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

ISOLATION AND MOLECULAR CHARACTERIZATION OF *MYCOBACTERIUM BOVIS* ISOLATED FROM CATTLE SLAUGHTERED AT HAWASSA UNIVERSITY AND MUNICIPAL ABATTOIRS, SOUTHERN ETHIOPIA

MVSc. Thesis

BY

Yibrah Tekle

Addis Ababa University, College of Veterinary Medicine and Agriculture

Department of Veterinary Microbiology, Immunology and Veterinary Public Health

June, 2016

Ethiopia, Bishoftu

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MVSc. Thesis

A Thesis Submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Veterinary Science in Veterinary Microbiology

BY

Yibrah Tekle

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College of Veterinary Medicine and Agriculture, Bishoftu

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Immunology and Veterinary Public Health

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As member of the examining board of the final MVSc. open defense, we certify that we have read and evaluated the thesis prepared by: **Yibrah Tekle** titled: "**Isolation, and Molecular Characterization of *Mycobacterium bovis* Isolated from Cattle Slaughtered at Hawassa University and Municipal Abattoirs, Southern Ethiopia"** and recommended that it be accepted as fulfilling the thesis requirement for the degree of Master Science in Veterinary Microbiology.

Dr. Hagos Ashenafi	_____	_____
Chairman	Signature	Date
Dr. Alemayehu Regassa	_____	_____
External Examiner	Signature	Date
Dr. Tilaye Demsie	_____	_____
Internal Examiner	Signature	Date
Dr. Gezahegn Mamo	_____	_____
Major Advisor	Signature	Date
Professor Gobena Ameni	_____	_____
Co-Advisor	Signature	Date
Dr. Gezahegn Mamo	_____	_____
Department Chairperson	Signature	Date

DEDICATION

This thesis manuscript is dedicated to my instructors, friends and families those who are behind my success.

STATEMENT OF AUTHOR

First, I declare that this thesis is my authentic work and that all sources of materials used for this thesis have been properly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for MVSc. 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 certificate, diploma, or degree.

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Name: Yibrah Tekle

Signature: _____

Date of Submission: _____

Advisors name

Main advisor: Gezahegn Mamo (DVM, MSc., PhD - Assoc. Prof., CVMA-AAU)

Signature: _____

Co-advisor: Gobena Ameni (DVM, PhD - Professor, ALIPB-AAU)

Signature: _____

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Immunology and Veterinary Public Health

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Title: "**Isolation and Molecular Characterization of *Mycobacterium bovis* Isolated from Cattle Slaughtered at Hawassa University and Municipal Abattoirs, Southern Ethiopia"**

Submitted by: Yibrah Tekle	_____	_____
Name of Student	Signature	Date

Approved for submittal to thesis assessment committee

1. Dr.Gezahegne Mamo	_____	_____
Major Advisor	Signature	Date
2. Prof. Gobena Ameni	_____	_____
Co- Advisor	Signature	Date
3. Dr. Gezahegne Mamo	_____	_____
Department chair person	Signature	Date

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LIST OF ABBREVIATIONS

AFB	Acid fast bacilli
BCG	Bacille calmette guérin
BCS	Body condition Scoring
bp	Base pair
BTB	Bovine tuberculosis
CDC	Centers for disease control
CI	Confidence interval
CIDT	Comparative intradermal tuberculin test
DNA	Deoxy ribonucleic acid
DR	Direct repeat
EDTA	Ethylene diaminetetraacetic acid
ELISA	Enzyme linked immunosorabent assay
FAO	Food and agricultural organization
HIV	Human immunodeficiency virus
HMCA	Hawassa city municipal abattoir
HPC	Hexadecyl pyridinium chloride
HUA	Hawassa University abattoir
IFN-γ	Gamma interferon
IS	Insertion sequence
LJ	Lowenstein-Jensen
LJG	Lowenstein-Jensen glycerol
LJP	Lowenstein-Jensen pyruvate
masl	Meter above sea level
MIRU-VNTR	Mycobacterium interspersed repetitive unit-variable number tandem repeats
mPCR	Multiplex polymerase chain reaction
MTBC	Mycobacterium tuberculosis complex

NTM	Nontuberculosis mycobacterium
OIE	Office des internationale epizooties
OR	Odds ratio
PCR	Polymerase chain reaction
PPD	purified protein derivatives
PPD-A	Avium purified protein derivatives
PPD-B	Bovine purified protein derivatives
RDs	Regions of differences
RFLP	Restriction fragment length polymorphism
rpm	Revolution per minute
rRNA	Ribosomal ribonucleic acid
SE	Standard error
SIT	Shared international spoligotype
SNNPRs	Southern Nations Nationalities and Peoples Regional state
TAE	Tris-acetate- ethylenediaminetetra acetic acid
TB	Tuberculosis
VNTR	Variable number tandem repeat
WHO	World health organization
ZN	Ziehl-Neelsen
μm	Micrometer
χ^2	chi-square

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ABSTRACT

A cross sectional study was conducted on 753 Cattle, selected using systematic random sampling technique, in Hawassa abattoirs, Southern Ethiopia from December 2015 to May 2016 to investigate the prevalence of bovine tuberculosis (BTB), identifying the risk factors as well as to isolate and characterize its causative agent. The methods used were postmortem examination, bacteriological culturing, RD4 deletion typing and Spoligotyping. The overall prevalence of the bovine tuberculosis was 5.8% (95%CI: 4.16-7.52) on the basis of detailed postmortem examination. Multivariable logistic regression analysis identified age, body condition; breed and market were statistically significant. The older cattle above 8 years were eighteen times (OR=18.25) more likely to have tuberculosis than the younger cattle. The cross breeds were nine times (OR = 9.75) more sensitive to *M. bovis* as compared to local breeds as well as the poor body conditioned cattle forty one times (OR = 41.21) more likely to have tuberculosis than the cattle have good body condition. Relatively, the occurrence of BTB was higher in cattle brought from Hawassa (OR = 13.17) and Borena (OR = 9.41) markets than Tula market. The lesions were found most frequently distributed in thoracic cavity lymph nodes (75%), which indicated that respiratory route was the main mode of infection in the study area. Out of 44 tissue samples cultured, 24 (54.5%) were culture positive and all were acid fast staining positive. However, using RD4 deletion only three isolates were confirmed as *M. bovis.*The further Spoligotyping characterizations of these three isolates revealed the strain SB1477. And the rest isolates that have never showed any signal (21/24) in the RD4 deletion typing should be characterized by using the mPCR in order to confirm either they are Mycobacterium tuberculosis complex or notuberculosis mycobacterium. In conclusion, the study confirmed that the low prevalence of BTB and the current *M. bovis* strain circulate in the cattle being SB1477 that indicate implement control program and investigate the zoonotic role of this strain.

Key words: *Culturing, Hawassa abattoirs, M. bovis, Postmortem, RD4 deletion, Spoligotyping,* ZN S*taining*

1. INTRODUCTION

Mycobacteria are slow-growing, acid-fast and aerobic organisms whose genus classified in to more than 90 different species (Watterson *et al*., 1998). A group of *Mycobacterium* species called *M. tuberculosis* complex (MTBC) that comprise *M. tuberculosis*, *M. bovis*, *M. microti* and *M. africanum*, is of utmost clinical importance since it causes tuberculosis in humans worldwide (Kidane *et al*., 2002; Haddad *et al.*, 2004; Marais, 2008; Rock *et al*., 2008). *Mycobacterium* species other than those of the tuberculosis complex, also called nontuberculous mycobacteria (NTM), are widely distributed in the environment and may colonize and occasionally cause infections in humans (Adle-Biassette *et al.*, 2003; Barne *et al*., 2004; Castro *et al*., 2007). Mycobacteria of the MTBC and NTM have been found to cause infections in immunecompetent and immunocompromised subjects and cause pathology in pulmonary and ext-rapulmonary sites (Scarparo *et al*., 2001; Sachdeva *et al*., 2002; Stout, 2006; Fanlo and Tiberio, 2007; Glassroth, 2008).

Tuberculosis (TB) is a chronic pulmonary disease which threats to animal and human causing high morbidity, economic losses and mortality (Smith *et al.*, 2006; WHO, 2007; Pal, 2013). Since TB is a major health threats particularly in developing countries, it has been declared as global emergency by the World Health Organization in 1995 (WHO, 2003). TB causes death in each year among millions of people as a result it ranked as globally a second leading disease from the infectious disease next to human immunodeficiency virus (HIV) (Zeweld, 2014). TB is a leading killer among adults in the most economically productive age groups and people living with HIV (Lopez *et al*., 2006), and even those cured from TB can be left with lifetime sequelae that substantially reduce their quality of life (Miller *et al*., 2009).

Bovine tuberculosis (BTB) is among the principal zoonotic diseases (Buncic, 2006) caused by *M. bovis* (Grange *et al.*, 1996) which affects many vertebrate animals (Grange *et al.*, 1996) and humans (Ayele *et al.*, 2004; Thoen *et al.*, 2009) and characterized by

progressive development of granulomas/ tubercles in tissues and organs (Amanfu, 2006; OIE, 2010; Hlokwe *et al.,* 2013; Pal *et al.,* 2014).

Bovine tuberculosis has been widely distributed throughout the world and has been recognized from 176 countries as one of the important bovine diseases causing great economic loss in animal production (Awah-Ndukum *et al.,* 2013) and the most frequent cause TB in man (Grange *et al*., 1996; Tenguria *et al.,* 2011). *M. bovis* is most frequently isolated from domesticated cattle (Grange *et al*., 1996; 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. The members of MTBC are characterized by 99.9% or greater similarity at nucleotide level, and are virtually identical at 16s rRNA sequence but they vary in host specificity (Brosch *et al.,* 2002).

Bovine tuberculosis affects broad range of mammalian hosts including humans, cattle, de er, pigs, domestic cats, wild carnivores and omnivores (De Lisle *et al*., 2002); it rarely affects equids or sheep (Delahay *et al*., 2002; Phillips *et al*., 2003). Moreover, human TB of animal origin caused by *M. bovis* is becoming increasingly evident in developing countries (Russel, 2003; Mamo *et al*., 2013a). The productivity efficiency of the infected animal reduced from 10-25%; the direct losses due to the infection decreases in milk production 10-18% and meat production 15 %. BTB has both the effect in animal production and public health importance (Radostits *et al.,* 1994; Müller *et al.,* 2013).

In developing countries like Ethiopia, the low standard living areas and socio-economic situation 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.,* 2010b; Ejeh *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 (Phillips *et al.,* 2003; Radostits *et al.,* 2007). Human infection by *M. bovis* is thought to be mainly through drinking of contaminated or unpasteurized raw milk. The potential for transmission of *M. bovis* and other mycobacteria between cattle and humans are the presence of close contact of animal and humans or the rural societies living together with

their animals in the same microenvironment and house, raw milk and meat consumption habit, the prevalence of HIV increasing and HIV patient's susceptibility to TB (Shitaye *et al*., 2007). In the areas where the bovine tuberculosis is common and the milk pasteurization is rare, *M. bovis* cases in human estimated 10-15 % (Ashford *et al.*, 2001; Mbugi *et al*., 2012). Thus, in cattle the main route of infection transmission: aerosol, close contact between animals (Neill *et al*., 1991) and ingestion of contaminated products (Menzies and Neill, 2000; Ameni *et al*., 2007; Cleaveland *et al*., 2007).

Bovine tuberculosis, is an endemic disease of cattle in Ethiopia with prevalence of 1.1%-24.7% in abattoir and 3.5–50% in crossbreed farms (Shitaye *et al*., 2007; Berg *et al*., 2009; Biffa *et al*., 2009; Regassa *et al.*, 2010), like human TB has not received more focus on research and its control strategies plus the test and slaughter control strategies not applied, due to its economic constraints, but its applicable effective method in developed countries (Chukwu *et al.*, 2013). Due to inadequate comprehensive abattoir surveillance and lack of diagnostic facilities the BTB has limited information (Cosivi *et al.*, 1998; Asseged *et al*., 2000), particularly on genotypic characteristics of *M. bovis*, a strain affecting the cattle population in Ethiopia (Biffa *et al*., 2010b). Knowing the *M. bovis* strains which are circulating in cattle population is using to examining host pathogen relationships to monitor transmission and spread of the disease among cattle (Gagneux and small, 2007; Berg *et al.*, 2011). Therefore, this study was designed with the following objectives:

- To assess the prevalence of bovine tuberculosis based on postmortem examination of slaughtered cattle at Hawassa University and municipal abattoirs
- To evaluate the association between the potential risk factors and BTB
- To isolate and characterize the causative agents of bovine tuberculosis using molecular techniques (RD4 Deletion typing and Spoligotyping)

2. LITERATURE REVIEW

2.1. Taxonomy of Mycobacteria

The genus *Mycobacterium* is classified under the Order Actinomycetales and Family Mycobacteriaceae (Karlson and Lessel, 1970; Quinn *et al*., 1999). The genus includes a number of species (Watterson *et al.,* 1998), 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* (Quinn *et al.,* 1999; Marie-France *et al*., 2009).

2.2. Characteristics

The mycobacteria most closely resemble the other myclolic acid producing bacteria, Corynebacterium, Nocardia and Rhodococcus. Mycobacteria are aerobic, non-motile, non-spore forming bacilli (Songer and Karen, 2005; Quinn *et al.,* 2011), in tissues they appear as rods, which may be strait, curved or in the form of clubs and measuring 1.0-4.0μm in length and 0.2-0.3μm in width (Quinn *et al.,* 1999), gram positive, acid fast, and occasional branching and sometimes a mycelium-like growth that fragments in to coccoid and rod forms (Carter and wise, 2004). 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 cell wall of mycobacteria is consisting of a peptidoglycan layer (basal), arabino-galactanmycolate complex (intermediate) and peptidoglycolipid layer, lipid rich rope like structure (outer) (Chukwu *et al.,* 2013). Mycobacteria when stained are acid fast as they resist decolorizing with strong acid/alcohol solutions (Carter and wise, 2004; Quinn *et al.,* 1999). They often stain irregularly and appear somewhat beaded. They are catalase positive (Songer and Karen, 2005). The species grows on medium containing serum, potato and egg. Lowenstein-Jensen media is the most commonly used one 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. The optimal growth temperature is 37^{o}c (Quinn *et al.,* 1999).

