaged which may lead to increase in the level of these enzymes (Khodke *et al*., 2009). Alkaline phosphatase is a widely distributed enzyme consisting of several different isozymes that hydrolyze a variety of phosphates at alkaline pH. Although the physiological function of alkaline phosphatase has not been identified, the enzyme is located primarily on cell membranes of tissues where active transport processes occur, e.g., brush border of small intestinal epithelium, brush border of proximal tubules of kidney, and canalicular domain of hepatocyte plasma membrane (Harris, 1989). It is observed

high level ALP enzyme activity in mastatic milk as well as non specific mastitis showed high milk enzymatic activity (Zeinhom *et al.*, 2013)

Alkaline Phosphatase is the enzyme found primarily in the epithelial cell membranes of the bovine mammary gland where the active processes take place (Lenhardt, 1984; Lenhardt, 1999). It was observed that the activity of ALP was significantly increased in cows affected with mastitis. Katsoulos *et al.* (2010) reported that the origin of elevated ALP activity was from leukocyte and mammary epithelial and interstitial cells damaged during inflammation, particularly from disintegrated leukocytes. Which may occur by physical and mechanical irritation by lactation, but Zeinhom *et al.* (2013) observed high level ALP enzyme activity in mastatic milk as well as non specific mastitis showed high milk enzymatic activity. Also Babei *et al.* (2007) reported that the mean activitiy of ALP was higher in the milk from udders with subclinical mastitis than in the milk from healthy udders. The results showed that the ALP test was a reliable parameter for the early diagnosis of subclinical mastitis.

Several decades back, it is known that there is close relationship between ALP activity and bovine chronic mastitis. Alkaline phosphatise is an enzyme which is found primarily in the cell membranes of the cow's glandular parenchyma. This is a very important part of the mammary epithelium, because of the active transport processes. The alkaline phosphatase activity in the epithelial cells of the alveoli of the diseased glandular tissue was significantly increased in comparison with the controls. The alkaline phosphatase activity was stated in the epithelial cells from cows from the diseased udder. In the group of animals with healthy mammary gland, the activity of ALP was significantly lower (Pukačova *et al.*, 2010).

Although the origin of the increased ALP activity in mastitic milk has not been determined, the results strongly suggest that the origin of the elevated LDH activity is from leukocytes found in mastitic milk and from mammary epithelial and interstitial cells that damaged during the inflammation process. Changes in enzyme activities in blood or other biological fluids such as milk can be a consequence of cell structural damage. Activities of LDH and ALP were higher in milk with high CMT scores than in normal milk. Similar results have been reported previously researchers in the last decades Babaei *et al.* (2007).

Babaei *et al.* (2007) revealed that higher LDH and ALP activities were observed in milk from subclinical mastitis udders but only the ALP test was sensitive and reliable enough for the early diagnosis of subclinical mastitis. It would appear that the CMT is still the gold-standard screening test for subclinical mastitis, but measurement of enzyme activities appears to be a suitable diagnostic method for identifying infected mammary glands in early lactation or selective dry cow therapy.

2.6. Diagnosis of mastitis

Early diagnosis of mastitis is important for reducing production losses and for enhancing the prospects of recovery. In addition, the identification of subclinically infected gland is urgently required for successful control of mastitis in dairy animals. While farmers can recognize clinical mastitis, subclinical mastitis can only be discovered by detecting of an inflammatory components and pathogens in the milk (Nielen *et al.,* 1995).

In the past many methods that have been developed for the diagnosis of mastitis include Visual method, Direct method, Indirect method, CMT method, SCC method, Simplified Resazurin Rennet Test, Stir cup test, Surf filled mastitis test, Bromothymol Blue test, Modified Whiteside test, Wisconsin Mastitis test, Electrical Conductivity test and Culture method test (Sandholm, 1995).

2.6.1. Visualization and Palpation of the udder

Regular examination of the udder especially during milking is the primary step in preventing mastitis. With one or two episodes of mastitis, the animal attendant would be able to monitor the physical changes due to mastitis or any other infection. In clinical mastitis, visually the udder may turn red, hard and n hot to touch. Udder may be painful to the cow at the time of palpation. These symptoms show the changes in vascularity and blood flow of the gland when inflamed. On collection of the sample, the presence of pathogen is screened (Milner *et al.,* 1996).

2.6.2. Qualitative examination of milk

According to Quinn *et al.* (1994) changes in color of milk can be caused by the presence of blood (red or brownish) or pus (yellow). The consistency may be increased, resulting in thicker, "sticky" milk, or it may be more than usually watery. Flakes and clots are always abnormal. The smell of the secretion may also be altered as a result of mastitis.

2.6.3. California Mastitis Test (CMT)

One of the oldest and best known is the CMT. It is based on the principle that the addition of a detergent to a milk sample with a high cell count will lyse the cells, release nucleic acids and other constituents and lead to the formation of a ‘gel-like’ matrix consistency. However, the interpretation can be subjective, and this might result in false positives and negatives (Viguier *et al*., 2009).

This practical test was developed in the 1950's during a California testing program; it gives a measure of the SCC of the sampled milk. The reagent (3% sodium lauryl sulphate is often used) is a detergent which ruptures somatic cells in the milk, thereby releasing Deoxyribonucleic acid (DNA). This forms a precipitate with other serum components, fat particles and the CMT reagent, causing visible gelling of the milk. A pH-indicator (for example bromcresol purple) may be added to the reagent. The test procedure is simple and straightforward: after the stripping milk is discarded, a few streams of (fore) milk from each quarter are milked into four plastic dishes set on a paddle. The paddle is then tipped nearly vertically to drain excess milk. An equal volume of the reagent is added from a plastic squeeze bottle and the two components mixed by swirling (Quinn *et al*., 2002).

2.6.4. Somatic cell count (SCC)

In a normal, non infected and non inflamed quarter, the somatic cell population consists of polymorphnuclear cells, macrophages, lymphocytes and epithelial cells (Concha, 1986). The majority of cells are macrophages (approximately 60 % of cells), lymphocytes are approximately

30 % of cells, and polymorphonuclear cells form approximately 10 % of the population, the epithelial cells are about 2 % (Sandholm, 1995).

Barkema *et al.* (1998) opined that bulk milk SCC was related to management practice. Herds, managed by farmers who worked precisely, paid more attention to individual cows and implemented measures to prevent mastitis, more often had a lower bulk milk SCC. Schukken *et al.* (2003) opined that the somatic cells in milk play an important role in the innate immunity of the uninfected mammary gland. A complete absence of cells would put cows at risk for disease and a very low concentration of somatic cells increases the risk of clinical mastitis.

In milk from healthy uninfected glands, the cell count is typically <200,000 cells per ml. Most of these cells are lost epithelial cells, macrophages, and neutrophils. With the onset of inflammation, dramatic increases in mastitic somatic cell count (MSCC) are accounted for by increases in neutrophils. For most dairies, mastitis monitoring depends on evaluation of apparent udder health at the time of milking i.e. appearance of the udder and foremilk, periodic (typically monthly) measurement of MSCC in milk samples collected as part of production evaluation programs (milk yield, as well as concentrations of protein and fat), and in some situations measurement of milk electrical conductivity and lactose concentration. The MSCC is valuable because increases in MSCC are typically the result of an influx of neutrophils into the milk from the blood stream. The relationship between MSCC and milk production loss was established by a many workers in the 1970s and 80s as illustrated in the Jones *et al*, (1984).

2.6.5. Culture method

The surest way of diagnosing mastitis is by directly isolating and identifying any pathogenic microorganisms which may be present in the milk. This can be achieved by cultural methods and a number of additional determinative tests. To obtain correct results and avoid contamination and hence bias, it is important to work as securely and as accurately as possible under the circumstances. Similarly, the procedure of routine mastitis testing should be standardized and work protocols instituted (Quinn *et al*., 1994).

2.6.6. Electrical conductivity (EC)

Electrical conductivity is now employed as a routine test for subclinical mastitis detection (Milner *et al.*, 1996). This is influenced by sodium, potassium, calcium, magnesium, chlorine and other ions. EC of the milk increases due to an increased concentration of Na+ and Cl- . However, factors other than mastitis, like breed, lactation stage, milking interval and milk composition may affect milk electrical conductivity. Moreover, many dairy producers especially those who still adopt hand milking, may not depend on electrical conductivity as a routinely test for detection of subclinical mastitis.

2.7. Prevention, treatment and control of mastitis

Effective and economical mastitis control programs rely on prevention rather than treatment. Nonetheless, therapeutic intervention is an important part of a control program for bovine mastitis. Therapy of infectious disease should either assist host defences in eliminating invading pathogens and/or reduce the patho-physiologic consequences of infection (Compton *et al*., 2007). Logically, research emphasis and clinical application of antibacterial for therapy of mastitis has focused on the elimination of infectious agents.

Selecting clinical mastitis treatment is generally based on clinical signs, number of episodes and the likelihood of response. Clinical mastitis can be scored by severity with a score of 1 given when only the milk is abnormal, a score of 2 given when milk and quarter appearance are abnormal, and a severe score of 3 given when the animal is sick (Sears and McCarthy, 2003). Clinical mastitis treatment should include supportive therapy, milk-out, and observation until culture results are available the following day. Antimicrobial therapy is pivotal for its containment and recovery. Despite the wide spread use of these drugs, antimicrobial treatment of mastitis has been less effective than desirable (Sears and McCarthy, 2003; Biffa *et al*., 2005).

The fundamental principle of mastitis control is that the disease is controlled by either decreasing the exposure of the teat ends to potential pathogens or by increasing resistance of dairy cows to infection. Controlling mastitis is not a matter of doing just one thing. Instead, it involves following a number of steps better referred to as a control program. To be acceptable, such a

program must be economical, practical, effective under most management conditions, and reduce new infections. The program should also shorten the duration of pre-existing infections, reduce the incidence of clinical mastitis, and be subject to easy modification as improved methods are developed through research. Fortunately, working toward the reduction of clinical mastitis is not in conflict with the long range objectives of a control program because most clinical cases are preceded by sub clinical cases. Thus, a reduction in the clinical mastitis is good evidence that the unnoticed sub clinical infections are also being reduced (Reinard and Riollet, 2006; Ferguson *et al.*, 2007).

3. MATERIAL AND METHODS

3.1. Study area and study animals

The study was under taken from November 2015 until May 2016 in Bishoftu, Addis Ababa and Adama towns. Farm level udder morphometrical study and collection of samples for isolation of bacterial pathogens from milk and ALP Enzyme activity measurement were conducted in Bishoftu town, whereas cow's udder gross examination and samples for histopathological characterization and mammary tissue bacterial culture were brought from Addis Ababa and Adama municipality abattoirs.

Bishoftu is located at 9°N and 40°E, in Oromia National Regional State about 47 km southeast of the capital city of Ethiopia, Addis Ababa. It has a human population of about 95,000. The altitude is about 1850 m. a. s. l. It experiences a bimodal pattern of rainfall with the main rainy season extending from June to September (of which 84% of rain is expected) and a short rainy season from March to May with an average annual rainfall of 800 mm. The mean annual minimum and maximum temperatures are 12.3 and 27.7^{o}C, respectively, with an overall average of 18.7°C. The highest temperatures recorded in May and the mean relative humidity is 61.3%. Bishoftu is the center of Ada'a-Liben Woreda. The majority of dairy producers in Bishoftu town are market oriented smallholder dairy farmers with average herd size of three cows which are organized under, one dairy cooperative called, Ada'a milk and milk products marketing cooperative share company. There is also few government and private owned large (commercial) scale dairy farms with milking heard size of over 50 in the town. Among these farms, several farms having crossbred (Hollistain Fressian X local) cattle, variable number of herd size and different managemental status were visited for the research purpose.

Addis Ababa Abattoirs Enterprise, located in capital city Addis Ababa, slaughters several species of animals including sheep, goat, cattle and pig. These animals were brought from all parts of the country majorly coming from Harar and Borana areas (mostly local breeds) and crossbred cows were most importantly brought to the abattoir from intensive and semi-intensive urban or peri-

urban dairy farms in Addis Ababa and neighbouring areas. The main reasons for slaughtering of female cattle at the abattoirs were old age, udder problem, reproductive and related problems.

Adama municipality abattoir is located at Adama town, central Ethiopia in Oromia regional state about 99 kilometres from Addis Ababa. Animals slaughtered were brought to the abattoir from in and around Adama town and Arisi as well as Bale areas.

3.2. Sampling and study Design

To pursue the intended objectives a cross-sectional study design with purposive sampling methods was followed. A total of 252 dairy cattle were examined for mastitis (clinically and by CMT) and udder was measured morphometrically from dairy farms in Bishoftu. The cattle visited at Bishoftu farms were crossbred (Holistain Fressian X local breed) with variable degree of exotic blood lever from 50% to high blood level. Moreover, a total of about 72 udders were collected from Addis Ababa and Adama municipality abattoirs for gross pathological, histopathological and bacteriological study during the study period. Among the total collected udders, about 19 were excluded from the study because of extra small size or heifer's udder.

Information about some parameters including age, parity, stage of lactation, milk yield and related once were collected by interviewing owners or persons directly responsible for handling of animals or recorded data (if any). Body condition score was measured at farm during visitation for morphometrical measurement and scored on scale from 1-5 categorised as good (BCS 3, 4and 5) or poor (BCS 1 and 2) according to annex II.

3.3. Macroscopic examination and biometric data collection

The udders of all animals in the farm were clinical examinations for clinical mastitis, presence of any gross lesion and morphometricaly evaluated before evening milking. For cows at abattoir, examination was conducted at antemortem and after slaughter the whole udder was removed from its base (Shukla *et al.*1997; Bhutto *et al*., 2010).

The morphological indices of the cow's udders with subclinical (CMT and SCC) mastitis were evaluated in relation to their physiological status. In this study stage of lactation, mastitis history, presence of udder lesion, parity, udder quarter location, milk yield and age were evaluated as independent variables.

Gross measurements of the mammary glandular morphometry (udder conformation or shape, glandular longitudinal length and glandular mid-circumference) and teat morphometry (teat length, diameter, end conformation and lesions) were carried out. In addition, teat end to floor distance, the distance between teats (fore, rear and right, left) and were measured from animals in selected dairy farms in Bishoftu. The mammary glands of all the animals were analyzed for presence of any gross lesions and recorded following Bhutto *et al*. (2010).

Udder shape and teat-end shape were evaluated through visual examination in the study. Udder shape was evaluated and classified into pendulous and regular or normal udder type. Whereas, Teat length and teat diameter were measured with vernier caliper for every quarter of experimental animals in the beginning of the investigation. Teat length was taken as the distance from the base of the teat to the end of teat while the diameter of teat was measured at the mid length of the teat (Bhart *et al.*, 2015)

The udder shape of each cow were evaluated and classified into pendulous and regular or normal udder type and teat-end shape was categorized as flat, inverted, and pointed according to Bhart *et al.* (2015). Glandular longitudinal length and glandular mid-circumference were also carried out using a measuring tape in centimetre (cm).

Teat length of all teats were taken as the distance from the base of the teat to the end of teat (cm) (Deniz, 2013) and teat diameter (all quarters) were measured with vernier calliper and measured at the mid length of the teat (mm). The shortest distance from teat end to the floor and the distance between teats (fore, rear and side) were also measured by measuring tape and recorded in centimetres (Deniz, 2013; Bharti *et al.*, 2015).

3.4. Sample collection and transportation

Milk samples were collected by thoroughly washing the udder with clean water and towels soaked with antiseptic solution were used for wiping off the udder and teats and allowed to dry and first 2-3 streaks of fore milk were discarded before collection of milk samples. Then, milk samples were collected aseptically in sterilized plastic bottles for milk total somatic cell and bacteriological culturing (Quinn *et al.,* 1999). The collected samples were brought to the laboratory immediately for further analysis by ice box.

In the abattoirs, after slaughtering, the udder was incised quickly and mammary parenchyma tissue samples were collected and divided into two parts, one part put in a sterile glass universal bottle containing saline water in an ice box under aseptic conditions for bacterial isolation and the second part was immersed in 10% neutral buffered formalin saline for histopathological evaluation. Any milking cows at abattoir will be screened for subclinical mastitis (CMT) (Hussain *et al.,* 2012).

*3.5.*Laboratory examinations

3.5.1. California mastitis test (CMT)

The California mastitis test was carried out as described by Quinn *et al*. (2002) as it is shown on annex III. As quirt of milk, about 2 ml from each half was placed in each of 2 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. Based on the thickness of the gel formed by CMT reagent-milk mixture, test results will be scored as 0 (negative/trace), +1 (weak positive), +2 (distinct positive), and +3 (strong positive). Positive CMT-cows were defined as having at least one CMT-positive quarter.

