

**ADDIS ABABA UNIVERSITY
COLLEGE OF HEALTH SCIENCE
SCHOOL OF VETERINARY MEDICINE**

**EPIDEMIOLOGY OF BOVINE TUBERCULOSIS IN EAST WOLLEGA ZONE,
WESTERN ETHIOPIA**

BY

MEZENE WOYESSA BUSSA

DEBRE-ZEIT, ETHIOPIA

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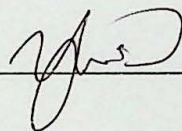
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DEDICATION

This dissertation is dedicated to my lovely mother, HULU-HAGERISH
DAGNACHEW who was 68 years old, when she passed away in June, 2009.
May GOD bless her life!!

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LIST OF ABBREVIATIONS

AFB	Acid Fast Bacilli
AIDS	Acquired Immune Deficiency Syndrome
ALIPB	Aklilu Lemma Institute of Pathobiology
AMP	Adenosine mono Phosphate
BCG	Bacillus of Calamette and Guerien
BCS	Body condition score
bp	Base Pairs
BTB	Bovine tuberculosis
CD	Cluster of diffentation
CIDT	Comparative intradermal skin test
CI	Confidence interval
CMI	Cell-mediated immunity
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside Triphosphate
DR	Direct repeat
DVM	Doctor of Veterinary Medicine
ECL	Enhanced chemoiluminesces
EDTA	Ethyl diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
ESAT-6	Early secreted antigen target 6-kDa protein
FAO	Food and Agriculture Organization
GC	Guanine and cytosine
HGCBIC	Horro-Guduru cattle breeding and improvement center
HIV	Human immunodeficiency virus
IFN- γ	Interferon-gamma
iNOS	Inducible nitric oxide synthase
ILRI	International Livestock Research Institute
IL	Interleukin

IS	Insertion sequence
IU	International unit
LAM	Lipoarabinomannan
LJ	Löwenstein Jensen
MHC	Major histocompatibility complex
MIRV	Mycobacterial interspersed repetitive variable
MoARD	Ministry of Agriculture and Rural Development
MoH	Ministry of Health
M Sc	Masters of science
MTBC	<i>Mycobacterium tuberculosis</i> complex
NVL	Non-visible lesions
OIE	Office International des Epizooties
M-PCR	Multi -Polymerase Chain Reaction
PGRS	Polymorphic GC-rich Repetitive Sequence
PPD	Purified protein derivative of tuberculin
RD	Regions of difference
RFLP	Restriction fragment length polymorphism
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
SNPs	Single Nucleotide Polymorphisms
SOP	Standard operation Procedure
SOD	Superoxide dismutases
TB	Tuberculosis
Th	Helper T cell
TNF	Tumor necrosis factor
UV	Ultra Violet
VNTR	Variable numbers of tandem repeats
WHO	World Health Organization

ABSTRACT

A cross-sectional study was conducted at Nekemte Abattoir and Horro-Guduru Cattle Breeding and Improvement Centre (HGCBIC) from September 2009 to May 2010 to estimate the prevalence of bovine tuberculosis (BTB), and characterize its causative agents using post mortem examination, comparative interadermal tuberculin (CIDT) test, bacteriological culturing, Zeihl Neelsen staining, multiplex polymerase chain reaction (PCR), and region of difference-4 (RD4) deletion typing. To accomplish these objectives, 1,186 cattle were recruited into the study from Nekemete Abattoir and 500 cattle from HGCBIC. The prevalence of BTB was 5.9% (70/1186) at Nekemete Abattoir on the basis of gross lesion while it was 1.8% (9/500) at HGCBIC using CIDT test. There was no significant difference ($\chi^2=4.75$; $P=0.079$) in prevalence of BTB between Horro-Jersey cross breed and zebu (Horro) breed at HGCBIC. At Nekemete Abattoir, 70% of the gross lesion was detected in the thoracic cavity while 25% of the lesion was found in the abdominal cavity. Only 31.4% (22/70) of the suspicious lesions yielded colonies of which 19 were acid-fast positive. Further identification of these 19 isolates using multiplex PCR revealed that 17 isolates belong to the Genus *Mycobacterium* while the remaining two isolates did not show signal to the Genus. Of the 17 isolates that showed signal to the Genus *Mycobacterium*, 7 were members of *Mycobacterium tuberculosis* (*M. tuberculosis*) complex while the remaining 10 isolates were members of the non-*M. tuberculosis* complex. Further identification and characterization of the *M. tuberculosis* complex members using RD4 deletion typing identified four isolates with intact RD4 which could be either *M. tuberculosis* or *M. africanum* and three isolates with deleted RD4 thus confirmed to be *M. bovis*. In conclusion, TB lesions were caused by both the members of *M. tuberculosis* complex and the non-*M. tuberculosis* complex *Mycobacteria*. Hence, as the majority of the isolates were the non-*M. tuberculosis* complex members, the pathogenicity of these members in cattle needs further study.

Key words: *Mycobacterium tuberculosis* complex, pathology, tuberculin test, prevalence, risk factors, Cattle, East Wollega, Ethiopia

1. INTRODUCTION

Tuberculosis (TB) is still accounts for a large number of death and great morbidity World wide. The disease is most common in parts of the developing nations of the world. These countries have nearly two-third of the world livestock population, but produce less than the developed worlds meat and milk production due to poor management and high prevalence of livestock diseases such as mastitis, respiratory diseases, tuberculosis, etc (FAO,1995).

Tuberculosis is one of an important disease not only due to its effect on animal production and productivity, but also due to its public health importance (O'Reily and Dabron, 1995). Tuberculosis is estimated to infect two billion people world wide with eight million new cases and two million deaths per annum. Ninety five percent of tuberculosis cases occur in the poorer parts of the world. According to WHO (1993), tuberculosis is very serious and global emergency disease. This is due to many factors, which contribute to such problem, which are: 1, the synergy that exists between AIDS and tuberculosis. Currently 8-10 % of the cases of tuberculosis are related to HIV infection; 2, the emergence of drug resistant and multi-drug resistant strain; 3, Many cases of tuberculosis occur in the third world where sanitation and health care are very poor; meaning the treatment of the disease is not carried out as effectively as in the rest of the world. In Africa, TB has received scanty attention mainly as a public health threat. The incidences of TB in human runs parallel to that of cattle, and it is increased by introduction of modern farming system together with risk of close contact with infected cattle in rural areas, the habitat of consumption of raw meat and milk from infected cattle and HIV/AIDS pandemic (Daborn and Grange, 1993).