2.3. Pathogenesis

None of the *Mycobacterium* has yet been shown to produce exotoxins. They have the concentration of lipids, 20-40% dry weight, which is thought to be in part responsible for their resistance to humeral defense mechanisms and to disinfectants, acids, alkalis. The thick cell wall of mycobacteia is rich in mycolic acid and other complex lipids (mycosides, glycolipids and sulfolipids), making it hydrophobic and impermeable to aqueous stains without heat (Carter and wise, 2004).

The entrance of tubercle bacilli to animal body through respiratory, alimentary, genital, cutaneous and congenital routs (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 sever course of the disease with haematogenous spreading as a result of lysis of macrophages that release bacteria in to the blood from the primary foci and secondary seeding to various tissues (Andersen, 1997; Songer and Karen, 2005).

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; Songer and Karen, 2005). 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 (Thoen and Bloom, 1995).

Mycobacterium bovis eludes the bacteriocidal activities of macrophages by escaping from fused phagolysosomes into nonfused vacuoles in the cytoplasma, this due to its cell wall lipids (mycosides, phospholipids, and sulpholipids) (Songer and Karen, 2005). In additio n 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 avirulent strains develop as clumps (Thoen and Bloom, 1995; Ereqat *et al*., 2013).

2.4. Immunity

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).

2.5. Epidemiology

Mycobacterium 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 (Grange, 1995; Gemechu *et al.*, 2013). However, the epidemiology of this organism is not well studied particularly in developing countries and the epidemiological data is necessary for its control strategy (Ali, 2006).

2.5.1. Transmission

Cattle are considered to be the main host of *M. bovis*, although it isolated from many other livestock and wildlife species and transmit to many mammalian species including man (Acha and Szytre, 2001; Ayele *et al*., 2004; Van Rhijn *et al*., 2008) because Cattle shed *M. bovis* in respiratory secretions, feces and milk, and sometimes in the urine, vaginal secretions or semen (O'Reilly and Daborn, 1995; Ameni *et al.*, 2001; Russell, 2003) which may act as source of infection to other animals since the excretions may contaminate grazing pasture, drinking water, feed, water and feed troughs or fomites (**Figure 1**). Due to the presence of *M. bovis* reservoirs in wildlife makes disease eradication difficult to achieve (Quinn *et al*., 2011).

Mycobacterium bovis can infect human primarily by the ingestion of unpasteurized dairy products, inhalation of aerosols and through breaks in the skin. The possibility of transmission of this Mycobacterium to humans from infected animals could be high in areas where there is close contact between human and animals (O'Reilly and Daborn, 1995). Main routs of infection by which tubercle bacilli gain entrance into the host are respiratory and alimentary tracks (O'Reilly and Daborn, 1995; Ameni *et al.*, 2001; Russel , 2003). Experimental studies indicated that infection of cattle by aerosol requires fewer than 10 organisms whereas approximately 10^7 organisms are necessary to establish infection by the oral route (Quinn *et al.*, 2011). The nature and extent of tuberculous lesions vary with the route of exposure and the anatomical location of the lesions, which can subsequently, affects how *M. bovis* is excreted from the infected host (Gavier-Widen *et al*., 2001).

Mycobacterium bovis caused as much as 25% of cases of human tuberculosis in developed country in the late 19^{th} and early 20^{th} centuries where as in the late 20^{th} centuries, only 1%–2% of human TB cases in developed countries are caused by *M. bovis* which usually affects persons who acquired the infection locally before the implementation of control measures or in developing countries where control measures have not been implemented (O'Reilly and Daborn, 1995).

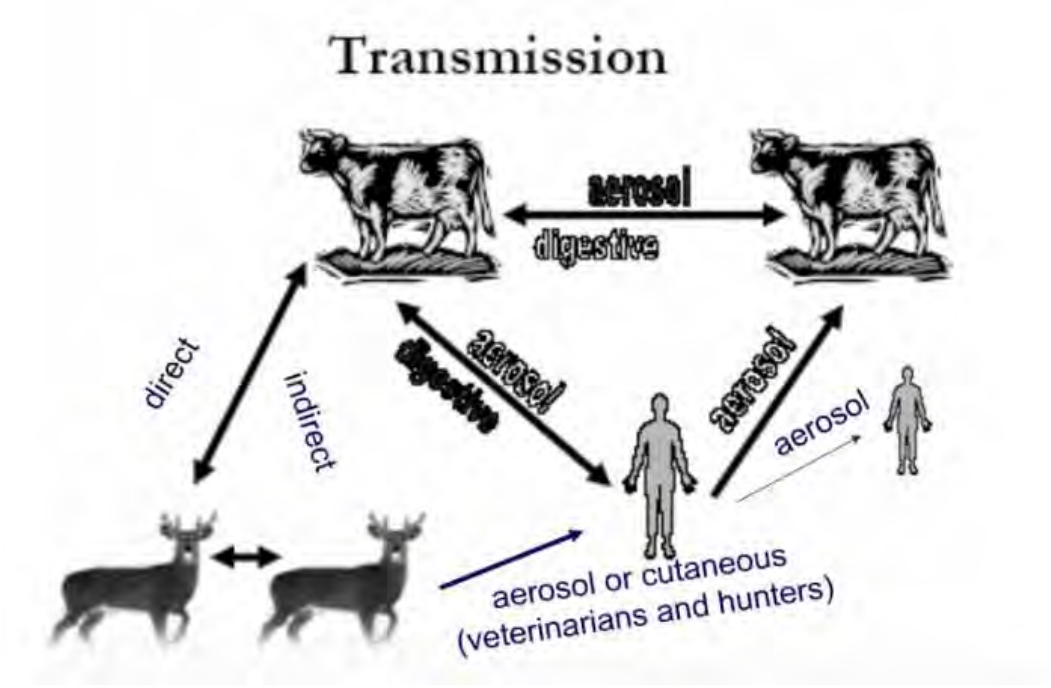


Figure 1: Cycle of *M. bovis* transmission between cattle and human.

Source: (Grange and Collins, 1987)

2.5.2. Risk factors in animal

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

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 (Regassa, 2005; Katale *et al.*, 2013).

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).

Host Range

Species reported to be spillover 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. 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 intratracheal inoculation and in crows after intraperitoneal inoculation. Some avian species, including mallard ducks, appear to be resistant to experimental infection (Thoen, 1984; Biffa *et al.,* 2009; Silaigwana *et al.,* 2012; Tadayon *et al.,* 2013). 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 tuberculous Zebu cattle (Regassa, 2005).

2.5.3. Risk factors in human

Milk consumption increase

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). This rise demand for milk consumption will be met by increasing number of productive animals and intensifying animal production (Regassa, 2005).

Feeding habit

Consumption of raw or soured milk is mainly practiced in some parts of the world. 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 (Acha and Szytres, 2001).

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 therefore 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).

HIV infection

In many developing countries, TB is the most frequent opportunistic disease associated with HIV infection (Cosivi *et al.,* 1998). HIV Seroprevalence rates greater than 60% have been found in TB patients in various African countries (WHO, 1997; WHO, 2006). 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 live. In the remaining 90% effective host defense prevent progression from infection to disease (Cosivi *et al.,* 1998).

Difficulty of implementing control mechanism

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

2.5.4. Distribution

Although BTB 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, the 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).

2.5.5. Bovine tuberculosis in Ethiopia

The knowledge of molecular epidemiology in the field of tuberculosis have been used to provide novel information about the spread of tubercle bacilli in outbreaks, to track the transmission dynamics of tuberculosis in the population and to distinguish exogenous re-infection from endogenous reactivation. In addition molecular epidemiology is also being used to identify the source of contamination, to determine the risk factors TB transmissions in a community, to investigate drug resistance pattern and to track the geographic distribution and spread of clones of *Mycobacteria* species or strains of public health importance (Mamo *et al.,* 2013b). Ethiopia is among the 22 high TB-burden countries that accounts for 81% of estimated cases (WHO, 2014). The prevalence of BTB

in Ethiopia is high and molecular typing of *M. bovis* has also indicated the existence of unique strains of tuberculosis (Zeru *et al.,* 2013).

The isolation and molecular characterization of the causative agent for BTB has been carried out in the last decade mainly in cattle and a number of isolates has been reported from different regions of the country (**Table 1**). The first *M. bovis* characterized in Ethiopia using spoligotype and VNTR was *EMbs 1 (*Ethiopian *M. bovis* strain 1) named later as SB1176 and its VNTR profile was 5254*33.1. This isolate was identified from cattle in Holleta government farm (Ameni *et al.,* 2007).

The work of Berg and his colleagues in 2009 has established important information in term of the molecular epidemiology of BTB and geographic distribution the causative agents of BTB in cattle of Ethiopia; out of the 32,779 Post mortem examination 135 isolates were characterized using molecular methods, of which 58 were *M. bovis*, 8 were *M. tuberculosis*, 53 were nontuberculous mycobacteria and 16 were not identified (Berg *et al.,* 2009).

The study carried out in central and Southern region of Ethiopia including Borena pastoral zone of Oromia Region identified 12 spoligotypes, of which SB1176 was the dominant spoligotype (41.2%) followed by SB0133 (14.7%). New spoligotypes have been reported to www.mbovis.org database including SB1517, SB1518, SB1519, SB1520, SB1521 and SB1522 (Biffa *et al.,* 2010b).

A more recent study in Central Ethiopia on dairy cattle have indicated that SB0134, SB1176, SB0133, and SB1477 spoligotype patterns circulates in the central Ethiopia and further characterization using MIRU-VNTR showed there is low *M. bovis* strain genetic diversity in the central Ethiopia (Firdessa *et al.,* 2013). And the *M. bovis* has been a predominant isolate in intensive dairy farms in central Ethiopia dairy cattle (Ameni *et al.,* 2010a; Firdessa *et al.,* 2013). The spoligotype pattern of 17 strains of *M. bovis*, isolated from a herd with a high prevalence of BTB was identical for all animals and recently published (Ameni *et al.,* 2007; Biffa *et al.,* 2010b; Berg *et al.,* 2011; Mamo *et al.,* 2011;

Arega *et al.,* 2013; Firdessa *et al., 2013;* Mekibeb *et al.,* 2013; Woyessa *et al.*, 2014) prov ided a comprehensive investigation on BTB in Ethiopia and showed a widespread distribution of the disease at an average prevalence of approximately 5% (Mekibeb *et al.*, 2013).

The studies in Ethiopia revealed a higher prevalence of BTB 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).

A number of studies confirmed that *M. tuberculosis* of different strains as a causative agents of tuberculosis in cattle and other domestic animals particularly in cattle grazing in the field under semi-intensive and/or extensive husbandry systems in central and southeast Ethiopia (Berg *et al.,* 2009; Ameni *et al.,* 2010a; Gumi *et al.,* 2012). Ameni and his colleagues revealed that 27% of the isolate from grazing cattle in central Ethiopia were *M. tuberculosis* and the potential route of transmission from human-to-cattle of *M. tuberculosis* was suggested to be the common local practice of farmers spitting chewed tobacco directly into mouths of the cattle (Ameni *et al*., 2010a) (**Figure 2**).

Figure 2: Direct discharging of tobacco juice (chewed) into oral cavity of cattle, Central Ethiopia.

Source: (Ameni *et al.,* 2011).

Table 1: Shows the molecular based studies of Bovine tuberculosis in some part of Ethiopia.

No.	Title	Molecular techniques used	Authors
1	A prevalence study of bovine tuberculosis by using abattoir meat inspection and tuberculin skin testing data, histopathological and IS*6110* PCR examination of tissues with tuberculous lesions in cattle in Ethiopia	mPCR	Shitaye *et al.* (2006)
2	Molecular typing of *Mycobacterium bovis* isolated from tuberculosis lesions of cattle in north eastern Ethiopia	RD4 deletion; PCR; Spoligotyping	Ameni *et al.* (2010b)
3	Molecular characterization of *Mycobacterium bovis* isolates from Ethiopian cattle	RD4 deletion; Accuprobe gene probe method; PCR; Spoligotyping	Biffa *et al.* (2010b)
4	Conventional and Molecular Epidemiology of Bovine Tuberculosis in Dairy Farms in Addis Ababa City, the Capital of Ethiopia.	RD4 deletion; mPCR; Spoligotyping; VNTR analysis	Tsegaye *et al.* (2010)
5	Mycobacteria and zoonoses among pastoralists and their livestock in South-East Ethiopia	Genus typing; RD4 and RD9 deletion; 16S rDNA sequencing; Spoligotyping	Gumi *et al.* (2012)
6	Prevalence study on bovine tuberculosis and molecular characterization of its causative agents in cattle slaughtered at Addis Ababa municipal abattoir, Central Ethiopia	PCR; RD4 deletion; Spoligotyping	Mekibeb *et al.* (2013)
7	Gross and Molecular Characterization of *Mycobacterium tuberculosis* Complex in Mekelle Town Municipal Abattoir, Northern Ethiopia	mPCR; Spoligotyping	Zeru *et al.* (2013)
8	Epidemiology of mycobacterial infections in cattle in two districts of Western Tigray Zone, northern Ethiopia	mPCR	Romha *et al.* (2013)
9	Cultural and molecular detection of zoonotic tuberculosis and its public health impacts in selected districts of Tigray region, Ethiopia	mPCR; RD4 deletion; Spoligotyping	Zeweld (2014)
10	Molecular Epidemiology of *Mycobacterium Tuberculosis* Complex at Nekemte Municipality Abattoir, Western Ethiopia	RD deletion; mPCR	Woyessa *et al.* (2014)
11	Epidemiology of bovine tuberculosis in Butajira southern Ethiopia: A cross sectional abattoir based study	mPCR	Nemomsa *et al.* (2014)

2.6. Diagnosis

The identification of the closely related members of the MTBC has remained a challenging task in diagnostic laboratories (David *et al*., 1978; Van Soolingen *et al.,* 1997 ; Niemann *et al.,* 2000). MTBC includes a variety of closely related Mycobacteria namely *M. tuberculosis*, *M. bovis*, *M. Canetti*, *M. africanum* and *M. microti*. A panel of classical tests based on microbiological features such as growth rate and phenotypic and biochemical characteristics has conventionally been utilized to distinguish members of M TBC (David *et al*., 1978). However, these tests are slow, cumbersome, unreliable and tim e consuming. The high degree of variability among these tests warrants the development of molecular biological tools for identification of MTBC members. In this regard, multiple gene targets have been used to detect and differentiate genetically identical species such as *M. tuberculosis and M. bovis* (David *et al*., 1978).