3.5.2. Somatic cell counts

SCC was done by direct microscopic method. Milk film preparation, staining and counting were done according to the standards set by International Dairy Federation (IDF, 1995). To obtain a uniform distribution of cells, milk samples were mixed by moving up side down gently 25 times and allowed to stand for 2 minutes to permit air bubbles and foam to disappear. Microscopic slides will be degreased with alcohol before milk film preparation. A 0.01ml of milk will be taken with a 50µl micropipette calibrated at 10 and spread evenly over one square centimetre (cm^2) area on a microscopic slide and allowed to dry at room temperature on a leveled table. One cm2 area was delineated by a slide template collected from Ethiopian meat and dairy development institute prepared from a cap board. Dried films were stained by Levowitz-Weber Modification of the Newman-Lampert Stain by submerging or flood slides in the stain for 2 minutes and drain off excess stain by resting edge of slide on absorbent paper . Thoroughly dry air dry and then, dip dry stained slides in 3 changes of tap water at 35-45ºC and finally drain and air dry slides before examining smears. Levowitz-Weber Modification of the Newman-Lampert Stain was prepared by using 0.6 g certified methylene blue chloride, 52 ml of 95% ethyl alcohol, 44 ml of tetrachloroethane and 4 ml of glacial acetic acid (Atasever and Erdem, 2013)

According Dhakal (2006) somatic cells were counted to under 1000X magnification using oil immersion, and calculated as follows: as 0.01 ml of milk was spread in 1 cm2 the possible number of such fields which could be counted in 1 cm2 was 4000. Milk volume represented by each field was 1/100 · 1/4000 ¼ 1/400 000. Hence microscopic field (MF) was 400 000. Total number of fields counted was 50. The working factor (WF) was 400 000/ 50 ¼ 8000.

Then, SCC/ml = 8000 X number of cells counted (50 fields per sample)

3.5.3. Serum ALP enzyme analysis

The enzyme activity of ALP in serum samples was done by collecting of ten ml of blood and centrifuged. ALP Enzyme activity was estimated using commercial kinetic assay test kits. The standardized protocol provided with the kit was followed for estimation. The final results were

reported in units per litre of blood serum (U/L). Serum activities were determined using a human 800 biochemical automatic analyzer as per Mohamed (2014).

3.5.4. Bacteriological culture

For the case of bacterial isolation mammary tissue and milk samples were collected. Tissue samples collected from parenchyma of the mammary gland at the abattoir were dipped in alcohol and sterilized with a flame three times. Subsequently, they were grounded in a sterile porcelain recipient containing sterile saline solution (0.85%) and then enriched with brain and heart infusion broth for 24 h at 37^0C. The collected specimens were cultured into nutrient broth for 24 h at 37°C. A loop full was inoculated onto the following media, 5% sheep blood agar, Mac-Conkey and nutrient agar (Quinn *et al.*, 1999).

Milk samples from CMT reactive quarters were used for culture due to the shortage of resources. Milk samples that had been refrigerated, dispersion of bacteria and fat were accomplished by warming the samples at room temperature (25°C) for about an hour and then mixed by shaking. The samples were allowed to stand for a while for the foam to disperse and just before inoculation the tube was inverted gently. One standard loop (0.01ml) of milk sample was streaked on 5% sheep blood. Identification of mastitis pathogens was carried out following microbiological procedures for diagnosis of bovine udder infection described in Quinn *et al.* (1999).

For primary identification, colony size, shape, color, hemolytic characteristics, Grams reaction and catalase production were used (NMC, 1990). Each culture was subjected to gram staining to determine their shape, and gram reaction. Catalase test using 3% Hydrogen per oxide (H_2O_2) was performed to identify catalase positive and catalase negative bacteria. Mannitol Salt Agar and purple base agar with 1% maltose were used to differentiate staphylococcus species and incubated at 37°C and examined after 24–48 hrs. for mannitol and maltose fermentation respectively. Tube coagulase test using rabbit plasma was used to identify the coagulase positive and coagulase negative *staphylococcus* species. Enterobactericae species were identified using

Oxidase test, indole test after addition few drops of kovacs reagent and motility test, TSI (Triple Sugar Iron) to detect sugar fermentation, MacConkey agar for lactose fermentation and colony characteristics and Simmon's citrate agar to differentiate bacteria based on citrate utilization.

For confirmation the suspected colonies were picked up and sub cultured onto the selective media for identification by microscopic examination and biochemical reactions using standard bacteriological methods according to (Quinn *et al*. 2004) indicated on annex IV. Then, Interpretation was made according to NMC (1990).

3.5.5. Histopathological investigations

Tissue processing was conducted following the procedure described by (Talukder, 2007) as it is indicated on the annex I. Tissue were trimmed, fixed in 10% buffered neutral formalin, dehydrate in ascending grade of ethyl alcohol, alcohols, clear with xylene andimpregnating with paraffin wax the tissue is infiltrated with the embedding agent, almost always paraffin. Then, applied tissue section at 5 μm thickness spread on warm water bath and attaching the tissue to glass slide. The slide was incubated in incubator at 60^0c to avoid paraffin wax. The sectioned tissues were deparaffinised in three changes of xylene, rehydrated in descending grades of alcohol and stained with Routine stain Haematoxylin-Eosin (HE) and finally examined under microscope using 10 x 20x, and 40x magnification and photomicrographs are considered.

3.6. Data management and statistical analysis

Data generated from morphometrical study and laboratory investigations were recorded and coded using Microsoft Excel spreadsheet (Microsoft Corporation) and analyzed using STATA 13.0 (Stata Corp LP, College Station, TX, USA). Descriptive statistics such as Mean, percentile, minimum, maximum and standard deviations were used for subclinical and clinical mastitis, SCC, udder and teat measurements and enzyme activity. The association of different risk factors with dependent variables such as matitits positivity and SCC was analyzed by using $\chi 2$ (Chi-square) technique for screening significance and Odd's ratio (OR) for correlation as univariate

and multivariate regression analysis (p- value consideration as significant was calculated at alpha 0.05 or 95% level of confidence).

4. RESULTS

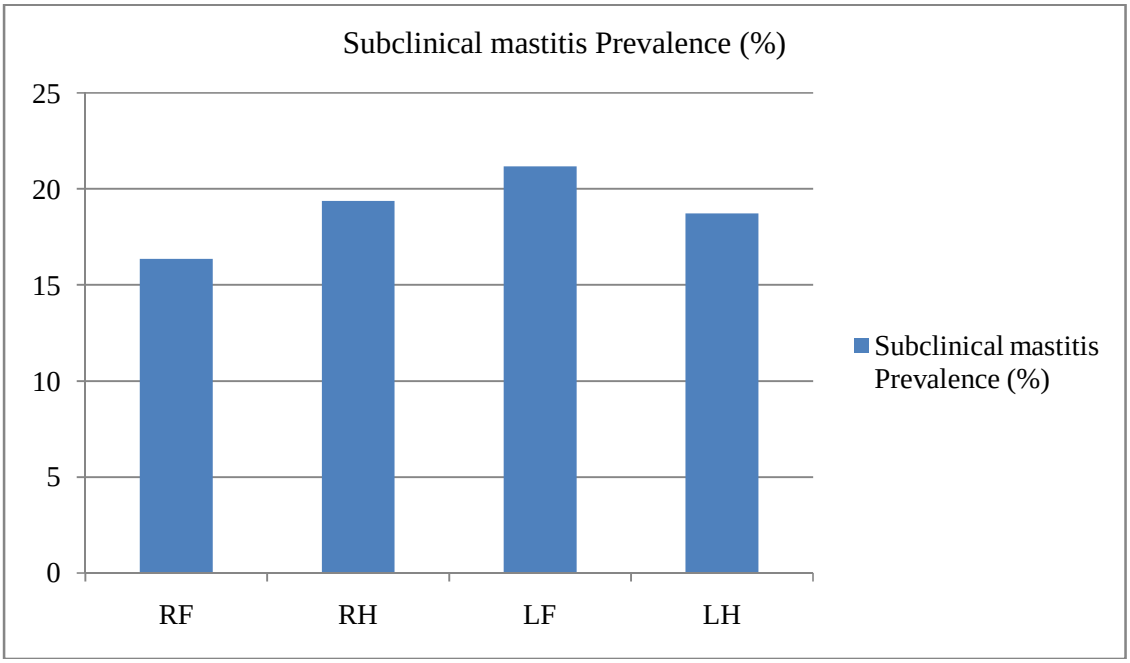
4.1. Prevalence of clinical and subclinical mastitis

From the total of 252 lactating cows examined by CMT and clinical examination, 112 (44.44%) were diagnosed with mastitis. The prevalence of clinical and sub-clinical mastitis was 16.11% and 36.67%, respectively (Table 1). Out of the 1008 quarter examined 59 (4.56%) were found to be blind teat. Clinical examination and CMT of the functional teats revealed a quarter of 49(4.86%) and 166(16.47%) affected by clinical and sub clinical mastitis respectively.

Table 1: Prevalence of clinical and sub clinical mastitis at cow and quarter levels.

Forms of mastitis	**Total examined cows**	**Total no. Of affected cows (%)**	**Total examined quarters**	**Total no. of affected quarters (%)**
Clinical	252	21 (8.33%)	1008	49 (4.86%)
Subclinical	252	92(36.51%)	1008	166(16.47%)
Total	**252**	**112(44.44%)**	**1008**	**232 (25.79%)**

In quarter prevalence of sub clinical mastitis, left front teats (RF) showed the highest rate of infection (21.17%) followed by the right hind (RH) which is 19.37%. The overall quarter prevalence of sub clinical mastitis was 18.92% (Figure 1).



RF= Right front RH=Right hind LF= Left front LH=Left hind

Figure 1: Quarter level prevalence of subclinical mastitis using CMT in Bishoftu dairy farms

Among risk factors indicated on table 2, parity, age, lactation stage and milk yield had significant effect on subclinical mastitis ($p<0.05$). The highest prevalence 54(58.7%) was found in animals having many parities (>3 parity), followed by 38 (40.3%) for cows of few parity (1-2 parity). The highest prevalence 71.3% was found in lactating cows of medium (8-21litre) milk yield per day, followed by cows of high (>21 litre) milking cows (14.7%), and the lowest prevalence (13.9%) was recorded in cows low milk (4-7litre) yield per day.

Table 2: Association between risk factors with occurrence of mastitis

Variables	Category	Total no of cows	Frequency	Percentage (%)	X^2	P value
Parity	Few (1-2 parity)	128	42	32.81	15.22	0.00
	Many (>3 parity)	124	71	57.26		
Mastitis history	No	203	87	42.86	1.66	0.191
	Yes	49	26	53.06		
Stage of lactation	Early (1-3 month)	73	29	39.73		
	Mid (3-6 Months)	166	72	43.37	12.76	0.02
	Late (>6 months)	13	12	92.31		
Presence of udder lesion	No	130	58	44.61	0.005	0.941
	Yes	122	55	45.08	5	
BCS	Poor	46	21	45.65	0.015	0.903
	Good	206	92	44.66		
Milk yield	Low (4-7)	35	14	40	10.31	0.006
	Medium (8-21)	209	91	43.54		
	High (>21)	8	8	100		
Age	Young adult (<6 years)	150	150	36	11.71	0.001
	Adult(>6 years)	102	102	57.84		

X^2 = Chi square BCS=Body condition score

4.2. Udder and teat morphometry and association with subclinical mastitis by CMT

4.2.1. Udder and teat end shape and association with subclinical mastitis

For the morphometrical study about 231 cows (excluding clinically 21 positive once) were used. Among these, 150 regular and 81 pendulous udder conformations were found and also pointed, flat and inverted teat end shape accounted for 105, 84 and 42 were recorded respectively. The

frequency analysis revealed that both udder conformation and teat end shape were not significantly different in mastiitic and healthy cows (table 3).

Table 3: Analysis of udder shape and teat end shape with mastitis in cows

Variables	**Category**	**Total examined**	**Positive frequency**	**Positive percentage**	**OR**	**P value**
Udder conformation	Regular	150	58	63.94%	1.07	0.626
	Pendulous	81	34	36.96		
Teat end shape	Pointed	105	39	42.39	1.03	0.845
	Flat	84	38	41.3		
	Inverted	42	15	16.3		

OR= Odds ratio

4.2.2. Various udder and teat measurements and association with subclinical mastitis

The results of teat measurements showed variation in average teat length from 2 to 12cm with a mean ± SD of 6.54±1.60. The mean of teat diameter was 23.44±4.14mm, ranging from 12.0mm to 37.0 mm. The mean distance between hind teats, front teats, left teats and right teats were 4.90±2.44cm, , 9.70± 3.33cm, , 6.63± 2.04cm and 6.73±1.88cm respectively as shown on table 5. The front teats (7.54±1.6cm) of the cows were averagely 0.83 times longer ($P<0.001$) than the hind teats (6.71±1.47cm) whereas front teats were 0.14 cm thicker than hind teats although the difference was not statistically significant ($P>0.05$).

The mean of some other udder and teat parameters such as glandular longitudinal length, front udder height, glandular circumference and teat end to floor distance were 39.48±7.65, 14.38±3.84, 88.52±16.37 and 51.53± 8.0 respectively.

Table 4: Mean with standard deviation, minimum and maximum of various udder/ teat measurements.

Variables	Mean ± SD	Minimum	Maximum
Teat length	6.54± 1.60	2	13
Teat diameter	23.44±4.12	12	37
Distance between hind teats	4.90± 2.44	0	14
Distance between front teats	9.70± 3.33	3	21
Distance between left teats	6.63± 2.04	2	15
Distance between right teats	6.73±1.88	2.5	12
Glandular mid circumference	88.52±16.37	50	139
Front udder height	14.38±3.84	6	44
Glandular longitudinal length	39.48±7.65	18	79
Teat end to floor distance	51.53± 8.0	22	72

SD= Standard Deviation

The length and diameter of the teats of the individual udder quarter's analysis showed that the longest and thickest teats were the left front and left hind teats respectively (table 5).

Table 5: The analysis of teat length and diameter for individual udder quarters

Teats	Teat length (Mean±SD)	Teat diameter
LF	7.12±1.52	23.57±4.19
LH	6.38± 1.56	23.58±4.03
RF	6.91±1.60	23.52±4.40
RH	5.75± 1.36	23.103±3.91

The bivariate analysis of teat diameter, teat length, and distance between hind, front, left and right teats, udder shape, glandular circumference, glandular longitudinal length and front udder height in infected and healthy cows were presented in Table 6. The results revealed that among these parameters teat length, teat diameter, distance between hind teats, glandular longitudinal

length had a significant association with mastitis. The mastitis was significantly increased in cows having longer teat length (P<0.05), large teat diameter (P<0.05), while higher distance between hind teats (P<0.05). Also cows having longer glandular longitudinal length and shorter teat end to floor distance (P<0.05) had high prevalence of subclinical mastitis. The distance between the front teats (9.7±3.7) was 1.85 times larger than the distance between the hind teats (5.25±2.7) (P<0.01) and mastitic cattle had shorter distance between hind quarters (P<0.05).

Table 6: Association of some udder and teat measurements with subclinical mastitis

Variables	**Non mastitic (n=139) M±SD**	**Mastitic (N=92) M±SD**	**X^2**	**P value**
Teat length	6.52±1.55	7.0±1.5	12.15	0.00
Teat diameter	23.38± 4.05	24.25±4.02	5.99	0.014
Distance between teats hind teats	4.6±2.14	5.32±2.75	9.96	0.041
Distance between front teats	9.65±3.36	9.8±3.28	0.11	0.54
Distance between left teats	6.67±1.99	6.61±2.2	0.05	0.521
Distance between right teats	6.83±1.92	6.6±1.91	0.91	0.908
Teat end to floor distance	53.92 ± 6.33	50.08± 6.73	18.01	0.00
Glandular circumference	88.28± 17.70	90.26±9.30	13.74	0.00
Glandular longitudinal length	38.89± 7.15	43.13± 9.02	2.28	0.134
Front udder height	13.99± 2.60	14.64±3.68	0.62	0.432

4.3. SCC and association with udder and teat morphometry

The SCC of all milk samples collected from a total of 217 quarters were counted and the overall mean, minimum and maximum values were 416,405.5, 0 and 7,296,000 respectively. The analysis of the SCC of the teats of the individual udder quarters showed that the highest and lowest mean number of SCC of the cows had studied in this study were of the Left front and right hind udder quarters respectively (table 7).

Table 7: The analysis of mean, SD, minimum, maximum SCC of individual udder quarters

Udder quarters	**Frequency of quarters**	**SCC (M±SD)**	**Minimum**	**Maximum**
LF	53	660,857.1±1363581	0	7,296,000
LH	52	241,428.6±667901.1	0	4,672,000
RF	56	348,830.2± 736923.1	0	5,472,000
RH	56	410,461.5± 1010157	8000	7296,,000

About eight risk factors assumed to affect SCC were considered in this study. The details of factors included and association were indicated in Table 8. Among the risk factors only yield had significant effect on SCC ($p<0.05$) which shows high milking cows had highest SCC compared to medium and low yielders.