Bovine tuberculosis (BTB) is described as a contagious, chronic and debilitating disease. The disease is caused by bacteria from the *mycobacterium tuberculosis* complex. It is a fastidious organism that causes tuberculosis in cattle, swine, goats, cats, dogs and many other domestic and wild animals. Among livestock species, cattle mostly are affected by Tuberculosis (TB), and the principal cause is *M. bovis* (Weinhaupl *et al.*, 2000). It also causes tuberculosis in human and non-human primates (Thoen and Barletta, 2004). The potential for transmission of zoonotic

tuberculosis, that is, tuberculosis from animals to human occurs directly by aerosol and through the food chain by consumption of milk and rarely meat from tuberculous cattle. Milk products such as yoghurt, cream and cheese were also noted to have contained tubercule bacilli several days after being manufactured from unpasturized milk. As the main route of entry is oral rout, tuberculosis of bovine origin in man is mainly extra pulmonary resulting in bone and joint tuberculosis as well as infection of the cervical and mesenteric lymph nodes (Daborn and Grange, 1993; Edelsten, 1996).

Diagnosis of BTB is helpful to reduce the risk of zoonosis, together with increasing public awareness and proper hygienic in food chain from animal source which may result in eradication (Acha and Szyfres, 2001). Cattle and other bovine species are considered the primary and most well known reservoirs or maintenance host in countries where maintenance hosts are present endemically in the wild, infection from this population to the domestic cattle or other farm animals is difficult to avoid (Ayele *et al.*, 2004). Diagnosis of BTB will be achieved in different ways in animal, human and food of animal origin either by history of contact, physical, chemical, immunological and/ or molecular methods (Quinn *et al.*, 2002). Human infection caused by *M. tuberculosis* and *M. bovis* are clinically and radiologically indistinguishable. Therefore definitive diagnosis can be achieved through isolating and typing the ethological agent through different diagnostic techniques (Acha and Szyfres, 2001).

However diagnosis in animals is achieved additionally by tuberculin test using purified protein derivative (PPD), milk bacteriology and meat inspection (Cosive *et al.*, 1995). The advent of genetic engineering has provided alternative DNA based strategies, which have the potential to overcome non-specific reactions. The development of agarose electrophoresis is for the size separation of DNA fragment, followed by the variability of restriction endonucleases which cleave DNA at defined sites lead to the development of restriction fragment analysis of bacterial DNA; this technique produce a pattern of fragments or finger prints, which uniquely characterizes the strain from which DNA was isolated (Butcher *et al.*, 1996).

Although national data is not available on the prevalence of BTB in Ethiopia, it is assumed that the incidence of the disease is rising because of the present private-oriented economic policy of

the government, which thereby promotes the expansion of dairy industry. A few studies (Ameni *et al.*, 2003a, b; Asseged *et al.*, 2001) have been conducted in central highlands of Ethiopia on the epidemiology of BTB, and the results of such studies have indicated that the disease is prevailing in these areas. Such studies were carried out most commonly using tuberculin skin testing, abattoir meat inspection and rarely on bacteriological techniques. Therefore, expansion of similar studies to the untouched regions of the country will be useful towards made to establish the epidemiology of the disease at the national level. Moreover, complementing such studies with the application of molecular tools so as to identify and characterize the species and strains of *Mycobacteria* that are pathogenic to cattle is of paramount importance. The current study was formulated to estimate the prevalence of BTB in cattle slaughtered at Nekemete Abattoir and in a dairy herd kept at HGCBIC, Eastern Wollega, and thereafter characterize the causative agents of the disease.

Objectives:

- To estimate the prevalence of BTB in cattle slaughtered at Nekemete Abattoir and in dairy herds kept at HGCBIC, Eastern Wollega.
- To evaluate the distribution of lesion of BTB in different tissues and organs of cattle slaughtered at Nekemete Abattoir.
- To isolate and characterize *Mycobacteria* from suspicious TB lesions of cattle slaughtered at Nekemete Abattoir.

2. LITERATURE REVIEW

2.1. Etiology

2.1.1. Classification

Mycobacteria belong to the kingdom bacteria; phylum *Actinobacteria*; order *Actinomycetales*; family *Mycobacteriaceae*, and the genus *Mycobacterium* (Quinn *et al.*, 2002). *Mycobacteria* can be classified into several major groups for the purpose of diagnosis and treatment; this includes the *Mycobacterium tuberculosis* complex (MTBC), which can cause tuberculous-like pathologies in different host species. Non-tuberculosis *Mycobacteria* are all the other *Mycobacteria* which can cause pulmonary disease resembling tuberculosis, lymphadenitis, skin disease, or disseminated disease. For example *M. leprae* causes Hansen's disease or leprosy. *Mycobacterium* species other than the *M. tuberculosis* complex that cause TB like diseases in man and animals are commonly called 'atypical *Mycobacteria*' (Quinn *et al.*, 2002). They are classified into four groups; photochromogenic, scotochromogenic, non-chromogenic and rapid growers based on growth rate and formation of pigments (Quinn *et al.*, 2002). Atypical *mycobacteria* are not pathogenic to man and animals except in certain situations such as direct inoculation into wounds or introduction into immunocompromised hosts due to immunosuppressive therapy or due to HIV/AIDS. However, they are very important during diagnosis as they sensitize man/ animals to tuberculin test (Theon *et al.*, 2006).

The *Mycobacteria* are a genus of more than 80 species, within a complex of related and poorly studied organisms. The genus *Mycobacterium* includes obligate parasites, saprophytes and intermediate forms differing in their nutritional requirement. The phylogenetic analysis of the genome of pathogenic *Mycobacteria* has shown that all (except *M. avium*) belong to a single genetic species: the *Mycobacterium tuberculosis* complex. The cause of bovine tuberculosis, *Mycobacterium bovis*, belongs to the "*M. tuberculosis* complex", which includes *M.*

tuberculosis, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. microti*, *M. canettii*, *M. bovis* sub spp *caprae* and *M. pinnipedii* (Haddad *et al.*, 2004; Pavlik *et al.*, 2005).

2.1.2. Morphology

The *Mycobacteria* are thin rods of varying lengths (0.2-0.6 by 1.0-10.0 μm) (Quinn *et al.*, 2002). Most of the members are slightly curved rods, sometimes branching filamentous or mycelium type growth may occur but usually gets fragmented into rods or coccoid cells. They are acid fast, non motile and do not form endospores, capsules. *M. tuberculosis* is straight or slightly curved rod, whereas *M. bovis* is usually straighter, Stouter and shorter (Gupte, 2006).

2.1.3. Staining

Though *Mycobacteria* are cytochemically gram positive, they often resist staining with gram Stain (Biberstein and Hirsh, 1999). Thus, they cannot be classified as either gram-positive or gram-negative bacteria. The high lipid content renders *Mycobacteria* hydrophobic and makes them difficult to stain with commonly used aniline dyes at room temperature. The bacteria, therefore, take up the stain with dyes only by prolonged application or by heating (Sharma and Adlakha, 1996). *Mycobacterias*' most noted staining property is their acid-fastness that depends on the integrity of the waxy envelope and is related to the resistance of *Mycobacteria* to the decolorizing effect of 95% ethyl alcohol containing 3% hydrochloric acid unlike other bacteria which quickly get decolorized. Usually the Ziehl-Neelsen technique of staining is employed for identification of acid-fast bacteria including *Mycobacteria*. Moreover, *Mycobacteria* can also be stained with fluorescent dyes like auramine, rhodamine (Biberstein and Hirsh, 1999).