A presumptive diagnosis of TB in cattle and other susceptible species is often made on history, clinical findings, tuberculin skin tests and/or necropsy findings. Invitro lymphocyte assays including an interferon gamma assay and enzyme linked immune-sorbent assays have been developed for the detection of the disease in cattle and so other animals exposed to *M. bovis* (Samuel, 2010; Nahar *et al.,* 2011; Špičić *et al.,* 2012; Sharifipour *et al.,* 2014).

2.6.1. Clinical examination

Because of the chronic nature of the disease and the multiplicity of signs caused by the variable localization of the infection, TB is difficult to diagnose on clinical examination (Radostits *et al.,* 1994). Enlarged superficial lymph nodes provide a useful diagnostic sign when lungs are extensively involved; there is commonly an intermittent cough. The principal sign of TB is commonly chronic wasting or emaciation that occurs despite good nutrition and care (Thoen and bloom, 1995; Smith *et al.,* 2006).

2.6.2. Tuberculin skin test

Since it is a screening test, results are not considered absolute proof that an animal or herd has BTB. Therefore, it is used to identify animals and herds that need to undergo further testing for BTB. The primary diagnostic test for TB in both humans and cattle is Tuberculin skin test, which may be performed using bovine tuberculin alone or as a comparative test using avian and bovine tuberculins (Angus, 1978; Radostits *et al.,* 1994; OIE, 2008). Tuberculins are crude antigen preparations derived from heat-killed cultures of mycobacteria and contain mixtures of proteins, polypeptides, nucleic acids, and substantial amounts of polysaccharides (Angus, 1978). The tuberculin test is usually performed on the shaved 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).

2.6.3. 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 (**Figure 3**). Some of epitheloid cells may fuse together and form multinucleated giant cells (Ulrichs and Kaufmann, 2006; Palmer, 2007; Russell, 2007; Gil *et al.,* 2010; Ramakrishnan, 2012). 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 mycobacterial growth (Cosma *et al.,* 2004; Volkman *et al.,* 2004; Gil *et al.,* 2010) and spread of infection. Since the lesions are not conclusive, it is necessary to demonstrate the etiological agent using Ziehl-Neelsen stain (ZN) (Thoen and Blooom, 1995).

Figure 3: Tuberculous lesion of *mycobacterium* from lymph node of infected bovine

Source: (Quinn *et al.*, 1999)

2.6.4. Bacteriological

Mycobacterium bovis can be demonstrated microscopically on direct smears from clinical samples and on prepared tissue materials (Quinn *et al.*, 1999). The presumptive diagnosis of mycobacteriosis can be made if the tissue has characteristic histological lesions (caseous necrosis, mineralization, epitheloid 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 (OIE, 2004; Shitaye *et al.*, 2006; Jovan *et al.*, 2011; Deressa *et al.*, 2013).

Differential staining

The preliminary examination of stained smears from lesions, sputum, milk, urine, pleural and peritoneal fluids, uterine discharges and feces is a basic for the confirmatory diagnosis of BTB through isolation and identification of the bacteria (Radostits *et al.*, 2007). In the smear (**Figure 4**), the organism appear red rods against a blue background

(ZN staining), while in the fluorochrome procedures, the acid fast organisms appear as fluorescent rods, yellow to orange (WHO, 2006).

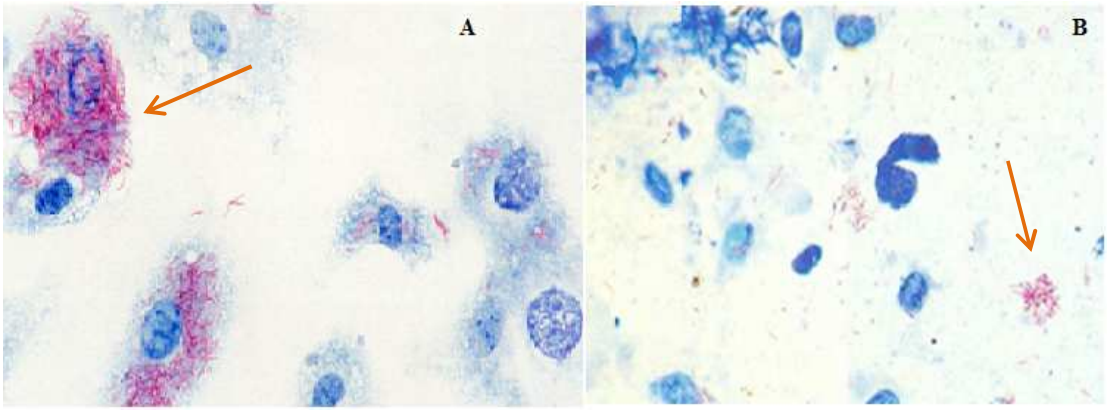


Figure 4: (A) *M. bovis* BCG stained pink in ZN stain as seen through a microscope and (B) *M. bovis* in a ZN stained smear of material from tubercule; the slender, beaded, red-staining (ZN-positive) rods tend to be more numerous in the lesions from deer and badgers compared to the low numbers in bovine lesions

Source: (Quinn *et al.*, 1999)

Culture media

To process specimens for culture, the tissue is first homogenized using a mortar and pestle; 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 on to a set of solid egg-based media, such as LJ, 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 (Cousins *et al.*, 1989; Ali, 2006).

Cultures are incubated for a minimum of 8 weeks (and preferably for 10–12 weeks) at 37°C with or without CO_2. 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 ZN 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 hospital and 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 (**Figure 5**) 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). Identification of *M. bovis* by culture and biochemical methods is important for definitive diagnosis (Corner, 1994). However, because of the technical problems and cost, they have not come into widespread use in veterinary diagnostic laboratories (Araujo *et al.,* 2005).

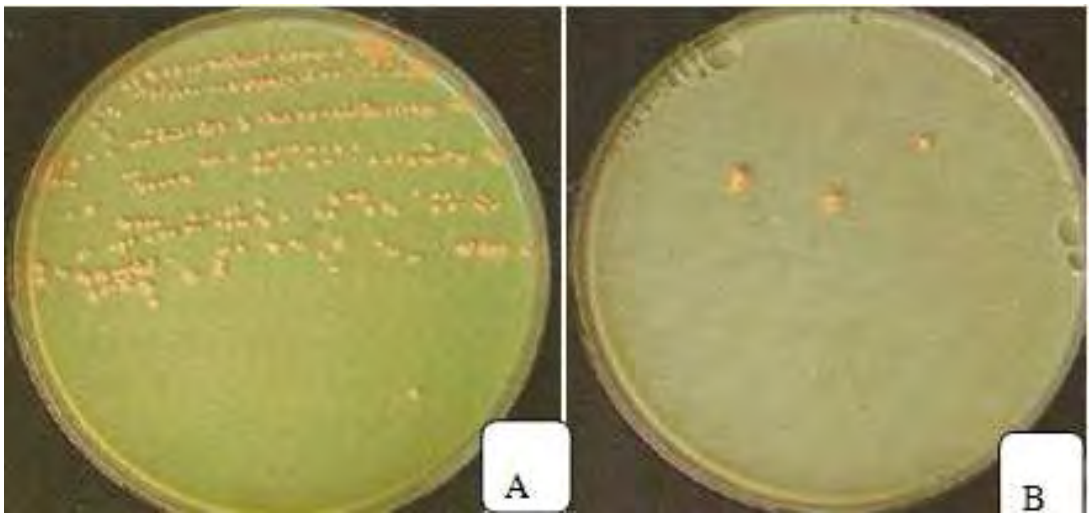


Figure 5: (A) *M. bovis* colonies on Lowenstein-Jensen medium with pyruvate (B) *M. tuberculosis* on LJ medium with glycerol showing the typical colonial morphology (Rough, tough and buff).

Source: (Quinn *et al.*, 1999).

2.6.5. Microscopic pathological lesion

A part of each lesion from meat and organs fixe in 10% formalin and then sections prepare by the paraffin embedding technique according to Banchroft *et al.* (1996). Paraffin sections cut at five microns in thickness and stain with hematoxylin and eosin stain (H&E) follow by microscopic examination. Histopathological examination, used commonly for routine diagnosis of bovine tuberculosis, permits rapid identification of lesions but may not differentiate between those caused by *M. bovis* and by other mycobacterial or closely related agents. The histopathological analysis positive specimens may be negative for both culture and PCR; so this suggesting that these histopathology results may represent false positives (De Lisle *et al.*, 1993).

2.6.6. Serological diagnostic methods

Besides the classical intradermal tuberculin test, a number of blood tests have been used (Haagsma, 1993). Due to the cost and the more complex nature of laboratory-based assays, they are usually used as auxiliary tests to maximize the detection of infected animals (parallel testing), or to confirm or negate the results of an intradermal skin test (serial testing). The serological diagnostic tests are gamma-interferon assay, lymphocyte proliferation assay and ELISA. Both the gamma-interferon assay and lymphocyte proliferation assay are measured cellular immunity, while the ELISA measures humoral immunity (Jovan *et al*., 2011)

Gamma interferon assay/Bovigam

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 16-24hours incubation period with specific antigen (PPD-tuberculin) (Wood *et al.,* 1990). The test makes use of the comparison of IFN-γ production following stimulation with avian and bovine PPD (Asiimwe, 2008). Because of the IFN-γ test capability of detecting early infections, the use of both tests in parallel allows 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 (Gormley *et al.,* 2006). In animals that are difficult or dangerous to handle, such as excitable cattle or other bovidae, the advantage of the IFN-γ test over the skin test is that the animals need be captured only once. The test is available as commercial kits for bovine species and primates (OIE, 2009).

Lymphocyte proliferation assay

This type of *in-vitro* assay compares the reactivity of peripheral blood lymphocytes to tuberculin PPD (PPD-B) and (PPD-A). The lymphocyte proliferation assay can be performed on whole blood (Buddle *et al.,* 2001) or purified lymphocytes from peripheral blood samples (Griffin *et al.,* 1994). Results are usually analyzed as the value obtained in

response to PPD - B minus the value obtained in response to PPD-A, the B-A value must then be above a cut-off point. The assay has scientific value, but is not used for routine diagnosis because the test is time-consuming and the logistics and laboratory execution are complicated (it requires long incubation times and the use of radio-active nucleotides). As with the IFN-γ test, the lymphocyte proliferation assay should be performed shortly after blood is collected (OIE, 2009).

Enzyme linked immunosorabent assay (ELISA)

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. 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).

2.6.7. Molecular techniques for diagnosis of bovine tuberculosis

Studies relied on bacteriological methods to differentiate *M. tuberculosis* from *M. bovis*. However, molecular typing of *M. tuberculosis* complex isolates provides a rapid means for discriminating members of the *M. tuberculosis* complex, a process which can often be difficult when using classical bacteriological methods. The identification of *M. bovis* is critical for determining the impact of zoonotic transmission of infection to humans, as it gives impetus to the adoption of public health measures such as the pasteurization of milk, cooking of meat, and control of tuberculosis in domestic animals. Genetic typing of *Mycobacterium tuberculosis* complex strains is now commonly used in the molecular epidemiology of tuberculosis (Barnes and Cave, 2003). Molecular techniques are used to track specific strains of pathogens and to determine more precisely the distribution of infectious diseases in populations, providing opportunities for more effective intervention s (Genewein *et al*., 1993).

Multiplex PCR

Molecular technique differentiates MTBC from *M. avium, M. intracellularae,* and other mycobacterial species. 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. intracellularae* (MYCINT-F) and *M. avium* (MYCAV-R), and the MTB70 gene specific for MTBC (TB-A, TB-1B) (Mamo *et al.,* 2013b).

RD deletion typing

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 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).

Spoligotyping

Another molecular typing method for MTBC is the PCR based on spacer oligonucleotide typing (Spoligotyping). This method was proposed as an alternative to hybridization based fingerprinting methods for diagnosis and epidemiology of tuberculosis (Aranaz *et al.,* 1996; Kamerbeek, *et al*., 1997; Haddad *et al.,* 2001; Njanpop-Lafourcade *et al.,* 2001; Cadmus *et al*., 2006; Diguimbaye-Djaïbe *et al*., 2006). Furthermore, the international designation of spoligotype patterns (Brudey *et al.,* 2006) has facilitated the comparison of results from different countries and helps elucidate the distribution and spread of strains. Assuming that spoligotype spacers can only be lost and not regained, phylogenetic relationships between strains can be suggested (Van Embden *et al*., 2000; Smith *et al*., 2006). A number of MTBC strain families are readily identifiable through Spoligotyping (Vitol *et al*., 2006; Rahim *et al*., 2007; Streicher *et al*., 2007).

Spoligotyping is based on the variability of spacer sequences interspersed with repeat sequences in the polymorphic chromosomal direct repeat (DR) locus (**Figure 6**). 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 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).

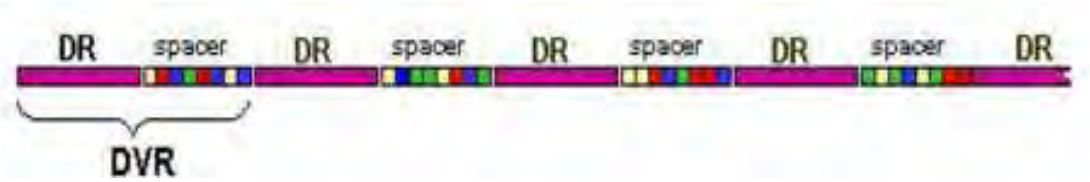


Figure 6: DR locus /fragment

Source: (Van Embden *et al*., 2000).

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 *et al.*, 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 *et al.*, 2000; Supply *et al.*, 2001). A set of 24 MIRU-VNTR loci was standardized to increase the discrimination power (Supply *et al*., 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. 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 IS*6110* RFLP genotyping for isolates with high-copy-number IS*6110* insertions but more discriminating than IS*6110* RFLP genotyping for isolates with low-copy-number IS*6110*. When more than 12 loci are used, or MIRU analysis is combined with Spoligotyping, the discriminatory power approximates that of IS*6110* 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 *et al.*, 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* transmissio n (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).

2.7. Public Health Importance

Ethiopia is among the 22 high TB-burden countries that accounts for 81% of estimated cases (WHO, 2014). Over a 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).

In Africa, the BTB is widespread and is affecting the animal industries and human health, posing serious public health threats (Cosivi *et al.*, 1998; Ayele *et al.*, 2004; Thoen *et al.*, 2009). Africa is assumed to bear the highest consequences of zoonotic TB worldwide because of the frequent and concurrent presence of multiple risk factors (Müller *et al.*, 2013).