Table 8: Association of risk factors with SCC

Variables	Category	Frequency	Mean ± SD	X^2	P value
Parity	Few (1-2 parity)	90	401600±1153496	0.03	0.854
	Many (>3 parity)	127	426897.6±863416.4		
Mastitis history	No	193	456994.8± 1042901		
	Yes	24	90000±172200.1		
Stage of lactation	Early (1-4 month)	58	137931±340663.7	4.05	0.054
	Mid (4-6 Months)	51	545568.6±1167644		
	Late (>7 months)	108	504963±1108186		
Presence of udder/teat lesion	No	130	445476.9±992505	0.07	0.786
	Yes	87	372965.5±994384.4		
BCS	Poor	128	434500±1128754	1.91	0.180
	Good	63	348190.5±604150.6		
Milk yield	Low (4-7)	32	509500±1052788	6.79	0.020
	Medium (8-21)	122	438163.9±1136397		
	High (>21)	63	326984.1±580979.4		
Quarter location	Front	108	506814.8±1115276	1.03	0.323
	Hind	109	326825.7±847305.7		
Age	< 6 years	112	411785.7±1099515	0.06	0.809
	>6 years	105	421333.3±867067.7		

4.3.1. Association of SCC and udder and teat morphometry

In the current study association analysis showed that there was no significant association ($p>0.05$) between SCC and teat length as well as teat diameter. The results of the mean ± SE of SCC revealed that teat-end shape and udder shape also had no significant ($p>0.05$) effect on SCC level (Table 10). Distance between hind teats, front teats, left teats and right teats were associated with SCC but only distance between front teats had significant effect on SCC ($p<0.05$). Association of SCC with teat end to floor distance, glandular longitudinal length, front udder

height and glandular mid circumference revealed only front udder height as significant effect on mastitis (P<0.05). Both distance between hind teats and front udder height had direct correlation with SCC

Table 9: Bivariate frequency analysis of different udder and teat end shape with SCC

Variables	**Category**	**No. of quarters**	**Mean±SD**	**X^2**	**P value**
Udder shape	Regular	135	538074.1± 1200468	3.21	0.084
	Pendulous	82	216097.6±415523.9		
Teat end shape	Pointed	82	245658.5±684408.1	0.23	0.635
	Flat	102	518117.6±1145865		
	Inverted	33	526303± 1089506		

4.3.2. The relationship of SCC and CMT scores

The mean SCC of healthy cows (CMT score 0) was 157,542.2 for CMT score 0 whereas other CMT scores (trace, 1, 2 and 3) had shown distinctively higher counts of somatic cells. The mean, standard deviation, minimum and maximum values of SCC and relationship of CMT scores and SCC is presented in table 10.

Table 10: Relationship of mean SCC and CMT scores

CMT score	Somatic cell count (SCC)				
	No. of observations	Mean	Stand. Dev.	Minimum	maximum
0	166	157,542.2	470667.8	0	5264000
T (Trace)	9	408,000	729273.6	0	2304000
1	17	544,941.2	549449.8	64000	2064000
2	13	1,711,385	1571525	40000	4672000
3	12	2,418,667	2252201	200000	7296000

4.4. Bacterial isolation and identification

Milk and mammary tissue samples were collected for the purpose of identifying major bacterial pathogens causing subclinical mastitis in dairy farms. Of the milk samples collected, CMT positive quarters, about two hundred seventeen (217) quarter milk samples were used for bacterial culture from Bishoftu farms. Out of these samples, 147 (67.74%) samples were culture positive for single colony, 46 (21.2%) showed mixed growth and 24 (11.06%) yield no bacteria. The predominant isolated bacteria were Coagulase Negative *Staphylococci* (CNS) with isolation rate of 66 (34.2%) followed by *S. aureus* 63 (32.8%) and *E.coli* 40 (20.7%) as shown on table 8.

On the other hand, bacteriological investigation from 53 mammary gland tissue samples collected from abattoirs revealed that the major isolated bacteria were *E. Coli (35cases), streptococci* (32 cases), *S. aureus* (23 cases), *Coagulase Negative Staphylococci* (CNS) (14 cases),, and *S. hyicus* (11 cases), as single and/or mixed infection as discus in Table 11.

Table 11: Frequency of isolated aerobic bacteria from milk and tissue of mastitic cattle

Bacteria isolated	**Milk sample N (%)**	**Tissue sample N (%)**
Staphylococcus aureus	63(32.8%)	23 (43.4%)
Staphylococcus hyicus	19(9.8%)	11(20.75%)
Staphylococcus Intermedius	9(4.7%)	1(1.89%)
Coagulase negative staphylococcus	66(34.2 %)	14 (26.41%)
Escherichia coli	19(9.8%)	35 (66.04%)
Streptococcus	40 (20.7%)	32(60.38%)
Citrobacter frundi	-	1(1.89%)
Others	25 (12.9%)	-

4.5. Serum ALP activity in mastititic and non mastitis cows

The recorded overall mean values of serum ALP in the study was 111.3±65.9. However, there was no statistically significant different at ($P< 0.05$) between mastitic and non mastitic dairy cows by CMT as indicated on table 12.

Table 12: The serum ALP activity in mastitic and non mastitic cows by CMT

Variable	No. Of cows	Mean	Stand. Dev.	Minimum	Maximum	P value
Non- mastitic	55	114.20	77.72	32.1	554.9	0.585
Mastitic	30	106.0	35.92	48.2	191.7	
Oveall	85	111.30	65.91	32.1	554.9	

4.6. Gross changes of mammary gland of cows

Among all cows in the study (farm and abattoir) investigated for any gross injury on udder or teat, 50.5 % (155/313) of them revealed udder or teat gross lesion as shown in table 13. There was no significant statistical association between mastitis and gross udder and teat lesion ($p>0.05$). Among the gross udder and teat lesions encountered in the present study, skin nodule and teat blindness were most prevalent with percentage of 71(22.68%) and 36 (11.5%), respectively (table 13).

Table 13: Gross udder/teat lesions of crossbred cows dairy farms

Udder /teat gross lesions	No. Of cows (N=313)	Percentage (%)
Skin abrasion	18	5.75
Skin nodule	71	22.68
Blind teat	36	11.5
Teat end injury	5	1.6
Hyperkeratosis	24	7.7
Chord formation	4	1.3
Udder edema	2	0.63
Tick infestation	5	1.6
None	155	49.5

Some of the main gross udder/teat lesions and teat end lesions observed during examination by naked eye were witnessed by pictures as labelled on figure 2 and 3. The cut sections of mammary gland of mastitic cow shows different gross pathological lesions as it is shown on figure 4 and also figure 5 reveals gross lesion and cut section of clinical mastitic cow.

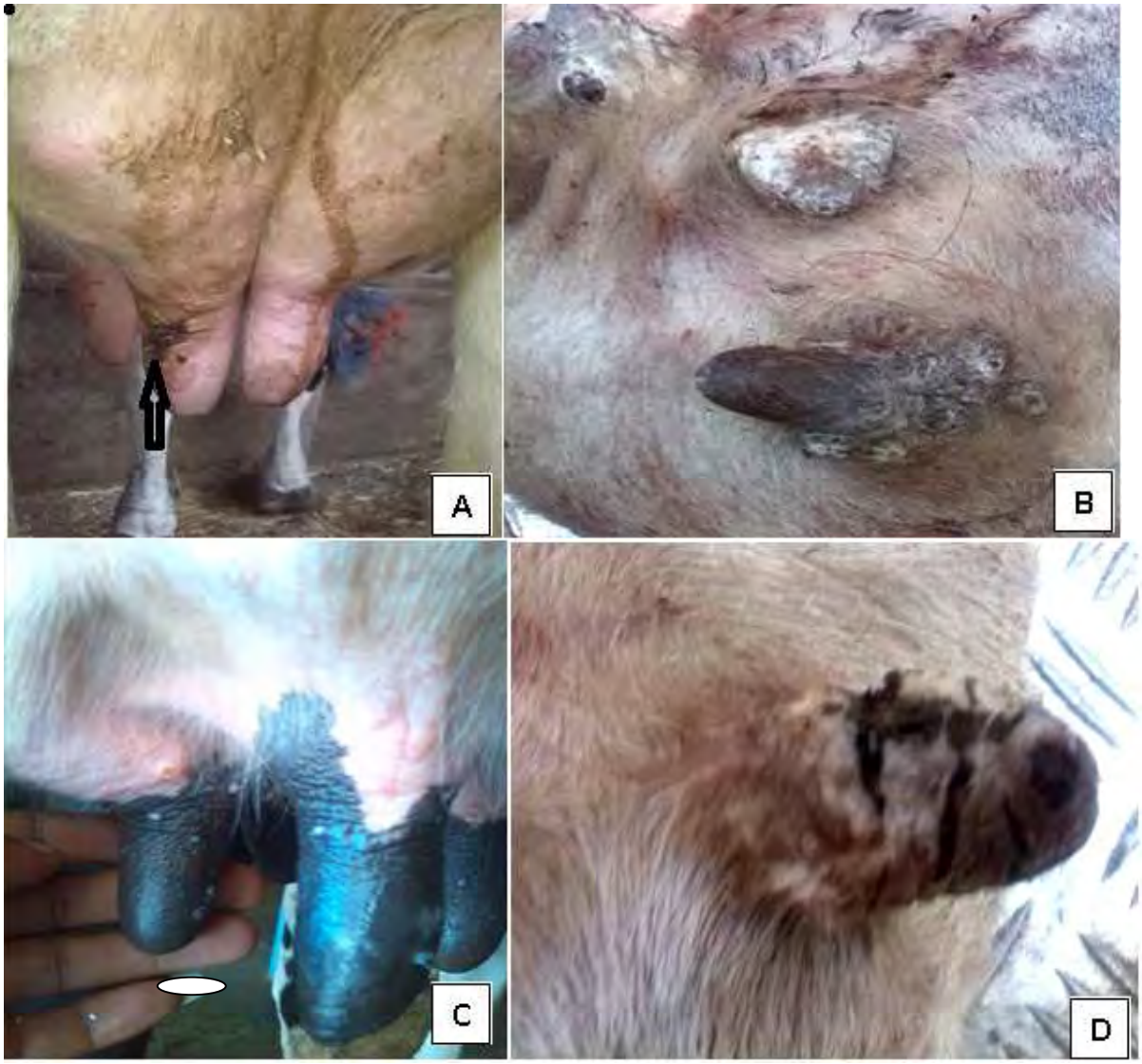


Figure 2: Gross teat and udder lesions.

Note the abnormal distance between the two hind quarters and laceration on one of the teat indicated by the arrow (A) and hyperkeratosis on teat and total loss of one teat (B) Hand milking induced nodules on the teat (C) Severe cuts of teat filled with debris (D)

The udder teats are the first line of defence against intra-mammary infection and the teat end lesions observed during the study period at farm and abattoirs showing the sphincter damaged and which may allow for bacterial pathogen to join the gland through teat canal as indicated on the figure 2.

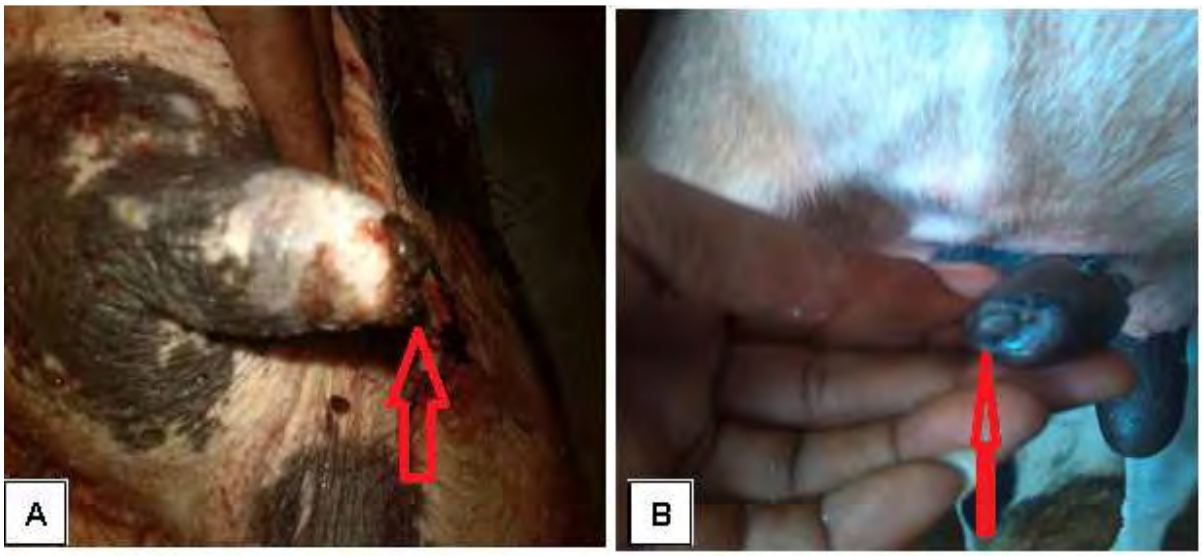


Figure 3: Gross teat end lesion.

Note the total blockage of the teat canal and outgrowth on the teat shown by the arrow (A) and teat end cut with leakage of milk indicated by the arrow (B)

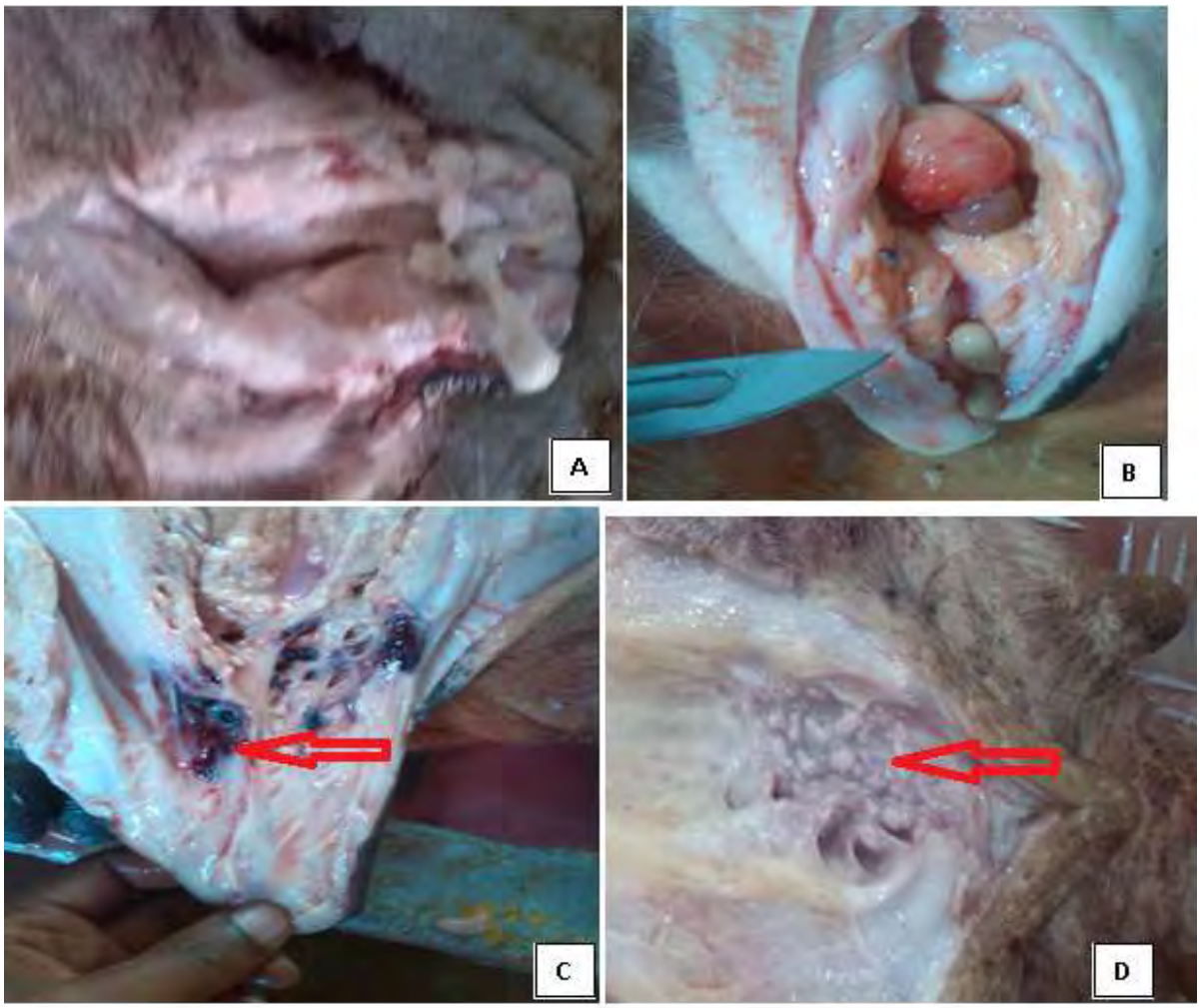


Figure 4: Cut section of streak canal of mammary gland

Puss accumulation in the gland (A) Tissue growth and puss in the streak canal (B) Haemorhagic mucosa of steat canal (C). nodular formation at the base of teat canal (D).