2.1.4. Structure and composition

Mycobacteria are rich in lipids (mycolic acids, waxes and phosphatides) and are also composed of proteins and polysaccharides. The lipids are largely bound to proteins and polysaccharides

(Brooks *et al.*, 2004). The cell wall of *Mycobacteria* contains N-glycol muramic acid in place of N-acetyl muramic acid and has (60%) lipid content (Sharma and Adahlaka, 1996). Lipids account for acid-fastness and pathogenic and immunogenic properties. Various structural components that are found in *Mycobacteria* includes surface mycosides (mostly glycolipids and peptidoglycolipids), mycolic acids and their esters, wax D, cord factor (dimycolil trehalose), sulfolipids (or sulfatides), phosphatidyl inositol mannoside, mycobactins, carotenoid pigments (in chromogens) and tuberculins (peptides liberated into culture media during growth) (Biberstein and Hirsh, 1999).

A major finding from the genome sequencing project has been that the 65.6% GC *M. bovis* genome (4,345,492 bp for the virulent bovine isolate AF2122/97) is a downsized version of the genome of *M. tuberculosis* (4,411,532 bp) for the human isolate H₃₇Rv), with more than 99.95% identity. Moreover, *M. bovis* does not have any new genetic material when compared to *M. tuberculosis*. Thus, DNA deletions in *M. bovis* are the major contributors to these differences, though more than 2000 single nucleotide polymorphisms have also been found. It is intriguing how these two microorganisms could have such differences in pathogenicity and host range. However, a comparative genomic analysis indicates that minor genome changes have a profound effect on the phenotype. For example, a point mutation in *M. bovis* is responsible for its resistance to pyrazinamide. The greatest sequence variations have been found in gene coding for cell wall and secreted proteins. Because these Proteins may be involved in adhesion functions (Theone and Barletta, 2004), it is possible that tissue tropism and host range are also affected.

Likewise, the TbD1 locus (present in *M. bovis*, but absent from most *M. tuberculosis* strains) encodes transporter proteins and synthetases involved in lipid trafficking and glycolipid biosynthesis. Other sequence changes involve master regulatory genes controlling the expression of multiple gene families. Similarly, deletions have been found to affect genes involved in transport, cell-surface structures, and intermediary metabolism. Finally, it is important to note that deletions may remove genes that are unnecessary for host adaptation and lead to a different or even wider host range (Sharma and Adahlaka, 1996).

2.1.5. Evolutionary relationships

The analysis of the *M. bovis* genome challenged the natural epidemiological hypothesis that *M. tuberculosis* is a human-adapted variety of *M. bovis* that was acquired from cattle. The irreversible loss of DNA material uncovered by the *M. bovis* genome sequencing project and the systematic analysis of polymorphisms in a large panel of strains indicate quite a different scenario (Brosch *et al.*, 2002; Fabre *et al.*, 2004). This analysis indicates that *M. canettii* is likely the ancestral species of the *M. tuberculosis* complex. Successive DNA deletions, starting by the loss of region RD9 (RD stands for regions of difference), originated *M. africanum*, *M. microti*, and *M. bovis*. Moreover, *M. bovis* BCG experienced further deletions during *in vitro* adaptation, and the loss of region RD1 has been implicated as the mechanism of virulence attenuation. In this view, modern *M. tuberculosis* strains originated later from ancestral *M. canettii* by loss of the TbD 1 locus (Fabre *et al.*, 2004)

2.1.6. Growth and cultural characteristics

Examination of bacteriological culture provides definitive diagnosis of tuberculosis. However, the usual microbiological techniques of plating clinical material on selective or differential media and sub-culturing to obtain pure cultures cannot be applied to tuberculosis bacteriology. For that reason, many different media have been devised for cultivating tubercle bacilli and three main groups can be identified, egg-based media, agar-based media and liquid media (WHO, 1998). In addition, tubercle bacilli may also be grown on chick embryos and in tissue culture (Gupte, 2006). The only media which allow abundant growth of tubercle bacilli are egg-enriched media containing glycerol/pyruvate and asparagines, and agar or liquid medium supplemented with serum or bovine albumin (WHO, 1998). To date, the most frequently used media for isolation of *M. bovis* are Löwenstein-Jensen (L-J) and Ogawa-medium (both containing eggs phosphate, magnesium) and the former contains asparagine. The media are prepared as solid slants in screw capped bottles. *M. tuberculosis* and *M. avium* prefer the caps on the culture media to be loose while *M. bovis* grows best in airtight containers (Quinn *et al.*, 2002). All the members of the

Mycobacteria complex are slow growers. Therefore, the inoculated media may have to be incubated at 37 °C up to 8 to 12 weeks (Quinn *et al.*, 2002). With regard to the colony characteristics, on solid media *M. tuberculosis* appears as dry, rough, raised and irregular colony. It is creamy white first and becomes yellowish or buff colored later on and is not emulsified easily. On the other hand, the colony of *M. bovis* is flat, smooth, white and breaking up easily when touched (Gupte, 2006).

2.2. Pathology

2.2.1. Route of infection and lesion distribution

Aerosol exposure of cattle to *M. bovis* is considered the most frequent route of infection; gross lesions usually involve the lungs and thoracic lymph nodes (Theon and Barletta, 2004). Cattle exposed by ingestion of food and water contaminated with *M. bovis* often develop primary foci in lymph tissues associated with the intestinal tract. Experimental investigations in animals comparing intravenous, intratracheal, intraperitoneal injection, as well as oral exposure to *M. bovis*, reveal that the nature and extent of disease varies with the route of exposure and dose of organisms. The characteristic gross lesion seen in an animal infected with BTB is the presence of “tubercles” (a tubercle is a white nodule usually 1 mm to 2 cm in diameter) within the body. In cattle, BTB most commonly will cause lesions in the lymph nodes of an infected animal. Therefore, during necropsy, the lymph nodes, especially those associated with the head, thorax and abdomen are closely examined. It should be remembered that an animal may be infected with BTB despite the absence of tubercles (Patterson and Grooms, 2000).

