

ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE



*BOVINE TUBERCULOSIS LESION DESCRIPTION WITH MOLECULAR
CHARACTERIZATION OF MYCOBACTERIUM SPECIES FROM CATTLE
SLAUGHTERED AT ABATTOIRS BISHOFTU, ETHIOPIA*

MSc THESIS

BY

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Addis Ababa University

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CHARACTERIZATION OF MYCOBACTERIUM SPECIES FROM CATTLE SLAUGHTERED
AT ELFORA AND MUNICIPAL ABATTOIR, BISHOFTU, ETHIOPIA*

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DEDICATION

I dedicate this MSc thesis to my father Wagari Ganati; I dreamed a little dream, Once upon a time. I dreamed we'd be together one day, Sweet Father of mine. Sadly that dream was not meant to be, and it's very difficult to know, that now you won't be coming to me. You weren't strong enough to thrive and grow, but I know that you're in heaven now and that's a very good place to be. And I know that when I get there, I'll recognize you, and you'll know me. We'll get to share the love we would have shared here on this earth. And then we'll know without a doubt what all this waiting was worth.

STATEMENT OF THE AUTHOR

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

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LIST OF ABBREVIATIONS

AIDS	Acquired immune-deficiency syndrome
BTB	Bovine tubercle bacilli
DNA	Deoxyribonucleic acid
DR	Direct repeat
ELISA	Enzyme linked immuno-sorbent assay
FAO	Food and agriculture organization
HIV	Human immune deficiency Virus
HPC	Hexadecylpyridium chloride
IFN	Interferon
<i>M. bovis</i>	<i>Mycobacterium bovis</i>
MIRU-VNTR	<i>Mycobacterial</i> interspersed repetitive unit's-variable number tandem repeat
MTBC	<i>Mycobacterium tuberculosis</i> complex
NMSA	National metrology agency
OIE	Office International des Epizooties
PCR	Polymerase chain reaction
PPD	Purified protein derivative
RD	Repeat deletion
RFLP	Restriction fragment length polymorphism
rRNA	ribosomal ribonucleic acid
SICCT	Single intradermal comparative cervical tuberculin test
TB	Tuberculosis
WHO	World health organization
Mm	Millimeter

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Abstract

*A cross-sectional study was conducted at ELFORA and Bishoftu Municipality Abattoir from November 2015 to April 2016 to describe the gross and microscopic lesion of bovine tuberculosis and characterize its causative agents. Post-mortem examination, pathological scoring, bacteriological culturing, Zeihl Neelson staining, histopathology and multiplex polymerase chain reaction (PCR) were used for investigation. Six hundred twenty six (626) Cattle were recruited for the study and the prevalence was found to be 2.9% (18/626) on the basis of gross lesion. There was statistical significant ($X^2=24.01$ and $P=0.000$) difference between age of the animal and tuberculosis lesion. Eighty eight percent (88%) of the gross lesion was detected in the thoracic cavity while 12% of the lesion was found in the abdominal cavity and head region. Only twenty eight percent (28%=5/18) of the suspicious lesions yielded colonies of which one were acid-fast positive. Severe depletion of lymphocytes, calcification, cellular debris and aggregation of macrophage were observed microscopically. Further identification of the isolates using multiplex PCR revealed that the isolates belong to the Genus *Mycobacterium*. Of the isolates that showed signal to the Genus *Mycobacterium*, it was the members of the non-tuberculosis *Mycobacterium* complex. In conclusion, TB lesions were caused by the non-tuberculosis *Mycobacterium* complex. Hence, as the isolates were the non-tuberculosis *Mycobacterium* complex members, the pathogenecity and public health importance of these members in cattle needs further study.*

Key words: Bishoftu, Bovine tuberculosis, Calcifications, Cattle, Non-tuberculosis *Mycobacterium*, PCR

1. INTRODUCTION

Tuberculosis is one of the world's deadliest communicable *Mycobacterial* disease caused by members of *Mycobacterium tuberculosis* complex (MTBC) (Pal, 2007; Tamiru *et al.*, 2013). *M. tuberculosis* is specifically adapted to humans while *M. bovis* is most frequently isolated from domesticated cattle (Smith *et al.*, 2006), although recent studies indicated that *M. tuberculosis* has been isolated from cattle and *M. bovis* from humans infected with BTB and TB, respectively (Malama *et al.*, 2013). In spite of variation in host specificity, the members of MTBC are characterized by 99.9% or greater similarity at nucleotide level and are virtually identical at 16S rRNA sequence (Brosch *et al.*, 2002).

TB is present in all regions of the world and the Global Tuberculosis Report 2014 includes data compiled from 202 countries and territories. This year's report shows higher global totals for new TB cases and deaths in 2013 than previously, reflecting use of increased and improved national data. In 2013, an estimated 9 million people developed tuberculosis and 1.5 million died from the disease; 360,000 of whom were HIV-positive. TB is slowly declining each year and it is estimated that 37 million lives were saved between 2000 and 2013 through effective diagnosis and treatment (WHO, 2014).

In 2014, 6 million new cases of TB were reported to WHO fewer than two thirds (63%) of the 9.6 million people estimated to have fallen sick with the disease. In 2014, TB killed 1.5 million people (1.1 million HIV-negative and 0.4 million HIV-positive). The toll comprised 890,000 men, 480,000 and 140,000 children. This means that worldwide, 37% of new cases went undiagnosed or were not reported. The quality of care for people in the latter category is unknown (WHO, 2015).

From 2016, the goal is to end the global TB epidemic by implementing the End TB strategy. Adopted by the world health assembly in May 2014 and with targets linked to the newly adopted SDGs (sustainable development goals), the strategy serves as a blueprint for countries to reduce the number of TB deaths by 90% by 2030 (compared with 2015 levels), cut new cases by 80% and ensure that no family is burdened with catastrophic costs due to TB (WHO, 2015).

Bovine tuberculosis is a contagious, chronic, granulomatous *Mycobacterial* infectious disease, caused mainly by *M. bovis*, which is a member of *Mycobacterium tuberculosis* complex; which can affect most warm-blooded animals, including human being (Radostits *et al.*, 2007). In cattle, exposure to this organism can result in a chronic disease that jeopardizes animal welfare and productivity and in some countries leads to significant economic losses by causing ill health and mortality. It is a chronic bacterial disease characterized by progressive development of tubercles in any tissue/organ of the body (Hlokwe *et al.*, 2013; Pal *et al.*, 2014). It has been recognized from 176 countries as one of the important bovine diseases causing great economic loss (Awah-Ndukum *et al.*, 2013).

Organisms are excreted in the exhaled air, in sputum, feces (from both intestinal lesions and swallowed sputum from pulmonary lesions), milk, urine, vaginal and uterine discharges; and discharges from open peripheral lymph nodes of infected animals (Radostits *et al.*, 2007).

Due to its economic impact on international trade, contagious nature and implications for human health, global programs to eradicate the disease were implemented worldwide (Marjorie and Golden, 2005).

Infected animal loses 10 to 25% of their productive efficiency. Direct losses due to the infection become evident by decrease in 10 to 18% milk and 15% reduction in meat production (Radostits *et al.*, 1994).

Apart from effects on animal production, it has also a significant public health importance (Müller *et al.*, 2013). Currently, the disease in human is becoming increasingly important in developing countries, as humans and animals are sharing the same micro-environment and dwelling premises, especially in rural areas and susceptibility of HIV-AIDS patients to tuberculosis (Shitaye *et al.*, 2007).

It is estimated that *M. bovis* causes 10 to 15% human cases of tuberculosis in countries where pasteurization of milk is rare and bovine tuberculosis is common (Ashford *et al.*, 2001).

In developing countries like Ethiopia, the socio-economic situation and low standard living area for both animals and humans are more contributing in TB transmission between human to human and human to cattle or vice versa (Ameni *et al.*, 2010; Ejeh *et al.*, 2013).

Human infection due to *M. bovis* is thought to be mainly through drinking of contaminated or unpasteurized raw milk. The high prevalence of TB in cattle, close contact of cattle and humans, the habit of raw meat and milk consumption; and the increasing prevalence of HIV may all increase the potential for transmission of *M. bovis* and other *Mycobacteria* between cattle and humans (Shitaye *et al.*, 2007).

Bovine tuberculosis is an endemic disease of cattle in Ethiopia, with a reported prevalence of 3.5–5.2 % in abattoir (mostly zebu) and 3.5–50% in crossbreed farms (Shitaye *et al.*, 2007; Berg *et al.*, 2009; Demelash *et al.*, 2009; Regassa *et al.*, 2010). Nevertheless, the available information is limited due to inadequate disease surveillance and lack of better diagnostic facilities (Cosivi *et al.*, 1998; Asseged *et al.*, 2000). In particular, information on genotypic characteristics of *M. bovis*, a strain affecting the cattle population in Ethiopia, is limited (Biffa *et al.*, 2010). Such information is critical to monitor transmission and spread of the disease among cattle (Berg *et al.*, 2011).

One of the results of bovine tuberculosis eradication programs has been a reduction in disease and death caused by bovine tuberculosis in the human population (Zeweld *et al.*, 2014).

Despite the large number of livestock population in the Oromia region, particularly in Bishoftu Town there is inadequate information on bovine tuberculosis. As well as, there are several risk factors that promote transmission of *M. bovis* from animals to animals and animals to humans, because of consumption of raw/poorly cooked milk, meat, close physical contact between human and livestock, frequent animal movements across the different districts, sharing of communal grazing lands and watering points by animals from different herds (Ameni *et al.*, 2011).

Bovine tuberculosis is endemic in many African countries, but economic constraints impede the use of skin test and slaughter control strategies, which have proved effective in the developed

world. Despite the fact that bovine tuberculosis is a public health threat and also leads to economic losses, in Ethiopia research on control of animal tuberculosis has not received much attention like human tuberculosis (Chukwu *et al.*, 2013).

This study will be a way to answer key questions on the pathological characterization of tuberculosis lesion in-relation to strains of *Mycobacterium* isolated, molecular identification of the causative agents of tuberculosis in cattle and thus could help in policy formulation regarding the control of animal tuberculosis in Ethiopia.

Objectives

- Characterization of lesion of bovine tuberculosis both grossly and microscopically; molecular identification and characterization of the *Mycobacterium* agent involved.

1.1. Research question

- Is there any variability in pathological lesion both grossly and microscopically among *Mycobacterium* species isolated in this study?
- Is there any variability among *Mycobacterium* species complex isolated in this study?

2. LITERATURE REVIEW

2.1. Etiology

2.1.1. Taxonomy of *Mycobacteria*

The genus *Mycobacterium* is classified under the Order Actinomycetales and Family Mycobacteriaceae (Quinn *et al.*, 1999). The genus includes a number of species, some being pathogenic to man and animals, some are opportunistic while others are essentially saprophytic (Thoen, 1984). The classic species of *Mycobacterium* that cause disease in man and animals include: *M. bovis*, *M. tuberculosis*, *M. paratuberculosis*, *M. avium*, *M. leprae* and *M. lepraemurium* (Marie-France *et al.*, 2009; Quinn *et al.*, 1999).

2.1.2. Physical and biochemical characteristics

2.1.2.1. Morphology and staining

Mycobacteria are non-motile, non-spore forming, non-capsular, pleomorphic bacilli or coccobacilli, obligate aerobic, thin rod usually straight or slightly curved having 1-10µm length and 0.2-0.6µm width, facultative intracellular microbe and has a slow generation time about 15-20 hours. They occur singly, in pairs or as small bundles. On laboratory media they may appear as cocci or rods measuring 6-8µm (Quinn *et al.*, 1999). The distinguishing features of pathogenic *Mycobacteria* are the formation of characteristics cords (Grange, 1995).

The *Mycobacterial* cell wall is triple layered comprising a basal peptidoglycan layer and an intermediate arabinogalactan mycolate complex. The outer layer is lipid rich; comprising surface rope like structure of peptidoglycolipid (Chukwu *et al.*, 2013). This waxy coat (mycolic acid) is also greatly contributing for the bacterium resistance to many disinfectants, common laboratory stains, antibiotics and physical injuries. It probably also contributes to the slow growth rate of some species by restricting the uptake of nutrients. *Mycobacteria* when stained are acid fast as they resist decolorizing with strong acid/alcohol solutions (Quinn *et al.*, 1999).

2.1.2.2. Growth requirement and cultural characteristics

Mycobacterium species grows on medium containing serum, potato and egg. The most commonly used media are Lowenstein-Jensen (LJ) that contains egg, glycerol, asparagines, mineral salt and malachite green and stone-brink's medium. *M. bovis* grows more slowly than *M. tuberculosis*, which needs more than 8 weeks to appear on primary culture as well as it needs media free from glycerol, but having pyruvate as a nutrient which enhance the growth. The optimal growth temperature is 37°C (Quinn *et al.*, 1999).

2.2. Pathogenesis

2.2.1. Infection

The methods by which tubercle bacilli gain entrance to the animal body include: the respiratory, alimentary, genital, cutaneous and congenital routes (Neill *et al.*, 1994). After infection the bacteria may localize in tissues related to the route of infection and associated lymph nodes. Miliary TB represents the most severe course of the disease with haematogenous spreading as a result of lysis of macrophages that release bacteria into the blood from the primary foci and secondary seeding to various tissues (Andersen, 1997).

2.2.2. Gross lesion

A primary lesion or focus of infection is established following the interaction of the host and the agent at the site of entry within 8 weeks of bacterial entrance (Radostits *et al.*, 1994). The *Mycobacterium* is then taken by the alveolar macrophages to the circulation and establishes in the lymph nodes. Cellular responses attempting to control the disease results in the accumulation of large number of phagocytes and lead to the formation of a macroscopic lesion referred as tubercle. These granulomas are usually yellowish and either caseous or calcified, they are often encapsulated. In some species the lesion tends to resemble abscesses rather than typical tubercles (Thoen and Bloom, 1995). In cattle, tubercles are found in the lymph nodes, particularly those of the head and thorax. Most of those lesions found in lymph nodes are associated with the

respiratory system. It is also common in the lungs, spleen, liver and the surfaces of body cavities (Regassa *et al.*, 2010). In disseminated case, lesions are sometimes found on the female genitalia, but are rare on the male genitalia. In countries with good control programs, infected cattle typically have few lesions at necropsy. However, small lesions can often be discovered in the lungs of these animals if the tissues are sectioned (Rieder *et al.*, 2007).