The current increasing incidence of tuberculosis in humans, particularly in immuno-compromized 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 is the

commonest source of protein to man, in the transmission of the disease remain significant. Moreover, zoonotic infection of BTB among dairy farm workers has been reported (Hassanian *et al*., 2009). 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 (Nwanta *et al.,* 2010; Tamiru *et al.,* 2013; Zeweld, 2014).

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 programmes (Shitaye *et al.,* 2006).

In contrast, spread from animals to humans in developing countries remains a very real danger, mostly from infected milk. This seems to be a danger, which is being entirely ignored (Gemechu *et al.,* 2013). The animal and public health consequences of *M. bovis* are grave. Disease surveillance programmes in animals and humans should be considered a priority, especially in areas where risk factors are present. Training of personnel at all levels of control programmes and the 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). Identification of the etiological agent belonging to MTBC is important for determination of the origin and reservoirs of infection and also for implementation of appropriate public health measures.

2.8. Control and Prevention

The efforts attempting to stamp out BTB has various reasons: the risk of infection to the human population; loss in productivity due to infected animals; and animal market restrictions set by countries with advanced eradication programmes. The priority will be given to these points vary depending up on the specific question to the country (Cousins, 2001).

In industrialized countries, control and eradication of BTB has been successfully carried out by regular testing and removal of infected animals under mandatory national bovine TB programmes, such programmes have been successful in many European Union member states and in seven central European countries between 1953 and 1980 (Pavlik *et al*., 2002) and pasteurization of animal products have greatly reduced the economic and public health significance of BTB, however, in developing countries bovine TB remains a major animal health problem, mainly because these countries cannot shoulder the financial burden required to implement a control programme and compensate for slaughtered animals. Limited access to education, poor information networks and lack of disease surveillance are other factors that limit the implementation of any such programme. And also due to the disease prevails in wide geographic distribution and has significant economic impact to the livestock sector and creates a zoonotic risk to the exposed human population, where control program is lacking or not implemented (Ayele *et al.*, 2004).

Due to the cattle movement and where the natural reservoirs of the disease in wild animals pose a serious risk of transmission to domestic livestock, as a result the eradication programme of the BTB makes difficult or unsuccessful. In most African countries, controlling free movement of animals within a country is difficult and movement between countries cannot be regulated, primarily due to a lack of border controls. In addition, spread of bovine TB amongst wildlife in game parks in Africa is increasingly being recognized as a serious problem, with consequences for domestic animals (OIE, 2009).

Bacille Calmette Guérinv (BCG) is an attenuated strain produced by continuous subculture of a wild-type *M. bovis* isolate from cattle, has played a crucial role in controlling human TB, particularly in children. However, its use for BTB is less effective. This has shown variable efficacy in cattle trials, which may be attributable to various factors including vaccine formulation, route of vaccination, and the degree of exposure to environmental mycobacteria (skinner *et al.,* 2001). In infected countries where there is no test and slaughter control scheme, BCG vaccination may be used to reduce the spread of infection in cattle (Daborn and Grange, 1993; OIE, 2009); however, there is no solid knowledge of long-term reduction in prevalence and safety for human beings and the environment. Before embarking on a vaccination programme, the vaccination schedule must be optimized for local conditions. Typical dosage would be from 104 to 106 colony-forming units given subcutaneously (OIE, 2009). Development and production of an effective vaccine with appropriate methods and strategies for delivery could therefore contribute to BTB control in Africa.

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted from December 2015 to May 2016 in Hawassa University and municipal abattoir in Southern Nations Nationalities and Peoples Regional state (SNNPRs), Southern Ethiopia. This region has an estimated total area of about 112,323.19 km^2. Hawassa city is located in Southern part of Ethiopia, in Sidama Zone, on the shores of Lake Hawassa in the Great Rift Valley and located 270 km South of Addis Ababa. The city serves as the capital of the SNNPRs, and its total area is 157.21 square kilometers. It bounded by Lake Hawassa on the West and North-west, Chelelaka swampy area on the East and South-East, Tikur Wuha River on the North and Alamura Mountain on the South. It lies on the Trans-African Highway for Cairo-Cape Town, and has a geographic coordinate of 7°3′ N latitude and 38°28′ E longitude and an elevation of 1708 m.a.s.l. (CSA, 2007).The livestock resource of the city is 61,123 cattle, 14,764 sheep, 17,735 goats, 5,544 equines and 56,961 poultry and the total population is estimated bout 304,479 (CSA, 2011).

Hawassa city has two abattoirs: one municipality abattoir and second Hawassa University abattoir, in main campus. The Hawassa municipal abattoir supplies the inspected meat to about 304,479 inhabitants (CSA, 2011) and based on the information obtained from personnel working in Hawassa University Registrar Office, the University's abattoir supplies for about 25,000 students (personal communication).

Even though the abattoirs were fenced, the places used to dispose condemned carcasses were not secured since they were easily accessed by hyenas, dogs and other animals. The overall hygiene and the internal facilities including the drainage were not good in the Municipal abattoir whereas the University's abattoir was good. Even though the Municipal abattoir has recently built abattoir inside the compound, currently the slaughtering is performing in the old house. The minimum and maximum numbers of cattle slaughtered per day during the study period in Hawassa city Municipal abattoir

were about 75 and 175 heads of cattle, respectively and about 8 and 12 heads of cattle were for Hawassa University abattoir, respectively. Few numbers of female cattle were also slaughtered in both abattoirs which have reproductive problems, poor performance and at the end of their reproductive life (aged). The municipal abattoir was operated by one veterinarian and three assistant meat inspectors but the University abattoir was operated only by one assistant meat inspector (Personal communication to the responsible bodies).

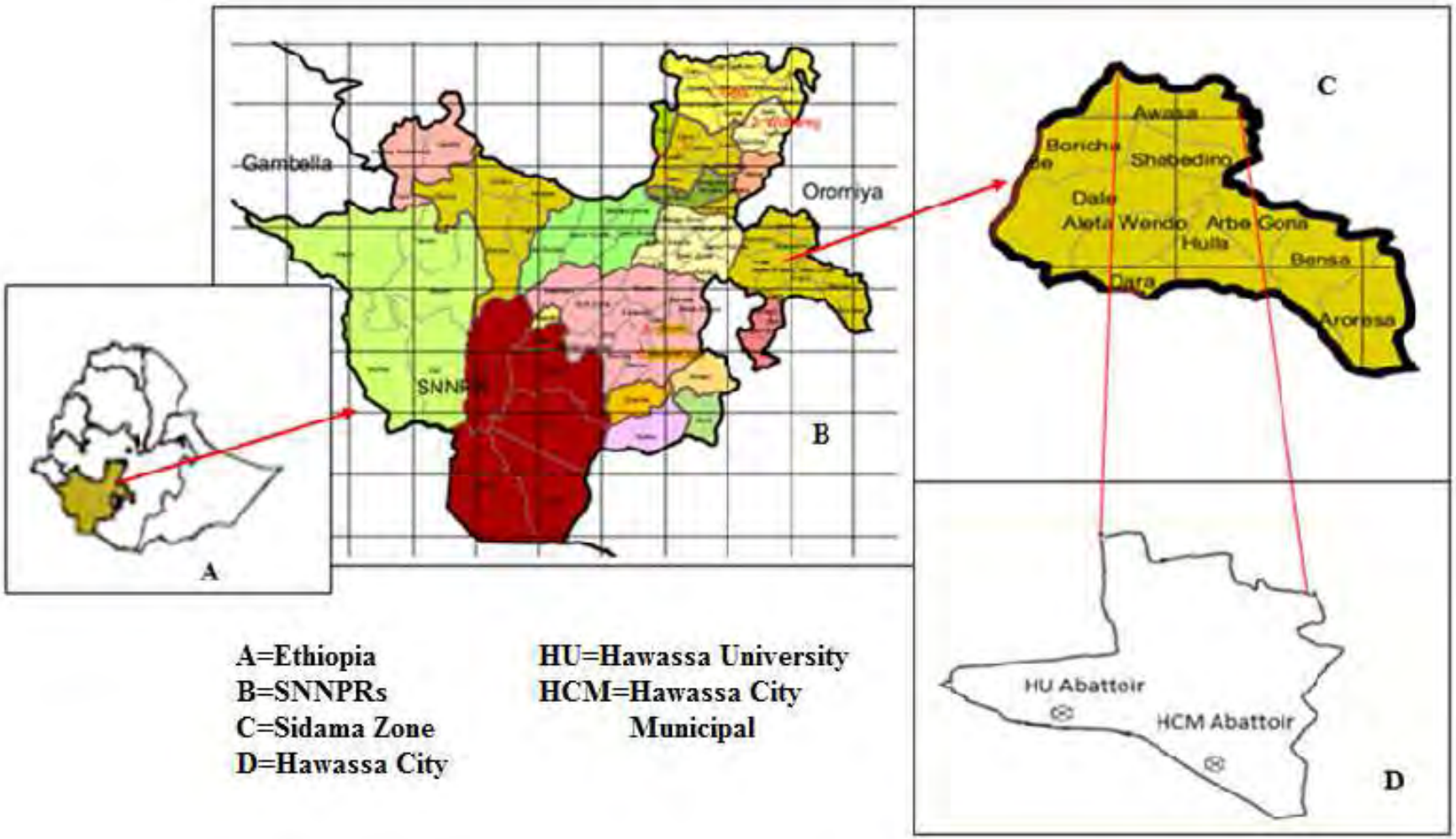


Figure 7: The of geographical location map of Hawassa city with some sort of modifications

Source: (Getamesay *et al.*, 2015)

3.2. Study Animal

A total of 753 apparently healthy adult cattle slaughtered in the abattoirs (Hawassa city municipal abattoir, n = 369 and Hawassa University abattoir, n = 384) regardless of sex, breed and origin were considered in the study. And the cattle used for this study were mainly originated from different markets (source of cattle); for the municipal abattoir were from Tula, Arsi Negele, Tukr Wuha, Wolayita, Harrar, Borena, Hawassa and Nazeriat (Adama) Markets and for University abattoir the origin were from Borena and Wolayita markets. Basic animal information on each study animals (such as sex, age, breed, body condition score, market) were collected and recorded during antemortem examination. All the study animals in this study were cross and local breed.

3.3. Study Design and Sampling Method

A cross sectional study with systematic random sampling was carried out to examine the carcass and sample suspected TB lesions from cattle slaughtered at Hawassa city municipal and Hawassa University abattoirs.

In these abattoirs, individual heads of cattle to be slaughtered were given identification number for proper recording of ante mortem and post mortem examination results. Selection of cattle population to be involved in the study was based on systematic random sampling, from among those cattle slaughtered each day, which was arranged in a systematic manner with each individual animal constituting a sampling unit.

In Hawassa city municipal abattoir on average 20 carcasses were decided to be inspected each day (range from 15 to 25) from among those slaughtered cattle whereas in Hawassa University abattoir the whole cattle slaughtered in each day were considered for postmortem examination because the number of slaughter cattle was often fewer (range from 6 to 12) compared the Hawassa city municipal abattoir. The work was carried out in all the weekdays except Wednesday and Friday when animals were not slaughtered due to fasting occasions according to the belief of Ethiopian Orthodox Christianity.

3.4. Sample Size Determination

All animals coming to the abattoirs from different markets during the study period were considered for sampling. The sample size calculation was done by using the Thrusfield (2007) formula:

$$n = \frac{1.96^2 \times p_{exp} (1-p_{exp})}{d^2}$$

Where n = required sample size

P_{exp} = expected prevalence, 8.8 % was for the Hawassa city municipal abattoir

d = desired absolute precision, 5%

The expected prevalence, Pexp, of Hawassa city municipal abattoir was (8.8%) used from the previous works of Biffa *et al.* (2010a). The calculated sample size for the municipal abattoir was 123 heads of cattle, but to increase the precision of the study this sample size multiplied three times, then n = 369 heads of cattle. Whereas the calculated sample size of the Hawassa University abattoir, Pexp considered as 50% because there was no previous study on bovine tuberculosis in this abattoir, was 384 heads of cattle. Thereafter, the total animals supervised in both abattoirs were 753 heads of cattle.

3.5. Study Methodologies

3.5.1. Ante mortem and post mortem examination

Clinical examination was conducted on the study animals before they slaughtered; this included examining of superficial lymph nodes, visible mucus membrane, and other basic parameters (Chauhan and Agarwal, 2006). Age determination based on dentition (teeth) as previously described (De-Lahunta and Habel, 1986; Torell *et al.*, 2003) (Appendix 3) and the body condition of each of the study animal was scored using the guidelines established by Nicholson and Butterworth (1986) and Maurya *et al.*(2009) (Appendix 2). Accordingly, on the basis of observation of anatomical parts such as vertebral column, ribs, and spines, the study animals were classified as poor (score, 1 to 3), medium (4 to

6), or good (greater than 6).The breed, sex and source of the animals (markets) were recorded during ante mortem examination.

Post mortem examination was carried our according to the OIE (2009) and Meat Inspection and Quarantine Division of the Ministry of Agriculture method (Hailemariam, 1975). All lymph nodes, livers, kidney and lungs were visualized, palpated, and incised into a size of 2 mm to facilitate the detection of tuberculous lesion from each animal. These include the mandibular, medial retropharyngeal, cranial and caudal mediastinal, left and right bronchial, hepatic, mesenteric lymph nodes as well as The seven lobes of the two lungs, including the left apical, left cardiac, left diaphragmatic, right apical, right cardiac, right diaphragmatic and right accessory lobes were investigated. The animal was classified as suspected tuberculos lesion when tuberculous lesion was found, and if not as non-lesioned.

The severity of gross lesions in individual lymph node was scored as follows; 0 = no gross lesion, 1 = small lesion at one focus, 2 = small lesions at more than one focus and 3 = extensive necrosis as developed by Vordermeier *et al*. (2002) and Ameni *et al. (*2006). And for organs, the pathological scoring was 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 = gross coalescing lesions. The scores for the individual lobes were added up to calculate the lung score.

The cut surfaces were examined under bright light for the presence of abscess, cheesy mass, and tubercles (Corner *et al*., 1990). In the presence of suspected tuberculous lesion, tissue samples were collected independently in sterile universal bottles containing 0.85% normal saline for culture and kept store froze at -20^0c at Hawassa University, School of Animal Science and Rang Management, Dairy section before being transporting to laboratory. The samples were labeled and pooled together and then transported at 4°C under cold chain by ice box with packed ice to Addis Ababa University, College of Veterinary Medicine and Agricultural (CVMA) and stored at -20^0c until bacteriological culture.