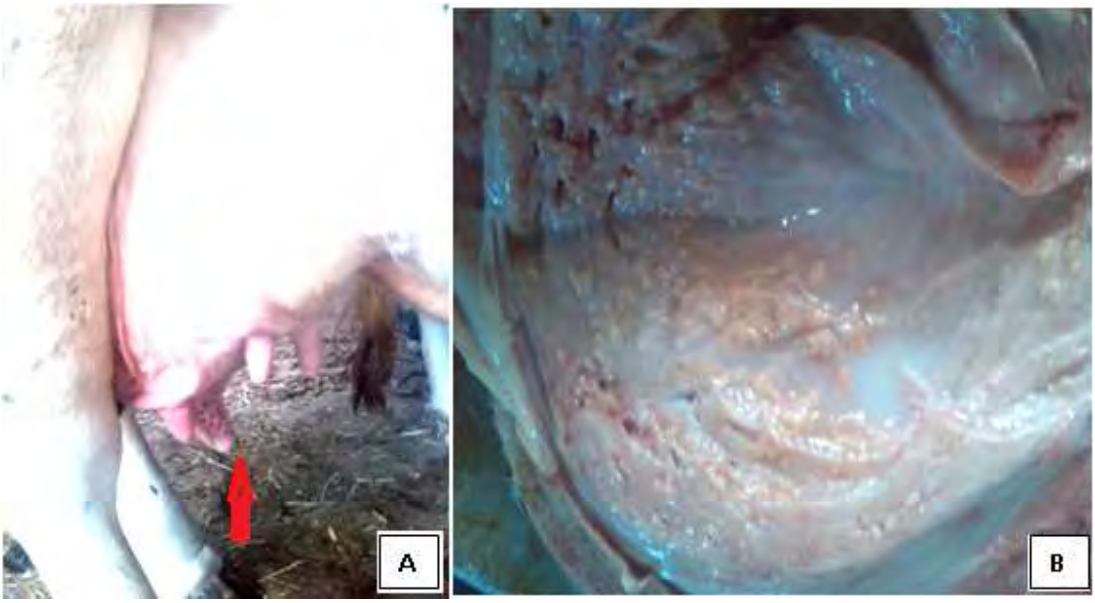


Figure 5: Swollen left hind teat from cows (arrow) with clinical mastitis

(A) and Cut section of glandular tissue from the same cow with extensive nodular formation (B)

4.7. Histopathological changes of mammary gland of cows

Histopathologically, udders from apparently healthy cows (mammary gland tissue culture negative) showed no pathological lesions with normal alveoli and retained normal glandular structure. However, histopathologic evaluation of bacterial culture positive mammary glands showed acute, sub-acute and chronic mastitis or inflammatory changes based on the type of exudates. The vast majority of acute mastitis was characterized by suppurative inflammation characterized by neutrophil infiltration. The non suppurative types were characterized by infiltration with lymphocytes and macrophage in the parenchyma. The details of microscopic lesions were indicated on figures 6to 9 below.

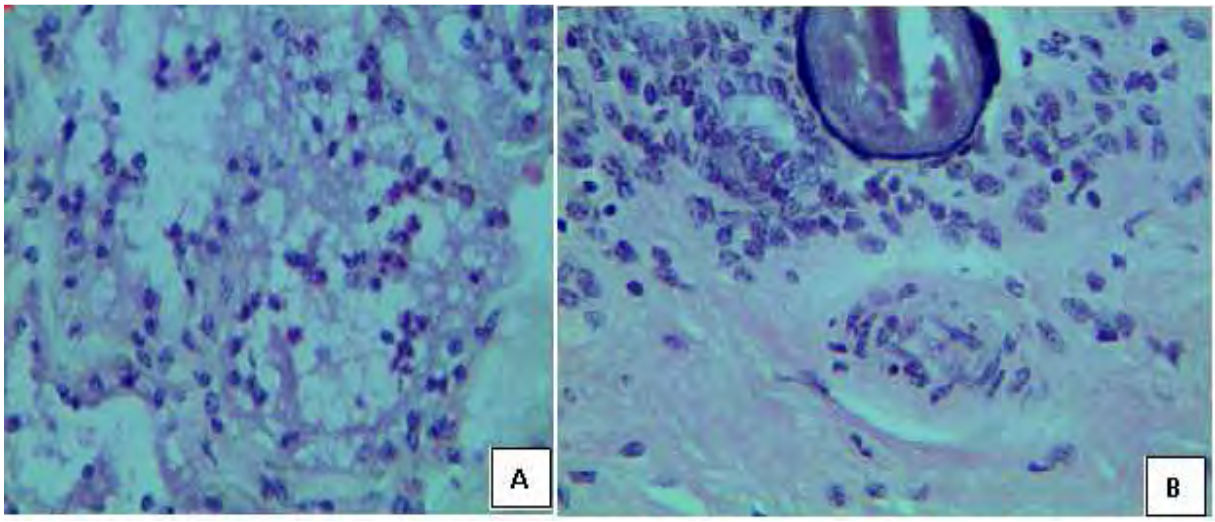


Figure 6: Microscopic lesion of mastitic cow.

Note higher magnification of suppurative mastitis with a large number of neutrophils per field (A) and mastitis with infiltration of mononuclear cells (lymphocytes &macrophages) and caseated milk in alveoli of the gland. H and E stain, 400X

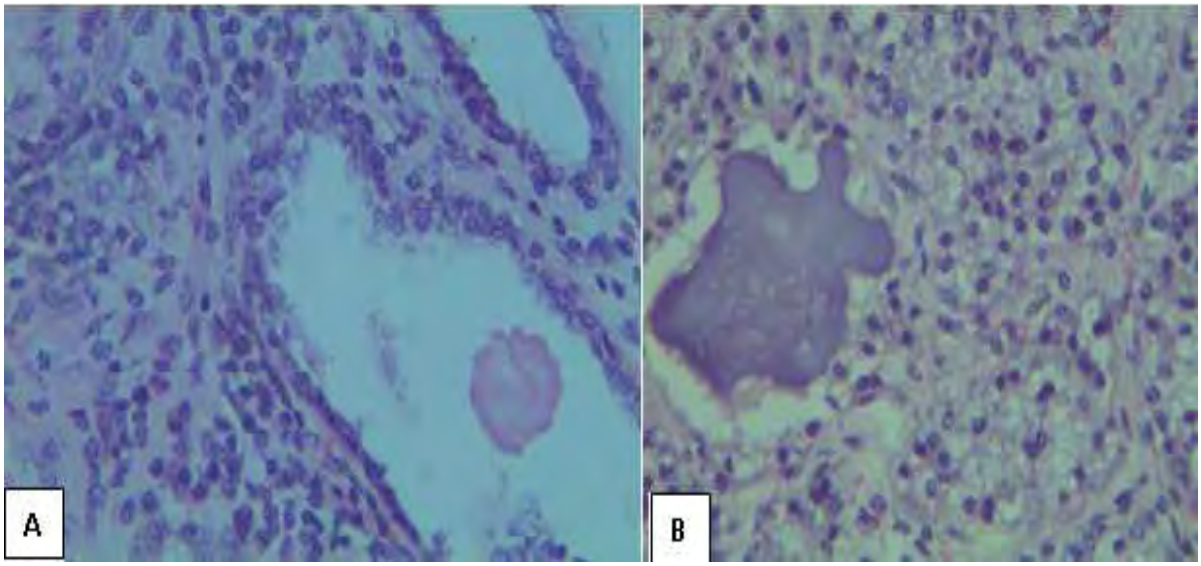


Figure 7: Dried milk and cellular debris in the lumen of the alveoli and large number of neutrophils in cow mammary galand (A&B). H and E stain, 400X

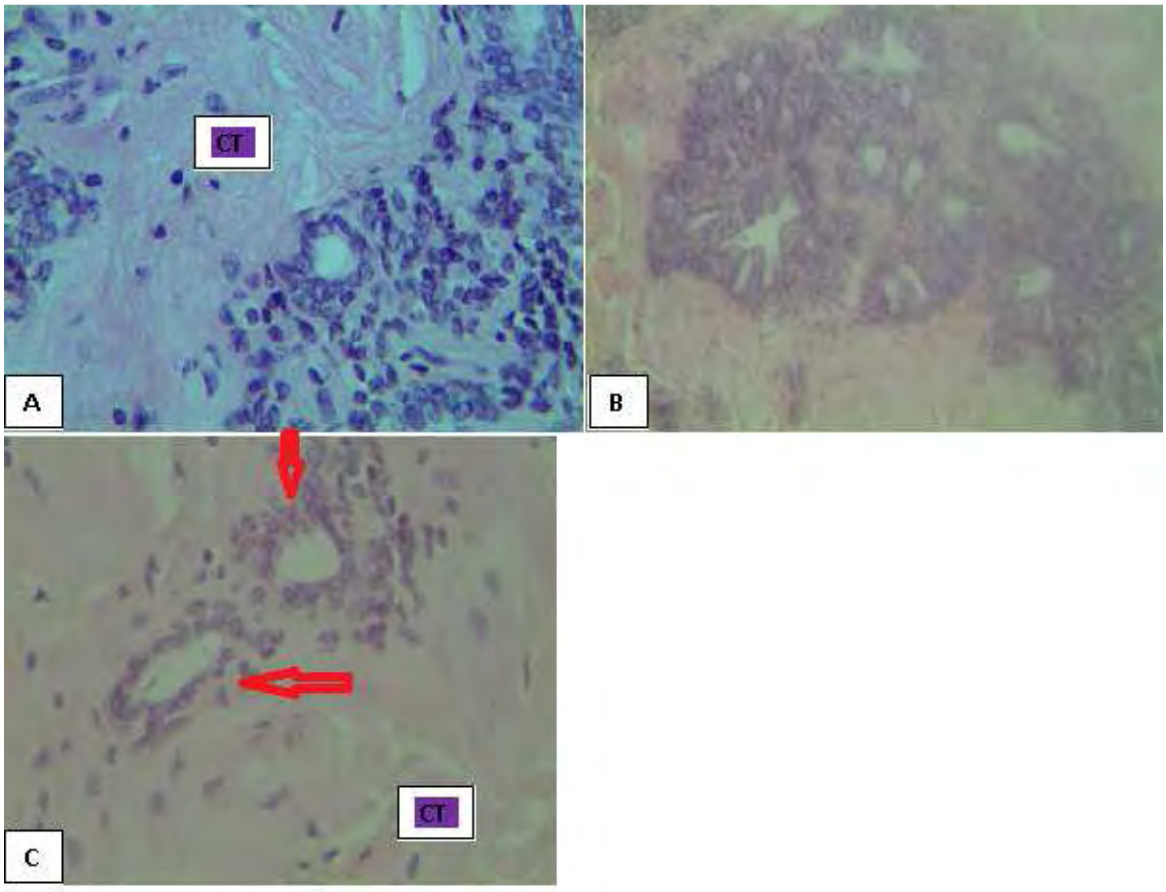


Figure 8: Chronic mastitis with excess fibrous connective tissue(CT)

(A) 400X, degeneration and necrosis of glandular epithelium 100X (B) Severe fibrosis (CT) and loss of most glands with only two of acini perfield (arrow) 400X (C)

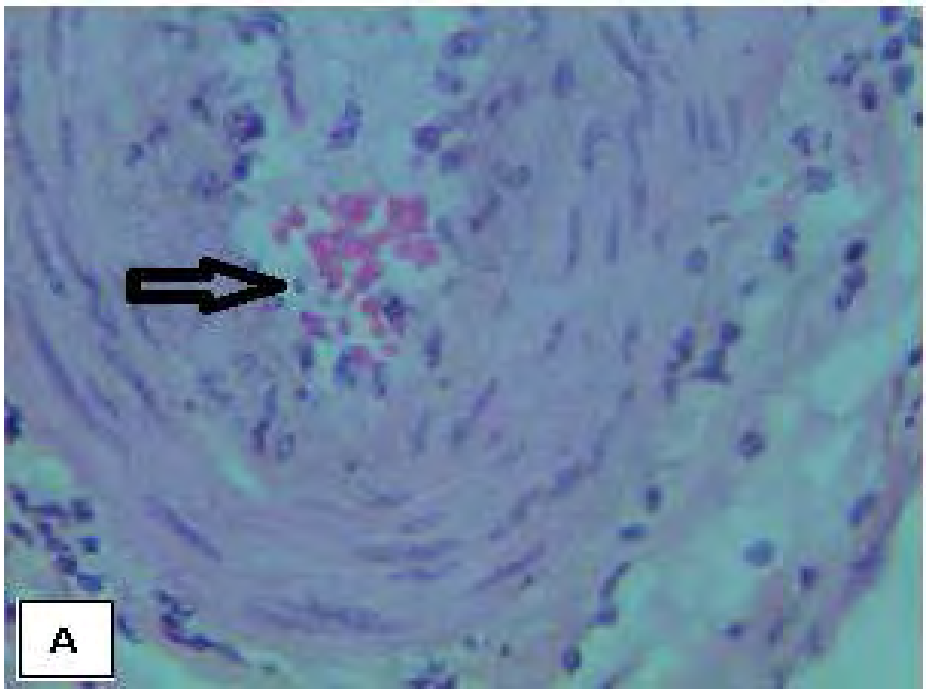


Figure 9: Section of mammary gland showing vascular response due to acute mastitis. Note hyperaemic dilated artery that contain red blood cells. H and E stain, 400X

5. DISCUSSION

Mastitis is a complex disease involving interactions of several factors, mainly of management, environment, 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 (Radostits, 2007).

In the present study from the total of 252 lactating cows examined, 112 (44.62%) were diagnosed as mastitic which is comparable with report of Girma (2010) who reported 44.1% in Zebu cattle at Doba district and 48.1% of prevalence at Adama by Mekonnen and Tesfaye (2010). This finding is slightly lower than those of Gizat *et al*. (2008), Sori *et al*. (2005), Mungube *et al*. (2005) and Abuna *et al*. (2013) who reported 56%, 52.78%, 52.3% and 52.27% respectively in different parts of Ethiopia. The result was found to be by far lower than 74.7% , 68%, 71%, 69.8% and 64.5% prevalence reported by Zeryehun *et al*. (2013) in Addis Ababa , Tilahun and Aylate (2015) in Addis Ababa, Regasa *et al*. (2010 b) at Holeta town, Bishi (1998) in Addis Ababa and vicinity and Matios *et al*. (2009) in dairy farms of and Asella respectively. In contrast, the current finding is greater than Nibret *et al*. (2011) who reported 32.6% and 33.6% prevalence by Getahun *et al.* (2008) at Selalle. The overall 21.1% (123/584) had mastitis in smallholder dairy farms in Zimbabwe (Katsande *et al.*, 2013).

The study revealed prevalence rate of 36.67% subclinical form of mastitis at cow level which is in line with Abuna *et al.* (2013), Aylate *et al.,* 2013, Matios *et al.* (2009) Mekebib *et al.* (2009), Sori *et al.* (2005), Workineh *et al.* (2002), and Girma (2010) with prevalence of 36.86%, 41.02%, 30.4%, 34.8, 40.6, 38.6 and 34.4% respectively in different parts of Ethiopia. However, the finding is higher than reports of Belayneh *et al.* (2014) estimated 25.0% small holder dairy farms at Akaki district; Biffa *et al.* (2005) in Southern Ethiopia accounted 23.0%; Lakew *et al.* (2009) in Khartoum revealed 9.81% and 16.3% (95/584) subclinical mastitis in smallholder dairy farms in Zimbabwe by Katsande *et al.* (2013). The high prevalence of sub-clinical mastitis may be attributed to improper milking hygiene, lack of post milking teat dipping and contact labours used, absence

of order in milking cows of different ages and milking of mastitic animals before the healthy ones all of which might have increased the prevalence (Radostits, 2007). This result is lower than 55.1% prevalence by Zeryehun *et al.* (2013) in smallholder dairy farms in Addis Ababa.

The present study showed clinical mastitis prevalence of 16.11% which is in agreement with the prevalence of 15.41%, and 16.11% by Abuna *et al.* (2013) and Sori *et al.* (2005) respectively. The current result is a bit lower than report of Tilahun and Ayano (2015) estimated 19.6% and 21.2% Zeryehun *et al.* (2013). The result is much greater than reports of Belayneh *et al.* (2014) (3.0%) and 4.8% in smallholder dairy farms in Zimbabwe (Katsande *et al.,* 2013).

The overall prevalence of clinical at quarter level found to 19.1% which is in conscience with the quarter level prevalence was 44.9% (192/428) (Mekibib *et al.,* 2010).The quarter level clinical mastitis is lower than 34.8% by Regasa *et al.* (2010a) and 34.8% (149/428) report of Mekibib *et al.* (2010). The quarter level clinical mastitis prevalence recorded in this study was greater than several previous studies including 10%,14.9%, 2.4%, 0.9%, 1.2%, 5.2% and 10.0% reported by Regasa *et al.* (2010b), Matios *et al.* (2009), Mekonnen and Tesfaye (2010), Getahun *et al.* (2008), Belayneh *et al.* (2014), Zeryehun *et al.* (2013) and Mekibib *et al.* (2010) respectively.

In this study, among the total quarters examined, 46 (4.6%) had blind teats. This finding agrees with Abuna *et al.* (2013), Etifu (2012), Matios *et al.* (2009), Getahun *et al.* (2008) and Mekonnen and Tesfaye (2010) who reported prevalence of 4.61%, 5.2%, 4.5%, 2.3% and 3.6% respectively. The present study also revealed quarter level sub clinical mastitis was 18.93% which is lower than 34.8% and 42.7% accounted by Mekibib *et al.* (2010) and Zeryehun *et al.* (2013) respectively. However, the finding was greater than result found from small holder dairy farms at Akaki district (912.7%) by Belayneh *et al.* (2014).