2.2.3. Virulence

Mycobacteria are intra-cellular organisms and their virulence appears to be related to their ability to survive and multiply within the macrophages. *M. bovis* eludes the bacteriocidal activities of macrophages by escaping from fused phagolysosomes into non-fused vacuoles in the cytoplasm. In addition to these survival mechanisms, an important aspect of pathogenicity of *Mycobacteria* is their ability to subvert the protective immune response (Grange, 1995). A characteristic feature of virulent strains of *Mycobacteria* is that they form cords when they grow in a liquid culture media whereas the a-virulent strains develop as clumps (Ereqat *et al.*, 2013; Thoen and Bloom, 1995).

2.3. Immunity against *Mycobacterial* infection

Both humoral and cell mediated immune responses can be induced to *Mycobacterial* infection, but the cell mediated immunity is generally accepted to have the most significant role in protection (Neill *et al.*, 1994). The macrophages have a central role in processing and subsequent presenting of *Mycobacterial* antigens to antigen specific T-lymphocytes (Ali, 2006; Fentahun and Luke, 2012).

2.4. Epidemiology of *Mycobacterium bovis* infections

M. bovis combines one of the widest host ranges of all pathogens with a complex epidemiological pattern, which involves interaction of infection among human beings, domestic animals and wild animals (Gemechu *et al.*, 2013; Grange, 1995). However, only little is done

particularly in developing countries on the epidemiology of this organism and the epidemiological requirements for its control (Ali, 2006).

2.4.1. Source of infection and mode of transmission

The main reservoir of *M. bovis* is cattle, which can transmit the infection to many mammalian species including man (Acha and Szytre, 2001). Organisms live in the host respiratory discharges, faeces, milk, urine, semen and genital discharges. These body excretions may contaminate grazing pasture, drinking water, feed, water and feed troughs or fomites, which may act as source of infection to other animals. Main routes of infection by which tubercle bacilli gain entrance into the host are respiratory and alimentary (Ameni *et al.*, 2001; Russell, 2003).

2.4.2. Risk factors: animal population

The probability of infection with *M. bovis* is influenced by factors, which are linked to environment, host and the pathogen itself (Ameni *et al.*, 2011; Regassa, 2005).

2.4.2.1.Environment

Housing predisposes to the disease, as high stocking intensity and a large number of animals on a farm so that the disease is more common and serious where these forms of husbandry are practiced. The closer the animals are in contact the greater is the chance that the disease will be transmitted. In spite of the low overall incidence in countries where cattle are at pasture all the year round, individual herds with 60-70% morbidity may be encountered (Katale *et al.*, 2013; Regassa, 2005).

2.4.2.2. Agent

The causative organism is moderately resistant to heat, desiccation and many disinfectants. It is readily destroyed by direct sunlight unless it is in a moist environment. In warm, moist, protected positions, it may remain viable for weeks (Corner *et al.*, 1990).

2.4.2.3. Host range

Cattle are the primary hosts for *M. bovis*, but other domesticated and wild mammals can also be infected. Known maintenance hosts include brush tailed opossums (and possibly ferrets), badgers, bison and elk, and kudu and African buffalo (Regassa, 2005).

Species reported to be overrun hosts include sheep, goats, horses, pigs, dogs, cats, ferrets, camels, llamas, many species of wild ruminants including deer and elk; elephants, rhinoceroses, foxes, coyotes, mink, primates, opossums, otters, seals, sea lions, hares, raccoons, bears, warthogs, large cats (including lions, tigers, leopards, cheetahs and lynx) and several species of rodents (Cadmus *et al.*, 2006). Most mammals may be susceptible. Little is known about the susceptibility of birds to *M. bovis*, although they are generally thought to be resistant. Experimental infections have recently been reported in pigeons after oral or intra-tracheal inoculation and in crows after intra-peritoneal inoculation. Some avian species, including mallard ducks, appear to be resistant to experimental infection (Demelash *et al.*, 2009; Tadayon *et al.*, 2013; Thoen, 1984).

Zebu (*Bos indicus*) type cattle are thought to be much more resistant to tuberculosis than European cattle; and the effects on these cattle are much less severe but under intensive feedlot conditions a morbidity rate of 60% and a depression of weight gain can be experienced in Zebu cattle (Regassa, 2005).

2.4.3. Risk factors: human population

2.4.3.1. Close physical contact

Close physical contact between humans and potentially infected animals is present in some communities, especially in developing region (Samuel, 2010). For example, in many African countries cattle are an integral part of human social life; they represent wealth and are at the center of many events and gatherings. In addition, with 65% of Africa, 70% of Asian and 26% of

Latin America and Caribbean population working in agriculture, a significant proportion of the population of these regions may be at risk for bovine TB (Cosivi *et al.*, 1998).

2.4.3.2. The increase in the demand for milk and meat

The demand for milk was increasing at estimated rate of 2.5% per year over the period of 1970-1988 in sub Saharan Africa (Nwanta *et al.*, 2010). For developing countries as a group, evidence suggests that income elasticities for animal proteins are relatively high: 0.8 to 1.7%. This means that a 10% increase in total income will lead to an 8–17% increase in demand for animal proteins. The income elasticities of meat and milk in tropical Africa have been estimated at 0.98 and 0.82 respectively, compared to 0.22 for cereals. Hence if real income increases in Africa, demand for animal products will rise faster than for cereals. This rise demand for meat and milk consumption will be met by increasing number of productive animals and intensifying animal production (Regassa, 2005; <http://ilri.org/infoserv/Webpub/fulldocs/Rolelive/Rolelive.htm>).

2.4.3.3. Feeding habit

Consumption of raw or soured milk/meat is mainly practiced in some parts of the world (Hailemariam, 1975). Approximately 90% of the total volume of milk produced in sub Saharan Africa is consumed fresh or soured and only a very small proportion follows official marketing channels (Tamiru *et al.*, 2013). It is known that consumption of milk contaminated by *M. bovis* is regarded as the principal mode of TB transmission from animals to humans, but meat also plays a role (Acha and Szytres, 2001).

2.4.3.4. HIV infection

In many developing countries, TB is the most frequent opportunistic disease associated with HIV infection (Cosivi *et al.*, 1998). HIV Sero-prevalence rates greater than 60% have been found in TB patients in various African countries (WHO, 1997; WHO, 2006; WHO, 2014, WHO, 2015). Persons infected with both pathogens have annual risk of progression to active TB of 5 to 15% depending on their level of immune-suppression; approximately 10% of non-HIV infected

persons newly infected with TB become ill at some time during their life. In the remaining 90% effective host defense prevent progression from infection to disease (Cosivi *et al.*, 1998).

2.4.3.5. Absence of control mechanism

Bovine tuberculosis can be controlled/eliminated from a country or region by implementing the test and slaughter policy. However, because of financial constraints, scarcity of trained man power, lack of political will, as well as the under estimation of the importance of bovine tuberculosis by national governments and donor agencies, control measures are not applied or are applied inadequately in most developing countries (Cosivi *et al.*, 1998).

2.4.4. Distribution

Although bovine tuberculosis was once found worldwide, control programs have eliminated or nearly eliminated this disease from domesticated animals in many countries (Regassa, 2005). Nations currently classified as tuberculosis free include Australia, Iceland, Denmark, Sweden, Norway, Finland, Austria, Switzerland, Luxembourg, Latvia, Slovakia, Lithuania, Estonia, the Czech Republic, Canada, Singapore, Jamaica, Barbados and Israel (Ali, 2006).

Eradication programs are in progress in other European countries, Japan, New Zealand, United States, Mexico and some countries of Central and South America. Bovine tuberculosis is still widespread in Africa, parts of Asia and some Middle Eastern countries (Ashford *et al.*, 2001).

2.4.5. Status of bovine tuberculosis in Ethiopia

Studies in Ethiopia revealed a higher prevalence of bovine tuberculosis in cattle kept indoors compared to free grazing animals and a higher susceptibility to *M. bovis* infection of exotic Holstein Bos Taurus cattle compared to local Zebu cattle (Bilal *et al.*, 2010).

Moreover, a study by Ameni *et al.*, revealed a better performance of SICCT in Ethiopia if the cut-off value for positive test interpretation was lowered from > 4 mm (OIE standard cut-off) to

> 2 mm. The spoligotype pattern of 17 strains of *M. bovis*, isolated from a herd with a high prevalence of bovine tuberculosis was identical for all animals and recently published (Firdessa *et al.*, 2013). Berg *et al.*, 2011, provided a comprehensive investigation on bovine tuberculosis in Ethiopia and showed a widespread distribution of the disease at an average prevalence of approximately 5% (Mekibeb *et al.*, 2013).

2.5. Diagnosis

Diagnosis of bovine tuberculosis infection in live animals is usually based on delayed hypersensitivity reactions. A presumptive diagnosis of TB in cattle is often made on history, clinical findings, tuberculin skin tests and/or necropsy findings. In-vitro lymphocyte assays, including an interferon gamma assay and enzyme linked immunosorbent assays have been developed for the detection of the disease in cattle (Nahar *et al.*, 2011; Samuel, 2010; Sharipour *et al.*, 2014; Špičić *et al.*, 2012). Infection is often subclinical when present, clinical signs are not specifically distinctive and can include weakness, anorexia, emaciation in the presence of good nutrition, dyspnoea, enlargement of lymph nodes and cough, particularly with advanced tuberculosis (OIE, 2009). However, diagnosis based on clinical signs is not conclusive as the signs are not specific to bovine tuberculosis. The most effective methods for diagnosis of bovine tuberculosis are those diagnostic methods carried after death/slaughter of suspected animals. These diagnostic methods include gross postmortem examination (necropsy), histopathology, mycobacteriological culture methods and various molecular techniques for identification of the isolates. *Mycobacterial* culture diagnostic method remains the gold standard method for routine confirmation of infection (OIE, 2009).

2.5.1. Clinical examination

Tuberculosis is usually a chronic debilitating disease, but it can occasionally be acute and rapidly progressive. Early infections are often asymptomatic. In late stages, common symptoms include progressive emaciation, fluctuating fever, weakness and in-appetence. Animals with pulmonary involvement usually have a moist cough that is worse in the morning, during cold weather, exercise and may have dyspnea or tachypnea. In terminal stage, animals may become extremely

emaciated and develop active respiratory distress (Radostits *et al.*, 1994). Bovine tuberculosis is usually a fulminating pulmonary disease that typically lasts two to six months. In the final stage of the disease, animals become disoriented, can't climb and may be seen wandering about in day light. The advantage of using clinical sign as one tool of diagnosis is that it requires less resource and equipment, but in other condition especially in early infection it is asymptomatic as well as long lasting (Radostits *et al.*, 2007). The principal sign of tuberculosis is commonly chronic wasting or emaciation that occurs despite good nutrition and care (Smith *et al.*, 2006; Thoen and Bloom, 1995).

2.5.2. Tuberculin skin test

Tuberculin skin tests are the international standard for ante-mortem diagnosis of bovine tuberculosis in cattle herds and individual animals (Smith *et al.*, 2006). It involves the intradermal injection of bovine tuberculin purified protein derivative (PPD) and the subsequent detection of swelling (delayed hypersensitivity) at the site of injection 72 hours later. Skin delayed type hypersensitivity is characterized by leukocyte infiltration that is dominated by macrophages and CD4+ and CD8+ T-lymphocytes. It is convenient, cost effective method for assessing cell mediated responses to a variety of antigens and it is “gold standard” for diagnostic screening for detection of new or asymptomatic *M. tuberculosis* complex infection (Tadesse *et al.*, 2015). This may be performed using bovine tuberculin alone or as a comparative test using avian and bovine tuberculin (OIE, 2008; Radostits *et al.*, 1994). Animals, which have been sensitized by environmental strains of *Mycobacteria*, may react positively to PPD-B, due to the presence of antigens common to virulent and non-virulent *Mycobacterial* strains. When this occurs, discrimination between cattle infected with *M. bovis* and those exposed to environmental strains is done using the comparative intradermal tuberculin test (Pollock *et al.*, 2005). The tuberculin test is usually performed on the skin of mid-neck, but the test can also be performed in the caudal fold of the tail. The skin of the neck is more sensitive to tuberculin than the skin of the caudal fold. To compensate for this difference, higher doses of tuberculin may be used in the caudal fold (OIE, 2009). The single caudal fold intradermal test, the single cervical intradermal test and the comparative cervical intradermal test are the tuberculin tests used in most bovine tuberculosis control or eradication programs (O'Reilly, 1995; Kaneene and Thoen, 2004). In-

spite of its wide use, intradermal tuberculin reactions present some important limitations, related to their sensitivity and specificity (Fra'guas *et al.*, 2008). Tuberculin skin test lacks sensitivity and can be confounded by exposure to non-tuberculosis *Mycobacteria* and cannot be repeated for 60 days due to desensitization (Palmer *et al.*, 2006). Delayed hypersensitivity may not develop for a period of 3–6 weeks following infection, hence testing using tuberculin test in this period may result false-negative. In addition, tuberculin test may be unresponsive in chronically infected animals with severe pathology (OIE, 2009).

2.5.3. *Postmortem examination*

Postmortem examinations should be supported by a histological examination of samples stained with haematoxylin and eosin (OIE, 2009). Typically, lesions caused by *M. bovis* in cattle are described as having a center of caseous necrosis with some calcification and a boundary of lymphocytes, neutrophils and epitheloid cells. Some of epitheloid cells may fused together and form multinucleated giant cells. An outer border fibrous of connective tissue is usually present, giving the lesion a focal appearance and providing encapsulation to some extent, which may limit the spread of infection ((Mihreteab and Indris, 2011). Since the lesions are not conclusive, it is necessary to demonstrate the etiological agent using Ziehl-Neelsen stain (Thoen and Bloom, 1995).

Tuberculosis is characterized by the formation of granuloma (tubercle), which is an organized pathological structure that consists of differentiated macrophages with a characteristic morphology, T-lymphocytes, some B-lymphocytes, dendritic cells, neutrophils, fibroblasts and extracellular matrix components (Flynn and Chan, 2001). The complex, dynamic interactions within granuloma lesions reflect a composite of macrophage and helper T-cell function, cytokine production and *Mycobacterial* activity that in-turn influence the morphological appearance of the granuloma. Lesion necrosis, liquefaction, mineralization and regression represent some of the outcomes of these interactions that dictate lesion size and appearance and ultimately the presentation of disease in the host (Peters and Ernst, 2003).