3.5.2. Mycobacteria culturing and acid fast staining

For bacteriological culture, tissue samples were macerated in sterile mortal by using surgical blades and forceps to get fine pieces and in order to initiate the release of mycobacteria organisms from body fluid and cells and then homogenized by pestle and mortar. Five milliliters of the homogenized tissue sample was transferred into centrifuge tube and decontaminated with equal volume 4% NaOH followed by centrifugation at 3,000 rpm for 15 minutes. The supernatant was discarded, while the sediment was neutralized with 1 % (0.1N) HCl using phenol as an indicator. Neutralization achieved when the color of the solution changed from purple to yellow (OIE, 2009). Thereafter, 0.1 ml of the suspension from each sample was inoculated on to LJ egg based media. Duplicates of LJ media were used; one enriched with sodium pyruvate, while the other was enriched with glycerol. The Cultures were incubated at 37°C for 1 week and in upright position for the rest 8-12 weeks. The media was in tightly closed tubes to avoid desiccation and slopes were examined for macroscopic growth at intervals during the incubation period for the presence of any mycobacterial colonies (OIE, 2009).

Whenever, colonies were seen, ZN staining was performed to confirm the presence of Acid fast bacilli (Quinn *et al*., 1999; OIE, 2009) (Appendix 6). In parallel to the ZN staining, Positive colonies were preserved with freezing media, and some portion of the colonies were heat killed in water bath maintained at 80 ^{0}C heat for 45 minutes, by mixing two loops full of colonies in 200µl distilled water (Brosch *et al.*, 2002; Cadmus *et al*., 2006). The frozen and heat killed isolates were stored at -20 ^{0}C for future Mycobacteriology and further molecular typing analysis was performed at Akililu Lemma Institute of Pathobiology according to the standard developed by Cadmus *et al*. (2006), Hewinson *et al*. (2006) and WHO (2012).

3.5.3. Region of difference (RD)-4 deletion typing

For the RD4 deletion typing, the procedure described by Cadmus *et al*. (2006) was used. Each sample was tested in a separate PCR tube. Primers directed against the RD4 were used to generate a deletion profile that would allow species identification of the isolate.

RD4 is 12.7 kb genetic segment that is deleted from *M. bovis BCG* strain, but present in *M. microti, M. africanum* and *M. tuberculosis* (Gordon *et al.,* 1999). RD4 internal F: 5´-ACA CGC TGG CGA AGT ATA GC-3´; RD4 flank R: 5´-AAG GCG AAC AGA TTC AGC AT-3´ and RD4 flank F: 5´-CTC GTC GAA GGC CAC TAA AG-3´ primers were used to check for the presence of RD4 locus.

Each PCR tube consisted of 7µl distilled water (Qiagen), 10µl Hot Star Taq Master Mix, 0.3µl of each of the three primers and 2.1µl of DNA template, thus making the total final volume of 20µl. *M. tuberculosis* H37Rv and *M. bovis* SB1176 were used as positive control, and distilled water was used as a negative control. The mixture was heated in a Thermal Cycler (VWR, International Ltd. Portsmouth, UK) using an initial hot start at 95°C for 15 minutes and then subjected to 35 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1minute and final extension step 72°C for 10 minutes to complete the cycle. The product was electrophoresed in 1.5% agarose gel in 1x TAE running buffer; SYBR Safe/Ethidium bromide at a ratio of 1:10, 100 bp DNA ladder and orange 6 x loading dye were used in gel electrophoresis. The gel was visualized using Syngene Bio Imaging System (Syoptics Group). Interpretation of the result was based on the detection of bands of different sizes. The presence of RD4 (RD4 is intact in *M. tuberculosis*, *M. africanum*) gives a product size of 335 bp (RD4 intF + RD4flankR), and its absence (*M. bovis*) gives a product size of 446 bp (RD4flankF + RD4flankR).

3.5.4. Spoligotyping

Spoligotyping was carried out using the commercially available kit according to the manufacturer's instructions and as previously described by Kamerbeek *et al.* (1997). Briefly, the direct repeat (DR) region was amplified with primers DRa (5'-GGT TTT GGG TCT GAC GAC -3' biotinylated at the 5′ end) and DRb (5'-CCG AGA GGG GAC GGA AAC -3'). PCR amplification was done for 30 cycles with denaturation and annealing for 1 min at 95°C and1 min at 55°C, respectively, and extension for 30s at 72 °C in each cycle. The amplified DNA was hybridized to inter-DR spacer oligonucleotides covalently bound to a membrane for 1 hour at 60°C. Reference strains of *M. tuberculosis (*H37Rv) and *M. bovis* SB1176 were used as positive control whereas sterile water was

used as a negative control. The amplified DNA was subsequently hybridized to a set of 43 oligonucleotide probes by reverse line blotting. The hybridized PCR products were incubated with streptavidin peroxidase conjugate and the signal detection was done with an enhanced chemiluminescence detection system followed by exposure to an X-ray film according to the manufacturer's instructions. The X-ray film was developed and washed using standard photochemical procedures. The results were entered in a computer-based strain identification system described in international online database website http://www .mbovis.org.

3.6. Statistical Analysis

Prevalence was calculated as the proportion of suspected lesion positive animals from the total number of animals sampled (Thrusfield, 2007). Data related with age, sex, breed, markets source of animals and body conditions scoring of each animal were recorded on a data sheet during ante mortem examination. The recorded data was entered, stored, classified, coded and filtered using Microsoft Excel computer program and was transferred and analyzed by STATA version 11 (STATA Corp. College station, TX). Presence or absence of TB like lesions and affected tissues (suspected lesions) were recorded during postmortem examination. The variations between different factors were analyzed using logistic regression and chi-square ($\chi 2$) was used for association of different risk factors. P-value less than 5% was considered statistically significant. In cases of estimating the effect of different risk factors in terms of odds ratio (OR), to assess the strength of association of different factors with the prevalence of BTB, with corresponding 95% confidence interval, statistical significance was assumed if the confidence interval did not include one among its values. In molecular epidemiology study of isolates from animal tissues, the spoligotype patterns were converted in to binary and entered to the online spoligotype database website http://www.mbovis.org. to retrieved the strain type.

4. RESULTS

4.1. Prevalence and Associated Risk Factors Analysis

Upon detailed post mortem examination of 753 cattle, an overall prevalence of BTB was 5.84% (95%CI: 4.58 - 8.42). Out of the total 753 slaughtered cattle in both abattoirs, 675 (89.6%) were local breed and 78 (10.4%) cross breed whereas the prevalence of tuberculous lesion was higher in cross breed, 17.9% than local breeds, 4.4% and this difference in prevalence of BTB was found to be statistically significant ($\chi 2 = 23.18$, P = 0.000). Associations of the risk factors, the difference in prevalence of BTB in relation to body condition score, age, and markets were statistically significant ($\chi 2 = 118.64$, p = 0.000; $\chi 2 = 54.67$, p = 0.000 and $\chi 2 = 20.9$, p = 0.004) respectively (Table 2).

Multivariable logistic regression analysis identified age, body condition; breed and market were statistically significant (Table 3). The cross breed cattle (OR = 9.75, 95%CI: 3.34 - 28.47) slaughtered in the abattoirs nine times more sensitive to *M. bovis* than the local breeds and also the cattle brought from Hawassa and Borena markets had more likely bovine tuberculosis lesions (Figure 8) thirteen times (OR = 13.17, 95%CI: 1.96 - 88.42) and nine times (OR = 9.41, 95%CI: 1.26-69.97) respectively than Tula market. Older cattle above 8 years had eighteen times the odds of being bovine tuberculosis lesion compared with the younger age group (5 or less than 5 years old) (OR=18.25; 95% CI: 2.12 - 157.06). In relation to sex and abattoirs, there were no statistical significance differences of bovine tuberculosis lesion positive between groups.

Table 2: Association of different risk factors with gross pathological lesion of bovine tuberculosis in Hawassa abattoirs, Southern of Ethiopia.

Variables	No of cattle examined	No of positive (%)	χ^2 Value	P-value
BCS				
Good	348	2(0.6)		
Medium	318	15(4.7)	118.64	0.000*
Poor	87	27(31)		
Breeds				
Local	675	30(4.4)		
Cross	78	14(17.9)	23.18	0.000*
Age				
≤ 5 years	210	1(0.5)		
>5 - ≤8 years	367	13(3.5)	54.67	0.000*
>8 years	176	30(17)		
Sex				
Male	664	38(5.7)	0.148	0.700
Female	89	6(6.7)		
Markets				
Tula	115	3(2.6)		
Tukur Wuha	59	0(0.0)		
Arsi Negele	62	2(3.2)		
Wolayta	153	9(5.9)	20.91	0.004*
Borena	305	25(8.2)		
Harrar	32	0(0.0)		
Nazeriat	2	0(0.0)		
Hawassa	25	5(20)		
Abattoirs				
HUA	384	31(8.1)	7.08	0.008*
HCMA	369	13(3.5)		

* = Statistically significant, BCS = body condition score, HUA = Hawassa University abattoir, HCMA = Hawassa city municipal abattoir

Table 3: Logistic regression analysis of bovine tuberculous lesion with various host related risk factors.

Variables	No of cattle Examined	Positive (%)	Crude OR (95% CI)	Adjusted OR (95% CI)
BCS				
Good	348	2(0.6)	1	1
Medium	318	15(4.7)	8.56(1.94 - 37.75) *	7.05(1.49 - 33.22) *
Poor	87	27(31)	77.85(18.04 - 335.97) *	41.21(8.27 -205.29) *
Breed				
Local	675	30(4.4)	1	1
Cross	78	14(17.9)	4.70(2.37 - 9.32)*	9.75(3.34 - 28.47)*
Age				
≤5 years	210	1(0.5)	1	1
>5 - ≤8 years	367	13(3.5)	7.68(0.99 - 59.09)	7.65(0.87 - 67.15)
>8 years	176	30(17)	42.94 (5.79 - 318.45) *	18.25(2.12 -157.06) *
Sex				
Female	89	6(6.7)	1	1
Male	664	38(5.7)	0. 84(0.34 2.05)	1.68(0.49 - 5.77)
Market				
Tula	115	3(2.6)	1	1
Tukur Wuha	59	-	-	-
Arsi Negele	62	2(3.2)	1.24 (0.20 - 7.65)	2.40 (0.31-18.51)
Wolayta	153	9(5.9)	2.33 (0.62 - 8.82)	3.89(0.56 - 26.81)
Borena	305	25(8.2)	3.33(0.99 -11.26)	9.41(1.26 - 69.97) *
Harrar	32	-	-	-
Nazeriat	2	-	-	-
Hawassa	25	5(20)	9.33 (2.07 - 42.18) *	13.17(1.96 - 88.42) *
Abattoirs				
HMCA	369	13(3.5)	1	1
HUA	384	31(8.1)	2.40 (1.24 - 4.67)*	0.45(0.11-1.87)

* = statistically significant, HUA = Hawassa University abattoir, HMCA = Hawassa city municipal abattoir; BCS = Body condition score

4.2.Distribution of Gross Pathological Lesions

Gross pathological lesions were observed in lymph nodes and organs of the slaughtered cattle; the majority of the lesions were considered typical of tuberculous lesions which characterized by central round, oval, or irregular, often coalescing areas of caseous necrosis and mineralization (calcification) (Figure 9). Large encapsulated nodules containing thick yellowish cheesy material were mostly observed in the thoracic lymph nodes. Whenever gross lesions of suggestive pathological lesions of TB noticed in any tissue; the tissue was classified as positive for TB.

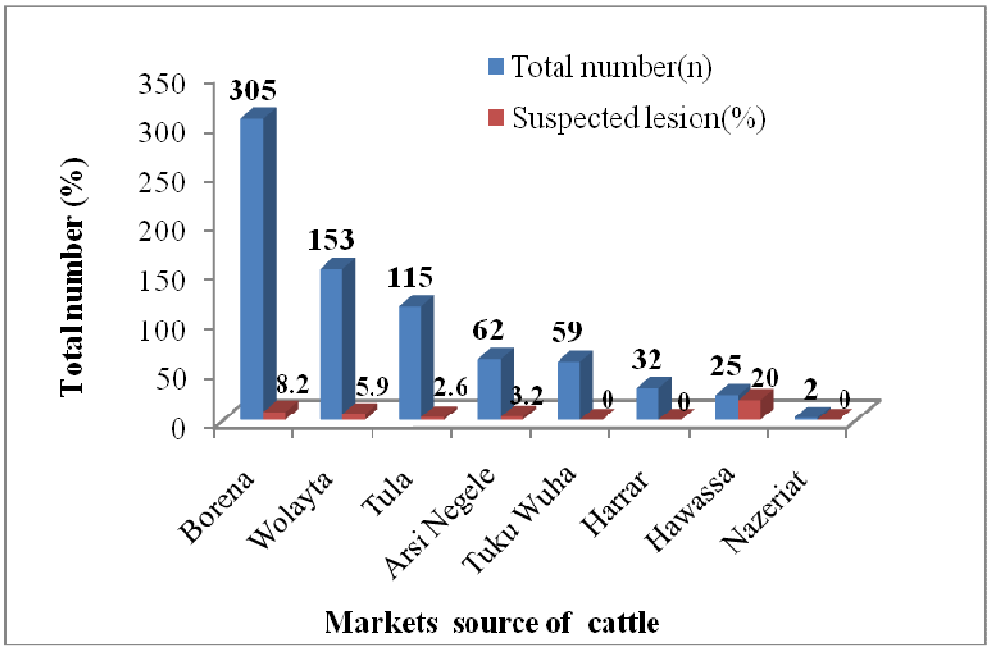


Figure 8: The markets total number of cattle slaughtered and their proportion of suspected lesions in Hawassa abattoirs, Southern Ethiopia.