Higher prevalence of sub clinical mastitis form was observed in comparison to clinical in the current study that may indicate the magnitude of subclinical mastitis problem and low level of attention that given to it in terms of diagnosis and treatment. In Ethiopia, the sub clinical form of mastitis received little attention and efforts have been concentrated on the treatment of clinical cases Hussein *et al.* (1997) while the high economic loss could come from sub clinical mastitis.

According to Radostits *et al.* (2007) an infected quarter showed 30% and a cow 15% reduction in milk yield. 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.

The results of analysis of the risk factors showed that parity, age, lactation stage and milk yield had significant effect on prevalence of sub clinical mastitis. The prevalence of mastitis was higher in adult cows (57.8%) than young adults (36%). Radostitis *et al.* (2007) have explained that older cows have largest teats and more relaxed sphincter muscles, which increase the accessibility of infectious agent in the cows' udder. Cows with many calves were greater at risk than those of cows having moderate and few calves. Zeryehun *et al.* (2013), Belayneh *et al.*, 2014 and Mekibib *et al.* (2010) presented similar findings. Also Abuna *et al.* (2013) recorded cows with parity number than greater three had significantly higher mastitis prevalence than lesser number of parity ($p<0.05$). This could be due to that fact that primiparous cows have more effective defence mechanism than multiparous cows (Enerike, 2001).

In associated with the environment and managemental factors, selecting dairy cows which are less susceptible to mastitis is also a control measure worthy of consideration (Chrystal *et al.*, 1999). Udder and teat morphology is very heritable (Seykora and McDaniel, 1985) and could serve as a marker trait for selection to reduce mastitis in dairy cattle (Nakov and Trajcev, 2012; Nakov *et al.*, 2014). It is well established that a favourable association exists between mastitis resistance and several udder type traits. The literature data are generally similar about the genetic correlation between udder depth, udder attachment to the cow's body, milk production and association of these factors with mastitis incidence (Sorensen *et al.*, 2000; Klein *et al.*, 2005; Ptak *et al.*, 2011).

The udder teats are the first line of defence against intra-mammary infection. The probability of mastitis occurring varies considerably between different teat shapes, sizes, teat placement and the morphology of the teat tip (Bardakcioglu *et al.*, 2011). In any case, there is no consensus in the literature about the influence of teat morphology on mastitis occurrence (Haghkhah *et al.*, 2011; Singh *et al.*, 2013). The effects of certain unfavourable udder characteristics on mastitis risk are

likely to appear when machine milking begins and several studies have identified udder and teat conformation as risk factors for mastitis (Rupp and Boichard, 2003; Bhutto *et al.,* 2010; Singh *et al.,* 2013).

In the present study, udder shape was not significantly different in mastitic and healthy cows ($p>0.05$) by both CMT and SCC. Similarly, Coban. *et al.* (2009) found insignificant effect of udder shape on SCC and a significant negative correlation between somatic cell number and udder depth has been also reported by Orban *et al.* (2009). However, many previous studies in different parts of the world has shown higher prevalence of mastitis in cattle having pendulous, round and bowl and long udder shape has been reported (Klaas *et al.,* 2004; Bhutto *et al.,* 2010; Hussain *et al.,* 2012) and cows with pendulous udders had the highest risk of mastitis (Uzmay *et al.,* 2003).

Different studies also indicated that deeper udders were found to be at higher risk of developing mastitis due to their increased tendency to become soiled (Lopez-Benavides *et al.,* 2005) and more susceptible to lesions (Bhutto *et al.,* 2010; Singh *et al.,* 2014). Several researchers reported the association of pendulous udder with teat/udder injuries (Klaas *et al.,* 2004; Shukla *et al.,* 1997; Breen *et al.,* 2009; Bhutto *et al.,* 2010; Hussain *et al.,* 2012). Hence, being contaminated with environmental pathogens and developed mastitis. Reports of Ahlawat *et al.* (2008) and Pranay Bhart *et al. (*2015) stated that cows with pendulous udders had the highest risk of mastitis and higher SCC in comparison of regular shaped udder and the effect of udder shape on degree of intra-mammary infection and SCC level was found significant ($p<0.01$) (Pranay Bhart *et al.,* 2015).

The probability of mastitis occurring varies considerably between different teat shape, sizes, and morphology of the teat-end (Bardakcioglu *et al.,* 2011). In current finding teat end shape variation as pointed, flat and inverted had no statistical significance ($p>0.05$) for CMT positivity and SCC. There are limited papers agreed with this finding as non-significant relationship between the SCC and teat-end shape was reported by Manzi *et al.* (2012).

According to Pranay Bhart *et al. (*2015) flat and inverted teat-end shape may be a risk factor for intra-mammary infection as highest SCC level found for flat teat-end followed by inverted and least for pointed teat-end and the effect of teat-end shape on SCC was significant ($p<0.01$). There are reports of the significant effect of teat-end shape with higher log SCC value for the inverted teat-end (Orban *et al.* 2009). Less pointed and more inverted teat-ends have been associated with increased susceptibility to mastitis as they retain milk, which can act as a substrate for bacterial growth (Chrystal *et al.,* 2001). Hence, it has been suggested that some teat-end shapes act as risk factors for infection, and, as they have high daughter-dam heritability, can be eliminated by selective breeding (Chrystal *et al.,* 1999)

The data analysis showed that both teat length and diameter had significant effect on mastitis as stated by CMT and but no statistically significant ($p>0.05$) with SCC. An increase in the degree of intra-mammary infection with an increase in teat length and teat diameter as log SCC was found to be significantly positively correlated with teat morphometry (Pranay Bhart *et al.,* 2015). Coban *et al.* (2009) observed a positive, despite low correlation between teat diameter and mastitis. An increase in mastitis incidence, with an increase in teat diameter had already been observed by Chrystal *et al.* (1999) and Kuczaj (2003). Some previous studies also revealed that the occurrence of subclinical mastitis was highest for cows with long and thick teats (Singh *et al.,* 2014; Haghkhah *et al.,* 2011) and are a potential risk factor for intra-mammary infection.

However association of these factors with occurance of mastitis has already been established different parts of the world (Chrystal *et al.,* 1999; Klaas *et al.,* 2004; Bhutto *et al.,* 2010), some study revealed no correlation between teat length and SCC ($P>0.05$). Other researchers also reported that the prevalence of mastitis was higher ($P<0.0001$) in quarters with small teat and large teat diameter (Hussain *et al.,* 2012). It could be due to the reason that the pathogens have to travel less distance to establish infection in mammary glands. Moreover, few literatures has been showed that the higher value of the somatic cell score for shorter teats (Nemcova *et al.,* 2007) and a negative correlation of somatic cell number with teat diameter (Orban *et al.,* 2009). Juozaitiene *et al.,* (2006) reported no effect of teat length and teat thickness on SCC. data suggested machine incompatibilities such as more frequent liner slips and increased likelihood of

teat end lesions in longer teats (Rogers *et al.,* 1991) and in teats with larger diameter (Mein *et al.,* 2004) which may act as a risk for intra-mammary infections.

Having performed the analysis of the teat length and diameter in relation to location, the result showed that the average length of front teats (7.54±1.6cm) were averagely 0.83cm longer ($P<0.001$) than the hind teats (6.71±1.47cm) whereas front teats were averagely 0.14 cm thicker than hind teats, however, it is not statistically significant ($P>0.05$). Kuczaj *et al.* (2000); Weiss *et al.* (2004) and Tilki *et al.* (2005) reported that the front teats were longer than the rear teats. Kuczaj *et al.* (2000) estimated that the rear teats were 0.05 cm thicker than the front teats. In contrary to our report Weiss *et al.* (2004) estimated that the rear teats were 0.1 cm thicker than the front teats.

The data analysis of length and diameter of the teats of the individual udder quarters revealed that left front and left hind udder quarters were longest and thickest teats of the cows the respectively. Weiss *et al.* (2004) also found the longest teats on the front right side of the udder quarters, and the shortest – on the rear right side of the udder quarters.
The analysis of the average measurements of the teats of the cow's udder showed that the teats of the cows we had tested were 0.58 cm longer and 0.45 cm thinner than the teats analyzed by Kuczaj et al. (2000). Tilki *et al.* (2005) carried out the study in which they estimated the teats which were 0.47 cm longer and 0.15 cm thinner than the teats of cows we had analyzed.

The distance between the front teats was 1.85 times larger than the distance between the hind teats compared to front teats ($P<0.01$) and mastitic cattle had shorter distance between hind quarters ($P<0.05$). This result is in between the report of the Turkish scientists (Tilki *et al.,* 2005) who reported 1.6 times larger the distance between the front teats than the distance between the rear teats and the Polish scientist Kuczaj (2003), who had investigated the characteristics of black and white cow's udders and estimated that the distance between the front teats was 2.5 times larger compared to the distance between the rear teats.

Influence of the difference between the distance of the front and rear teats was statistically significant for the productivity of cows ($P<0.05$). Cows with more even udders had 12.8% higher

difference of the distance between the front and rear teats. The examination showed that 5.94% less positive samples of milk according to mastitis were found in the udders of cows whose difference of the distance between the front and the rear teats was up to 5 cm than in the udders of cows whose difference of the distance between the front and the rear teats was greater than 5 cm (Slyzius *et al.*, 2014).

As stated in the result part, cows having longer glandular longitudinal length shorter teat end to floor distance (by CMT) ($P<0.01$) and front udder height (by SCC) ($P<0.05$) had high prevalence of subclinical mastitis. Some studies have reported that decreasing teat-end to floor distance is a risk factor for mastitis (Singh *et al.*, 2013). Also, an increasing proportion of teat lesions, with decreasing teat end to floor distance is a well-documented risk factor for mastitis (Bhutto *et al.* 2010). There was a negative correlation between SCC and teat end to floor distance ($p<0.001$) (Porcionato *et al.*, 2010).

The mean SCC of healthy cows (CMT score 0) in the study was 157,542.2 which is within the range of standard SSC by international dairy federation (Barbano, 1999) that declares SCC greater than 200,000 cells/ml as subclinical mastitis and the quarter is likely to be infected, and the milk has reduced manufacturing properties such as reduced shelf life of fluid milk, and reduced yield and quality of cheese. The result slightly lower but comparable with the report of Alma (2004) who estimated mean SCC of 168,944.8 in crossbred at Bahir Dar town. This implies that SCC can be used as an alternative method to diagnose subclinical mastitis in the study and at country level.

Bacteria involved in mastitis have been considered of great significance in the epidemiology of mammary gland disease (Waller *et al.*, 2009). In present study Staphylococci and *Streptococci* were the major pathogens recovered from milk from dairy farms in Bishoftu and also mammary tissue of slaughtered dairy cattle revealed higher prevalence of *E. coli* comparable to the prevalence of *staphylococcus* and *streptococcu*s. *Staphylococcal* and *streptococcal* isolation as major pathogens is in line with the earlier reports (Hussain *et al.*, 2012; Tempelmans Plat-Sinnige *et al.*, 2009) and may be attributed to their routine presence on various body parts and

survival in teat and skin lesions. Previously, it has been reported that S. aureus and *Streptocuccus* agalactiae show a rapid spread and, therefore, these pathogens cause mastitis in a high number of dairy animals (Karahan *et al.,* 2011; Nazifi *et al.,* 201).

The highest prevalent bacteria were found to be contagious bacteria of which CNS was found to be dominant in this study agrees with the result Etifu (2012), Mekonnen and Tesfaye (2010) and Gizat *et al.* (2008) who found CNS as the predominant bacteria among isolates in Alage, Adama and Bahirdar dairies, respectively. Other researchers, on the other hand, found *Staphylococcus aureus* as the most frequently isolated bacteria in different parts of Ethipia (Abuna *et al.*,2013; Workineh *et al.,* 2002); Kerro and Tareke (2003); Regassa *et al.*, 2010b); Matios *et al.* (2009); Getahun *et al.,* 2008).

The relative high prevalence of *S. aureus* in this study could be associated with lack of effective udder washing, hand washing before milking, use of separate towel, post milking teat dipping and disinfection routine milking area (Kerro and Tareke, 2003; Abuna *et al.,* 2013). As reported by Abuna *et al.* (2013) streptococci species were also among the dominant bacterial population as mastitis pathogens accounting (16.3%) that agrees with the present finding of 40 (20.7%) from milk and 32 (60.38%) from tissue samples.

Eschershia coli, environmental bacteria, isolated from milk was high proportion (9.8%) in congruent with the 9.4% report of (Etifu, 2012) and 7.5% of the total isolates from both Mekonnen and Tesfaye (2010), Matios *et al.* (2009). In contrast, this figure is higher than isolates reported by Regassa et al. (2010b), Sori *et al.* (2005) and Getahun *et al.* (2008) who reported 4.57%, 0.75% and 0.5% in different parts of Ethiopia, respectively. However, the prevalence of the pathogen from mammary tissue sample recorded was by far higher than most of the reports. The presence of environmental bacteria might be an implication of unhygienic milking practice and contamination of cows' teats and environment with their dung in the study area and origin of the animals for cows slaughter at abattoir.

The invasion of polymorphonuclear leukocytes and macrophages is one of the essential body defenses against clinical and subclinical mastitis. During the inflammatory process, these cells

and damaged cells of the udder's epithelial and interstitial cells secrete products that include hydrolytic enzymes (Oliszewski *et al.,* 2002). Some enzymes including ALP are secreted by the epithelial cells of mastitic mammary gland. In mastitis, muscle and tissues of mammary gland are damaged which may lead to increase in the level of these enzymes (Khodke *et al.,* 2009). Several previous researchers found statistically different level of enzymatic activity in mastitic and non mastitic, however the current finding is not in agreement with these studies. ALP test was sensitive and reliable enough for the early diagnosis of SCM (Babaei *et al.*, 2007).

ALP activity in milks with subclinical mastitis were significantly higher than those from healthy normal ewes. This indicates that determination of enzymes activities in serum milk is a sensitive and dependable method for detection of ovine subclinical mastitis (Hassan and Yousif, 2014). Batavani *et al.,* (2007) found that the increased in milk ALP enzyme in mastitic animals, might be linked with mammary tissue damage. Bogin and Ziv (1973) found approximately a six fold increase in the level of ALP after infusion of E. coli endotoxin into the bovine udder. Batavani *et al.,* (2003) showed that the increment ALP in milk of udders shows the presence of tissue damage, these parameters might be suitable for use in the early diagnosis of subclinical mastitis.

The recent finding showed that udder/teat detected overall prevalence of gross lesions 50.5% (158/313) and there was no significant statistical difference among mastitic and non mastitic when associated with udder/teat lesion ($p>0.05$). The result was lower that report of Biffa *et al.* (2005) about 68.8% of cows with udder/teat injuries in southern Ethiopia.

Among the gross lesions, skin nodules and teat blindness were the top ranking having percentage of 22.68% and 11.5% respectively in this research. However, high occurrence of mastitis-induced blind mammary quarters, like the report of Biffa *et al.* (2005), which has a direct influence on milk production, signifies the importance of the problem. Lack of screening and treatment of subclinical mastitis and inadequate follow-up of clinical and chronic cases coupled with persistent challenges of the mammary glands by microbial pathogens could be the main predisposing factors to quarter blindness. This hidden and gradual destruction of the mammary tissues would end with non-functional quarters.

Microscopical examination of tissue sections from teat of infected cows exhibited severe histological changes. Histological analyses have been widely used since the 1970s and are still being used today for assessing damage to secretory tissue in the bovine mammary gland caused by mastitis pathogens (Stabemfeldt and Spencer, 1965; Zarkower and Norcross, 1966; Jubb *et al.*, 1993; Benites *et al.*, 2002). The main histopathological findings in the present study were the epithelial degeneration and necrosis, atrophy of alveoli, fragmented alveoli, acute to sub-acute suppurative inflammatory response marked by neutrophil infiltration, hyperaemia, congestion, increased stromal tissue (fibrosis) and presence of non-suppurative inflammation marked by lymphocytic infiltration in alveoli. The present microscopic lesions of the mammary gland were in line with many of the previous works. The infected mammary gland showed inflammatory lesions of different types ranging from the disappearance of the alveolar lumen, through fibrosis to the complete destruction of the parenchyma (Kheira and Abdellatif, 2014). Benites *et al*. (2002) examined 131 mammary parenchyma from 184 slaughtered dairy cows for existence of microorganisms and histopathological changes. Of all the samples from which microorganisms were isolated, 96.9% of samples showed inflammatory response. According to Hussai *et al.* (2012) the tissue sections from mastitic animals revealed mild, moderate or severe atrophy of alveoli with cellular exudate in the lumen of the alveoli. The existence of acute and chronic inflammation in mammary parenchymal tissues was confirmed and fibrous tissue proliferation was seen in the mammary gland. Moreover, lesions of the breast tissue reduces the number and activity of epithelial cells and therefore contributes to lower milk production with increasing proportions of lymphocytes and macrophages was reported by Zhao and Lacasse (2007).