2.2.2. Pathogenesis

Tubercle bacilli were identified more than 100 years ago; however, definitive information on the pathogenesis of *M. bovis* in cattle and other bovidae is not available (Theon and Barletta, 2004). The mucociliary clearance by mucus and epithelial cilia in the upper respiratory passages provides a defense against infection by inhalation of *Mycobacteria*. However, microorganisms on

small particles such as dust and water droplets that do not impinge against the mucociliary layer can pass through terminal bronchioles, thus gaining access to alveolar spaces. The estimated size of terminal endings of bronchioles is about 20µm as compared to 1–4µm for an acid-fast bacillus. Following aerosol exposure, *M. bovis* is carried to the small air passages, where it is ingested by phagocytes. The phagocytes pass through the lining of the bronchioles, enter the circulation, and are carried to lymph nodes, parenchyma of lungs, or other sites.

After ingestion of the bacillus, the mononuclear macrophages attempt to kill the organism; however, virulent tubercle bacilli possess the ability to escape killing. Ingestion of the tubercle bacilli by the phagocytes into phagosomes or intra cytoplasmic vacuoles protects the organisms from bactericidal components in serum. Following ingestion into phagocytes, *Mycobacteria* effectively prevent phagolysosome fusion and acidification (Beatty *et al.*, 2000). *Mycobacterial* lipids such as lipoarabinomannan and phosphatidyl inositol mannoside have been shown to intercalate within endosomal membranes and contribute to the arrest in phagosome maturation (Beatty *et al.*, 2000; Vergne *et al.*, 2004). In addition, *Mycobacterial* proteins of the antigen 85 complex have been shown to localize within cytoplasmic vacuoles free of *Mycobacteria*. By this mechanism, *Mycobacteria* survive and multiply within the phagosomes and eventually destroy the phagocytes (Vergne *et al.*, 2004).

Mycobacterium marinum, a close relative of *M. tuberculosis* and *M. bovis*, may lyse the phagosome and enter into the cytoplasm and use actin polymerization to spread from cell to cell (Stamm *et al.*, 2003), a phenomenon that has not been observed with *M. tuberculosis* or *M. bovis*. Nonetheless, phagosomes containing *M. tuberculosis* or *M. bovis* BCG display certain degree of permeability, allowing entrance of cytosol components of up to 70 kDa in size (Chua and Deretic, 2004). These findings led to the hypothesis that these membrane-permeable phagosomes may allow a bidirectional transfer of *Mycobacterial* products such as peptides, cord factor, or other toxic products from the phagosomes into the cytoplasm. This process may have implications for the role of cytotoxic T cells and class I-mediated antigen presentation in the pathogenesis of *Mycobacterial* infections (Stamm *et al.*, 2003). On the basis of these findings, pathogenic *Mycobacteria* may even gain access to the cytoplasm. Following the stage described above, other phagocytes then enter the area and ingest the increasing numbers of tubercle bacilli.

A small cluster of cells referred to as a granuloma develops. Cellular responses attempting to control the disease result in the accumulation of large numbers of phagocytes, and finally the formation of macroscopic lesions. After 10–14 days, cell-mediated immunity (CMI) responses develop, and macrophages of the host have an increased capacity to kill the intracellular bacilli. The CMI responses are mediated by T lymphocytes, which release lymphokines (messenger proteins secreted by lymphocytes) that attract, immobilize, and activate additional blood-borne mononuclear cells at the sites where virulent *Mycobacteria* or their products exist (Chua and Deretic, 2004).

The cellular hypersensitivity that develops, contributes to cell death and tissue destruction (caseous necrosis). In some instances, liquefaction and cavity formation occur as a result of enzymatic action on proteins and lipids. Rupture of these cavities into the bronchi allows aerosol spread of bacilli. Activated macrophages migrate to blind endings of lymphatic vessels and course to one or more of the thoracic lymph nodes, either bronchial or mediastinal. Lymph nodes are more commonly infected than other tissues because fluids in an animal eventually pass through the nodes, where the meshwork of trabeculae entraps the microorganisms. The enlargement and presence of macrophages in impenetrable passageways between reticular cell fibers of the lymph node provide an environment for *Mycobacterial* growth and development of the granulomatous lesion in the node. On occasion, some phagocytized *Mycobacteria* remain in the lung, and both lung and thoracic nodes are affected. Primary lesions often become localized in a node or nodes and may become large and firm. Fibrous connective tissue development in the dynamics of granuloma formation probably contributes to the localization of lesions (Sharma and Adlakha, 1996; Kaneene and Theon, 2004).

Granuloma formation is an attempt by the host to localize the disease process and to allow inflammatory and immune mechanisms to destroy bacilli. A few lesions may appear to be regressing while becoming encapsulated by well-organized connective tissue; however, such lesions may contain viable bacilli. Typically, the microscopic appearance of a granuloma (tubercle) is focal and has some caseous necrosis in a central area encircled by a zone of epithelioid cells, lymphocytes, and some granulocytes. Mineralization may be present in necrotic centers; in more advanced lesions, several foci of mineralization may coalesce. Multinucleated

giant cells, which contain several nuclei, often in a horseshoe or ring shape near the cytoplasmic border, are often seen in the zone near the necrotic area. An outer boundary of fibrous connective tissue is usually present between the lesions and normal tissue (Brosch *et al.*, 2002; Fabre *et al.*, 2004). Occasionally, fibrous tissue is not apparent, and the lesion assumes a more diffuse appearance. Lesions caused by *M. bovis* may have few, if any, organisms that can be found by microscopic examination (Whipple *et al.*, 1996).

2.2.3. Virulence

The *Mycobacteria* do not produce the classic exotoxins and endotoxins (Sharma and Adlakha, 1996). Most of the virulence factors of *M. bovis* are the same as those of *M. tuberculosis*, as both organisms can cause identical clinical disease in humans and are genetically very similar. Virulence appears to reside in the lipids of the cell wall. Mycosides, phospholipids and sulpholipids are thought to protect the tubercle bacilli against phagocytosis. Glycolipids cause granulomatous response and enhance the survival of phagocytosed *Mycobacteria*. Wax D and various tuberculo proteins induce a delayed hypersensitivity reaction detected in the tuberculin test (Quinn *et al.*, 2002).

The development of disease in an animal depends on the ability of *M. bovis* to multiply within phagocytic cells and induce a host response while escaping the host bactericidal action associated with this response. Pathogenicity of *Mycobacteria* is a multifactorial phenomenon requiring the participation and cumulative effects of several components, including complex lipids and proteins in both the cell wall and the cytoplasm of tubercle bacilli (Cousins *et al.*, 2003). Complex lipids, extracted from both virulent and attenuated strains of *Mycobacteria*, have been extensively evaluated by *in vivo* or *in vitro* systems to obtain information on their significance in disease (Stamm *et al.*, 2003; Chua and Deretic, 2004).