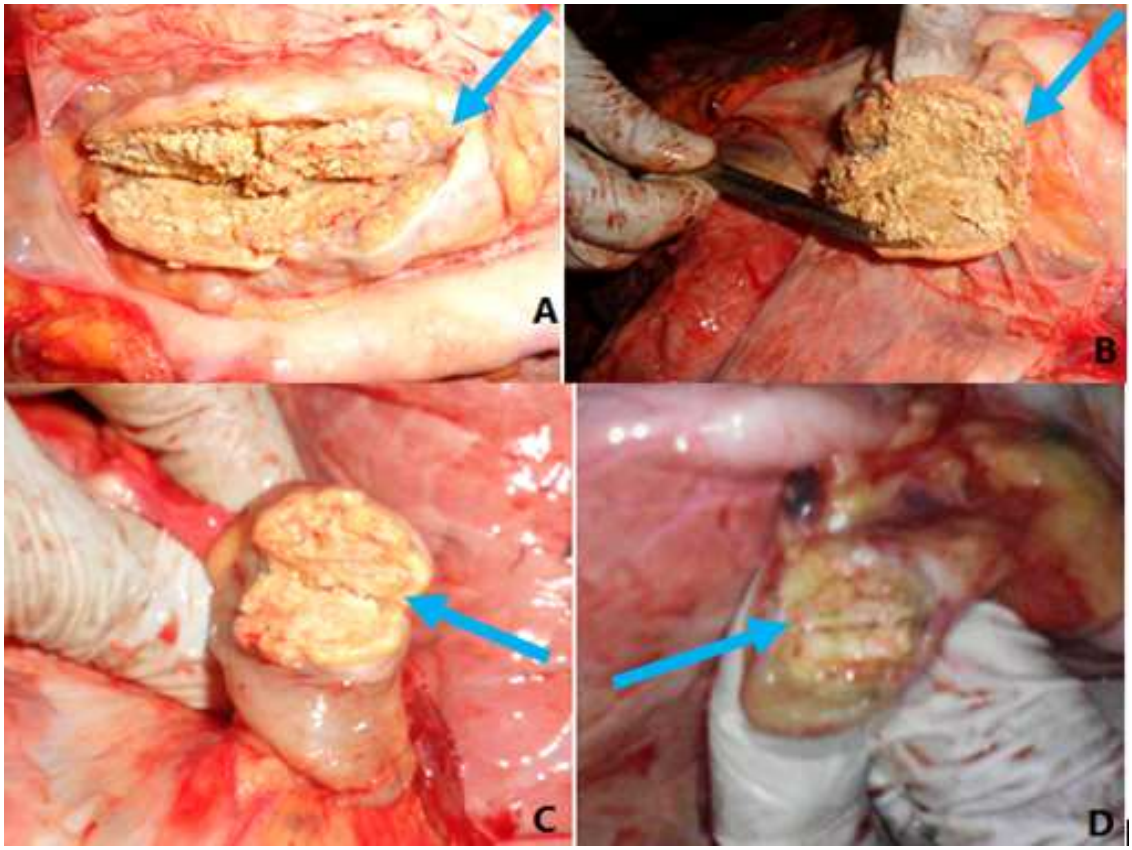


Figure 9: The typical TB lesions of cattle slaughtered in Hawassa city municipal abattoir (B & D) and Hawassa University abattoir (A & C); A & D = calcified and granulomatous lesion in mediastinum lymph nodes, B = granulomatous lesion from mediastinum lymph nodes, C = Caseous and granulomatous necrosis from mediastinum lymph nodes (lesions indicated by white arrow)**.**

According to the anatomical site, 75% (33/44) of the gross lesions were sampled from thoracic cavity followed by abdomen cavity and head region 13.64% (6/44) and 15.91% (5/44) respectively. 70.45% (31/44) of the gross lesions were collected from Mediastinal l ymph nodes whereas only 11.36% (5/44) where obtained from mesenteric lymph nodes (Table 4 & Figure 10).

Table 4: Lesion distribution in different organs and anatomic parts

Anatomical Site	Affected organs	Frequency (%)	Total (%)
Head	Retropharyngeal LN	4 (9.09%)	**6(13.64%)**
	Mandibular LN	2(4.5%)	
Thorax	Mediastinal LN	31(70.45%)	**33(75%)**
Abdomen	Mesenteric LN	5(11.36%)	**7(15.91%)**
	Livers	2(4.55%)	
Total		44(100%)	44(100%)

LN = lymph node

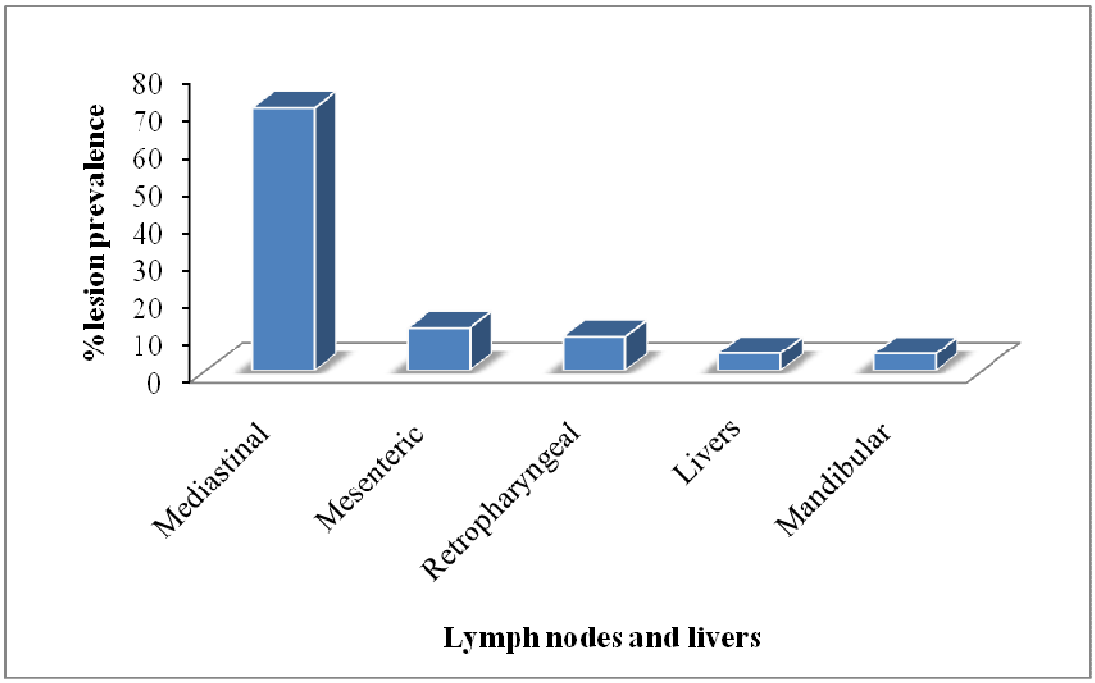


Figure 10: Proportion of lymph nodes and livers of TB suspected in 44 cattle with visible lesions.

The pathological scoring analysis of the lymph nodes and organs described by the mean severity of lesion; retropharyngeal lymph nodes (3±0.408) was higher followed by mediastinal lymph node (2.9 ±0.149), Liver (2.5±0.50), mesenteric lymph nodes (2±0.447), mandibular (1.50±0.50) (Table 5 & Figure 11).

Table 5: Mean pathology scoring of lesion from different lymph nodes and livers

Tissues	**Number Examined**	**Number (%)**	**Mean ± SE**
Retropharyngeal	753	4(0.53%)	3±0.41
Mandibular	753	2(0.27)	1.50±0.50
Mediastinal	753	31(4.12%)	2.9±0.15
Liver	753	2(0.27%)	2.50±0.50
Mesenteric	753	5(0.66%)	2±0.45

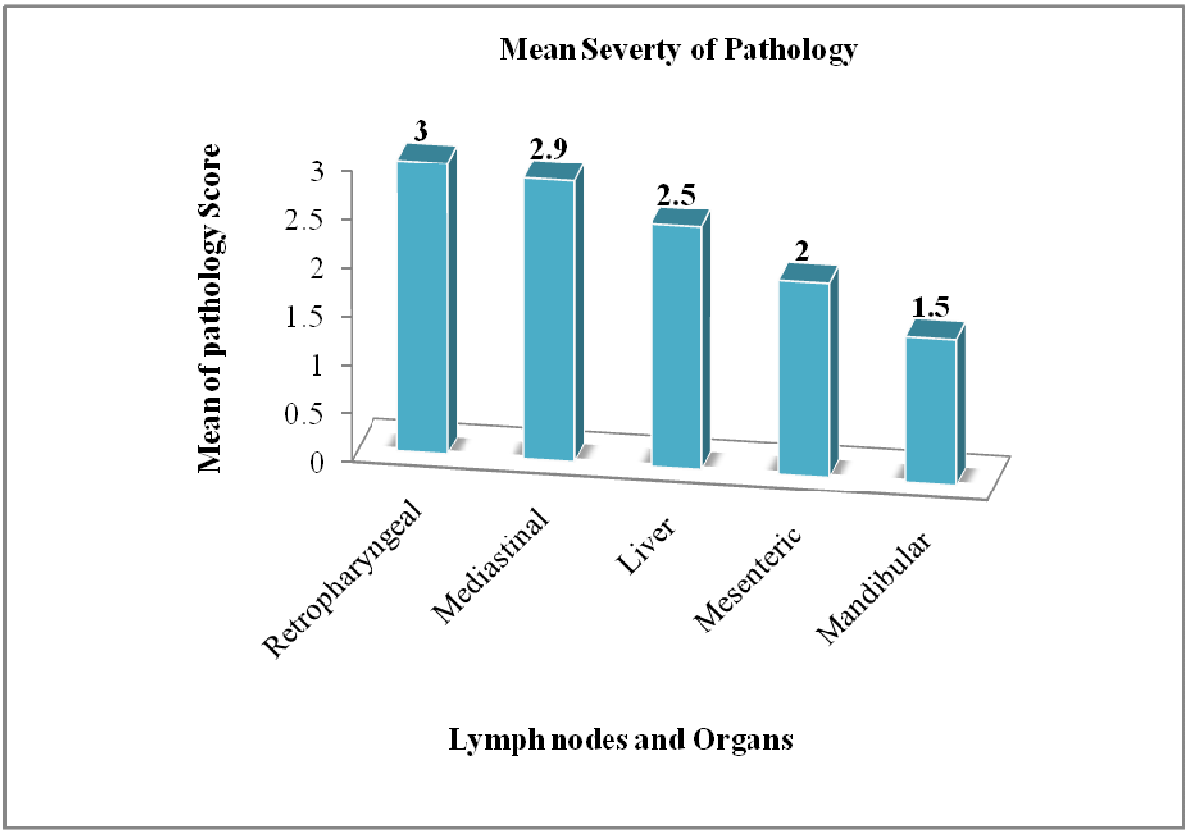


Figure 11: Mean severity of lesion in different lymph nodes and livers

4.3. Mycobacteriological Culture and Acid Fast Stain Results

Out of the total 44 suspected BTB lesions mycobacteriological cultured; growth was observed in 24 (54.55%) tissue samples on LJ medium culture (Figure 12) and all of them were found to be AFB positive. From this growth, colonies collected at a higher frequency from pyruvate supplemented LJ medium, 12/24(50%) than glycerol supplemented LJ medium, 8/24 (33.33%) and the remained 16.77% (4/24) from both LJ medium (Table 6). The observed colony morphology was smooth whitish or yellowish color colony (Table 7). These colonies positive for the ZN staining were cocci, short and long rod shape and also found in single and clump (Figure 13).

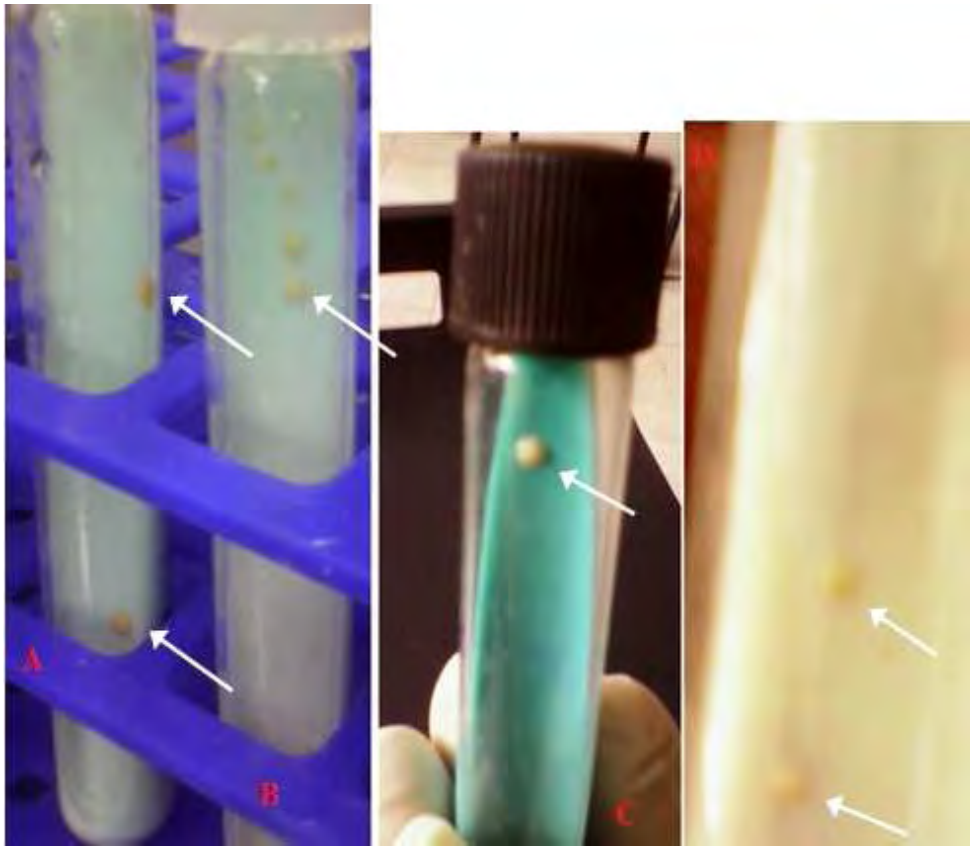


Figure 12: (A & C) Colonies grown on LJ medium glycerol supplemented (B&D) LJ medium pyruvate supplemented (Arrows indicate the colonies growth which are smooth, whitish or yellowish in color)

Table 6: Mycobacteriological culture result from suspected TB lesion on LJ medium and ZN staining positivity

Abattoirs	No. of Sampled	Lesions (%)	Growth on LJ medium			ZN stain positive (%)
			LJP	LJG	Both	
Hawassa University Abattoir	384	31(8.07)	9	6	2	17(70.83)
Hawassa City Municipal Abattoir	369	13(3.51)	2	3	2	7(29.17)
Total	**753**	**44(5.84)**	**11**	**9**	**4**	**24(100)**

Table 7: Time appearance of mycobacteria colony growth on the LJ medium and characteristics.