6. CONCLUSION AND RECOMMENDATIONS

In this study significant number of lactating dairy cows had either clinical or sub clinical mastitis which might have significant loss on dairy farmers. It was also revealed that the chances of mastitis were higher in cows with longer and thicker teat. Cattle having longer glandular longitudinal length and shorter teat end to floor distance were more prone to subclinical mastitis. Likewise, distance between hind teats and front udder height had significant associations with somatic cell count. The result shows that some udder morphometry and teat parameters were found risk factors for subclinical mastits. *Staphylococci* (CNS and S. *aurous*), E. *Coli* and *Streptococci* were the major microorganisms of mastitis in area of study. Frequent histopathological lesions of the mammary glands were suppurative inflammation with degeneration and necrosis of glandular epithelium in acute cases and interstial fibrosis, and glandular atrophy in chronic cases. These lesions especially of chronic may decrease the secretary ability of the glands and could result in low productivity.

The following few points are pointed out as recommendation in line with the aforementioned conclusive mark:

- Mastitis is complex multi-causative agent disease that needs strategic control program through early detection. Continuous screening of cows at farm level and appropriate treatment should be implemented to reduce cows with mastitis and hence reduce cross contamination. There was shortage of CMT reagent was seen at local market to screen cows with subclinical mastitis; however, the prevalence of mastitis was high in the study area. So, concerned bodies should address the problem in order to minimize loss due to subclinical mastitis

- The use of other diagnostic methods like bacterial culture, and SCC should be encouraged.

- Udder and teat morphometry showed significant association on sub clinical mastitis, it may be implicated that udder and teat morphometry could be considered as selection traits of dairy animals among others, however further studied should be conducted.

- The bacterial isolated from mammary tissue or milk of mastitic cows were either contagious or environmental contaminants that may indicate managemental problems in the study area. Therefore, it could be recommended that cow and the environment should keep clean through improving farm management to reduce the risk.

7. REFERENCES

Abera, M., Demie, B., Aragaw, K., Regassa, F. and Regassa, A. (2010): Isolation and identification of *Staphylococcus aureus* from bovine mastitic milk and their drug resistance patterns in Adama town, Ethiopia. *J. Vet. Med. Anim. Health,* **2**: 29–340

Abunna, F., Fufa, G., Megersa, B. and Regassa, A. (2013): Bovine mastitis: Prevalence, risk factors and bacterial isolation in smallholder dairy farms in Addis Ababa city, Ethiopia. *Glob. Vet.*, **10** (6): 647-652.

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

Alma, G. (2004): A cross-sectional study of bovine mastitis in and around Bahir Dar and antibiotic resistance patterns of major pathogens. Msc. thesis, Addis Ababa University, Faculty of veterinary Medicine, Debrezeit, Ethiopia.

Aylate, A., Hiriko, F., Molla, A., and Yohannes, A. (2013): Prevalence of subclinical mastitis in lactating cows in selected commercial dairy farms of Holeta district. *J. Vet. Med. Anim. Health,* **5**(3), pp. 67-72

Babaei, H., Mansouri-Najand, L., Molaei, M. M., Kheradmand, A. and Sharifan, M. (2007): Assessment of lactate dehydrogenase, alkaline phosphatase and aspartate aminotransferase activities in cow's milk as an indicator of subclinical mastitis. *Vet. Res. Comm.*, **31**: 419-425

Bagnicka, E., Winnicka, A., Jozwik, A., Rzewuska, M., Strzalkowska, N., Kosciuczuk, E. et al. (2011): 'Relationship between somatic cell counts and bacterial pathogens in goat milk', *Small Ruminant Research,* **100**(1): 72–77.

Barbano, D. M., (1999): Influence of mastitis on cheese manufacturing. In: National Mastitis Council (edi.): Practical guide for Control of Cheese Yield, International Dairy Federation, Brussels, Belgium, Pp. 19–27.

Bardakcioglu, H. E., Sekkin, S., Toplu, D. H. (2011): Relationship between some teat and body measurements of Holstein cows and sub-clinical mastitis and milk yield. *J. Anim. Vet. Advances,* **10**, 1735-1737.

Barkema W. H., Y.H. Schukken, T.J.G.M. Lam, M.L. Beiboer, H. Wilmink, G. Benedictus, A. Brand (1998): Incidence of clinical mastitis in dairy herds grouped in three categories by bulk milk somatic cell counts. *J. Dairy Sci.,* 81 pp. 411–419

Batavani, R. A., Mortaz, E., Falahian, K. and Dawoodi, M. A., (2003): Study on frequency, etiology and some enzymatic activities of subclinical ovine mastitis in Urmia, *Iran. Small Rum. Research,* 50, 45–50

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. *J. Vet. Med. Anim. Health,* **6**(4): 116-122.

Bengtsson, B., Unnerstad, H., Ekman, T., Waller, K., Lindberg, A., Artursson, K., Jovanovic, J. and Nilsson Ost, M. (2005): Prevalence and antimicrobial susceptibility of bacteria causing acute clinical mastitis in dairy cows in Sweden 2002-03, Pp. 888-889.

Benites, N. R., Guerra, L. J. and Melville, A. P. (2002): Etiology and Histopathology of bovine mastitis of a spontaneous occurrence. *J. Vet. Med. B.,* **49**: 366-370.

Bharti, P., Bhakat, C., Pankaj, K. P., Bhat, A. S., Prakash, A. M., Thu, R. M. and Japheth, P. K. (2015): Relationship of udder and teat conformation with intra-mammary infection in crossbred cows under hot-humid climate. *Vet. World*, 8: 2231-0916.

Bhutto, A. L., Murray, D. R., Woldehiwet, Z. (2010): Udder shape and teat-end lesions as potential risk factors for high somatic cell counts and intramammary infections in dairy cows, *Vet. J.,* **183**, 63-67.

Biffa, D., Debela, E. Beyene, F. (2005): Prevalence and Risk Factors of Mastitis in Lactating Dairy Cows in Southern Ethiopia. *Intern. J. Appl. Res. Vet. Med.,* **3**:3.

Bishi, A. B (1998): Cross-sectional study and longitudinal prospective study of bovine clinical and subclinical mastitis in peri-urban and urban dairy production system in Addis Ababa region, Ethiopia. MSc. thesis, Addis Ababa University, Free University, Berlin, Germany.

Bitman, J., Wood, D. L. And Bright, S. A. (1991): Lipid composition of teat canal keratin collected before and after milking from Holstein and Jersey cows. *J. Dairy Sci.,* **74**, 414-420.

Blood, D. C., and O. M. Radostits (eds) (1991): Mastitis. In: Veterinary Medicine, 7th edn, Baillie`re Tindall, London. Pp. 501–559.

Bogin, E. And Ziv, G. (1973): Enzymes and minerals in normal and mastitic milk. *Cornell Veterinarian,* **63:** 666-676.

Bramley, A. J., Cullor, J. S., Erskine, R. J. and Fox, L. K. (1996): In: Harmon, R. J., Hogan, J. S. (eds): The mastitis problem. Current concepts of bovine mastitis. 4th ed. Madison: National Mastitis Council edition, National. Mastitis Council, Inc., Madison, Wisconsin, Pp. 1-64.

Burvenich, C, Paape, M. J., Hill, A. W. (1994): Role of the neutrophil leucocyte in the local and systemic reactions during experimentally induced E. coli mastitis in cows immediately after calving. *Vet. Q.,* **16**, 45-50.

Capuco, A. V., Akers, M. R. and Smith, J. J. (1997): Mammary growth in Holstein cows during the dry period: Quantification of nucleic acids and histology. *J. Dairy Sci.,* **80:** 477–487.

Chandler, R. L. and Reid, M. I. (1973): Ultrastructure and associated observations in clinical cases of mastitis in cattle. *J. Comp. Pathol.,* **83**: 233-24.

Chrystal, M. A., Seykora, A. J., Hansen, L. B., Freeman, A. E., Kelley, D. H. and Healey, M. H. (2001): Heritability of teat end shape and the relationship of teat-end shape with somatic cell score for experimental herd of cows. *J. Dairy Sci.,* **84**: 2549-2554.

Chrystal, M.A., Seykora, A.J. and Hansen, L.B. (1999): Heritabilities of teat end shape and teat diameter and their relationships with somatic cell score. J. Dairy Sci., 82(9): 2017-2022.

Coban, O., Sabuncuoglu, N. and Tuzemen, N. (2009): A study on relationships between somatic cell count (SCC) and some udder traits in dairy cows. *J. Anim. Vet. Adv.,* **8**: 134-138.

Colditz I. G. and Watson D.L. (1982): Effect of immunisation on the early influx of neutrophils during staphylococcal mastitis in ewes, *Res. Vet. Sci.* **33:**146-151.

Compton, C. W.R., Heuer, C., Parker, K. And McDougall, S. (2007): Epidemiology of Mastitis in Pasture-Grazed Peripartum Dairy Heifers and Its Effects on Productivity. *J. Dairy. Sci.,* **90:** 4661-4669

Concha, C. (1986): Cells types and their immunological functions in bovine mammary tissues and secrections. A review of the literature. *Nor. Vet. Med*., **8:** 252-272.

Costa, E. O. (1998): Importancia da mastite na projecao leiteira do pais. *Rev. Ed. Cont. CRMV-SP,* **1:** 3-9

Deniz A. U. (2013): The Relationships Among Some Udder Traits and Somatic Cell Count in Holstein-Friesian Cows. *Kafkas Univ. Vet. Fak. Derg.,* **19** (4): 601-606.

EI-Rashidy, A. A., Sobhy, M. M., Kadry, R. M. and Kholeaf, Z. M. (1996): Studies On Different Causative Agents Of Bovine Mastitis. *Zag. Vet. J.*, **24** (4): 51 – 59.

El-Metwally, A. E. and Asfour, H. A. E. (2008): Mastitis pathogens in relation to histopathological changes in buffalo udder tissues and supramammary lymph nodes. *Egypt J Comp Path & Clinic Path.*, **21**(4): 190-208.

Eriskine, R. J. (2001): Intramuscular administration of ceftiofursodiu versus intrammamary infusion of penicillin/novobiocin for treatment of *Streptococcus agalactiae* mastitis in dairy cows. *J. Am. Vet. Med. Assoc.*, **208**: 258-260.

Erskine R. J. (2001): Mastitis control in dairy herds. In: Radstits, O. M. (Ed.): Herd following diagnosis of mastitis detected by a change in the electrical conductivity of milk. *J. dairy sci.*, **80**:859-863.

Etifu, M. (2012): Study on prevalence of mastitis and associated risk factors with isolation and anitimicrobial susceptibility of major pathogens in Alage state dairy farm, Ethiopia. MSc. Thesis, Addis Ababa University, Bishoftu, Ethipia.

Ferguson, J. D., Azzaro, G., Gambina, M., Licitra, G. (2007): Prevalence of mastitis pathogens in Ragusa, Sicily, from 2000 to 2006. *J. Dairy Sci*., **90**: 5798-5913.

Frost, A. J. and Brooker, E. B. (1986): Hyperacute Escherichia Coli mastitis of cattle in the immediate post -partum period. *Aust. Vet. J.*, **63**: 327-331.

Getahun, K., Kelay, B., Bekena, M. and Lobago, F. (2008): Bovine mastitis and antibiotic resistance pattern in Sellale smallholder dairy farms, Ethiopia. Trop. *Anim. Health Product.*, **40**: 216-268.

Girma, D. D. (2010): Study on prevalence of bovine mastitis on cross breed dairy cows around Holeta areas, west Shewa zone of Oromia, Ethiopia. *Glob. Vet.*, **5** (6): 318-323.

Green, M. J., Bradley, A. J., Medley, G. F. and Browne, W. J. (2008): Cow, farm, and herd management factors in the dry period associated with raised somatic cell counts in early lactation. *J. of Dairy Sci.*, **91**, 1403–1415.

Guha, A., Gera, S. and Sharma, A. (2012): Evaluation of Milk Trace Elements, Lactate Dehydrogenase, Alkaline Phosphatase and Aspartate Aminotransferase Activity of Subclinical Mastitis as Indicator of Subclinical Mastitis in Riverine Buffalo (*Bubalus bubalis*). *Asian-Aust. J. Anim. Sci.*, **25** (3): 353 – 360.

Guidry, A .J. (2007): Mastitis and the immune system of the mammary gland. In: Larson, B.L. (eds): *Lactation*. The Iowa State University Press, Ames, Iowa, USA, Pp. 229-262.

Gulyas, L. and Ivancsics, J. (2002): Relationship between the somatic cell count and certain udder-morphologic traits. *Acta Vet. Hung.*, **50:** 373–383.

Haghkhah, M., Ahmadi, R. M., Gheisari, R. H., Kadivar, A. (2011): Preliminary bacterial study on subclinical mastitis and teat condition in dairy herds around Shiraz. *Turkish J. Vet. Anim. Sci.*, **35**: 387-394.

Harby, H. A., Jakee, E. J. and Refai, M. (1991): The etiology and diagnosis of mastitis in Oman. *J. Egypt. Vet. Med. Asso.,* **51**: 783-795.

Harris, H. (1989): The human alkaline phosphatases. *Clin. Chim. Acta.,* **186**:133.

Hassan, S. M. and Yousif A. A. (2014): Evaluation of some biochemical indices in "milk whey" for diagnosis of subclinical mastitis caused by some *Enterobacteriaceae* family. *MRVSA* 3(1), 1-7.

Hill, A. W. (1981): Factors influencing the outcome of Escherichia coli mastitis in the dairy cow. *Res. Vet. Sci.,* **31**:107-112.

Hogan, J. S. (eds): The mastitis problem: Current concepts of bovine mastitis. 4th ed. Madison: National Mastitis Council edition, National. Mastitis Council, Inc., Madison, Wisconsin, Pp. 1-64.

Hussain, R., Javed T. M., Khan A., Mahmood, F. and Kausar R. (2012): Mastitis and Associated Histo-pathological Consequences in the Context of Udder Morphology**.** *Int. J. Agric. Biol., Vol.* **14** (6): 947-52.

Hussein, N., Yehualashet, T. and Tilahun, G., (1997): Prevalence of mastitis in different local and exotic breeds of milking cows. *Eth. J. Agr. Sci.*, 16: 53-60.

Ibrahim, A. M., Al-Humiany, A. A., Mansour, A. M. A., and Zaki, M. H. (2011): Epidemiology and microbiological studies on mastitis in she-camels. *Int. J. Microbiol. Res.*, **2**: 18–27.

IDF (1995): Enumeration of somatic cells in milk , Microscope method. pp 1 – 7.

Ingalls, W. (2009): Somatic cells, mastitis and milk quality. http://www.jds.fass.org/cgi/content/full

Javaid, S. B., Gadahi, A., Khaskeli, M. J., Bhutto, B. M., Kumbher, S. and Panhwar H. A. (2009): Physical and chemical quality of market milk sold at Tandojam. Pakistan. *Pakistan Veterinary J.*, **29**: 27-30.

Jones, G. M. and Bailey, T. L. (2010): Understanding the basics of mastitis. *Virginia Cooperative Extension*.

Jones, G. M., Pearson, R. E., Clabaugh, G. A. And Heald, C. W. (1984): Relationships between somatic cell counts and milk production. *J. Dairy Sci.*, **67**:1823–31.

Jubb, K. V. F., Kennedy, C. P., and Palmer, N. (1993): The female genital system. In: Kennedy, P. C., and Muller, B. R. (eds): Pathology of Domestic Animals. Academic Press, California, Pp. 454–470.

Juozaitiene, V., Juozaitis, A. and Micikeviciene, R. (2006): Relationship between somatic cell count and milk production or morphological traits of udder in black and white cows. *Turk. J. Vet. Anim. Sci.*, **30**: 47-51.

Katsande, S., Matope, G., Ndengu, M. and Pfukenyi, D. M. (2013): Prevalence of mastitis in dairy cows from smallholder farms in Zimbabwe. *J. of Vet. Research*, **80**(1): 523-527

Katsoulos, P. D., Christodoulopoulos, G., Minas, A., Karatzia, M.A., Pourliotis, K. and Kritas, S. K. (2010): The role of lactate dehydrogenase, alkaline phosphatase and aspartate aminotransferase in the diagnosis of subclinical intramammary infections in dairy sheep and goats. *J. Dairy Res.* **77**: 107-111.

Kerro, D. O. and Tareke, F. (2003): Bovine mastitis in selected areas of southern Ethiopia. *Trop. Anim. health and prod.*, **35:** 197 – 205.

Kheira, G. and Abdellatif, N. (2014): Impact of Subclinical Mastitis on the Health of The Mammary Gland. *Global Veterinaria*, **12** (2): 193-196.

Khodke, M. V., Bonde, S. W. and Ambade, R. B. (2009): Alteration of Enzyme Aspartate Transaminase in Goat milk related to Udder Health Status. *Veterinary World.*, **2** (1): 24-26.

Klaas, I. C., Enevoldsen, C., Vaarst, M., Houe, H. (2004): Systematic clinical examinations for identifi cation of latent udder health types in Danish dairy herds. *J. Dairy Sci.*, **87**, 1217-1228.

Klein, D., Flock, M., Khol, J. L., Franz, S., Stuger, H. P. and Baumgartner, W. (2005): Ultrasonographic measurement of the bovine teat: breed differences, and the significance of the measurements for udder health. *J. Dairy Res.*, **72**: 296-302.

Klein, D., Flock, M., Khol, L. J., Franz, S., Stuger, P. H., Baumgartner, W. (2005): Ultrasonographic measurement of the bovine teat: breed differences, and the significance of the measurements for udder health. *J. Dairy Res*., **72**: 296-302.