A presumptive diagnosis of bovine tuberculosis can be made following the macroscopic detection of these granulomatous lesions in different organs of infected animals (Corner, 1994).

Macroscopically, a tuberculous granuloma usually has a yellowish appearance and is caseous, caseo-calcareous, or calcified in consistency. Occasionally, its appearance may be more purulent in cervids and camelids (OIE, 2009).

At necropsy, tubercles are most frequently seen in bronchial, mediastinal, retropharyngeal and portal lymph nodes and may be the only tissue affected (Tefera, 2014). In addition, the lung, liver, spleen and the surfaces of body cavities are commonly affected. In disseminated cases, multiple small granulomas may be found in numerous organs. Early nodular pulmonary lesions can often be detected by palpation. Lesion size ranges from small enough to be missed by the unaided eye, to involvement of the greater part of an organ. Serial sectioning of organs and tissues may be required to detect the small lesions contained within the tissue. Non-visible lesion reactors may be due to early infection, poor necropsy technique or infection with *Mycobacterium* other than *M. bovis* (Corner, 1994).

In cattle, tubercles are found in the lymph nodes, particularly those of the head, thorax and abdominal cavity. They are also common in the lung, spleen, liver and the surfaces of body cavities. In disseminated cases, multiple small granulomas may be found in numerous organs. Lesions are sometimes found on the female genitalia, but are rare on the male genitalia. In countries with good control programs, infected cattle typically have few lesions at necropsy. Most of these lesions are found in the lymph nodes associated with the respiratory system. However, small lesions can often be discovered in the lungs of these animals if the tissues are sectioned. In general, postmortem examination should be supported by bacteriological examination of lesions for definitive diagnosis of tuberculosis in animals (OIE, 2008).

2.5.4. Bacteriology

Mycobacterium bovis can be demonstrated microscopically on direct smears from clinical samples and on prepared tissue materials (Quinn *et al.*, 1999). The acid fastness of *M. bovis* is normally demonstrated with the classic Ziehl Nielsen stain, but a fluorescent acid fast stain may also be used. Immunoperoxidase techniques may also give satisfactory results (Regassa, 2005).

The presumptive diagnosis of tuberculosis can be made if the tissue has characteristic histological lesions (caseous necrosis, mineralization, epithelioid cells, multinucleated giant cells and macrophages). As lesions are often paucibacillary, the presence of acid-fast organisms in histological sections may not be detected, although *M. bovis* can be isolated in culture (Jovan *et al.*, 2011; Deressa *et al.*, 2013; OIE, 2004; Shitaye *et al.*, 2006).

2.5.4.1. Differential staining

Final confirmatory diagnosis of bovine tuberculosis depends on isolation and identification of the bacteria, but preliminary examination of stained smears from lesions, sputum, milk, urine, pleural and peritoneal fluids, uterine discharges and feces is very important (Radostits *et al.*, 2007).

In the smear, the organism appear red rods against a blue background (Ziehl Nielsen staining), while in the fluorochrome procedures, the acid fast organisms appear as fluorescent rods, yellow to orange (OIE, 1996; WHO, 2006).

2.5.4.2. Culture media

Cultures of *Mycobacteria* require only 10 to 100 organisms to detect *M. tuberculosis* complex. Cultures increase the sensitivity for diagnosis of *M. tuberculosis* complex and allow specification, drug-susceptibility testing and, if needed, genotyping for epidemiologic purposes (Brodie and Schluger, 2005). *Mycobacteria* grow on protein enriched artificial media. There are three types of culture media: solid media, which includes egg-based media (Löwenstein-Jensen) and agar-based media (Middlebrook 7H10 and 7H11); and liquid media (Middlebrook 7H12). The most frequently used solid media is the Löwenstein-Jensen (L-J) containing eggs, phosphate buffer and magnesium salts; and asparagines. The bacteriological culture differentiation of *Mycobacteria* is based on growth rate, temperature of growth and production of pigments in light and darkness (Biberstein and Hirsh, 1999) and colony characteristics. The surface mycosides (glycolipids and peptidoglycolipids) determine the colony characteristics and serologic specificities (Quinn *et al.*, 1999). With regard to the colony characteristics, on solid media the

colony of *M. bovis* is flat, smooth, white and breaking up easily when touched. *M. bovis* can also be identified based on specific biochemical and metabolic properties, since it requires pyruvate as a growth supplement and it is negative for niacin accumulation and nitrate reduction and is generally resistant to pyrazinamide (Cole, 2002; Kubica *et al.*, 2006).

In the laboratory, *M. bovis* is microaerophilic, i.e. it grows preferentially at a reduced oxygen tension. The temperature and hydrogen ion concentration ranges, in which the bacillus is able to multiply, are relatively narrow (Mamo *et al.*, 2012). All the members of the MTBC are slow growers. Therefore, the inoculated media may have to be incubated at 37⁰C up to 8 to 12 weeks (Quinn *et al.*, 1999).

To process specimens for culture, the tissue is first homogenized using a mortar and pestle, stomacher or blender, followed by decontamination with either detergent (such as 0.375 – 0.75% hexadecylpyridiniumchloride [HPC]), an alkali (2–4% sodium hydroxide) or an acid (5% oxalic acid). The alkali or acid mixture is shaken for 10–15 minutes at room temperature and then neutralized (Regassa, 2005).

Neutralization is not required when using HPC. The suspension is centrifuged, the supernatant is discarded; and the sediment is used for culture and microscopic examination. For primary isolation, the sediment is usually inoculated onto a set of solid egg-based media, such as Lowenstein–Jensen, Coletsos base or Stone-brinks; these media should contain either pyruvate or pyruvate and glycerol. An agar-based medium such as Middle brook 7H10 or 7H11 or blood based agar medium may also be used (Ali, 2006).

The media should be in tightly closed tubes to avoid desiccation. Slopes are examined for macroscopic growth at intervals during the incubation period. When growth is visible, smears are prepared and stained by the Ziehl Neelsen technique. Growth of *M. bovis* generally occurs within 3–6 weeks of incubation depending on the media used (Silaigwana *et al.*, 2012).

Liquid culture systems are used routinely in some veterinary laboratories; in these systems growth is measured by radiometric or fluorometric means (Bilal *et al.*, 2010).

If gross contamination of culture media occurs, the culture process should be repeated using the retained inocula with an alternative decontaminating agent. Characteristic growth patterns and colonial morphology can provide a presumptive diagnosis of *M. bovis*; however every isolate needs to be confirmed. It is necessary to distinguish *M. bovis* from the other members of the ‘tuberculosis complex’, i.e. *M. tuberculosis* (the primary cause of tuberculosis in humans), *M. africanum* (occupies an intermediate phenotypic position between *M. tuberculosis* and *M. bovis*), *M. microti* (the ‘vole bacillus’, a rarely encountered organism), *M. pinnipedii* and *M. caprae* (Hlokwe *et al.*, 2013).

2.5.4.3. Immunological/serological diagnostic methods

Besides the classical intra-dermal tuberculin test, a number of blood tests have been used. Due to the cost and the more complex nature of laboratory-based assays, they are usually used as ancillary tests to maximize the detection of infected animals (parallel testing), or to confirm or negate the results of an intra-dermal skin test (serial testing) (OIE, 2008). There is also evidence that when an infected animal is skin tested, an enhanced blood test can occur during the following week. This allows for better separation of in-vitro blood test responses leading to greater test accuracy. The gamma-interferon assay and the lymphocyte proliferation assay measure cellular immunity, while the ELISA measures humoral immunity (Jovan *et al.*, 2011).

2.5.4.4. Gamma interferon assay/Bovigam

Serological assays provide an important tool for testing for exposure to *M. bovis*. Among these tests, gamma-interferon assay is commonly used in conjunction with tuberculin skin testing as a confirmatory test following a positive response to the tuberculin skin test (Palmer *et al.*, 2004).

In this test, the release of a lymphokine gamma interferon (IFN- γ) is measured in a whole-blood culture system. The assay is based on the release of IFN- γ from sensitized lymphocytes during a 16–24 hour incubation period with specific antigen (PPD-tuberculin). The test makes use of the comparison of IFN- γ production following stimulation with avian and bovine PPD (Asiimwe, 2008).

IFN- γ diagnostic test is a rapid whole blood assay. The detection of plasma gamma-interferon is carried out by means of a sandwich-ELISA using specific monoclonal antibodies. This test in its current form will not generally be acceptable as a direct substitution for skin testing, but could be applied rather as an ancillary test. Benefits of this test include accelerated elimination of tuberculosis from infected herds and the possibility of the test to be performed as soon as 10 days after the application of a tuberculin skin test. Other application of the IFN- γ test is that confirmation of the immunological status of skin test reactors and the investigation of fraudulent intervention into the skin test (Vordermeier *et al.*, 2001).

It is recommended that the blood samples be transported to the laboratory and the assay set up as soon as practical, but not later than the day after blood collection. Because of the IFN- γ test capability of detecting early infections, the use of both tests in parallel allows the detection of a greater number of infected animals before they become a source of infection for other animals as well as a source of contamination of the environment (Soolingen, 2008).

2.5.4.5. Enzyme linked immunosorbent assay

There have been numerous unsuccessful attempts to develop clinically useful sero-diagnostic tests for tuberculosis. The ELISA appears to be the most suitable of the antibody detection tests and can be a complement, rather than an alternative, to tests based on cellular immunity. It may be helpful in anergic cattle and deer. An advantage of the ELISA is its simplicity, but sensitivity is limited mostly because of the late and irregular development of the humoral immune response in cattle during the course of the disease. Specificity is also poor in cattle when complex antigens such as tuberculin or *M. bovis* culture filtrates are used. However, a comparison of antibody levels to PPD-B and PPD-A has been shown to be useful in increasing specificity in the ELISA (OIE, 2009).

2.5.5. Molecular techniques for diagnosis of BTB

2.5.5.1. Multiplex PCR

Multiplex PCR as molecular technique differentiates MTBC from *M. avium*, *M. intracellulae*, and other *Mycobacterial* species by *Mycobacterial* genus typing (Mamo *et al.*, 2011). Heat killed AFB positive sample DNA was used as source of DNA template. The PCR targets the sequence of the genus *Mycobacterium*, within the 16S rRNA gene (G1,G2) sequences, within the hyper variable region of 16S rRNA, that is known to be specific to *M. intracellulae* (MYCINT-F) and *M. avium* (MYCAV-R) and the MTB70 gene specific for MTBC (TB-A, TB-1B). In this method, it is possible to differentiate all members belonging to genus *Mycobacterium* and further more characterizes the groups belonging to the *M. tuberculosis* complex and *M. avium* complex. On the gel electrophoresis result all members of the genus *Mycobacterium* produce a band of 1030bp, members of *M. avium* complex produces a band of 850bp (*M. avium* subspecies *intracellulae*) and a band of 180bp (*M. avium* subspecies *avium* and *M. avium* subspecies *paratuberculosis*), while members of *M. tuberculosis* complex including *M. bovis* produces a band of 372bp (Mamo *et al.*, 2013).

2.5.5.2. RD Deletion typing

Region of difference (RD) deletion typing is a PCR-based typing method that makes use of the MTBC chromosomal regions of difference deletion loci (Huard *et al.*, 2003). The regions of difference represents the loss of genetic material that arise due to errors in DNA replication, movement of mobile genetic elements, mycobacteriophage-mediated transduction, or recombination between adjacent homologous DNA fragments with loss of the intervening sequence (Cole, 2002). Some of these large sequence polymorphisms (LSPs) have been found to be restricted to one MTBC strain or subspecies while others appeared to be differentially distributed among the MTBC groupings (Parsons *et al.*, 2004; Huard, *et al.*, 2003; Huard, *et al.*, 2006). Several PCR primer pairs specific to the loci were used which include; 16S rRNA, Rv0577, Rv1510 (RD4), Rv1970 (RD7), Rv3877/8 (RD1), Rv3120 (RD12), Rv2073 (RD9), Rv1257 (RD13), IS1561 (MiD3) and TbD1 (Huard *et al.*, 2003; Huard, *et al.*, 2006). Selective amplification of the 16S rRNA gene was performed on several MTBC and non-*tuberculosis*

Mycobacteria strains and the primers amplified a DNA fragment from all the tested *Mycobacteria*. This gene was therefore chosen to provide the positive control when evaluating *Mycobacteria* by PCR (Huard, *et al.*, 2003; Huard, *et al.*, 2006). The Rv0577 gene was found to be an MTBC restricted gene. Primers were designed that could specifically and consistently amplify the Rv0577 coding region and this could therefore be used as a genotypic marker for the MTBC and could be used to distinguish the MTBC species from non-tuberculosis *Mycobacteria* species (Huard, *et al.*, 2003; Huard, *et al.*, 2006). IS1561 (MiD3) a transposase pseudogene fragment was found to be positive for all MTBC isolates except *Mycobacterium microti* (*M. microti*) (Huard, *et al.*, 2003, Huard, *et al.*, 2006). Its absence therefore could serve as a good indicator for *M. microti*. Deletion of a 12.7kb Rv1510 gene (RD4) could serve as an indicator for *M. bovis* while it is present in *M. tuberculosis*, *M. africanum* and *M. microti* (Gordon *et al.*, 1999) and on gel electrophoresis *M. bovis* shows a band of 446bp while *M. tuberculosis* shows a band of 335bp. The Rv3877 and Rv3878 (RD1 locus) was selectively absent in *M. bovis* BCG (Huard, *et al.*, 2003; Huard, *et al.*, 2006). In general, this MTBC PCR typing panel provides an advanced approach to determine the subspecies of MTBC isolates and to differentiate them from clinically important non-tuberculosis *Mycobacteria* species. Primers directed against the RD4, RD9 and RD10 loci are used to generate a deletion profile that would allow species identification of the isolates (Cadmus *et al.*, 2006). *Mycobacterial* genomic DNA will be obtained by heat killing, the isolates at 80 °C for 45-60 min and stored at -20 °C until it will be subjected to PCR. Isolates will be confirmed as *M. bovis* by deletion typing of the RD4 region according to a PCR protocol (Brosch *et al.*, 2002).