The cell wall core of *Mycobacteria* is composed of three covalently attached molecules: peptidoglycan, arabinogalactan, and mycolic acid. Glycolipid complexes present in the cell wall of virulent and attenuated strains of *Mycobacteria* have been extensively examined to obtain information on their significance in granuloma formation. An important property of virulent bovine tubercle bacilli is their ability to form cords when grown in liquid culture medium. These

cords are consistently demonstrated in smears. Lipids or lipid complexes present in the cell wall of the virulent tubercle bacilli appear to contribute to the formation of these “ropelike” cords, which represent bacilli aligned in parallel form. Several lipid components have been isolated, chemically characterized, and evaluated in certain *in vivo* and *in vitro* systems to determine their importance in pathogenicity. Cord factor, a glycolipid, extracted with petroleum ether from viable tubercle bacilli, identified as trehalose-6, 6-dimycolate does not induce tuberculin sensitivity, but it does inhibit the migration of leukocytes and is leukotoxic. Moreover, cord factor has been found to induce swelling and disruption of liver mitochondria; the swollen mitochondria have decreased respiratory and phosphorylative activity (Edwards *et al.*, 2001). Also, cord factor was reported to induce disintegration of the rough endoplasmic reticulum and detachment of ribosomes in liver cells (Braunstein *et al.*, 2002). Furthermore, gene deletion mutants unable to synthesize cord factor, have normal initial replication but fail to persist within and kill infected mice (Glickman *et al.*, 2000), which underlies the importance of this virulence factor.

Improved biochemical techniques for purifying cell wall lipids have permitted the isolation of sulfur-containing glycolipids (sulfolipids, sulfatides) from *M. tuberculosis*. However, sulfated lipids are absent from the envelope of *M. bovis* because the glycolipid sulfotransferase and arylsulphatase genes are disrupted or deleted (Garnier *et al.*, 2003). This difference may also contribute to determine the host range and tissue tropism of *M. bovis*. As described above, LAM, the major *Mycobacterial* glycolipid, may contribute to arrest phagosome maturation. In addition, LAM is a powerful scavenger of oxygen (reactive oxygen intermediates, ROI) and nitrogen (reactive nitrogen intermediates, RNI) reactive intermediates (Stamm *et al.*, 2003; Chua and Deretic, 2004). Moreover, virulent strains of *M. tuberculosis* and *M. bovis* BCG contain mannosylated LAM. Mannosylated LAM provides another way for *Mycobacteria* to enter phagocytes via mannose receptors, and it serves as a ligand for the interaction with dendritic cells (a major antigen-presenting cell). The macrophage mannose receptor is involved in the presentation of LAM to T cells via CD1 molecules. However, no definitive correlation of mannosylated LAM to *Mycobacterial* virulence has yet been shown (Fenton *et al.*, 2005).

Proteins and protein complexes (i.e., lipoproteins) of *M. bovis* and other pathogenic *Mycobacteria* also play an important role in pathogenesis. Of particular interest are the secreted proteins in the antigen 85 complex, as they may play a role in development of CMI and disease in the host. Moreover, these proteins have been shown to possess enzymatic activity and catalyze mycolyltransfer reactions involved in the final stages of *Mycobacterial* cell wall assembly. Regarding binding activities, fibronectin binds to antigen 85 complex and the release of large amounts of this antigen could inhibit binding of fibronectin to tubercle bacilli. Although little or no evidence exists that fibronectin directly mediates phagocytosis of *Mycobacteria*, there is some evidence that fibronectin enables monocytes to phagocytose C3b-sensitized cells (Edwards *et al.*, 2001). This finding may be of importance, as it has been shown that complement receptors also mediate phagocytosis of *M. tuberculosis*. Proteins encoded by the *mce* operons seem to play a role in the entry and survival of *Mycobacteria* within phagocytic cells, as well as in the invasion of epithelial cells. There are four operons in *M. tuberculosis*, each encoding five to six proteins, whereas the *mce3* operon is absent from *M. bovis* (Garnier *et al.*, 2003).

Several stress proteins have been identified as major immunodominant antigens of *Mycobacteria*. The elevated synthesis of these proteins in response to changes in physiological conditions within the phagosome may protect the *Mycobacteria* from hydrolytic enzymes, ROI (i.e., superoxide anion), and RNI (i.e., nitric oxide-NO). Superoxide dismutases (SODs) are produced and released by several *Mycobacterial* pathogens (Chan *et al.*, 2001). *M. bovis* and *M. tuberculosis* possess a redundant system of two SODs. The iron manganese dependent SOD (Sod A) is secreted and seems the more fundamental enzyme for resistance against reactive oxygen intermediates. Another membrane-associated copper-zinc-dependent SOD (Sod C) may play an additional role to protect tubercle bacilli against the oxidative burst of activated macrophages (Vergne *et al.*, 2004). In contrast, protection against reactive nitrogen intermediates is provided by two alkyl hydroperoxidases denominated AhpC and AhpD (Kehres and Maguire, 2003). In addition, housekeeping functions such as enzymes involved in major metabolic pathways are also important for the virulence of *M. bovis*. For example, it has been shown that *M. tuberculosis* persistence in infected mice and macrophages requires the glyoxylate enzyme isocitrate lyase that is also present in *M. bovis*. Furthermore, mutations in many metabolic genes also result in the attenuation of the virulence of tubercle bacilli (Nathan and Shiloh, 2000).

2.3. Immunity

2.3.1. Immune response

The natural and acquired cellular immune response mechanisms of bovines are often successful in limiting proliferation of tubercle bacilli and development of progressive disease. Elimination of *M. bovis* from host tissues depends, in part, on killing the bacillus in mononuclear macrophages that have been activated by lymphokines. Immunity against *Mycobacteria* is multifactorial and dependent on the balance between an inflammatory response that allows the host to develop a granuloma, which contains the microorganism and an anti-inflammatory response that restricts the extent of the granuloma and allows contact of effector T cells with the infected cells, which results in the killing of the infecting pathogen (Villarreal-Ramos *et al.*, 2003).

In cattle, both humoral and cell-mediated responses can be induced following *M. bovis* infection. Although the importance of the latter in the pathogenesis of the illness is very clear, the role played by antibodies in immunity still remains unclear (Silva, 2001). The immune response to *M. bovis* is a T helper type-1 response as evidenced by IFN- γ , interleukin (IL)-2, and tumour necrosis factor (TNF)- α production to pathogen-associated antigen (Flynn, 2004). All of the main T-cell subsets ($\gamma\delta$ T-cells, CD4, CD8, and $\alpha\beta$ T-cells) have been shown to be involved in the anti-*Mycobacterial* immune response in cattle. The humoral immune response to *M. bovis* infection in cattle is characterized by highly heterogeneous antigen recognition. Six proteins, ESAT-6, 14-kDa protein, MPT63, MPT70, MPT51, and MPT32, were identified as major sero-reactive antigens in bovine tuberculosis (Pollock *et al.*, 2005).