Kremer, W. D. J., Burvenich, C., Noordhuizen-Stassen, E. N. (1993): Severity of experimental Escherichia coli mastitis in ketonemic and nonketonemic dairy cows. *J. Dairy Sci.*, **76:** 3428-3436.

Kuczaj, M. (2003): Analysis of changes in udder size of high yielding cows in subsequent lactations with regard to mastitis. *Electron J. Pol. Agric. Univ. Ser. Anim. Husb*., **6**: 1.

Kuczaj, M., Kruszyński, W., Pawlina, E. and Akińcza, J. (2000): Relations between milk performance and udder dimensions of Black-White cows imported from Holland. *Electron. J. Pol. Agric. Univ. Ser. Anim. Husb.*, **3:** 2.

Kumar, A. H. S., Singh, N. K. (2011): Compromised cytology of bovine udder during mastitis: therapeutic potential of adult stem/progenitor cells; In: Sharma, N., Bacic, G., Singh, N. K. (eds.): Production diseases of dairy animals. Satish Serial Publishing House, Delhi, India, Pp. 391-411.

Lakew, M., Tolosa, T. and Tigre, W. (2009): Prevalence and major bacterial causes of bovine mastitis in Asella, south eastern Ethiopia', *Trop. Anim. Health and Prod.*, **41**: 1573–7438.

Lenhardt, L. (1984): Histochemical Observation of the Activity of some Enzymes in Cows with Mastitis (In Slovak). Dissertation thesis, Košice, SR, Pp. 71.

Lenhardt, Ľ., Švicky, E., Dudrikova, E., Mozeš, Š. (1999): Histochemical study of inflammation-affected mammary gland in lactating dairy cows (In Slovak). *Vet. Med.-Czech*, **44**:109-113.

Lopez-Benavides, M. G., Williamson, J. H., Cursons, R. T., Lacy-Hulbert, S. J. and Woolford, M. W. (2005): *Streptococcus urberis* population dynamics in the New Zealand pastoral dairy farm. In: Hogeveen, H. (edi): Proceedings of the 4th IDF International Mastitis Conference, Maastricht, Wageningen Academic Publishers. Pp. 649-655.

Lundberg, A. (2015): Genotypes, Spread, and Infection Outcome of Three Important Udder Pathogens Doctoral Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Manzi, M. D. P., Nobrega, D. B., Faccioli, Y. M., Troncarelli, Z., Menozzi, B. D. and Langoni, H. (2012): Relationship between teat-end condition, udder cleanliness and bovine subclinical mastitis. *Res. Vet. Sci.*, **93**: 430-434.

Matios, L., Tadele, T and Worku, T. (2009): Prevalence and major bacterial causes of bovine mastitis in Asella, South Eastern Ethiopia. *Trop. Anim. Health Prod.*, **41**:1525–1530.

Mein, G. A., Reinemann, D. J., Schuring, N. and Ohnstad, I. (2004): Milking machines and mastitis risk: A storm in a teat-cup. In: Proceedings of the 43rd annual meeting of the National Mastitis Council, USA.

Mekbib, B, Furgasa, M, Abunna, F., Megersa, B., Regassa, A. (2009): Bovine mastitis: Prevalence, risk factors and major pathogens in dairy farm of Holeta town Central Ethiopia. *J. Vet. World*, **3**(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 *J. Vet. Med,* **161**: 12, 574-579.

Milner, P. K. L., Page, A.W. and Hillerton, J. E. (1996): Detection of clinical mastitis by changes in electrical conductivity of fore milk before visible changes in milk. *J. of Dairy Sci.,* **79**: 83-86.

Milner, P., Page, K. L. and Hillerton, J. E. (1997): The effects of early antibiotic treatment following diagnosis of mastitis detected by a change in electrical conductivity of milk. *J Dairy Sci* **80**, 859-863.

Mohamed E. A. G. (2014): Investigation of some enzymes level in blood and milk serum in two stages of milk yield dairy cows at Assut city. *Assiut Vet. Med. J.,* **60**: 142.

Mungube, E.O. (2001): Management and economics of dairy cow mastitis in the urban and periurban areas of Addis Ababa (Addis Ababa milk shed), MSc Thesis, Faculty of Veterinary Medicine, Addis Ababa University, Debre Zeit.

Nakov, D., Hristov, S., Andonov, S. and Trajchev, M. (2014): Udder-related risk factors for clinical mastitis in dairy cows. *Vet. Arhiv.,* **84** (2): 111-127.

Nemcova, E., Stipkova, M., Zavadilova, L., Bouska, J. and Vacek, M. (2007): The relationship between somatic cell count, milk production and six linearly scored type traits in Holstein cows. *Czech J. Anim. Sci.,* **52**(12): 437-446.

Nibret, M., Yilkal, A., Kelay, B. and Abebayehu, T. (2011): Antimicrobial susceptibility of mastitis pathogen from smallholder dairy herds in and around Gondar, Ethiopia. *Anim. vet. Advance,* **12**: 1616-1622.

Nickerson, S. C. (2009): Control of heifer mastitis: antimicrobial treatment-an overview. *Vet Microbiol.,* **134**: 128-35.

Nielen, M., Schukken, Y. and Brand, A. (1995): Detection of subclinical mastitis from on-line milking parlor data. *J. of Dairy Sci.,* **78:** 1039-1049.

NMC (1990): Microbiological procedures for the diagnosis of bovine udder infection, 3 ed Arlington VA: National Mastitis Council Inc.

NMC (1996): Current Concepts of Bovine Mastitis. 4th Meeting, National Mastitis Council. Pp. 94-99.

Nyman, A. K., Ekman, T., Emanuelson, U., Gustaffson, H. A., Holtenius, K., Waller, P. K., Sandgren H. C. (2007): Risk factors associated with the incidence of veterinary-treated clinical mastitis in Swedish dairy herds with a high milk yield and a low prevalence of subclinical mastitis. *Prev. Vet. Med.,* **78**: 142-160.

O'Connor, J. S. and Laws, E. R. J. (1969): Changes in histochemical staining of brain tumor blood vessels associated with increasing malignancy. *Acta Neuropath.,* **14**: 161—173.

Ogorevc, J., Kunej, T., Razpet, A. and Dovc, P. (2009): Database of cattle candidate genes and genetic markers for milk production and mastitis. *Anim. Genet*., **8**:8.

Oliszewski, R., Nunez De Kairuz, M. S., Gonzalez De Elias, S. N. and Oliver, G. (2002): Assessment of β glucuronidase levels in goat's milk as an indicator of mastitis: comparison with other mastitis detection methods. *J. of Food Prot.,* **65**, 864–866.

Orban, M., Gulyas, L., Nemeth, S. and Gergacz, Z. (2009): Morphometric evaluation of udders in jersey cows. *Sci. Pap. Anim. Sci. Biotechnol.,* **42**(2): 327-332.

Oviedo-Boyso, J., Valdez-Alarcón, M., Cajero-Juarez, A., Ochoa-Zarzosa, J. E., López-Meza, A., Bravo-Patiño, V. M. Baizabal-Aguirre (2007): Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis. *J. Infect.,* **54**: 399–409.

Peeler, E. J., Green, J. M., Fitzpatrick, L. J., Morgan, L. K., Green, E. L. (2000): Risk factors associated with clinical mastitis in low somatic cell count British dairy herds. *J. Dairy Sci.* **83**: 2464-2472.

Piepers, S., De Vliegher, S., de Kruif, A., Opsomer, G. and Barkema, W. H. (2009): Impact of intramammary infections in dairy heifers on future udder health, milk production, and culling. *Vet. Microbiol.*, **134:** 113–120.

Porcionat, D., M. de Felicio, Weber Soares, V. B., dos Reis, M. B. C., Cortinhas, S. C., Mestieri. L. and dos Santos, V. M. (2010): Milk flow, teat morphology and subclinical mastitis prevalence in Gir cows. Pesq. agropec. bras., Brasília, **45**(12): 1507-1512.

Pösö, J. and Mäntysaari, E. (1996): Relationships between clinical mastitis, somatic cell Score, and production in the first three lactations of Finnish Ayrshire. *Journal of Dairy Sci.*, **79**:1284-1291.

Ptak, E., Jagusiak, W., Żarnecki, A. And Otwinowska-Mindur, A. (2011): Heritabilities and genetic correlations of lactational and daily somatic cell score with conformation traits in Polish Holstein cattle. *Czech J. Anim. Sci.*, **56**: 205-212.

Pukačova, J., Dudrikova, E., Lenhardt, Ľ. and Poľakova, L. (2010): Histological and histochemical picture of alkaline phosphatase in *Staphylococcus aureus* mastitis. *Folia veterinaria*, **54**(4): 173-177.

Quinn, P. J. Carter, M. E. Markey, B. K. and Carter, G. R. (2002): Veterinary Microbiology and Microbial Diseases, Bacterial Causes of Bovine Mastitis, 8th Edition, Mosby International Limited, London, pp. 465-475.

Quinn, P. J., Carter, M. E., Marker, B. K., and Carter, G. R. (1994): Clinical Veterinary Microbiology. 4th Edi.,Mosby-Year Book, Europe Limited, Spain.

Radostits, O. M. (2007): Veterinary medicine: a textbook of the diseases of cattle, horses, sheep, pigs and goats. 10th edition. Edinburgh, Saunders Elsevier, Pp. 1120-30

Radostits, O. M., Gay, G. C., Blood, D. C., Hinchillif, K. W. (2000). Mastitis In: Veterinary Medicine, 9th Ed. Harcourt Limited, London, Pp. 603-607.

Radostits, O. M., Leslie, K. E., Fetrow, J. (1994): Herd Health: Food Animal Production Medicine. 2nd ed. Toronto: W.B. Saunders Company, Pp. 229-273.

Rainard, P. and Riollet, C. (2006): Innate immunity of the bovine mammary gland. *Vet. Res.* **37**: 369-400.

Rajala-Schultz, P. J. and Grohn, Y. T. (1999): Culling of dairy cows. Part III. Effects of diseases, pregnancy status and milk yield on culling in Finnish Ayrshire cows. *Prev. Vet.Med.*, **41**: 295–309.

Refai, M .K. (1988): Mastitis, aetiology, diagnosis and control. *J. Egypt. Vet. Med. Ass.,* **48**: 521-545.

Regassa, A. Abera, M., Demie, B., Aragaw K., and Regassa, F. (2010a): Isolation and identification of *Staphylococcus aureus* from bovine mastitic milk and their drug resistance patterns in Adama town Ethiopia. *J. Vet. Med. and Animal Health*, **3**: 29-34.

Regassa, A., Mekibib, B., Furgasa, M., Abunna, F. and Megersa, B. (2010b): Bovine Mastitis: Prevalence, risk factors and major pathogens in dairy farms of Holeta town, central Ethiopia. *Vet. World,* **3**: 397-403.

Reinard, P., Riollet, C. (2006): Innate immunity of the bovine mammary gland. *Vet. Res.*, **37**:369–400.

Rodenburg, J. (1990): Mastitis prevention: Environmental control: Ontario. Ministryof Health. Food Animal Production Medicine. W. B. Saunders Company, Pennsylvania, USA.

Rogers, G. W., Hargrove, G. L., Lawlor, T. J. J. and Ebersole, J. L. (1991): Correlations among linear type traits and somatic cell counts. *J. Dairy Sci.,* **74**: 1087-1091.

Rupp, R., D. Boichard (2003): Genetics of resistance to mastitis in dairy cattle. *Vet. Res.,* **34**: 671-688.

Sandholm, M. (1995): Detection of inflammatory changes in the milk. In: Gum, J. (eds.): The Bovine Udder and Mastitis. Finland, Pp. 98-104.

Schukken, H., David, Y., Wilson, J., Welcome, F., Garrison-Tikofsky, L., Gonzalez N. (2003): Monitoring udder health and milk quality using somatic cell counts. *Vet. Res*. **34:** 579–596.

Sears, P.M., McCarthy, K.K. (2003): Diagnosis of mastitis for therapy decisions. *Vet. Clin. Food Anim*., **19**: 93–108.

Seffner, W. and Pfützner, H. (1980): Mycoplasma mastitis of cattle: Pathological anatomy and histology of experimental Mycoplasma bovis mastitis. *Archiv. fur. Experimentelle Veterinarmedizin*, **34**(6):817-826.

Seykora, A. J. and McDaniel, T. B. (1985): Udder and teat morphology related to mastitis resistance: A review: *J. Dairy Sci.,* **68**: 2087-2093.

Sharma, N., Gupta, S. K. and Sharma, U. (2007): Treatment of clinical mastitis in buffalo-A case report. *Buffalo Bull.,* **26**(2): 56-58.

Sharma, N. and Maiti, S. K. (2010): Incidence, etiology and antibiogram of sub clinical mastitis in cows in Durg, Chhattisgarh. *Indian J. Vet. Res.*, **19**(2): 45-54.

Shukla, S. K., Dixit, V. P., Thapliyal, D. C., Garg, S. K. and Kumar, A. (1997): A note on the incidence of bovine mastitis in relation to teat shape size and quarters affected. *Indian Vet. J.*, **74**: 989-990.

Singh, K., Erdman, R. A. and Swanson, K. M. (2010): Epigenetic Regulation of Milk Production in Dairy Cows. *J. Mammary Gland Biol Neoplasia.*, **15**: 101-112.

Singh, R. S., Bansal, K. B. and Gupta, K. D. (2013): Udder health in relation to udder and teat morphometry in Holstein-Friesian × Sahiwal crossbred dairy cows. *Trop. Anim. Health Prod.*, Published Online, 13 August 2013.

Slettbakk, T., Jerstad, A., Farver, B. T. and Holmes, C. J. (1995): Impact of milking characteristics and morphology of udder and teats on clinical mastitis in first and second lactation Norwegian cattle. *Prev. Vet. Med.,* **24:** 235–244.

Slyzius, E., Juozaitiene, V., Juozaitis, A., Zilaitis, V., Slyziene, B. and Cereskiene, E. (2014): Udder quarters morphological and milking traits risk factors influencing productivity and Subclinical mastitis in dairy cows. *Bulgarian J. of agri. Sci.*, **20** (6): 1502-1507.

Smith, K. L. and Hogan S. J. (1993): Environmental mastitis. *Vet. Clin. of N. Amer: Food Animal Pract.*, **9**:489-498.

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. *J. Dairy Sci.,* **77**: 75-79.

Sordillo, L. M. and Streicher, L. K. (2002): Mammary gland immunity and mastitis susceptibility. *J Mammary Gland Biol Neoplasia,* **7**: 135-146.

Sorensen, M. K., Jensen, J. and Christensen, G. L. (2000): Udder conformation and mastitis resistance in Danish first-lactation cows: heritabilities, genetic and environmental correlations, *Acta Agr. Scand,*. **50**: 72-82.

Sori, H. Zerihun, A. and Abdicho, S. (2005): Dairy cattle mastitis in and around Sebeta, Ethiopia *Intern. J. Appl. Res. Vet. Med.*, **3:**332-338.

Stabemfeldt, G. H., and Spencer, R. G. (1965): The lesions in bovine udders shedding hemolytic coagulase-positive staphylococci. *Pat. Vet.,* **2:** 585–600.

Szencziová, I., Strapák, P., Stádník, L., Ducháček J. and Beran, J. (2013): Relationship of udder and teat morphology to milking characteristics and udder health determined by ultrasonographic examinations in dairy cows. *Annals of Animal Science*, **13**: 783-79.

Talkuder, S. (2007): Histopathological techniques: Tissue processing and staining. In: *www.Talukderb.com*.

Tancin, V., Uhrinča M., Mihina, Š., Sudzinová, J., Foltys, V. and Tančinová, D. (2007): Somatic cell count and quarter milk flow parameters from udder of dairy cows. *Slovak J. Anim. Sci.,* **40**: 79–82.

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 J. of Med. Research,* **15** (2): 0975-5888.

Tilki, M., Inal, S., Colak, M. and Garip, M. (2005): Relationships between milk yield and udder measurements in Brown Swiss Cows Turk. *J. Vet. Anim. Sci.,* **29:** 75–81.

Timms L. L. (1990): Can Somatic Cells Get Too Low? Proceeding 29th Annual Agriculture and food Factsheet. AGDEX 410/662.

Trinidad, P., ickerson, S. C., N and Adkinson, W. R. (1990): Histopathology of staphylococcal mastitis in unbred dairy heifers. *J. Dairy Sci.*, **73**: 639-647.

Uzmay, C., Kaya, Y., Akbas, Y. and Kaya, A. (2003): Effects of udder and teat morphology, parity and lactation stage on subclinical mastitis in holstein cows. *Turk. J. Vet. Anim. Sci.*, **27**: 695-701.

Valde, J. P., Lystad, L. M., Simensen, E. and Osteras, O. (2007): Comparison of feeding management and body condition of dairy cows in herds with low and high mastitis rates. *J. Dairy Sci.,* **90:** 4317-4324.

Van Dorp, R. T. E., Martin, W., Houkri, M. S., Noordhuizen, M. T., Dekkers, M. C. J. (1999): An epidemiologic study of disease in 32 registered Holstein dairy herds in British Columbia. *Can. J. Vet. Res.*, **63:** 185-192.