2.5.5.3. Spoligotyping

Another molecular typing method for *M. tuberculosis* complex is the PCR based spacer oligonucleotide typing (Spoligotyping). This method was proposed as an alternative to hybridization based fingerprinting methods for diagnosis and epidemiology of tuberculosis (Kamerbeek, *et al.*, 1997). Spoligotyping is based on the variability of spacer sequences interspersed with repeat sequences in the polymorphic chromosomal direct repeat (DR) locus. This locus contains multiple, well-conserved 36-bp long direct repeats (DR) (Hermans, *et al.*, 1991). Strains vary in the number of DRs and in the presence or absence of particular spacers

and *M. bovis* characteristically lacks spacers 39 to 43 in the spoligotype system (Kamerbeek, *et al.*, 1997). Spoligotyping is thus not only useful for differentiation of *M. bovis* strains but can also be used to distinguish these from *M. tuberculosis*, a distinction which is often difficult to make by using conventional bacteriological techniques (Wayne, 1984).

2.5.5.4. MIRU-VNTR

This is a PCR based method that analyses multiple independent loci containing variable numbers of tandem repeats (VNTR) of different families of interspersed genetic elements collectively called *Mycobacterial* interspersed repetitive units (MIRU) (Supply, 2000). In its original format, the PCR primers are each run in separate reactions and the sizes of the products are analyzed by gel electrophoresis. Currently, the most widely used version of MIRU-VNTR analysis includes 12 tandem repeat loci (Supply, 2000; Supply, 2001). A set of 24 MIRU-VNTR loci was standardized to increase the discrimination power (Supply, 2006).

The advantages of MIRU-VNTR analyses are that the results are intrinsically digital and analysis can be applied directly to culture without the need for DNA purification (Roring *et al.*, 2004). The discriminatory power of MIRU-VNTR analysis is typically proportional to the number of loci evaluated; in general, when only the 12 loci are used, it is less discriminating relative to IS6110 RFLP genotyping for isolates with high-copy-number IS6110 insertions but more discriminating than IS6110 RFLP genotyping for isolates with low-copy-number IS6110. When more than 12 loci are used, or MIRU analysis is combined with spoligotyping, the discriminatory power approximates that of IS6110 RFLP analysis. A comparative study of genotyping methods aimed at evaluating novel PCR-based typing techniques found VNTR analysis to have the greatest discriminatory power among amplification based approaches (Kremer, 2005).

MIRU-VNTR genotyping has been used in a number of molecular epidemiologic studies, as well as to elucidate the phylogenetic relationships of clinical isolates (Warren *et al.*, 2004; Kremer *et al.*, 2005). VNTR analysis has also been used to evaluate *M. bovis* transmission (Roring *et al.*, 2004). In addition to their use for tracing TB transmission at the strain level, MIRU-VNTR

markers can also provide useful predictions for classifying strains into genetic lineages (Allix *et al.*, 2004).

2.6. Zoonotic importance of bovine tuberculosis

The current increasing incidence of tuberculosis in humans, particularly in immunocompromized persons, has given a renewed interest in the zoonotic importance of *M. bovis*, especially in developing countries (Pal, 2007). The role of meat and milk as the commonest source of protein to man, in the transmission of the disease remain significant. Due to the grave consequences of *M. bovis* infection on animal and human health, it is necessary to introduce rigorous control measures to reduce the risk of the disease in human and animal populations. The institution of proper food hygiene practices and stronger inter-sectoral collaboration between the medical and veterinary professions is vital to the control of the disease (Zeweld, 2014; Tamiru *et al.*, 2013; Nwanta *et al.*, 2010).

Animal and human health is inextricably interwoven and food animals, especially cattle serve as a reservoir of diseases of public health importance (Müller *et al.*, 2013; Pal *et al.*, 2014). The safety of food of animal origin with regard to infection by *M. bovis* is worth giving consideration, taking into cognizance the current tuberculosis crisis ravaging the world. Though animals with tuberculosis pose some risk to humans, this risk is extremely remote in developed countries due to introduction of milk pasteurization and effective bovine tuberculosis control programs (Shitaye *et al.*, 2006).

In contrast, spread from animals to humans in developing countries remains a very real danger, mostly from infected milk and meat. This seems to be a danger, which is being entirely ignored (Gemechu *et al.*, 2013). Disease surveillance programs in animals and humans should be considered a priority, especially in areas where risk factors are present. Other recommendations made by the WHO (2014) in its global tuberculosis reports include: Training of personnel at all levels of control programs and the urgent need for further research on the diagnosis and control, immunological, epidemiological and socioeconomic aspects of the disease. International

cooperation in all aspects of zoonotic tuberculosis remains essential in the fight against this disease (Nwanta *et al.*, 2010).

2.7. Molecular epidemiology of bovine tuberculosis in Ethiopia

Ethiopia is among the 22 high TB-burden countries that accounts for 81% of estimated cases. Over a one third of the population has been exposed to TB. An estimated 377,030 Ethiopians (0.62% of the population) have active TB of all kinds. In 2005 alone, the number of deaths in all cases of TB, including HIV positive patients was 56,456 and that of all cases, excluding HIV positive patients, were 42,508 deaths (WHO, 2005). The prevalence of bovine tuberculosis in Ethiopia is high and molecular typing of *M. bovis* has also indicated the existence of unique strains (Arega *et al.*, 2013; Biffa *et al.*, 2010; Mamo *et al.*, 2011; Mekibeb *et al.*, 2013; Mezene *et al.*, 2014). Human infection due to *M. bovis* is thought to be mainly through drinking of contaminated or unpasteurized raw milk. The high prevalence of TB in cattle, close contact of cattle and humans, the habit of raw milk and meat consumption; and the increasing prevalence of HIV may all increase the potential for transmission of *M. bovis* and other *Mycobacteria* between cattle and humans (Zeweld, 2013).

2.8. Prevention and control

The effective control and eradication of bovine tuberculosis from herds and/or farms of cattle depend on identifying and isolating potential sources of infection from the herds, through test-and-slaughter-strategy. However, there are also various modifications of eradication and control programs adopted in different countries. In developed countries bovine tuberculosis has nearly been eradicated or drastically reduced in farm animals to low levels by control and eradication programs. In Ethiopia these measures, however, cannot be adopted in practice due to various reasons such as: lack of knowledge on the actual prevalence of the disease, the prevailing technical and financial limitations, lack of veterinary infrastructures, cultural and/or traditional beliefs and geographical barriers, though certain control measures are in place like identification of animals, improvement of management and hygienic practices; legislation, insurance, sound testing and meat inspection, establishment of areas and/or farms free of bovine tuberculosis should have to applied (Cosivi *et al.*, 1998).

3. MATERIAL AND METHODOLOGY

3.1. Study area

The current study was conducted from November, 2015 to April, 2016, in Bishoftu town in the Eastern Shoa Zone of Oromia Regional State, Ethiopia. Six hundred twenty six (626) apparently healthy heads of cattle were slaughtered both at ELFORA and Bishoftu municipal abattoir. ELFORA is privately owned cattle abattoir located in Bishoftu. Animals slaughtered in the abattoir come from different parts of the country, including Wollo and Gonder for ELFORA while from Adama, A/Negelle and Godino areas for municipality. The abattoirs are found in the town of Bishoftu, which is located at 9⁰N and 40⁰E with an altitude of 1920meters above sea level in the central, highlands of Ethiopia at 47.9km South East of Addis Ababa. It has annual rainfall of 1151.6mm of which 84% falls down during the long rainy season that extends from June to September and the remaining during the short rainy season that extends from March to May. The mean annual minimum and maximum temperatures are 8.5⁰C and 30.7⁰C respectively; and the mean relative humidity is 61.3% (NMSA, 2015). The total population was estimated around 147,100 (CSA, 2015). Each day on average, 5-14 (ELFORA) and 8-30 (Municipal) heads of cattle were slaughtered starting from the early morning in ELFORA and midnight in municipal, except those fasting days. The history of all the cattle slaughtered at the abattoir about TB infection is not known and are not tested by the tuberculin test before slaughter. The TB status of the herd or the region from which cattle were brought to abattoir is also not known. Most of the cattle slaughtered in the abattoir were male adult local zebu, 4-7 years of age and above which had entered in the feedlots late in their life. Very few numbers of female cattle were also slaughtered in the abattoir. These were cows culled, because of reproductive problems, poor performance and at the end of their reproductive life. The two abattoirs supply meat and edible offal's for local market consumption.

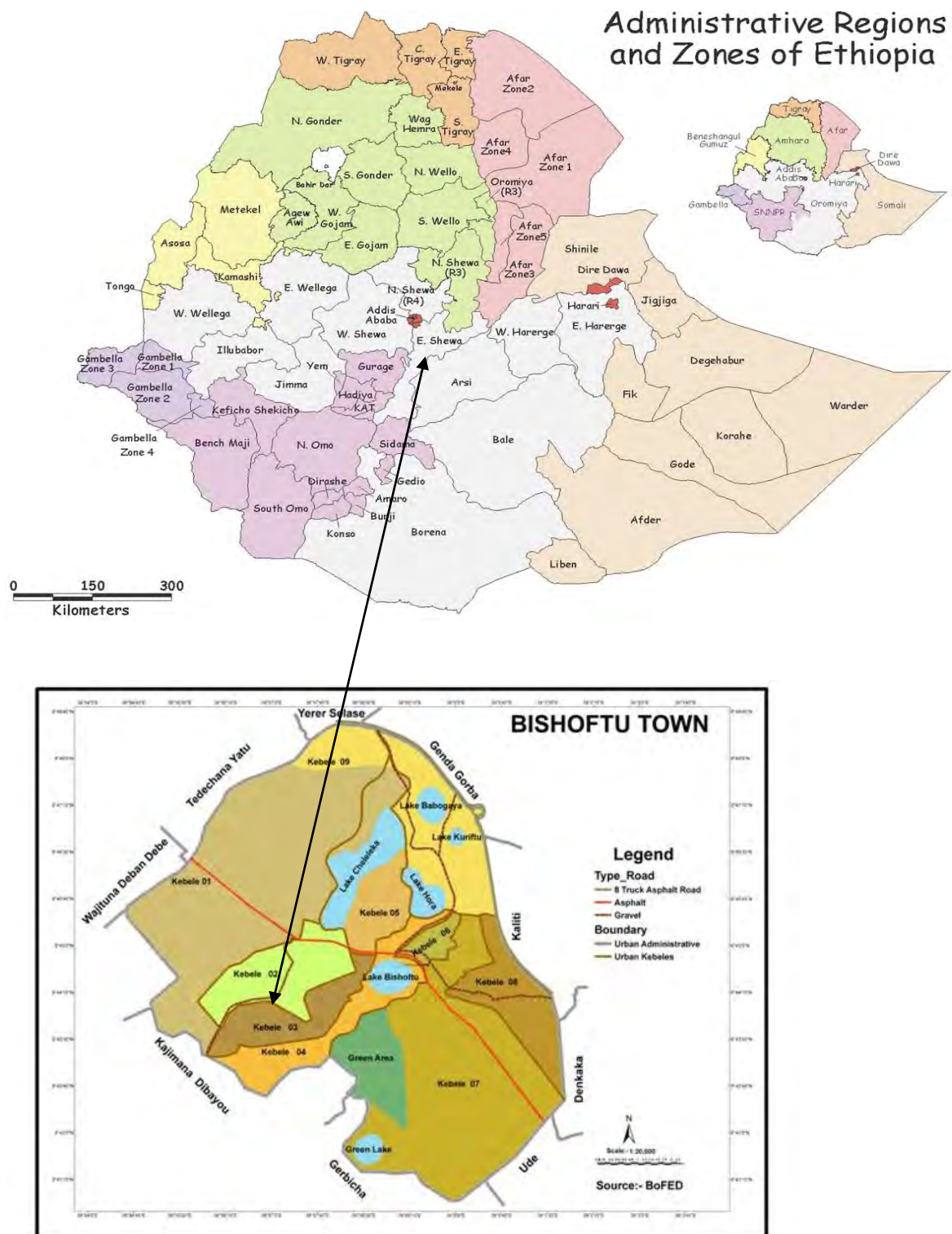


Figure 1: Study area (East Shoa Zone, Bishoftu Town)

Source: (<https://www.google.com.et/search?q=debre%20zeyit%20map&newwindow>)

3.2. Study population

Cattle brought to ELFORA and Bishoftu abattoirs for slaughter purpose were included in the study population without discrimination of their age, sex, breed, but body condition should have to be $\geq 250\text{Kg}$ at ELFORA; and physiological status if they pass the ante-mortem examination.

3.3. Study design

The study design followed was a cross-sectional study in which the prevalence of bovine tuberculosis was determined with purposive inclusion of carcass suspected of TB lesions was conducted on six hundred twenty six heads of cattle. This number includes six hundred twenty four (624) male animals and twelve (12) female animals. As well as gross and microscopic lesion description, pathological scoring, bacteriological and molecular characterization of tuberculosis lesions were conducted.

3.4. Sampling method and sample size determination

The sampling method was purposive and non-probability one (Thrusfield, 2005) and hence all gross bovine tuberculosis lesion samples during the study period was included. Accordingly six hundred twenty six (626) cattle were examined and eighteen (18) positive gross tuberculous lesions were sampled.

3.5. Sample collection, transportation and processing

All animals coming to the slaughter house from different areas during the study period was sampled. Before slaughter, the general physical examination was conducted on each cattle in the lairage house. Animals were clinically examined for any sign of illness both at static and in motion and following the judgments passed by FAO (1994). Animal fit for human consumption

was allowed for slaughter. All the information was preceded by age, sex, breed, body condition and other related information.

3.5.1. Ante and postmortem examination

Physical examination of animals was carried out according to Shitaye *et al.*, 2007 before they will be slaughtered. These include examining superficial lymph nodes, visible mucus membrane; and other related clinical parameters. Body condition scoring and age determination of the study animals was done according to the standards developed by Canadian food inspection agency.

Detailed postmortem examination was conducted in accordance with the method developed by Ethiopian meat inspection and quarantine division of the Ministry of Agriculture (Hailemariam, 1975). Mandibular, retropharyngeal, bronchial, mediastinal, mesenteric and hepatic lymph nodes were examined and organs including lungs, liver, small intestine and kidneys were examined in detail during post-mortem in the abattoir under a bright-light source. The lobes of the left and right lungs were inspected and palpated externally. Then, each lobe was sectioned to facilitate the detection of lesions with sterile surgical blades. Similarly, lymph nodes were sliced into thin sections (about 2mm thick) and inspected for the presence of visible lesions. Whenever gross lesions suggestive of TB were detected in any of the tissue, the tissue was classified as having lesions. The gross appearance, location, consistency and size of the lesions were recorded during the examination.