Tuberculostatic murine macrophage functions are enhanced following exposure to interferon (IFN) gamma, whereas IL4 and tumor necrosis factor do not increase these activities. However,

IFN-gamma and tumor necrosis factor act synergistically to induce increased production of NO and RNI in mouse macrophages that are able to inhibit growth and kill intracellular *M. tuberculosis* and *M. bovis* BCG. Significant studies have been performed on the role of RNI and ROI in *Mycobactericidal* action. Mouse macrophages release abundant NO production on infection with *Mycobacteria*. This release is mediated by IFN-gamma-inducible nitric oxide synthase (also referred as iNOS). Several studies indicated that cultured human macrophages did not produce iNOS upon *Mycobacterial* infections. However, *in vivo* studies have shown significant induction of NO production (Kehres and Maguire, 2003).

Current evidence indicates that human macrophages do possess iNOS activity, but that the levels of NO production are highly dependent on the state of differentiation of the phagocytes and the tissue of origin. For example, alveolar and peritoneal macrophages display significant NO production. In summary, RNI, especially NO, may play the more fundamental role in the control of *Mycobacterial* infections. This view is supported by the fact that NO has both bactericidal and immunomodulatory effects, in contrast to ROI, which are only bactericidal. Moreover, RNI and ROI may also act synergistically by the formation of the highly bactericidal compound peroxynitrite. In cattle, iNOS activity and NO production have been shown to occur in cultured bovine macrophages in response to *Mycobacterial* infections, but they do not seem to require stimulation with bovine IFN- γ . Thus, iNOS synthesis in cattle seems to differ in its regulation by lymphokines (Nathan and Shiloh, 2000).

Processing of *Mycobacterial* antigens by macrophages and presentation to T lymphocytes also play a key role in the release of the appropriate lymphokines necessary for full activation of the bactericidal mechanisms of phagocytic cells. T lymphocytes can be divided in T-helper (CD4) and T-cytotoxic cells (CD8,). T-helper cells are further subdivided on the basis of their lymphokine profiles. Type 1 cells (Th1) produce IFN-gamma and interleukin 2, which stimulate macrophage activation, whereas type 2 cells (Th2) produce interleukin 4, which leads to a humoral response. Th1 and Th2 cells operate in a reciprocal fashion, whereby cellular and humoral immune responses are mutually antagonistic. Thus, Th2 cells are associated with exacerbation and rapid lesion formation in several chronic infectious disease models. Therefore, the existence or absence of an immune response does not predict protection; this is dependent on

the balance between the various types of host responses and the virulence of the bacterial strain. The availability of transgenic “knockout” mice with disruptions in genes critical for immunological functions has provided new tools for delineating the role of the various immune responses in protection from infection. Mice lacking the gene for beta-2 microglobulin are unable to express the major histocompatibility complex class I (MHC class I). MHC class I molecules are required for presenting antigens to cytotoxic lymphocyte; hence, these mice lack the ability to generate cytotoxic lymphocyte. It was assumed that only CD4 T cells capable of making appropriate lymphokines were involved in protection against tuberculosis. However, it has been shown that mice that are unable to generate MHC class I restricted T cells die extremely rapidly from *M. tuberculosis* infection. Interestingly, these mice can survive *M. bovis* BCG infection, indicating that cytotoxic lymphocyte is necessary for protection against tuberculosis (Chan *et al.*, 2001).

Deficiencies of T lymphocyte and macrophage functions have been associated with opportunistic *Mycobacterial* diseases (Haile *et al.*, 2005). These abnormalities may be mediated by increased conversion of arachidonic acid into prostaglandins via the cyclooxygenase pathway. In this context, lymphocyte responses to a purified protein derivative from *M. avium* and *M. intracellulare* improved in cultures containing indomethacin, an inhibitor of cyclooxygenase. Activated mononuclear macrophages exhibit marked morphological changes, including increases in the number of mitochondria, spreading ability, and phagocytic and microbicidal activities. Activated alveolar macrophages from rabbits receiving *M. bovis* BCG (heat killed in oil) showed increases in tricarboxylic acid cycle and hexose monophosphate shunt activities in comparison to normal cells (Garnier *et al.*, 2003).

Macrophage functions such as chemotaxis, phagocytosis, enzyme secretion, and cytotoxicity are also influenced by intracellular nucleotide levels. Studies on the activation of adenylate cyclase in isolated macrophage membranes revealed that guanine-5-monophosphate, present in the macrophage membrane, regulates adenylate cyclase activation (Vergne *et al.*, 2004). Furthermore, certain chemical mediators such as prostaglandins can exert their effect on cyclic adenosine-5-monophosphate production by stimulating membrane-bound adenylate cyclase. Macrophages infected with live *M. bovis* BCG have increased cyclic adenosine-5-

monophosphate levels and reduced phagolysosome formation. Concerns when conducting tuberculin skin tests in animals are related most often to the failure of some *M. bovis*-infected animals with advanced lesions to respond to purified protein derivative tuberculin. The analytical sensitivity of these tests in detecting tuberculosis varies for animals in a population. In animals with healed lesions, dormant infections, or advanced disease, test responses may be diminished. The problem of non responsiveness can be minimized by removing all test-responder animals on the first test. Repeated testing appears to increase the number of *M. bovis*-infected animals failing to respond to *M. bovis* purified protein derivative in populations in which infection persists. Changes in responsiveness to tuberculin in skin tests have been attributed in part to lymphokine-induced immunosuppression. *M. bovis* and *M. tuberculosis* are formidable pathogens that appear to require an orchestrated immune response to engender protection (Fabre *et al.*, 2004).

In summary, both CD₄-helper T cells producing lymphokines such as IFN gamma that can activate bactericidal macrophage effectors mechanisms and CD₈ MHC class I restricted cytotoxic T lymphocytes are necessary. The model would hold that activated macrophages can kill or inhibit the growth of small numbers of invading organisms. However, if *Mycobacteria* escape this immunological surveillance and reach a critical number, an individual macrophage is no longer capable of killing or inhibiting growth of the microorganism and is likely to spread the infection. Thus, only by lysing macrophages overwhelmed by the infection and creating inflammation is it possible to reduce the number of invading bacilli to a level that can be resisted by new infiltrating mononuclear phagocytes. This ultimately raises the question of whether pathogenesis and tissue damage are the price one pays for protection against tuberculosis. However, it is not known whether protective immunity requires a strong delayed-type hypersensitive response. In this context, vaccines eliciting strong hypersensitive responses may be less protective than vaccines eliciting weaker responses (Nathan and Shiloh, 2000).