No of Isolates	Source	Time to appear in week		Colony characteristics
		LJP	LJG	
5	Cattle	3		Pinpoint, smooth, whitish, yellowish
13	Cattle	4	5	Pinpoint, smooth, whitish, yellowish
6	Cattle		6	Pinpoint, smooth, whitish, yellowish

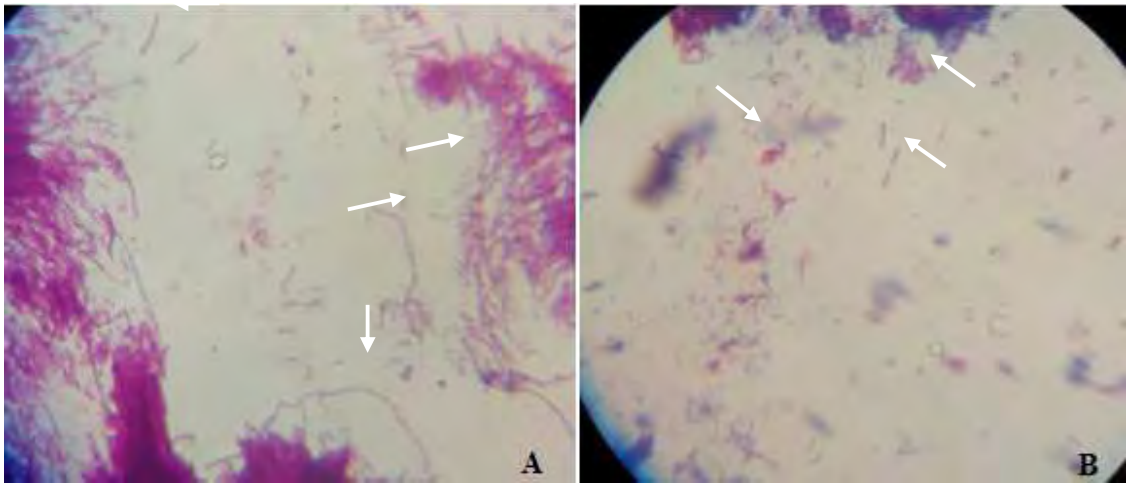
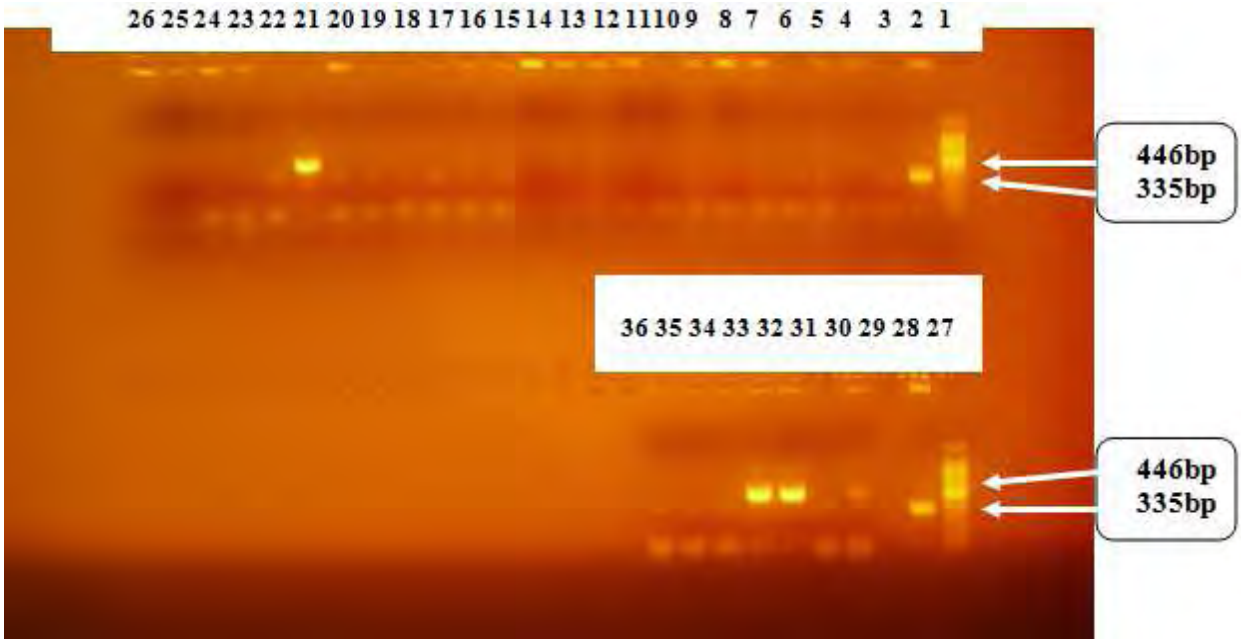


Figure 13: AFB staining positive isolates from mycobacteriological culture colony grows from lymph nodes TB lesion (arrows indicate acid fast bacilli which a short and long, in single or clump).

4.4. Molecular Characterization of Mycobacteria Isolates

The RD4 deletion typing of isolates was confirmed only three isolates to be *M. bovis* (Figure 14) that indicated a product size of 446bp; the rest of 21 isolates did not show any signal in RD4 deletion typing.



Electrophoretic separation of PCR products by RD4 deletion typing of mycobacteria isolates from 24 isolates sampled culture. Lane 1- Ladder (100bp), Lane 2- *M. tuberculosis H37Rv* positive control, Lane 3-Distilled water negative control, Lane 4-*M. bovis SB1176* positive control, Lane 5-26 and 31- 36 isolates from tissue culture positives, Lane 21, 32 and 33 positive for *M. bovis* and Lane 5-20, 22-26,31,34-36 were negative for *M. bovis*.

The Spoligotyping of the three *M. bovis* isolates, which were positive in RD4 deletion, showed the typical *M. bovis* spoligotype pattern with similar pattern and based on the database website http://www.mbovis.org analysis of the spoligotype results isolates were identified as SB1477 (Figure 15).

Code	Strain	Binary Format
Positive control (*M. bovis)*	SB1176	
Negative control (distile water)		
Ap 39	SB1477	
H41Med	SB1477	
H39 Med	SB1477	

Spoligotype patterns of *M. bovis* isolates recovered from TB lesions of cattle from Hawassa abattoirs. The filled boxes (blacks) represent the presence of spacers and the empty boxes (white) represent the absence of spacers. AP39. H41Med and H39Med are isolates from sample

5. DISCUSSION

Bovine tuberculosis is a chronic infectious disease of animals characterized by the formation of granulomas in tissues and its detection is carried out most commonly on the basis of tuberculin skin testing, abattoir meat inspection and rarely on bacteriological techniques. Bovine tuberculosis has serious economic significance to the livestock sector and public health hazard to human. Tuberculosis caused by *M. bovis* is clinically indistinguishable from tuberculosis caused by *M. tuberculosis* and the proportion of human tuberculosis caused by *M. bovis* is estimated to 10-15% (Ashford *et al.*, 2001; Mbugi *et al*., 2012).

Bovine tuberculosis, caused by *M. bovis,* is known to be endemic of most developing countries including Ethiopia although the magnitude varies between livestock production system and breed of animals (Ameni *et al.*, 2006; Ameni *et al.*, 2007) still nationwide epidemiological surveillance and control activities are often inadequate or unavailable in most developing countries (Cosivi *et al.*, 1998).

In the present study, the overall prevalence of BTB was 5.84% (95%CI: 4.58 - 8.42) which is comparably in agreement with the findings of various researchers who reported prevalence of BTB 4.2% in Yabello municipal (Biffa *et al.*, 2010a), 4.5% in Hosaana abattoir (Teklu *et al.*, 2004), 5.16% in Adama Municipality abattoir (Ameni and Wudie, 2003), 5.1% in Nekemte Municipality abattoir (Gudeta, 2008), 5% in Kombolch ELFORA abattoir (Desta, 2008), 6.4% in Mekelle town municipal abattoir (Zeru *et al.*, 2013) and 6.79% in Adama municipal abattoir (Dechassa, 2014) as well as it was the same (5.8%) with research report of Romha *et al.* (2013) in western Tigray Zone. However, the total prevalence of this study was lower than previous studies carried out by other authors; 11.50% by Abdurohaman (2009) in Butajira, 9% Nemomsa *et al.* (2014) in Butajira abattoir, 8.8% by Biffa *et al.* (2009) in Hawassa municipal abattoir, 7.96% by Regassa (1999) in Wolayta, Southern Ethiopia and 24.7% (Biffa *et al*. 2009) in Adama municipal abattoir. On the other hand, the finding of this study was higher than

the results of Regassa *et al*. (2010) in Hawassa municipal abattoir (1.1%), Gebremedhin *et al.* (2014) in Dilla Municipal Abattoir (2.6%), Asseged *et al*. (2004) in Addis Ababa (1.48%) and Shitaye *et al.* (2006) in Addis Ababa (3.46%). This lower prevalence recorded in the present study could be due to the fact that animals slaughtered in the abattoirs were mainly local breeds (Zebu) (675 out of 753) which are relatively resistant to BTB (Ameni *et al.*, 2007). This the variations in prevalence could be due to the possible difference in the epidemiology of the disease in the animal populations, markets sources of animal (from which they brought to abattoirs either from high BTB prevalent or their local BTB burden), body condition score of the animals and types of production system; The intensive livestock management system could contribute the development of mycobacterial infections than the extensive livestock management system (Radostits *et al*., 2007; Ameni *et al.*, 2007; Mamo *et al.*, 2013a).

The prevalence of BTB among the markets (source of cattle) and between the two abattoirs was statistically significant (P = 0.004, p = 0.008 respectively). Based on the post mortem inspection, the prevalence of TB lesions showed marked variation between the two abattoirs; the cases recorded in cattle slaughtered in Hawassa university abattoir was higher, 8.1% (31/384) than Hawassa municipal abattoir, 3.5% (13/369). Because most of the animal slaughtered at Hawassa University abattoir were from Boren area which might show the high prevalence in the source (Biffa *et al.*, 2010b).The abattoirs has no effects in the development of the TB infection within that short period of time since TB is a chronic disease that needs long period of time. For this reason, the statistically significance of the abattoirs indicated that to the prevalence variation of the market source of cattle.

The prevalence of BTB of cattle brought from various markets which were source of cattle to both abattoirs was different; higher prevalence was observed in the cattle come from Hawassa market 20% (5/25); followed by the markets of Borena 8.2% (25/305), Wolayta 5.9% (9/153), Arsi Negele 3.2% (2/62) and Tula 2.6% (2/115) but there was no BTB in the cattle brought from the Tukur Wuha, Harrar and Nazeriat markets. The source of cattle for Hawassa University abattoir were from Borena (275/305) and Wolayta

(109/153) markets and also the source of cattle for Hawassa city municipal abattoir were from Borena (30/305), Wolayta (44/153), Tula (115/115), Tukur Wuha (59/59), Harrar (32/32) and Nazeriat (2/2) markets. The cattle purchased from Hawassa market was (OR =13.17) thirteen times more likely to have the BTB than those cattle bought from Tula market. The infection rate in cattle has been found to differ greatly from place to place (Shitaye *et al*., 2006) and the difference most probably linked to the type of production system (extensive), which is unlikely to favor the spread of the disease in contrast to the intensive dairy farms (Ameni *et al.,* 2006; Shitaye *et al*., 2006). Grazing on the open field reduces the level of confinement and in turn minimizes the rate of infection in the herd (OIE, 1996).

The prevalence of bovine tuberculosis in this study showed a statistically significant difference among the age groups (P = 0.000). This result is in consistent with the reports of Gebremedhin *et al.* (2014) in Dilla Municipal Abattoir, Nemomsa *et al.* (2014) and Hussein (2006) in Butajira abattoir. The occurrences of TB lesions in cattle were 68.2% (30/44) in the older cattle (greater than 8 years) than younger cattle, 2.3% (1/44) and adult cattle, 29.5% (13/44), age groups; this indicted as the age of the cattle increased the prevalence also increased. The older cattle (OR = 18.25) were eighteen times more likely to have the gross pathological lesions than the younger cattle. The results of the current research also agreed with findings of Barwinnek and Taylor (1996), Ameni *et al.* (2007), Regassa *et al*. (2010) and Biffa *et al.* (2011) that explained as the age of the cattle increases the probability of acquiring TB infection also increases. The reason can be by declining of protective capability in aging animals, have weaker immune system (O'Reilly and Daborn, 1995); this is due to the fact that stresses, malnutrition and immunosuppression increase with age (Humblet *et al.*, 2009).

The difference in the prevalence of BTB among animals having different body condition scores was statistically significant (P = 0.000) and the prevalence was highest in cattle with poor body condition (31%) as compared to cattle with medium body condition (4.7%) and good body condition cattle (4.5%) which is in agreement with study resulted by Nemomsa (2014). The poor body conditioned cattle were (OR = 41.21) forty one

times more likely to have the BTB compare to the good body conditioned cattle. This could due to related to the weak protective immune response in poor body conditioned cattle compared to good ones that may result extensive lesions and wasting of the body condition as well as its chronicity nature of the disease. The present result is in consistent with previous reports which indicated that animals with good body condition have relatively good immunological response to the infectious agent than animals with medium and poor body condition score (Collins and Grange, 1994; Radostits *et al.,* 1994; Radostatit *et al.,* 2007).

Variation among breeds in susceptibility to tuberculosis has been documented in Ethiopia (Ameni *et al.,* 2006) and elsewhere (Bonsu *et al.,* 2000; Omer *et al.,* 2001; Kazwala *et al.,* 2001) and the result of this study agreed; was statistical significant between cattle breed and tuberculous lesion (P = 0.000). The prevalence of the BTB was being highest in cattle cross breed 10.36% (14/78) compared to cattle local breeds, 4.4% (30/675). The cross breeds (OR = 9.75) were nine times more sensitive to *M. bovis* (owned BTB lesions) as compared to local breeds. The finding of lower prevalence in the local/Zebu breed is in line with other previous studies (Radostits *et al.,* 1994; O'Reilly and Daborn, 1995; Ameni and Roger, 1998; Kiros, 1998; Biffa *et al.,* 2009; Zeru *et al.,* 2013) which showed different breeds could result in difference in susceptibility to BTB infections. The difference susceptibility of BTB infection between breeds is likely to be related to differences in management (Elias *et al.,* 2008): those genetically improved cattle are more prone to BTB infection than local breeds since they may suffer more severely from deficient housing and malnutrition.