Pathological scoring was conducted on tissues with abscesses and tubercle lesions to determine the severity of the lesions based on semi quantitative procedures. Briefly, lesions in the lobes of the lungs were scored separately as follows: 0=no visible lesions; 1=no gross lesions but lesions apparent on slicing of the lobe; 2=fewer than five gross lesions; 3=more than five gross lesions; 4= Greater than 1 distinct gross lesion of >10mm in diameter 5=gross coalescing lesions. The scores for the individual lobes were summed and generated lung score. Similarly, the severity of gross lesions in individual lymph nodes was scored as follows: 0=no gross lesions; 1=small lesion at one focus; 2=small lesions at more than one focus; 3=extensive necrosis. Individual lymph node scores were summed and generated the lymph node score. Total pathology score per animal was obtained from the sum of the two total scores.

In the presence of suspected tuberculous lesion, tissue samples were aseptically collected in sterile universal bottles containing phosphate buffer saline about 5 ml of 0.9% (PBS) for culture and transported in cold chain by using ice packed to the College of Veterinary Medicine and Agriculture (CVMA) for further processing and stored at -20°C until mycobacteriological culturing was carried out in TB laboratory.

The samples were further processed for isolation of *Mycobacterium* in accordance with the Office International des Epizooties. The specimens were sectioned using sterile blades, minced with scissors and homogenized with a sterile mortar and pestle under a biological safety cabinet. The homogenates were decontaminated by adding an equal volume (5ml by 5ml) of 4% NaOH on the sample in order to remove contaminants. There-after, centrifuged at 3,000 rpm for 15 minutes to concentrate the *Mycobacterium*. The supernatant were discarded; and the sediments were neutralized by 1% (0.1N) HCl acid using phenol red as an indicator. Neutralization was achieved when the color of the solution changed from purple to yellow. Next, 0.1 ml of suspension from each samples were spread onto a slant of Löwenstein Jensen (LJ) medium. Duplicate slants were used, one enriched with sodium pyruvate and the other enriched with glycerol. Cultures were incubated aerobically at 37°C for 8–12 weeks with weekly observation for growth of colonies. Positive cultures were confirmed with Ziehl-Nelsen staining and preserved with freezing media while at the same time heat killed in water bath at 80°C for 45 minutes. The frozen and heat killed isolates were stored at (-20°C) for further mycobacteriology and molecular typing analysis.

At the same time tissues of approximately 6mm thick were collected in buffered formalin (10%) from lesions in the respiratory system and from the corresponding bronchial lymph nodes for histopathology and taken to pathology laboratory of the National Animal Health Diagnosis and Investigation Center (NAHDIC).

3.5.2. Isolation, identification and characterization of mycobacteria

On arrival at laboratory, tissue samples were macerated in sterile mortal by using surgical blades and forceps to get fine pieces and then homogenized with pestle for 10 min in 5 ml of phosphate-buffered saline. The cultures were incubated at slant position aerobically at 37 °C for 1 week in

upright position, with weekly observation for growth. Initial identification of *Mycobacterial* species were made on the rate of growth and colony morphology as described in OIE (2009). On visible colonies, Ziehl Neelsen staining was performed to confirm the presence of acid-fast bacilli. Further identifications were performed at Aklilu Lemma institute of Pathobiology.

The RD (particularly RD4) deletion typing was applied to isolates that was suspected, but showed a band for non-tuberculosis *Mycobacterium* by multiplex PCR. *Mycobacterial* genomic DNA was isolated by heating the isolates at 80 °C for 45 min and stored at –20 °C and subjected to PCR. But during this study the isolates did not show a band for MTBC, rather it shows the band for genus non-tuberculosis *Mycobacterium*; which cause similar gross lesion on animal and having similar growth characteristics with that of MTBC.

Samples collected for histopathological study was dehydrated in graded alcohol, cleared by three passes in xylene, impregnated by molten paraffin, embedded, sectioned at 5 micro meter thickness and stained with Haematoxylin-Eosin for routine examination Bancroft and Harry (1994).

3.6. Data management and analysis

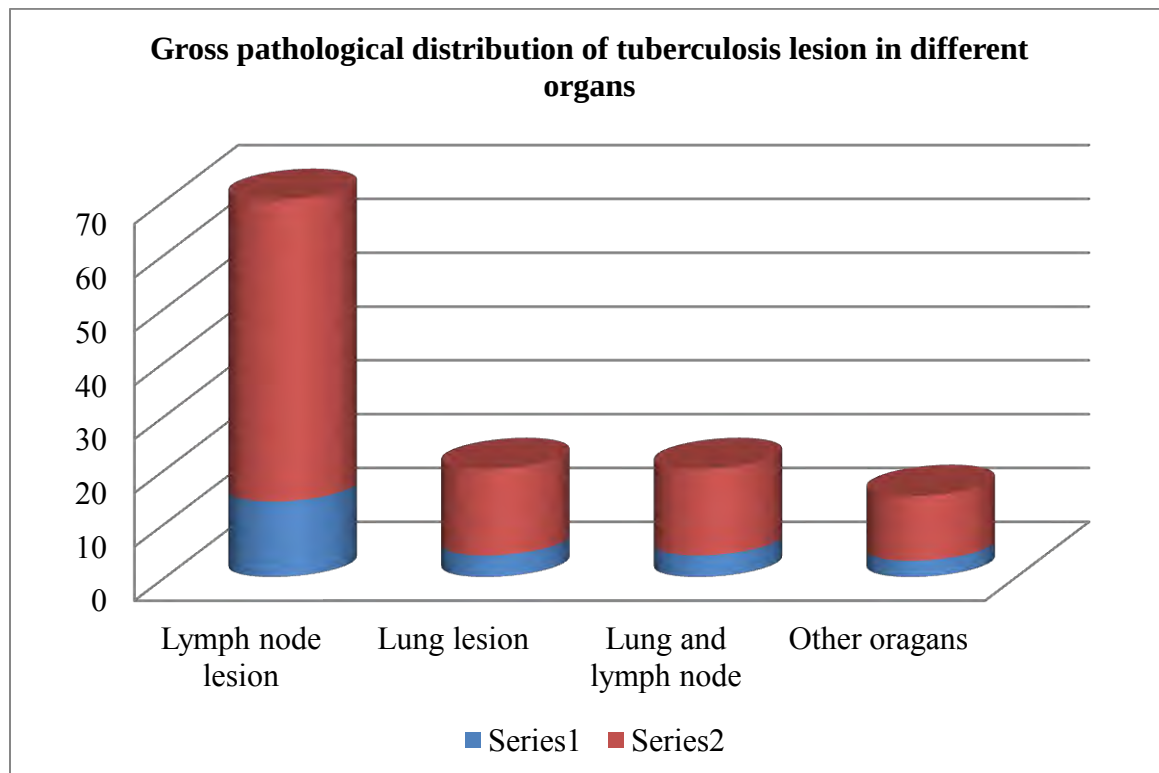
Data related to age, breed, body condition, sex and origin were classified, filtered and coded using MS Excel 7 and were transferred to STATA version 13 for statistical analysis. Presence or absences of tuberculous lesion affected tissue(s) were recorded on postmortem examination. Frequency determinations were used to summarize pathology scores. Similarly, proportions were used to summarize categorical exposure and outcome measures. Firth logistic regression analyses were used to assess the strength of associations of selected factors and prevalence of cattle TB. Effects were reported as statistically significant if p-value was less than 5%, coefficient and 95% confidence intervals were used to measure the strength of associations.

4. RESULT

4.1. Gross tuberculosis lesion description

The distributions of tuberculous lesions in tissues of positive animals were presented in Figure 2. From six hundred twenty six (626) cattle examined in both abattoir 56% (totally 14 lesions) of gross tuberculosis lesion was observed on different lymph node, 16% (totally 4 lesions) of gross tuberculosis lesion was observed on lung, 16% (totally 4 lesions) in both lung and lymph node; and 12% (totally 3 lesions) in addition in other organs like spleen, intestine and thorax.

Almost the entire lesions observed were localized lesions involving frequently a single organ. A high proportion (88%) of the lesions was located on the thoracic cavity lymph nodes, while 12% of the lesions were found in the abdominal cavity.



*Series 1(total lesion) and Series 2 (percent)

Figure 2: Gross pathological distribution of tuberculous lesion in different organs

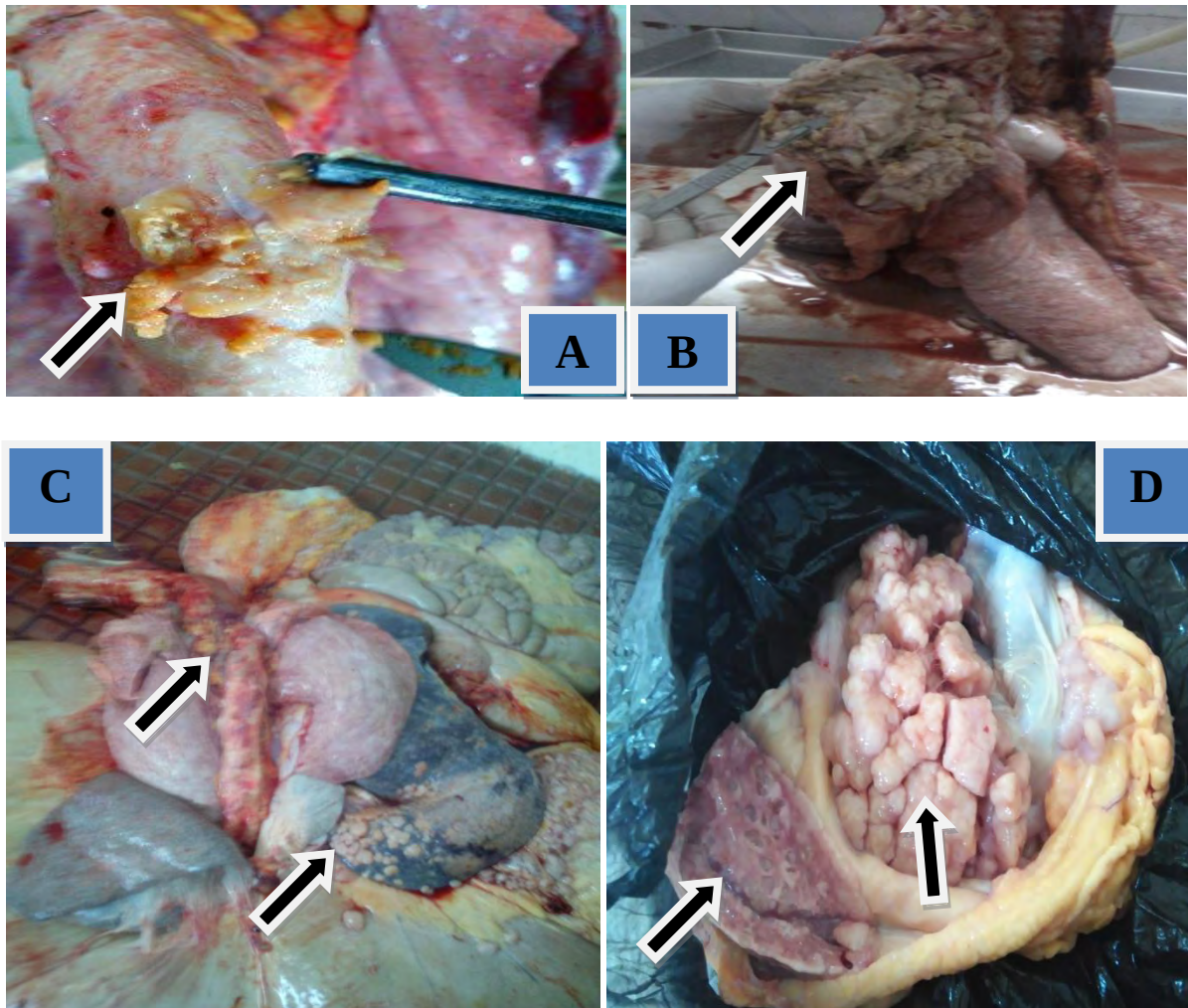


Figure 3: Gross TB lesion. **Note:** Calcification of left bronchial lymph node with typical caseous exudates (A), apical lobe of lung filled with abscess and creamy material (B), generalized TB lesion (lung, intestine, spleen and pancreas) with lesion on different organ (C) and lung lesion with caseous material from thoracic cavity (D)

Table 1 and 2 shows the frequency of gross lesion in different lymph node and lung lobes based on pathological score. Pathology scoring was conducted on tissues with abscesses and tubercle lesions to determine the severity of the lesions based on semi quantitative procedure. Briefly, lesions in the lobes of the lungs were scored separately as follows: 0=no visible lesions; 1=no gross lesions but lesions apparent on slicing of the lobe; 2=fewer than five gross lesions; 3=more than five gross lesions; 4= Greater than 1 distinct gross lesion of >10mm in diameter 5=gross coalescing lesions. The scores for the individual lobes were summed and generated lung score. Similarly, the severity of gross lesions in individual lymph nodes was scored as follows: 0=no gross lesions; 1=small lesion at one focus; 2=small lesions at more than one focus; 3=extensive necrosis. Individual lymph node scores were summed and generated the lymph node score. Total pathology score per animal was obtained from the sum of the two total scores.

Table 1: Frequency of lesion in different lymph node based on pathological score

Lymph nodes	Total pathological score	Frequency	Percent
RBL	3	1	0.16
Man L	3	9	1.44
Retro L	3	3	0.48
LBL	3	7	1.12
CuML	2	1	0.16
MeL	2	2	0.32

*Total pathological score 2 represents (pathological score 1) cattle having one lesion after slicing while score 3 (pathological score 2) represents cattle having gross lesion not greater than 5 in number.

*RBL-right bronchial lymph node; Man L-mandibular lymph node; Retro L-retropharyngeal lymph node; LBL-left bronchial lymph node; CuML- caudal mediastinal lymph node; MeL-mediastinal lymph node

Table 2: Frequency of lesion in different lobes of lung based on pathological score

Lobes of lung	Total score	pathological	Frequency	Percent
LA		-	-	-
LC		8	2	0.32
LD		3	1	0.16
RA		8	3	0.48
RC		3	1	0.16
RD		3	1	0.16

*Total pathological score 3 (2 pathological score) represents lymph node having small lesion at more than one focus, while total pathological score 8 (those lymph node having score 2+3+3) represents extensive necrosis.