Viguier, C., Arora, S., Gilmartin, N., Welbeck, K. and O'Kennedy, R. (2009): Mastitis detection: current trends and future perspectives. *Trends Biotechnol.,* **27:** 486–493.

Waller, K. P., Bengtsson, B., Lindberg, A., Nyman, A. and Ericsson, U. H. (2009): Incidence of mastitis and bacterial findings at clinical mastitis in Swedish primiparous cows influence of breed and stage of lactation. *Veterinary Microbiology*, **134**: 89–94.

Weiss, D., Weinfurtner, M. and Bruckmaier, M. R. (2004): Teat anatomy and its relationship with quarter and udder milk flow characteristics in dairy cows. *J. Dairy Sci.*, **87**: 3280–3289.

Workineh, S. Bayleyegn, M. Mekonnen, H. and Potgieter, L. N. D. (2002): Prevalence and etiology of mastitis in cows from two major Ethiopian dairies. *Trop. Anim. Health Prod.*, **34**: 19-25.

Yousaf, M., Muhammad, G., Khan, Z. M. and Rahman, U. S. (2010): Effect of infusions of non-antibiotic antibacterials alone and in combination with Cephradine on milk yield of buffaloes affected with clinical mastitis. *Pakistan Vet. J.*, **30**: 39–43.

Zarkower, A. and Norcross, L. N. (1966): Histological changes in the bovine mammary gland after infusion with Streptococcus agalactiae. *Cornell Vet.*, **56:** 555–566.

Zeinhom, M. M. A., Abed, A. H. and Hashem, K. S. (2013): A contribution towards milk enzymes, somatic cell count and bacterial pathogens associated with subclinical mastitis cows milk. *Assiut Vet. Med. J.*, **59**: 138.

Zeryehun, T., Ayaand, T. and Bayecha, 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. *J. Anim. Plant Sci.* **23**(1): 50-55.

Zhao, X. and Lacasse, P. (2007): Mammary tissue damage during bovine mastitis: Causes and control. *J. Anim Sci.*, **86**: 57-65.

Zwald, A. G., Ruegg, P. L. and Kaneene, J. B. (2004): Management practices and reported antimicrobial usage on conventional and organic dairy farms. *J Dairy Science*, **87:** 191-201.

8. ANNEXES

Annex 1 : Histopathological procedures (Takulder, 2007)

1. Fixation of tissue by 10% neutral buffered formaldehyde
2. Trimming part of the tissue in a way that the lesion we require be included or not missed and to fit standard histological processing tissue cassettes (5mm thickness).
3. Tissue specimen processing: fixation of tissue by formalin, dehydrating tissue by increasing alcohols concentration, clearing of tissue by xylene, and impregnation of tissue by paraffin wax.

   Formalin-I 2hr - ---Formalin-II 2hr - --70% Alcohol 1hr --- 95% Alcohol-100% Alcohol-I 1hr--- 100% Alcohol-II 2hrs ----- 100% Alcohol-III 2hrs ----- Xylene-I 1:30hrs ------- Xylene-II 1:30hrs ------ Xylene-III 1:30hrs ------ Paraffin-I 2hrs ---- Paraffin-II 3hrs.
4. Embedding of processed tissue: impregnated tissue is placed in a mould with their labels and then fresh melted wax (54-60c^{o}) is poured and allowed to settle and solidify.
5. Sectioninig: sectioning of tissue in 3-5 micron thickness and put on water bath to straighten the ribbon, and then adhere on the surface of frost ended and clear slide. Later label and put an incubator over night.
6. Staining: Hematoxyline eosine stain procedure
   a. Deparaffinize slides in 2 changes of xylene for 5minutes.
   b. Hydrate slides in 3 changes of 100% alcohol each for 3minutes and 1 changes of 95% alcohol for a minute and 1 change of 70% alcohol for 3minutes
   c. Rinse in distilled water until repples disappear from slides.
   d. Place in heamatoxyline (mayer's hematoxline) for 10-15 minutes
   e. Rinse in tap water until water runs clear
   f. Decolorize in 1% acid alcohol, 3-6 qiuck dips. Check differentiation microscopically: Nucleic should be distinct; cytoplasm should be uncolored.
   g. Rinse in tap water until ripples disappear from slides.
   h. Stain in eosin, 3 dips.
   i. Rinse in tap water until water runs clear.

j. Dehydrate in 95% alcohol of 3dips and 100% alcohol, 3 changes each for 3minutes.
k. Clear in 3 changes of xylene for 5 minutes each.
l. Mount cover glass with DPX.
m. Examination of the prepared slides under the microscope.

Annex 2: Body condition score 1-5 scale of dairy cattle

Score	Interpretation	Judgment
1	Deep cavity around tail head. No fatty tissue felt between pins. Pelvic bone easily felt. Skin is supple. Ends of short ribs sharp to touch. Upper surfaces can be felt easily. Deep depression in loin.	Poor
2	Shallow cavity lined with fatty tissue at tail head. Some fatty tissue felt under pin bone. Pelvis easily felt. Ends of short ribs feel rounded. Upper surface felt with slight pressure. Depression visible in loin.	Poor
3	No visible cavity around tail head. Fatty tissue is easily felt over whole rump. Skin appears smooth. Pelvis is felt with slight pressure.	Good
4	Folds of fatty tissue are visible around tail head. Patches of fat are present around the pin bones. Pelvis is felt only with firm pressure. Short ribs cannot be felt even with firm pressure. No depression is visible in loin between backbone and hip bone.	Good
5	Tail head is buried in fatty tissue. Skin is distended. No part of pelvis can be felt even with firm pressure. Folds of fatty tissue over short ribs. Bone structures cannot be felt. These cows are good candidates for fat cow syndrome	Good

Sources: (Parker et al. 1989)

Annex 3: California Mastitis Test (CMT) scores and interpretation

CMT score	Interpretation	Visible reaction	Total cell count
0	Negative	Milk fluid is normal	0-200,000 (0-25% neutrophils)
T	Trace	Slight precipitation	(1.5-5) x 105 (30-40% neutrophils)
1	Weak positive	Distinct precipitation but not gel formation	(4-15) x 105 (40-60% neutrophils)
2	Distinct positive	Mixture thickens with gel formation	(8-50) x 105 (60-70% neutrophils)
3	Strong positive	Strong gel that is cohesive with a convex surface	>5,000,000(70-80% neutrophils)

Source: Quinn et al. (1999)

Annex 4: Methods used to identify different bacteria (Quinn et al., 2002)

Blood agar base

Composition (g/l): hear muscle, infustion from(solid)2.0; pancreatic digest of casein13; yeast extract 5.0; sodium chloride 5.0; agar 15.0. Direction: Suspend 40g of powder in 1 litre of distilled water. Mix thoroughly and heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121 ocfor 15 minutes and cool the base to 45-50^{o}c and 5-7% sterile sheep blood. Colony growth on blood agar base and haemolysis formation were observed.

Gram staining reagent

Procedure: Applying a primary stain (crystal violet) for 60 second to a heat-fixed smear of a bacterial culture. Then wash off with tap water. Then addition of iodide which remain for 60 second. Then wash off with tape water. Rapid decolorization with ethanol or acetone for only 15-

30 seconds. Then wash off with tape water. Finally, counterstaining with safranin for 60 seconds. Then wash off with tap water and dried with bloating paper.

Catalase test

Principle: the breakdown of 3% hydrogen peroxide in to oxygen and water is mediated by the enzyme catalase.

Procedure: a loop of bacterial growth is taken from nutrient agar medium. Then the bacterial cell placed on a clean microscopic slide and a drop of 3% hydrogen peroxide is added. An effectiveness of oxygen gas, within a few seconds, indicates a positive reaction.

Coagulase test

Tube coagulase test was used for identification of coagulase positive and coagulase negative staphylococcus species based on the reaction of pathogenic staphylococcus species reacts with coagulase reacting factor (CRF) in plasma to form a complex, thrombin, and then converts fibrinogen to fibrin resulting in clotting of plasma.

Procedure: 0.5 ml of rabbit plasma was poured in to 10 mm test tube and equal amount of overnight grown presumptive Staphylococcus bacteria was added in the tube and mixed, then incubated at 37^{o}C. The tests were read by slowly tilting the tube. A positive test results in a highly viscous clot formation in the plasma. Once a coagulum, no matter how small, has formed the test is considered positive (usually within 4 hours). A negative test results in the plasma remaining free flowing with no evidence of a clot, were incubated overnight before a test is called negative, but prolonged incubation (over 24 hours) may result in the dissolution of a formed clot. Depending on the formation of clot for positive reaction S. *aureus* and *S. intermidius* and *S. hyicus* showed positive result otherwise considered as CNS.

Mannitol Salt Agar

Procedure: Mannitol Salt Agar media was prepared, then all gram positive bacteria were inoculated in to the medium and the result was recorded after 24 hours incubation period at 37 degree Celsius. *Staphylococci* species produced yellow colony and yellow medium that indicates mannitol fermentation other bacterial species could not ferment mannitol.

Manitol salt agar

Procedure: All the colonies that were collected through the necessary identification tests (catalase, O-F and coagulase test) were streaked on manitol salt agar which is selective media for members of *Staphylococci* and the bacterium were incubated at 37oc for about 24 hr. A positive result showing growth and a clear media change from red to yellow.

Purple agar base

Principle: Purple agar base contains maltose as a substrate and bromocresol purple indicator used to identify bacterium that can ferment maltose (1% maltose sugar).

Procedure: Purple agar base was pepared and the bacterium was inoculated and placed in the incubator at 37oc for 24 hour. A yellow color (acid) is a positive reaction for fermentation of the carbohydrate incorporated into the medium. Bubbles in the inverted fermentation vials are an indication of gas production.

Buffered peptone water

Composition (g/l): Pancreatic digest 10.00; Disodium Phosphate 3.50; Sodium Chloride 5.00; Monopotassium Phosphate 1.50. Final pH: 7.0 ± 0.2 at 25ºC; Distilled water 1 liter

Preparation: Dissolve 20 grams of the medium in one liter of distilled water. Mix well and dissolve by heating with frequent agitation. Boil for one minute until complete dissolution. Dispense into appropriate containers and sterilize at 121°C for 15 minutes.

Tryptone Soya Broth

Composition (g/l): Pancreatic digest of casein 17.00; Enzymatic digest of soya bean 3.00; Sodium Chloride 5.00; Dipotassium hydrogen phosphate 2.50; Glucose 2.50. Final PH: 7.3 ± 0.2 at 25ºC, Distilled water 1 liter

Preparation: Suspend 30 grams of the medium in one liter of distilled water. Mix well. Heat slightly until complete dissolution of the medium if necessary. Dispense in tubes and sterilize by autoclaving at 121°C for 15 minutes. Larger quantities may require longer sterilization time, but the temperature should not be increased.

Simmons Citrate Agar

Composition (g/l): Ammonium Dihydrogen Phosphate 1.00; Dipotassium Phosphate 1.00; Sodium Chloride 5.00; Sodium Citrate 2.00; Magnesium Sulphate 0.20; Bacteriological Agar 15.00; Bromthymol Blue 0.08. Final PH: 6.8 ± 0.2 at 25ºC, Distilled water 1 litre

Preparation: Suspend 24.28 grams of the medium in one liter of distilled water. Heat to boiling till the dissolve the medium completely. Dispense in tubes and sterilize in the autoclave at 121ºC for 15 minutes. Cool the tubes in a slanted position so that the base is short (1-1.5 cm. deep).

Triple Sugar Iron Agar

Composition (g/l): Peptone Mixture 20.00; Lactose 10.00; Sucrose 10.00; Sodium Chloride 5.00; Beef Extract 3.00; Yeast Extract 3.00; Glucose 1.00; Ferrous Ammonium Citrate 0.30; Sodium thiosulphate 0.30; Phenol Red 0.024; Bacteriological Agar 12.00. Final pH: 7.4 ± 0.2 at 25ºC, Distilled water 1 litre

Preparation: Suspend 65 grams of the medium in one liter of distilled water. Bring to the boil to dissolve completely. Mix well and distribute in tubes. Sterilize by autoclaving at 121º C for 15 minutes and cool in

Methyl Red-Vogues Proskauer Medium

Composition (g/l): Peptone mixture 7.00 (Peptic digest of animal tissue 5.00); Dextrose 5.00; Dipotassium Phosphate 5.00. Final pH: 7.5 ± 0.2 at 25ºC, Distilled water 1 litre

Preparation: Suspend 15 grams of the medium in one liter of distilled water. Heat to dissolve and distribute into tubes in 1 ml amount and sterilize by autoclaving at 121. Dispense in tubes in 10ml amounts and sterilize at 121ºC for 15 minutes. Reagents required Alpha-Naphthanol (5%) (1-Naphihol 6gm; Ethanol, 96% (volume fraction) 100ml, Potassium hydroxide solution (40%) (Potassium hydroxide 40gm; distilled water 100ml) *Preparation* dissolve the potassium hydroxide in the distilled water. Methyl Red alcoholic indicator solution (Dissolve 0.1g of methyl red powder in 300ml 95% ethanol and 200ml distilled water), 1% solution of 2, 3, 5-Triphenyl Tetrazolium Chloride (Dissolve 500mg of triphenylTetrazolium chloride in dehydrated alcohol tomake100ml)

Indole test

Principle: Organisms those posses the enzyme tryptophanase can break down the amino acid tryptophan to indole. When indole reacts with para-dimethylaminobenzaldehye (Kovac's reagent) a pink -colored complex is produced. Tryptophan is plentiful in most media, but growth on blood agar or chocolate agar produces the best effects.

Procedure: Take loopful of inoculum by touching the 3-5 representative colonies with inoculating loop from pure colonies and inoculate Tryptone soya broth tube. Incubate the tube at 37°C for 24 hours and cap left loosen to aerate the tube. After incubation, add 5-10 drops (0.5ml) of Kovac's reagent to the culture broth and agitate gently. Then observe the tube for color change within 5 minutes.

Citrate utilization test

Principle: Citrate contains carbon. If an organism can use citrate as its only source of carbon the citrate in the media will be metabolized. Bromthymol blue is incorporated into the media as an indicator. Under alkaline conditions this indicator turns from green to blue. The utilization of citrate in the media releases alkaline bicarbonate ions that cause the media pH to increase above 7.4 cause the media blue.

Procedure: Take loopful of inoculum by touching the center of 3-5 representative colonies with inoculating loop and streak it onto the surface of a Citrate slant. Incubate the tube aerobically at 35°C with cap left loosen for 22 hours. After 22 hrs incubation observe the tube for growth and color change.

Triple sugar iron (TSI) test

Principle: Bacteria that ferment any of the three sugars in the medium will produce by products which will change the color of the red pH-sensitive dye (phenol red). A bacterium that is a non-lactose fermenter and ferments glucose, initially causes a yellow slant/yellow bottom (acid/acid reaction) after 8 hours, but then converts to a red slant/yellow bottom after 24 hours (alkali/acid reaction). Where as if it ferments both lactose and glucose, it results in a yellow/yellow tube and remains that way due to the large amount of acid produced in the reaction.

Procedure: By sterile inoculating loop touching the center of colony from isolated pure colony take loop full of inoculum. Streak the inoculum back and forth on TSI agar in tube along the surface of the slant. Incubate the tube with the cap loosened at 35 ºC for 22 hours.

Methyl red and Vogues Proskauer test

Principle: Some organisms produce acid from the metabolism of glucose in a sufficient quantity to produce a pH of 4.4 in the media. These acids are not further metabolized and are said to be stable acids. At a pH of 4.4 or less the pH indicator methyl red is a bright cherry red. While also some organisms initially produce acid from glucose metabolism but further metabolize the acid produced to neutral end products, such as acetoin, and 2, 3-butanediol. Initially the pH may drop to 4.4 but the neutral end products raise the pH so the methyl red test will be negative. Acetoin and 2, 3-butanediol under alkaline conditions will react with alpha-naphthol (1-naphthol) to produce a mahogany red color.

Procedure: Take loopful of inoculum by touching the centre of 3-5 representative colonies with inoculating loop from the pure isolated colonies and inoculate MR-VP broth with inoculum, incubated 37 ºC for 48 hours. Aseptically from incubated broth after 48 hrs transfer aliquot to two clean test tubes each with two ml of broth culture with sterile pipette. Add 5 drops of methyl red to one tube. The result read immediately. The tube didn't mix. Add 15 drops of Voges-proskauer reagent A (5% alpha naphthanol) shake it and follow adding of 5 drops of Voges-proskauer reagent B (40% KOH) to the other tube containing transferred broth and shake the tube gently to aerate. Then observe tube for appearance of red color within 20 minutes

Enterobacteriaceae, method of classification

	Methods of identification						
Entero bacteriaceae	Gram staining	Citrate	Motility	Indole	Oxidase	sugar fermen tation (TSI)	Colony characteristics on (Macconkey Agar)
Escherichia. coli	G-ve rod	+	Motile	+	-	+	Red, not mucoid
Klebsiella pneumoniae	G-ve rod	+	Non motile	-	-	+	Large, not mucoid
Enterobacte r aerogenes	G-ve rod	+	Motile	-	-	+	Pink