The difference in the prevalence of BTB lesion between the two sexes was statistically insignificant (P = 0.700). BTB suspected slaughtered female and male cattle were 6.7% (6/89) and 5.7% (38/664) respectively. This insignificant result is in consistent with previous studies (Omer *et al.,* 2001; Teklu *et al.,* 2004; Asseged *et al.,* 2004; Cleaveland *et al.,* 2007). The possible reason might be due to in proportionality in the number of female and male animals compared in specific variable; less number of female animals was come to the study abattoir to be slaughtered.

The best evidence of the transmission route of *M. bovis* to cattle is the pattern of lesions observed in slaughtered animals (Phillips *et al*., 2003). In the present study, gross tuberculous lesions were found most frequently in lymph nodes of the thoracic cavity, 75% (33/44); followed by the lymph nodes of the head region, 13.64% (6/44) and the lymph nodes of the abdominal cavity 11.36% (5/44). The occurrence of tuberculous lesions in thoracic cavity was lower than the results of previous studies which reported greater than 84% TB lesions occurrence in the respiratory system (Corner, 1994; Neill *et al*., 1994; Collins, 1996; Whipple *et al*., 1996; Teklu *et al*., 2004); whereas it was higher than the report of Dechassa (2014) (67.7%), Firdessa (2006) (70%), Miliano-suazo *et al.* (2000) (49.2%) and Regassa *et al*. (2010) (50%). As a result, this study indicated the main route of transmission and infection being respiratory route and this finding agreed with the previous researchers who reported the same route of transmission and infection, respiratory route (Corner, 1994; Goodchild and Clifton-Hadley, 2001; Phillips *et al*., 2003; Ameni and Wudie, 2003; Teklu *et al., 2004;* Regassa *et al*., 2010; Tigre *et al.*, 2012).

In the current study, the growth rate of mycobacteria on culture media was 54.5% (24/44). *M. bovis* grows poorly on standard LJ medium (Amanfu, 2006; Cleaveland *et al.*, 2007). Furthermore, the presence of caseous and/or calcified lesions and miscategorization of tuberculous lesions resembling nontuberculous lesions as tuberculous lesions (Teklu *et al*., 2004) may not always found to be of mycobacterial origin; viable mycobacteria may not be present in calcified lesions (Pritchard, 1988; Diguimbaye-Djaibe, 2006). However, the result of this study, 54.5%, was higher than the previous report (47%) of Ameni *et al*. (2010b), (23.6%) Araujo *et al*. (2005), (32%) Shimeles (2008), (35%)Müller *et al.* (2008) and (31.4%) Woyessa *et al.* (2014) culture positivity from the lesion positive samples; but this finding was found slightly the same with the report of (56%) Ameni *et al.* (2007).

RD4 deletion typing of the isolates from 24 animal tissue samples; only three isolates were confirmed to be *M. bovis* indicating product size of 446bp while the remained isolates were not showed any signal in RD4. Further molecular characterization using spoligotyping on the three *M. bovis* isolate has designated the isolates as strain SB1477. The outcome was revealed the same spoligotype patterns (clustered stating) were recognized among these *M. bovis* isolates and defined as of type SB1477 at the international spoligotyping database www.mbovis.org. This spoligotype pattern of the strains has similarities to the strains previously described in Addis Ababa farm, Ethiopia (Firdessa *et al.,* 2012; Mekibeb *et al.,* 2013). This similar spoligopattern show that the strain has been circulating and transmitting in the cattle population of Borna and wolayta area. Warrants further investigation on their epidemiological and zoontic role in the area.

6. CONCULSION AND RECOMMENDATIONS

The output of this study has indicated that an overall BTB prevalence of 5.8% of which 20%, 8.2%,5.9% 3.2% and 2.6% were recorded in Hawassa, Borena, Wolayta, Arsi Negele and Tula markets respectively. Even though the overall prevalence was low, high prevalence was found in animals which are older, poor body condition and cross breeds cattle. Similarly, relatively high occurrence of BTB was in cattle brought from Hawassa and Borena markets than Tula market. This could be indicating the presence of BTB infection in certain geographical areas. This research also shown that the respiratory route was the major means of BTB transmission among the cattle population and the higher mean severity of pathology among associated lymph nodes and organs was recorded in retropharyngeal and mediastinal lymph nodes. The isolation and molecular characterization of this study confirmed that the clustered *M. bovis* strains (SB1477) circulating in the cattle population of the study area. The rest 21 isolates did not showed any signal in RD4 deletion will be characterize by mPCR (Genus typing) soon. In conclusion, in this study Gross pathological lesions, bacteriological culture, ZN staining and the molecular characterization findings indicated the occurrence of BTB in apparently healthy cattle in the study area and *M. bovis* has been confirmed as a causative agent of BTB with moderately low prevalence, a threat to livestock production and also for public health.

Basis of findings the present study the following points are recommended:

- Further investigation should be done by including wide geographical areas and large sample size to elucidate the epidemiological information on circulating strains, potential risk factors, ways of transmission and molecular diversity of the *M. bovis* strains as well as their zoonotic role in human.

- A proper postmortem meat inspection should be practiced efficiently in the abattoirs before taking beef to the retail markets to reduce the public health risk.

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8. APPENDICES

Appendix 1: Ant and post mortem recording format

	Anti mortem data						**Postmortem data**				
Code	Abattoir	Market	Age	sex	Breed	BCS	Retropha. LN	Mesenetric LN	Mediastinal LN	Mandibular LN	Liver

LN= lymph node, Retropha. = Retropharyngeal

Appendix 2: Description of body condition scores (BCS)

Poor

1. Clearly defined bone structure of shoulder, ribs, back, hooks and pins easily visible.
2. Little muscle tissue or fat present.
3. Small amount of muscling in the hindquarters. Fat is present, but not abundant.
4. Space between spinous process is easily seen.
5. Fat begins to cover loin, back and fore ribs. Upper skeletal structures visible. Spinous process is easily identified.

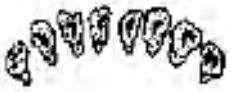
Medium/ Borderline (Optimum)

1. Fore ribs becoming less noticeable. The transverse spinous process can be identified by palpation. Fat and muscle tissue not abundant, but increasing in fullness.
2. Ribs are visible only when the animal has been shrunk. Processes not visible. Each side of the tail head is filled, but not mounded.
3. Ribs not noticeable to the eye. Muscling in hindquarters plump and full. Fat around tail head and covering the fore ribs.

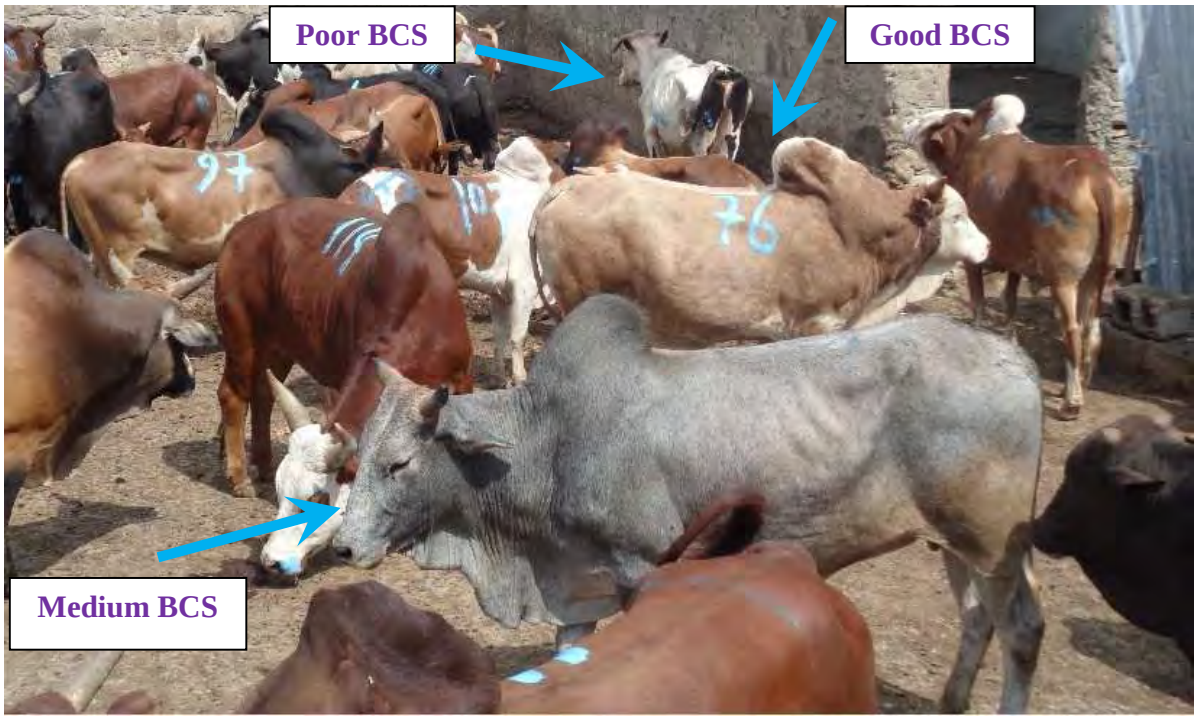
Good/ fat

1. Spinous process can only be felt with firm pressure. Fat cover in abundance on either side of tail head.
2. Animal smooth and blocky appearance; bone structure difficult to identify. Fat cover is abundant.
3. Structures difficult to identify. Fat cover is excessive and mobility may be impaired.

Appendix 3: Age of cattle determining by teeth

	At birth to 1 month	Two or more of the temporary incisor teeth present. Within first month, entire 8 temporary incisors appear.
	2 years	As a long-yearling, the central pair of temporary incisor teeth or pinchers is replaced by the permanent pinchers. At 2 years, the central 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 corners fully developed.
	5 to 6 years	The permanent pinchers are leveled, both pairs of intermediates are partially leveled, and the corner incisors show wear.
	7 to 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 6th year, the arch gradually loses its rounded contour and becomes nearly straight by the 12th 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.

Appendix 4: Picture of different breeds and body condition score of animals during the study period



Appendix 5: Löwenstein–Jensen egg based media preparation

LJ Pyruvate (LJP):

- Weigh 37.2g of LJ medium base powder
- Weight 6.65g of sodium pyruvate powder.
- Measure 600 ml of distillate water into a 1000 ml beaker.
- Pour the Lowenstein-Jensen medium base and the pyruvate powder in the sterile distillate water.
- Mix well and bring to boil in a microwave oven with constant agitation until the reagents are completely dissolved.
- Transfer the solution to 1 liter glass flask.
- Autoclave the mixture at 121ºC for 15 minutes.
- Let cool to room temperature.

LJ Glycerol (LJG):

- Weigh 37.2g of LJ medium base powder
- Measure 600 ml of distilled water into a 1000ml beaker.
- Dissolve the Lowenstein-Jensen Medium base in the water.
- Mix well and bring to the boil into a microwave oven with constant agitation until the reagents are completely dissolved.
- Using a single sterile pipette (25ml), measure 12ml of glycerol and add it to the mixture.
- Mix and autoclave the mixture at 121^{O}C for 15 minutes.
- Let cool to room temperature.

Egg Fluid

- Break 1000ml of eggs into a sterile beaker (2000ml).
- Add the fluid eggs to the autoclaved mineral solution.
- Using a Homogenizer, mix until homogeneous.
- Add 20ml of 2% Malachite Green.
- Stir for at least 10 minutes before dispensing.
- Filter the mixture through sterile sieve/muslin into a sterile round glass flask (2000ml) containing a sterile magnetic bar.
- Add antibiotics: 3.0ml of Polymyxin B (100, 000 iu/ml), 0.75ml of Carbenicillin (0.2g/ml), 15ml of Fungizone (5mg/ml) and 3.0ml 1% Trimethoprim.
- Mix well and slowly on a magnetic mixer for at least one hour under UV light in a biosafety cabinet (without running the cabinet).
- Decant into two separate sterile Duran glass flasks and close each flask with sterile lids surmounted by a sterile dispenser.

Aliquoting the medium

- Dispense 8ml into each sterile test tube or 10 ml into each universal tube. Avoid air bubbles.
- Clean the dispenser top with paper tissue impregnate with 70% ethylic alcohol after every set of 10 tubes.
- Transfer the tubes containing the medium into a sterile crate.
- After dispensing the entire medium, range the tubes on a special rack and lean them to give a slope when placing in an oven.
- Tighten screw caps, slant them and coagulate by inspissations at 85^{o}C for 50 min.
- Let cool.

- Give the medium batch a lot number and label the racks with this number.
- Label the universal lids or culture tube with color codes.

Appendix 6: Ziehl-Neelsen (Acid Fast) Staining

- Label new, clean, unscratched microscope slide at one end with the relevant sample name/number.
- Thoroughly mix the specimen with a pipette (1ml) and place about one drop (or 2 – 3 loop full) on the slide.
- Using a loop spread the smear over a surface of about 1.5cm x 1cm.
- Allow the smear to air dry completely in the BSC.
- Heat-fix the slide either by passing it through a flame three to four times with the smear side up.
- Let the slide cool before staining.
- Put the glass slides with the fixed smears on a staining rack (don't stain more than 12 smears at a time).
- Flood the entire slide with basic fuchsine solution.
- Heat the slides slowly until slight steam rises.
- Maintain steaming for 3-5 minutes (do not let slides dry, add basic fuchsine onto the slides if you note too much of evaporation).
- After 5 minutes of basic fuchsine reaction, gently wash off all free stain completely from each slide with distilled water (using forceps, slope the slide one by one to remove the water completely).
- Distain the smear by covering the glass slides with acid-alcohol solution and incubate for a maximum of 3 minutes.
- Wash the slides with distilled water (using forceps, slope the slide one by one to remove the water completely).
- Counter stain the smear by covering the glass slides with methylene blue solution and incubate for 2 minutes.
- Wash the slide thoroughly with distilled water (using forceps, slope the slide one by one to remove the water completely).

- Leave the smear to dry before examination under the microscope.

Thus, identification of AFB is done by examining the stained smears under a microscope using the (100x) oil immersion objective. Mycobacteria and other acid-alcohol resistant bacteria appear pink or red colored, whereas the background and other nonacid-alcohol resistant material will appear blue colored. *M. bovis* will appear as short thick acid-fast rods with cord formation being a characteristic property of strains of the *M. tuberculosis* -complex.