* LA- left apical; LC- left cardiac; LD- left diaphragmatic; RA- right apical; RC- Right caudal; RD- right diaphragmatic.

From six hundred twenty six (626) cattle examined at the study period (18 male cattle), (14 cattle having poor BCS and 4 cattle having good BCS), (2 cattle being categorized as adult and 16 old), (18 cattle being local) and ((14 cattle came from Wollo), (2 cattle from Adama and 2 cattle from A/Negelle)) each develop gross TB lesion during postmortem examination. This indicate that majority of the cattle presented for slaughter were almost all male. As well as having poor body condition also contribute as a factor for having TB lesion. But also when the animal get older, having TB lesion were statically significant; finally cattle came from Wollo, Adama and A/Negelle has developed gross TB lesion during postmortem examination (Table 3).

From the risk factors considered (Table 3), only age were found significantly associated with bovine tuberculosis infection ($P=0.000$) and the rest were not found significantly associated with the presence of gross tuberculosis lesions ($P>0.05$). Animal's ≥ 7 years of age were found to have (8.29) log odd of being positive to gross tuberculous lesions compared to animals within 4-7 years of age. The coefficient for both breed and sex were negative indicating that, the log odd of having or developing gross tuberculous lesion for cross breed compared to local ones and

female animal compared to male animals were, respectively found with fewer gross tuberculous lesions.

Table 3: Uni-variate and multivariate firth logistic regression of association of different risk factor in relation to postmortem lesion findings

Uni-variate				Multivariate		
Variable	No cattle	No positive	X ²	P-value	Coefficient	P-value
Sex						
Female	12	-	0.36	0.000	-3.15	0.123
Male	614	18				
BCS						
Poor	194	14	18.96	0.000	*	*
Good	432	4				
Age						
(4-7 years)	610	2	554.62	0.000	8.29	0.000
(>7 years)	16	16				
Breed						
Cross	2	-	0.05	0.000	-3.67	0.124
Local	624	18				
Origin						
Adama	336	2			-	-
A/Negelle	26	2	27.05	0.000	2.53	0.268
Godino	70	-			1.27	0.533
Gonder	17	-			2.99	0.135
Wollo	177	14			2.35	0.120

*Due to multicollinearity effect with age, BCS were not included during multivariable firth logistic analysis.

From a total of six hundred twenty six (626) cattle examined during the study period eighteen (18) cattle develop gross TB lesion which result 2.9% prevalence. Cattle came from Wollo ($14/177 \times 100 = 8\%$) has high percentage of having gross TB lesion, those which came from Adama and A/Negelle (0.6% having gross TB lesion) however large number of cattle were from Adama area as compared to A/Negelle. But cattle came from Godino and Gonder has no any gross TB lesion as compared to other cattle came from Adama, A/Negelle and Wollo area (Table 4).

Table 4: Prevalence of bovine tuberculosis in different origin of cattle examined

Origin	No. of animals examined	Positive	Percentage	p-value
Adama	336	2	0.6	0.010
A/Negelle	26	2	0.6	
Godino	70	-	-	
Gonder	17	-	-	0.000
Wollo	177	14	8	

4.2.Histopathology

Microscopic characterizations of tissue sample being positive for TB lesion during postmortem examination as well as culture positive were conducted on 5 samples. Tissues of approximately 6mm thick were collected in buffered formalin (10%) from lesions in the respiratory system and from the corresponding bronchial lymph nodes for histopathology and were taken to Pathology Laboratory of National Animal Health Diagnosis and Investigation Center (NAHDIC). Then tissue sample were trimmed, processed, embedded, sectioned, stained and finally subjected for microscopic examination. The tuberculous granuloma in the lymph nodes and lungs were microscopically classified into 4 categories of development according to criteria previously described for cattle. The lesions were evaluated for their size, type and extension of necrosis and mineralization. Briefly, the granuloma were classified into 4 categories, namely: stage I (initial or early lesions); stage II (solid granuloma); stage III (minimal necrosis); or stage IV (necrosis

and mineralization) (Mamo *et al.*, 2011) (The detail of each step was discussed within Appendix 5).

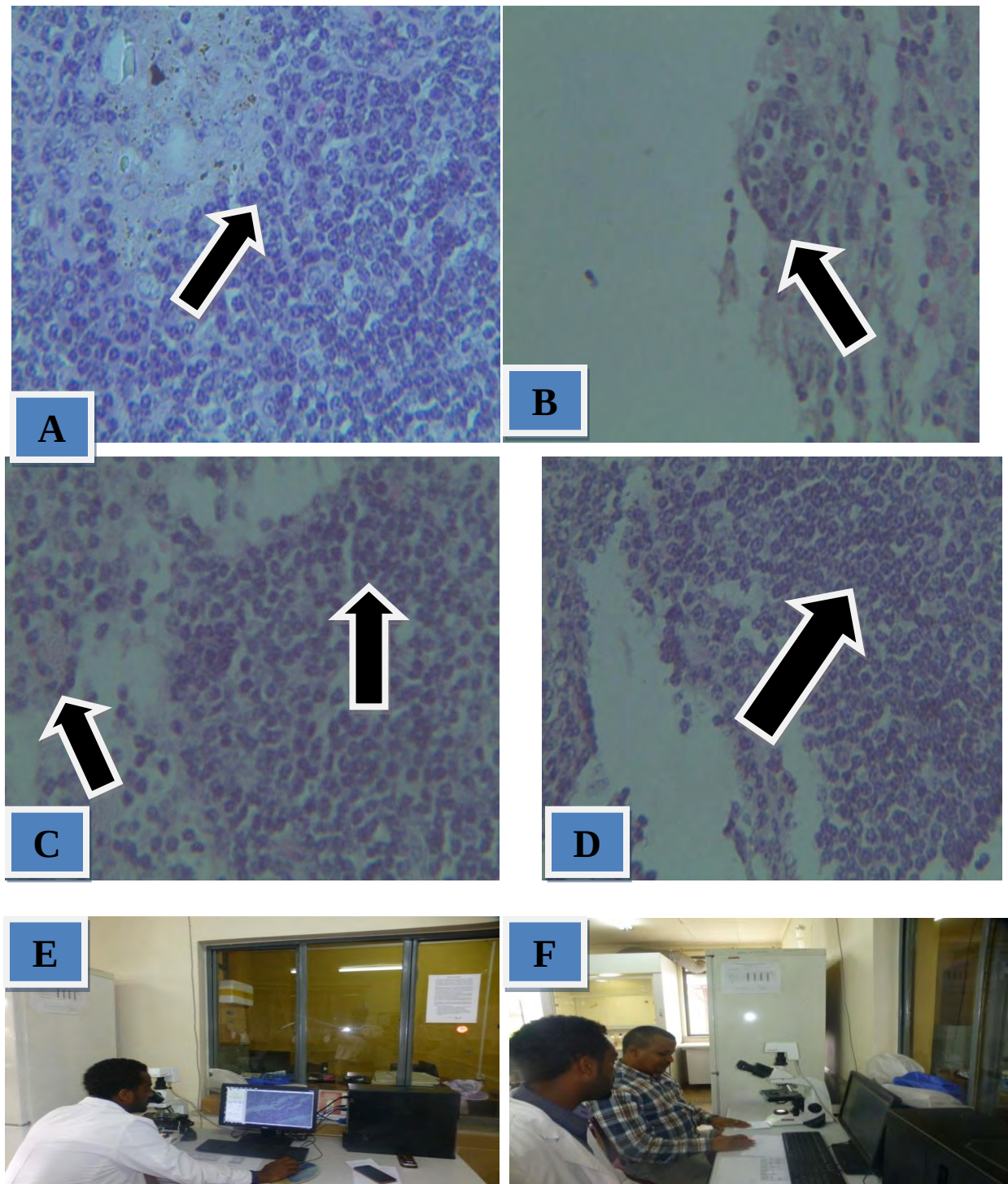


Figure 4: Microscopic TB lesions.

Note: The severe depletion of lymphocytes resulting in cystic structure in the cortex of the lymph node; also calcification and cellular debris (A) (stage II- solid granuloma). Aggregation of macrophage forming single layer (stage I-early lesion) with one giant macrophage (B, C and D (Black arrow)), and microscopic examination (E and F)

Stage I represent the initial stage and contain clusters of epithelioid macrophages with multinucleated Langhans-type cells and a thin rim of lymphocytes. Necrosis is absent at this stage. Neutrophil infiltration in the centers of the granulomas is not a feature of the lesions in lymph nodes, but neutrophils are often observed in early granulomas in cattle. While stage II represents, epithelioid macrophages, multinucleated Langhans-type cells and lymphocytes are more numerous and caseous necrosis starts to develop in the centres of the tubercles.

4.3.Bacteriological culturing and Zeihl Nielsen staining

A total of eighteen (18) TB lesion suspected tissue samples on postmortem inspection were transported to CVMA and cultured on primary culture media of which showed 28% (5/18) growth on primary culture media. The outcome of the culturing activity is indicated in table (5). Among those cultures which showed visible grows, only 11 % (2/18) were on L-J media enriched with pyruvate and the rest 89 % (3/18) were on L-J media enriched with glycerol (Table 5). The total of 5 grown cultures were subjected to Zeihl-Neelsen staining technique in the laboratory in order to check for the presence of acid fast *bacilli* organisms (A typical acid fast bacilli stain procedure involves dropping the cells in suspension onto a slide, then air drying the liquid and heat fixing the cells. The slide is flooded with Carbol Fuchsin, which is then heated to dry and rinsed off in tap water. The slide is then flooded with methanol (acid alcohol) to remove the carbol fuchsin, thus removing the stain from cells that are unprotected by a waxy lipid layer. Thereafter, the cells were stained in methylene blue and viewed on a microscope under oil immersion). Out of these, 80% (4/5) were acid fast negative. The remaining 1 grown culture media were confirmed for the presence of acid fast *bacilli*. (The detail of each step while culturing and staining was discussed within appendix 6).

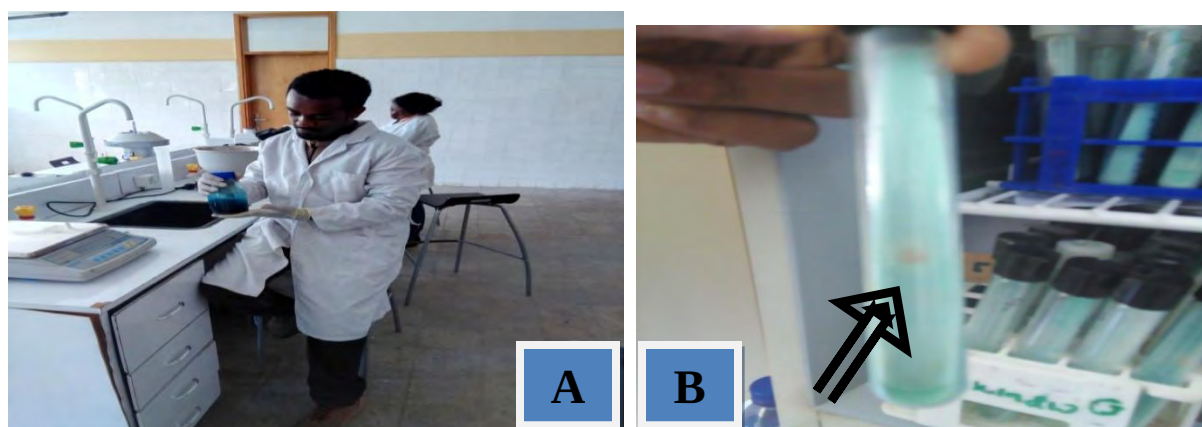


Figure 5: Media preparation and (A) culture positive (B)

Table 5: Bacterial culture result from tissues suspected tuberculosis lesions

Sample type	Growth on			
	L-J media with glycerol		L-J media with pyruvate	
	Total	Positive (%)	Total	Positive (%)
Lung	4	1(25)	4	-
Retropharyngeal LN	5	2(40)	5	1(20)
Mandibular LN	3	-	3	-
Bronchial LN	4	-	4	1(25)
Mediastinal LN	2	-	2	-
Total	18	3(89)	18	2(11)

4.4. Molecular characterization

4.4.1. Gene multiplex PCR

From 1 culture, positive colonies obtained were from tuberculosis tissue samples, up on multiplex PCR, it showed for the presence of the genus *Mycobacteria* (Figure 5), by using the genus specific primers MYCGEN-F (5'-AGA GTT TGA TCC TGG CTC AG-3') and MYCGEN-R (5'-TGC ACA CAG GCC ACA AGG GA-3'). The result indicate that the isolated *Mycobacterium* were NTM (Non-tuberculosis *Mycobacterium*).