2.3.2. Natural resistance to Mycobacterial infections

Some animals and humans display increased natural resistance to *Mycobacterial* infections. The molecular bases of this effect have been extensively investigated, but contradictory results across

and within host species have raised substantial controversy. In mice, it seems that the Nramp1 protein plays an important role in resistance. This protein is a divalent cation transporter and has affinity for both iron and manganese. Definite polymorphisms leading to non conservative changes in the amino acid sequence of the host Nramp1 protein have been associated with increased natural resistance to *Mycobacterial* infections, with the resistant allele being dominant. More recently, an Nramp1 orthologue (e.g., genes in different species that derive from a common ancestor) has been found in *M. bovis* and *M. tuberculosis*. This finding led to the hypothesis that the resistant Nramp1 protein in the host may compete favorably with the *Mycobacterial* counterpart (Mramp) and decrease the availability of iron and manganese for the bacilli (Vergne *et al.*, 2004). However, this hypothesis is difficult to reconcile with the finding that disruption of the bacterial gene in *M. tuberculosis* does not affect virulence in mice (Haile *et al.*, 2005).

Nramp1 orthologue is also present in cattle, but in this case, polymorphisms associated in mice with resistance failed to protect cattle against tuberculosis. This indicates that in bovines, Nramp1 polymorphisms cannot account for natural resistance. In humans, some evidence predicts a role of Nramp in resistance, but more extensive studies are needed. Moreover, in all animal species, there is a family of Nramp proteins, making it difficult to sort out individual effects. Thus, extensive investigations are necessary to reach a more thorough understanding of the resistant phenotype in the various hosts. (Chan *et al.*, 2001; Haile *et al.*, 2005)

2.4. Epidemiology

Mycobacterium bovis combines one of the widest host ranges of pathogens with a complex epidemiological pattern, which involves interaction of infection among human beings, domestic animals and wild animals. However, only little is done particularly in developing countries on the epidemiology of this organism and the epidemiological requirement for its control (Menzies and Neill, 2000).

2.4.1. Source of infection and mode of transmission

Mycobacterium bovis infected cattle are the main source of infection for other cattle. However, some wild animal populations such as the European badger in Ireland, the brush tail possum (*Trichosurus vulpecula*) and the ferret (*Mustela furo*) in New Zealand, the feral pig (*Sus scrofa*) and feral Asian water buffalo (*Bubalus bubalis*) in Australia, white tailed deer (*Odocoileus virginianus*) in Michigan, and African buffalo in South Africa are sources of infection for domestic livestock and humans (Michel, 2006). The disease can be transmitted either by the respiratory route or ingestion. In cattle, aerosol spread is more common (OIE, 2005). The dynamics of *M. bovis* transmission is poorly understood and the conditions under which a tuberculous animal becomes an effective disseminator of infection are currently not defined although environmental contamination appears to be a less effective method of disease transmission. Field studies indicate a wide spectrum of transmission rates but generally the spread of *M. bovis* infection is still considered to be a relatively slow process (Menzies and Neill, 2000).

2.4.2. Disease distribution

Tuberculosis has been found world wide. European countries as well as the United States, Canada, Japan, and New Zealand report a prevalence of bovine infection lower than 0.1%. A few countries including Australia, Denmark, Sweden, Norway, and Finland are considered to be free of bovine tuberculosis. However, the disease is endemic in almost all countries of Africa (Acha and Szyfer, 2001; OIE, 2005)

Status in Africa

In sub-Saharan Africa, humans and animals share the same microenvironment and water holes, especially during drought and the dry season, thereby potentially promoting the transmission of *M. bovis* from animals to humans. According to Cosivi *et al.*, (1998), 60% of the African, 47% of the Asian, and 38% of the Latin American and Caribbean countries have reported the occurrence of BTB from sporadic to enzootic levels. Approximately 85% of the cattle and 82% of the human populations of Africa live in areas where BTB is either partly controlled or not controlled at all. In such countries, where BTB is still common and pasteurization of milk is not practiced, an

estimated 10 to 15% of human TB cases are caused by *M. bovis*. Of 55 African countries, 25 reported sporadic/ low occurrence of bovine TB; six reported enzootic disease; two Malawi and Mali, were described as having high occurrence; four did not report the disease; and the remaining 18 countries did not have data. Of the nations in Africa, only seven apply disease control measures as part of a test and slaughter policy and consider bovine TB a notifiable disease ; the remaining 48 control the disease inadequately or not at all. Almost 15% of the cattle population is found in countries where bovine TB is notifiable and a test and slaughter policy is used (Ashford *et al.*, 2001).

Status in Ethiopia

Ethiopia is one among the nations that possesses the largest livestock population in the African continent. The distribution and the quantity of each species are different according to the type of prevailing animal production systems and agro-ecological zones. In contrast to the huge livestock resource, the livestock productivity is, however, found to be very low. The major biological and socio-economical factors attributing to the low productivity includes: the low genetic potential and performance, poor nutrition (in quality and quantity terms), the prevailing of different diseases, traditional way of husbandry systems and inadequate skilled manpower among others (Ameni, 2008).

Ethiopia is one of the African countries where BTB is considered as prevailing disease in animals. Detection of BTB in Ethiopia is carried out most commonly on the basis of tuberculin skin testing, abattoir meat inspection and rarely on bacteriological techniques. However, the current status on the actual prevalence rate of BTB at a national level is yet unknown. In Ethiopia, screening of cattle by the tuberculin skin test was sporadic until 1984. But, while the higher prevalence rate of the disease has been observed after the two year survey in government and some “parastatal” dairy farms, then it was decided to embark on a routine BTB survey, on these dairy farms, in particular using single and comparative intradermal skin tests (Alemu, 1992). Since then tuberculin skin test and abattoir meat inspection surveillances have been undertaken in different parts of the country at various times. However, still there is lack of knowledge about the actual prevalence and distribution of the disease at a national level. Despite

this, the economic impacts and zoonotic importance of the BTB infection are either not well studied or documented as cited by Regassa, 2005.

A study undertaken in previous years, 1984–1986, has shown the prevalence rate of 16.7% of BTB based on tuberculin skin tests on the government state farms and other “parastatal” dairy farms (Alemu, 1992). Among the recently undertaken studies, the prevalence rate of BTB ranges from 3.4% in a small holder production system to 50% in intensive dairy productions has been reported in various places of the country (Ameni and Roger, 1998; Ameni *et al.*, 2003a,b; Asseged *et al.*, 2001; Regassa, 2005) as indicated in Tables 2, 3 and 4.

In Ethiopia, exotic breeds were found to be more susceptible than cross and local breeds to *M. bovis* with manifestation of high incidence and prevalence rates (Kiros, 1998; Ameni *et al.*, 2006). In addition, a herd prevalence rate of 42.6% to 48.6% was found to be higher than the prevalence rate of individual animals (7.9% to 18.7%), that may indicate that the herd size can favor the transmission of BTB in intensive dairy farms in particular (Shitaye *et al.*, 2006)

Table 1: Prevalence of bovine tuberculosis detected by tuberculin skin test in a traditionally managed extensive production system

Areas of study	No of cattle			Reference
	Examined	Positive	%	
Assella*	281	25	8.9	Teshome, 1986
Debre-Birhan*	76	11	14.5	Tadele, 1998
Kombolcha	53	12	22.6	Tadele, 1998
Dessie	34	4	11.8	Tadele, 1998
West Wellega	353	12	3.4	Regassa, 2001
North Shewa *	1041	169	16.2	Regassa, 2005
Total	1838	233	12.9	

*A comparative intradermal tuberculin skin test

Table 2: Prevalence of BTB in small holder dairy farms based on tuberculin skin test.