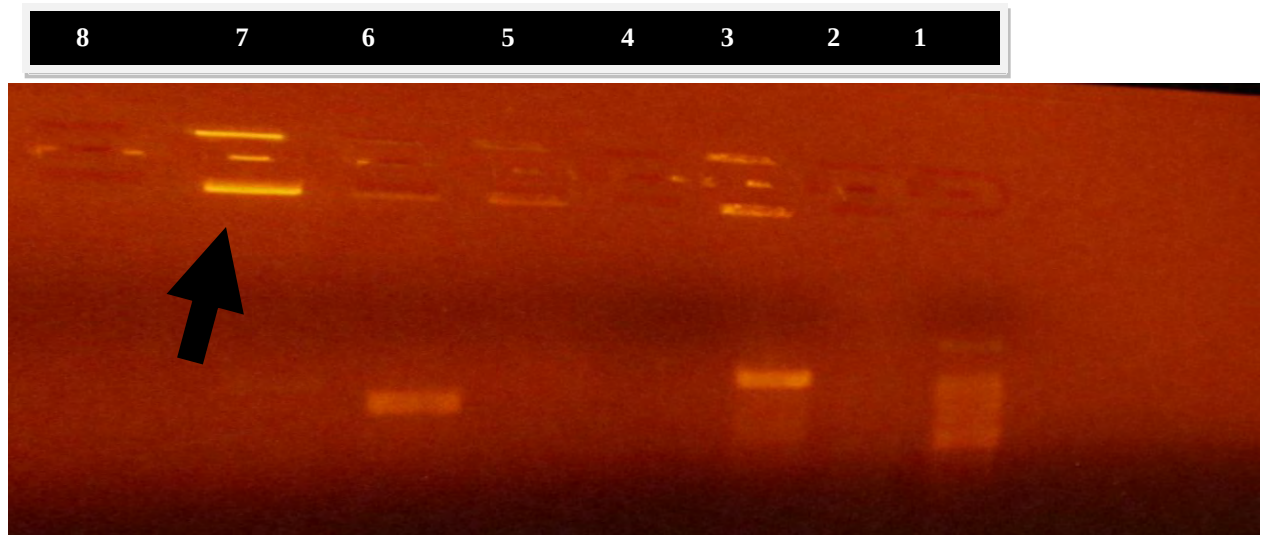


Figure 6: Gene multiplex's PCR result. **Note:** Electrophoretic separation of PCR products by multiplex typing of Mycobacteria isolated from tissue samples (the black arrow-are (**line 7**) positive samples for Genus *Mycobacterium* (1030bp)

5. DISCUSSION

The recognition of the gross and microscopic lesions of tuberculosis in cattle has aided the understanding of the disease process and has helped to eradicate the infection from herds and countries. Detection of tuberculous lesions in slaughterhouses takes place by observation of the visible tuberculous lesions in infected cattle; however, the level of the quality of such practices may vary from place to place and/or abattoir to abattoir in the country. Hence, the probability of carcasses to escape the abattoir inspection is likely to be high when a large number of animals are examined in large city abattoirs in particular. The failure to detect a lesion during abattoir inspection has the greatest significance in cattle with single lesion, since if the lesion is missed there is no further chance of detecting the disease in the animal (Domingo *et al.*, 2014).

The risk of the (bovine) meat-borne transmission of tuberculosis to humans is currently also considered negligible, at least in industrialized countries (EFSA, 2013). However, the recognition of bovine tuberculosis during meat inspection is still highly relevant for the surveillance and control of this infection in animals and herds, both in countries with high to low prevalence's of infection and in countries that are officially infection-free, where meat inspection is a cornerstone for granting and sustaining the official TB-free (OTB) status. Therefore, knowledge of the pathology of tuberculosis is a keystone for the recognition and identification of bovine tuberculosis-infected animals and herds.

Currently, most infected animals are found in the initial stages of infection. Thus, advanced forms of bovine tuberculosis are currently rarely found in countries with on-going eradication campaigns (Liébana *et al.*, 2008, Menzies and Neill, 2000 and Neill *et al.*, 2001). Advanced forms of bovine tuberculosis usually appear only in infected herds that have not been previously subjected to the tuberculin skin test. Regardless of the terms that are applied to tuberculous lesions and the stages of the disease, the pathological changes associated with infection by pathogenic mycobacteria are inseparable from the interplay between the host defense mechanisms and the mycobacterial virulence factors, which can be considered a delicate equilibrium between the immunological protective responses and the inflammatory damaging processes (Pollock and Neill, 2002 and Welsh *et al.*, 2005).

The very few studies in Ethiopia have indicated that not all cattle infected with *M. bovis* have visible tuberculous lesions at slaughter (Asseged *et al.*, 2004; Teklu *et al.*, 2004). This may limit the sensitivity of this detection technique at abattoirs, although detection of tuberculous lesions through abattoir inspection is so far the common procedure in Ethiopia. Among the undertaken abattoir studies, prevalence rates of 5.2% (Ameni and Wudie, 2003), 4.5% (Teklu *et al.*, 2004) and 3.5% (Shitaye *et al.*, 2006) have been reported in different abattoirs in the country.

The overall prevalence of bovine tuberculosis (2.9%) obtained in the current study at Bishoftu abattoir was almost parallel with a finding of Gebremedhin, *et al.*, 2014 (2.6%) in Dilla municipal abattoir. However the current finding was high when compared with Regassa *et al.*, 2009 (1.1%) at Hawassa, Asseged *et al.*, 2000 (1.48%) in Addis Ababa. But, the presently recorded prevalence was low as compared to previous report by Shitaye *et al.*, 2006 (3.46%) in Addis Ababa and Teklu *et al.*, 2004 (4.53%) at Hossana through similar diagnostic methods as well as previous reports of Ameni and Wudie, 2003 (5.16%) from Adama Municipality abattoir, Gudeta, 2008 and Mezene *et al.*, 2014 from Nekemte Municipality abattoir (5.1%) and (5.9%) respectively, Desta, 2008 (5%) at Kombolch meat processing plant, Southern Wollo, based on postmortem inspection, Shimels, 2008 at Debre Brihan, Central Ethiopia, Reggasa, 1999 (7.96%) at Wolaita Sodo, Nemomsa *et al.*, 2014 (9%) at Butajira, Dechasa, 2014 Adama Municipality abattoir (6.79%) and Romha *et al.*, Western Tigray 2013 (5.8%). But the current finding was very low as compared Mamo, 2007 (24%) at Adama municipality abattoir. The lower prevalence in the abattoir could be due to the fact that animals slaughtered in the abattoir are mainly Zebu, which are relatively resistant to bovine tuberculosis. The infection rate in cattle has been found to differ greatly from place to place (Shitaye *et al.*, 2006) and the difference might be most probably linked to the type of production system (most notably in extensive), which is unlikely to favor the spread of the disease in contrast to the intensive dairy farms as cited by Ameni *et al.*, 2006 and Shitaye *et al.*, 2006. But also these variations in prevalence could be due to the differences in the epidemiology of the disease in the animal populations and/or interventions taken to control it. There was statistical significant ($X^2=24.01$ and $P=0.000$) difference between age of the animal and tuberculous lesion. Radostitis *et al.*, 1994 has indicated that young animals are relatively resistant for bovine tuberculosis than older ones. But also as the age of the cattle increases owing to increased chances of exposure and infection with bovine tuberculosis, Humblet *et al.*, (2009) explicated that those stressors, malnutrition and immune-suppressants

increases with age; thus, older animals are more likely to have been exposed than younger ones. It has been suggested that increased incidence of bovine tuberculosis in older animals can be explained by a declining of protective capability in aging animals (O'Reilly and Dabron, 1995). The remaining assumed risk factors in abattoir revealed no statistical significant difference. The possible reason might be due to in-proportionality in the number of the animal compared in specific variable. For example, less number of female animals was presented to the study abattoir to be slaughtered and the proportions of animal with lean body condition encountered in abattoir were also low. However, there is biological association as observed from the coefficient value. In the present study, gross tuberculous lesions were found most frequently in lymph nodes of the thoracic cavity (88%); followed by lymph nodes of the head region and the lesser frequency was found in the lymph nodes of the abdominal cavity (12%). The occurrence of tuberculous lesions in thoracic cavity was lower than the results of previous studies, where greater than 90% occurrence of TB lesions in the respiratory system were reported in developed countries (Neill *et al.*, 1994; Collins, 1996; Whipple *et al.*, 1996). However it is higher than the report of Regassa *et al.*, 2009 which implies that inhalation is the most important route of infection. Husbandry factors such as enclosures of the animals overnight may facilitate respiratory transmission of the infection (WHO, 2005). The followed high proportion in lymph nodes of the abdominal cavity indicate, the other important route of transmission is ingestion of the agent which may happen during feeding or drinking contaminated materials (Hardie and Watson, 1992; Morris *et al.*, 1994). The microscopic features of tuberculous granulomas in lymph nodes have been described in detail and are used to stage these granulomas according to morphological criteria, such as the presence or absence of necrosis, mineralisation and fibrous capsules (Wangoo *et al.*, 2005). The motivation behind this classification system is to provide a framework within which the development and chronicity of lesions can be interpreted, which helps the identification of stages and the recognition of deviations from the normal granuloma progression, such as those induced by vaccination, immunosuppression and *mycobacterial* virulence variation. In the current study, the chance of growing *Mycobacteria* were less than 30% which might be due to either loss of the agent during freezing or delayed transportation from the site of collection. WHO (1998) indicated loss of 5-10% due to contamination resulting from prolonged preservation, which in turn resulted into, overgrowth of *M. bovis* with environmental *Mycobacteria* and a loss of up to 60% due to decontamination procedure. Besides, *M. bovis*

grows poorly on standard Löwenstein-Jensen medium (Cleaveland *et al.*, 2007). Therefore, the use of proper time in culturing and application of standard laboratory technique could increase the chance of recovery of acid fast bacilli. Molecular analyses of the isolates were revealed that the lesions were caused not by the members of *M. tuberculosis* complex rather by members of the non-tuberculosis *Mycobacterium* group. The isolates belonged to the later group. An extensive study on the Ethiopian cattle confirmed that about one third of the isolates from tuberculous lesions are non *tuberculosis Mycobacterium* species, similar results were reported by other workers in Ethiopia (Berg *et al.*, 2009), Shimelis, 2008, Nemomsa *et al.*, 2014 and Mezene *et al.*, 2014. In the present study, we have isolated non-tuberculosis *Mycobacterium* (NTMB) species from the gross lesion suggestive of TB in slaughtered cattle. Previous studies in Ethiopia (Ayana *et al.*, 2013) and in Tanzania revealed the association of non *tuberculosis Mycobacterium* with granulomatous lesions in cattle and humans (Kazwala *et al.*, 2002; Berg *et al.*, 2009). The isolation of non-tuberculosis *Mycobacterium* showed the importance of the strains in the epidemiology of bovine tuberculosis to cause tuberculous like lesions. Other African countries like Uganda and Chad have also reported the isolation of non *tuberculosis Mycobacterium* strains from more than 40% of the animals with tuberculous lesions (Diguimbaye-Djaïbe *et al.*, 2006; Oloya *et al.*, 2007). Moreover, non *tuberculosis Mycobacterium* is associated with tuberculous compatible lesions in camel and wildlife populations in Ethiopia (Ameni *et al.*, 2010; Tschopp *et al.*, 2010; Mamo *et al.*, 2011). But also, non- *tuberculosis Mycobacterium* had been isolated from milk and nasal swab of tuberculin reactor animals in Chifra pastoral district of Afar region, North Eastern Ethiopia (Ashenafi *et al.*, 2013). The techniques employed by the present study could not further identify these isolates to the species level.

6. CONCLUSION AND RECOMMENDATIONS

The result of the present study has shown that *Mycobacterium* other than *M. tuberculosis* complex members were isolated from the suspicious lesions in our study although *M. bovis* has been known to be the major cause of bovine tuberculosis in cattle as well as bovine tuberculosis is prevalent in cattle slaughtered at Bishoftu abattoir. Even though, the isolates were non-*tuberculosis Mycobacterium* members, it was observed that the lesions were caused by *Mycobacterium*. This may be due to contamination of an environment in which they belong (particularly pasture and water body), through faces and air droplet. Which indicate the isolation of non-*tuberculosis Mycobactrium* from tuberculous lesions highlights their importance in causing granulomatous lesions and the epidemiology of the disease in cattle. In addition to this people of the area have the habit of consuming raw meat and milk and share the same microenvironment with their livestock. This further disseminates the causative agent, both through inhalation and ingestion resulting in high economic loss and public health effect. This study reveals a high proportion of tuberculous lesion in the thoracic cavity lymph nodes (88%). It implies that respiratory route is the major means of transmission.

Therefore based on the above conclusion the following recommendations are forwarded:

- Similar studies, in the slaughterhouse across the country so as to estimate the national prevalence of BTB as well as identification and characterization of the non-*tuberculosis Mycobacterium* complex and evaluation of their pathogenicity in bovine is essential.
- Characterization of these isolates to specific species level and further investigation aiming at identification of the source of non-*tuberculosis Mycobacterium* infections, transmission routes in animals and their public health significance in the study area should have studied.
- Detailed abattoir inspection should be implemented by focusing on lymph nodes of the thoracic, mesenteric and head region.
- Finally public education to increase the awareness of the community about the potential risk of consumption of raw animal products is necessary.

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8. APPENDICES

Appendix 1: Format for recording post mortem examination data/results at the abattoir

No	Date	ID of Animal	Age (Y/A/O)	Any gross lesion (Y/N)	Type and description of the gross lesions detected

- **Age:** **Y**= Young, **A**= Adult, **O**= Old
- **Lesion:** **Y**= Yes, **N**= No
- **ID of animal**= Identification of animal (1, 2, 3...)

NB: If gross lesions are identified tissue samples will be coded using ID of the animal, species, age and any gross lesions, example, 1CYY means ID=1, Species = cattle, Age=Young, Any lesion= Yes.

Appendix 2: Format for recording histopathological examination results

No	Code of the tissue sample on the microscopic slide	Its gross pathological lesion description	Histopathological findings/descriptions

NB: The code on the tissue container during sample collection remains the same during tissue processing and when the tissue is put on the microscopic slide (code on the microscopic slide is the same as that of code given on tissue sample container).

Appendix 3: Format for recording bacteriological and molecular evaluation results

No	Code of sample during evaluation	Bacteriological characterization	Molecular characterization

NB: The code during sample collection remains the same after molecular characterization.

Appendix 4: Format for recording summary of the results

No	ID of Animal	Age (Y/A/O)	Any gross lesion findings	Any histopathological Findings	Bacteriological and Molecular characterization

Appendix 5: Histopathological technique

1. **Fixation of tissue** by 10% formaldehyde



2. **Trimming tissue** to fit in to standard histological processing tissue cassettes (5mm thickness)



3. **Tissue processing:** dehydration, clearing and impregnating

- Dehydrating tissue by using increasing strength of alcohol; e.g. 70%, 95% and 100%.

- ❖ Dehydration 70% alcohol 1 hour

95% alcohol 1 hour

100% alcohol I 1 hour

100% alcohol II 2 hour

100% alcohol III 2 hour

- Clearing of tissue by Xylene

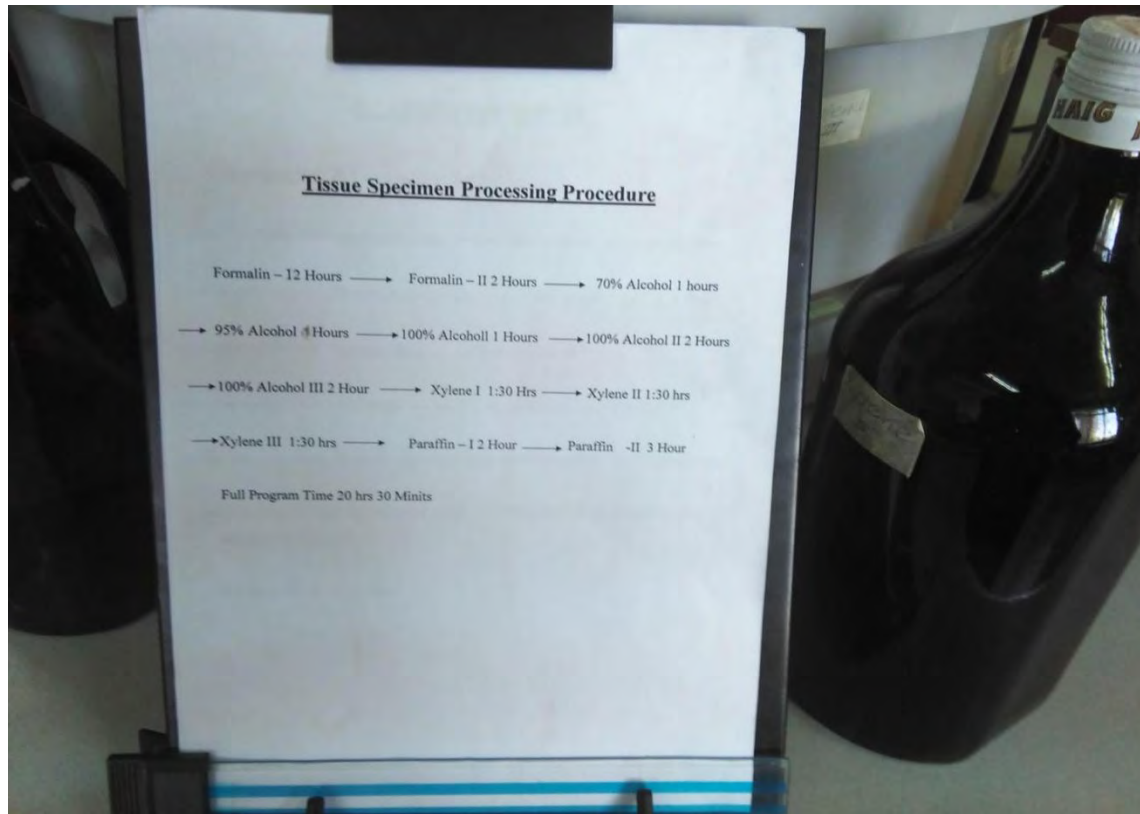
Xylene I 1:30 hours

Xylene II 1:30 hours and Xylene III 1:30 hours

- Impregnation tissue with Paraffin wax

Paraffin wax I 2 hour

Paraffin wax II 3 hour



Tissue specimen processing procedure at NAHDIC (Pathology laboratory)

4. **Embedding or Blocking:** Impregnated tissues are placed in a mold with their labels and then fresh melted wax (58⁰C) is poured in it and allowed to settle and solidify.



5. **Section:** sectioning of tissue in to 5 micron thickness and adhere on the surface of clear slide.

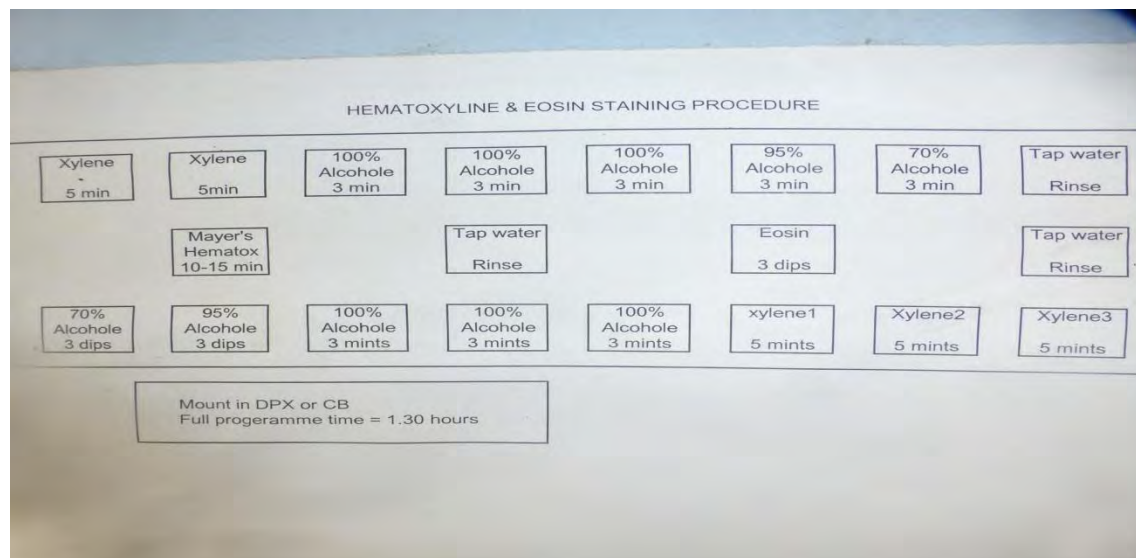


6. **Staining:** manual staining with Haematoxylin and Eosin to give color for sectioned tissue.





Staining procedure:



*HE staining procedure used at NAHDIC (pathology laboratory)

Source: (Asegedeche, 2007) **Note:** taken from NAHDIC pathology laboratory

7. **Microscopic examination:** stained slide is examined under microscope.



Appendix 6: Media preparation, procedures of culturing inoculums and staining.

Media preparation

- ✓ Measure 4.17 g LJ media for each Pyruvate and Glycerol labeled flasks
- ✓ Then pour 67.5 ml distilled water and mix by heating as well as stirrer
- ✓ Then add 1.23g Pyruvate in “P” labeled flasks and add 1.35ml glycerol in “G” labeled flasks
- ✓ Then cover using aluminum and autoclave the solution at 121°C for 30minutes
- ✓ Then cool at room temperature
- ✓ Then prepare 112.5ml homogenized whole egg (2-2.5egg) for each flask after soaking with alcohol. And filter the solution.
- ✓ Then mix with the autoclaved media in a well manner to prevent bubble formation
- ✓ Then pour around 8-10ml of the media into sterile screw capped test tubes
- ✓ Then put the media in autoclave at 85°C for 45minute in slanted position for drying purpose.
- ✓ Then the media was dated and incubated at 37°C for 24hrs to check for sterility.

Then for culturing the sample was taken from the deep freeze, then trimmed, then using pistol and mortal homogenized the tissue, then add 5ml saline water in the falcon tube having the homogenized tissue, then add 5ml NaOH for decontamination, then centrifuge at 3000rpm for 15 minute, then discard the supernatant and use the sediment by adding a drop of phenol red as indicator, then add a drop of HCl to see any color change (from red-yellow), then pour 1ml of the sample to the prepared media.

Inoculation

- The inoculum is distributed evenly over the surface of the slants.
- The tubes are allowed to remain in a slanted position at 37°C for approximately 1 week with screw caps loose.
- The tubes are returned to a vertical position when the free moisture has evaporated from the slants.
- The lids are tightened and the tubes are placed in baskets in an incubator at 37°C.

- The slants are incubated for at least 2 months and observed weekly for the presence of any growth from the week one onwards.
- Cultures will be considered positive based on the colony characteristics and presence of acid fast bacilli in the smear.

Staining

- Fix the smear of the specimen over the glass slide by heating.
- Then pour carbol fuschin over smear and heat gently until fumes appear and allow it to stand for 5 minutes and then wash it off with water.
- Then pour methanol (acid alcohol), wait for one minute and keep on repeating this step until the slide appears light pink in color. Wash off with water.
- Then pour methylene blue, wait for two minutes, again wash with water
- Allow it to air dry and examine under oil immersion lens.

Source: (OIE *Terrestrial Manual* 2014)

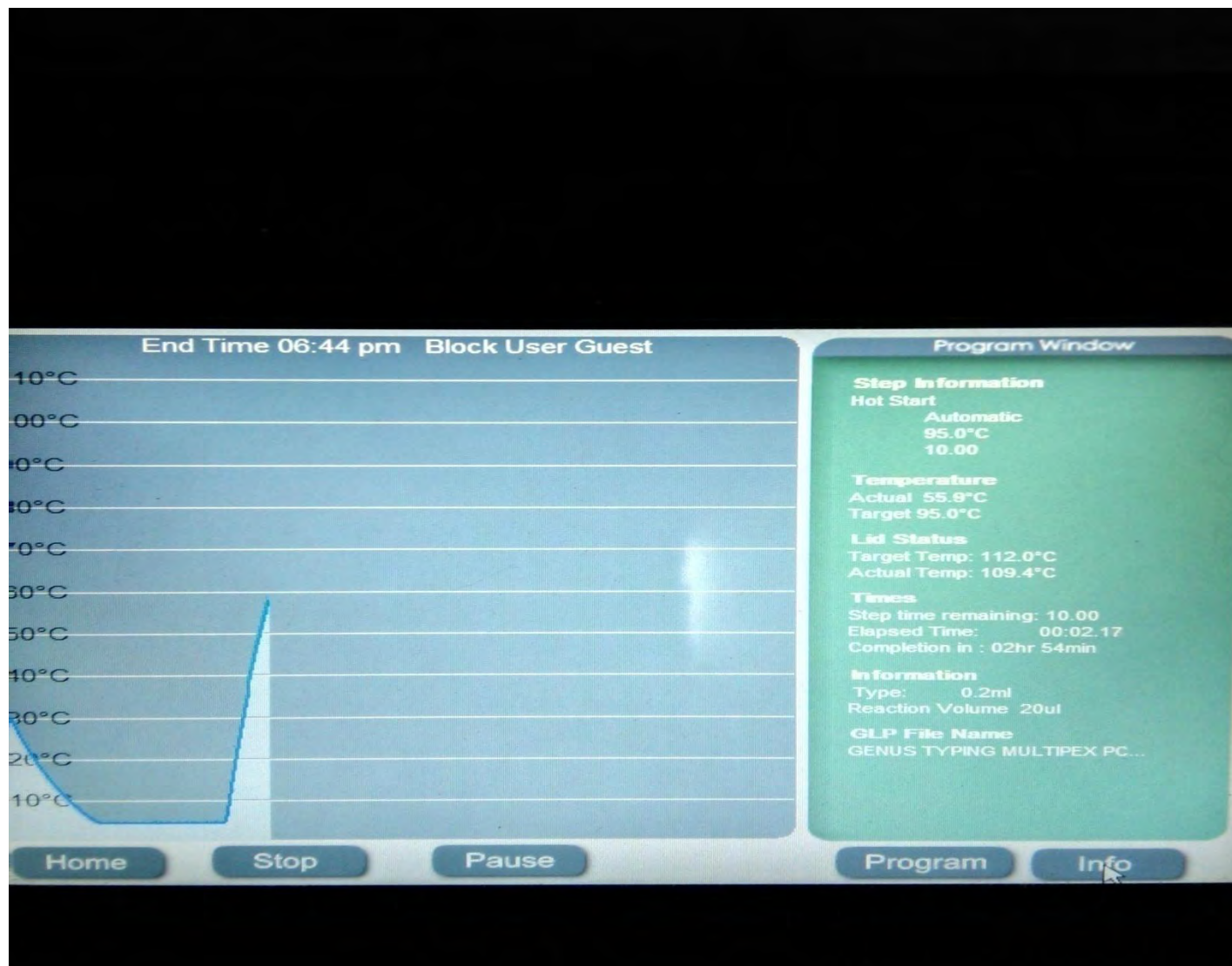
Appendix 7: Genus multiplexes PCR



The control that we use as a reference that indicate the Genus as well as different species



Gel electrophoresis and PCR machine while operating



the screen of PCR machine showing different adjustment for example time of processing will last for more than 2hr.

Source: (Taken from AKLIPB laboratory while we are working)

Appendix 8: Body condition scoring for beef cattle

BCS	% Body fat	Detailed description
Thin		
1	3.77	Clearly defined bone structure of shoulder, ribs, back, hooks and pins easily visible. Little muscle tissue or fat present.
2	7.54	Small amount of muscling in the hindquarters. Fat is present, but not abundant. Space between spinous process is easily seen.
3	11.30	Fat begins to cover loin, back and fore-ribs. Upper skeletal structures visible. Spinous process is easily identified.
Borderline		
4	15.07	Fore-ribs becoming less noticeable. The transverse spinous process can be identified by palpation. Fat and muscle tissue not abundant, but increasing in fullness.
Optimum		
5	18.89	Ribs are visible only when the animal has been shrunk. Processes not visible. Each side of the tail head is filled, but not mounded.
6	22.21	Ribs not noticeable to the eye. Muscling in hindquarters plump and full. Fat aroundtail head and covering the fore-ribs.
7	26.38	Spinous process can only be felt with firm pressure. Fat cover in abundance oneither side of tail head.
Fat		
8	30.15	Animal smooth and blocky appearance; bone structure difficult to identify. Fat cover is abundant.
9	33.91	Structures difficult to identify. Fat cover is excessive and mobility may be impaired.

Appendix 9: Age determination for cattle

At birth to 1 month	Two or more the temporary incisors teeth present. Within first month, entire 8 temporary incisors appear.
2 years	As long-yearling, the central pair of temporary teeth or pinches is replaced by the permanent incisors attain full development.
2-1/2 years	Permanent first intermediates. One on each side of the pinchers, are cut. Usually these are fully developed at 3 years.
3-1/2 years	The second intermediates or laterals are cut. They are on a level with the first intermediates and begin to wear at 4 years.
4-1/2 years	The corner teeth are replaced. At 5 years the animal usually has the full complement of incisors with the corner incisors show wear.
5-6 years	The permanent pinchers are leveled, both pairs of intermediates are partially leveled, and the corner incisors show wear.
7-10 years	At 7 or 8 years the pinchers show noticeable wear; at 8 or 9 years the middle pairs show noticeable wear; and at 10 years, the corner teeth show noticeable wear.
12 years	After the animal passed the 6 th year, the arch gradually loses its rounded contour and becomes nearly straight by the 12 th year. In the meantime, the teeth gradually become triangular in shape, distinctly separated and show progressive wearing to stubs. These conditions become more marked with increasing age.

9. APPROVAL SHEET

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College of Veterinary Medicine and Agriculture

Department of Pathology and Parasitology

Thesis title: Bovine tuberculosis lesion description with molecular characterization of *Mycobacterium* species from cattle slaughtered at ELFORA and municipal abattoir, Bishoftu, Ethiopia

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