Areas of study	No of cattle			Reference
	Examined	Positive	%	
Holleta*	389	25	6.4	Teshome, 1986
Selale*	1528	78	5.1	Teshome, 1986
Wuchale-Jida*	763	60	7.9	Ameni <i>et al.</i> , 2003a
Assella*	514	18	3.5	Redi, 2003
Addis Ababa	473	61	12.9	Alemu, 1992
Total	4818	332	6.9	

*Comparative skin tests with bovine and avian tuberculin

Table 3: Prevalence of BTB detected by tuberculin skin tests in intensive dairy farms.

Area of study	No of cattle			Reference
	Tested	Positive	%	
Addis Ababa	2098	392	18.7	Shitaye <i>et al.</i> , 2006
Ambo	133	37	27.8	Ameni <i>et al.</i> , 2003b
Asella	281	23	8.2	Alemu, 1992
Debre-Birhan*	51	3	5.9	Tadele, 1998
Debre-Zeit	788	234	29.7	Kiros, 1998
Dessie*	121	89	73.6	Ameni <i>et al.</i> , 2003b
Holleta	70	17	24.3	Ameni <i>et al.</i> , 2003b
Repi	481	310	64.4	Anonymous, 1999
Sebeta	37	4	10.8	Ameni <i>et al.</i> , 2003b
Sellale	44	3	6.8	Ameni <i>et al.</i> , 2003b
State dairy farms*	6940	1217	17.5	Alemu, 1992
Ziway*	205	56	27.3	Ameni <i>et al.</i> , 2003b
Total	14454	3560	24.6	

*Test conducted only by a single intradermal skin test with bovine tuberculin.

Table 4: Prevalence of BTB detected by abattoir meat inspection in cattle.

City abattoir	No of cattle			Reference
	Inspected	Positive	%	
Addis Ababa	1350	20	1.48	Asseged <i>et al.</i> , 2004
Addis Ababa	984	34	3.46	Shitaye <i>et al.</i> , 2006
Ghimbi	758	116	15.3	Woyessa, 2007
Hossana	751	34	4.53	Teklu <i>et al.</i> , 2004
Hawassa	1023	11	1.1	Regassa, <i>et al.</i> , 2009
Nekemte	940	48	5.1	Gudeta, 2008
Nazareth	1125	58	5.16	Ameni and Wudie, 2003
Total	246611	1517	0.62	

Source: Shitaye *et al.*, 2006 (for Tables 2, 3, 4 and 5)

2.4.3. Risk factors

Environmental risk factor

Several husbandry practices and environmental factors were reported to influence the occurrence of BTB in cattle. Factors that play role in predisposing animals to infection include type of production (dairy cattle are at risk because husbandry methods allow close contact between animals at milking and when housed during winter months), suckling (calves become infected by ingesting contaminated milk), and presence of wild reservoirs of *M. bovis* infection for grazing cattle (Radostits *et al.*, 1994). The prevalence of BTB infection was higher in the intensive than pastoral production systems and in the small-scale dairy sector than in other dairy-production systems in the eastern Zone of Tanzania (Shirima *et al.*, 2003). Cattle movement, purchase, grazing field boundaries, spreading cattle slurry on the fields are a potential risk for transmission of the disease between cattle.

There is evidence that the transmission of the disease between cattle following movement accounts for 10–15% of 17 outbreaks in the British and that transmission can occur across farm boundaries (Phillips *et al.*, 2003). Recent introductions from market, Provision of water from mud holes in dry river beds during dry season, and the presence of monkeys or warthogs are risk factors associated with BTB infection of cattle identified in transhumance areas of Uganda (Oloya *et al.*, 2007).

Circumstantial evidence suggests that an adequate intake of mineral, vitamin and protein reduces the susceptibility of cattle to BTB infection. Weather patterns have been implicated in the susceptibility of herds. A high reactor rate has been observed among cattle grazing in the hot and dry low lands (14%) than in those grazing in the cool and wet high lands (8%) indicated that higher infection rate was attributed to the congregation of animals during the dry period when grazing becomes scarce. The existence of a widespread intermediate host is also reported as the greatest contributor to infection in cattle (Kazawala *et al.*, 2001; Phillips *et al.*, 2003).

Host factors

Many individual animal and herd level characteristics are reported to be associated with BTB infection. Breed susceptibilities to BTB infection differ: zebu cattle are more resistant than European breeds. The higher prevalence of BTB in dairy than beef cattle may reflect closer confinement, longer life spans, and greater productivity stress among dairy cows. Exemption from pregnancy and lactation may explain the lower disease prevalence in bulls than cows (Biberstein and Hirsh, 1999).

Risk of a positive reaction varies with an animal's age and body condition. Besides, immunological suppression in the periparturient period can produce anergic reactors, which may act as a constant source of infection for cattle-to-cattle transmission (Foster *et al.*, 2002). Kazawala *et al.* (2001) identified increased skin test reactivity in older animals than yearlings and calves, in animals with exotic blood than short horn zebu, in bulls often used for drought power than the entire bulls mainly used for breeding, in lactating than not lactating cows and in males than females. Herds with the following risk factors had a significantly greater prevalence of intra

dermal test positives; greater than 50 cattle in the herd; herds housed inside at night and herds in contact with wildlife. Furthermore, villages that experienced annual flooding had a higher prevalence of infection (Cleaveland *et al.*, 2007). The herds of those households with a reported human TB cases are more likely to have a tuberculin positive animal than herds in households without a reported human TB case (Regassa, 2005).

Pathogen risk factors

The tubercle bacilli of various types are quite sensitive to heat and are killed at 60 °C in 15 minutes. They are also susceptible to ionic detergents and are killed by sunlight, ultraviolet irradiation, and pasteurization. However, tubercle bacilli are usually resistant to drying, to most disinfectants, acids and alkalis. This resistance is due to hydrophobic lipid surface. They also remain viable for long period in the dark, putrefying material, faces, sputum and soil (Sharma and Adlakha, 1996; Biberstein and Hirsh, 1999). *Mycobacterium bovis* is an obligate pathogen and is highly susceptible to sunlight (UV radiation, but it can survive for several months in the environment, in moist soil and in darkness and remains viable for long periods (6 months) in frozen tissues (Jahans and Worth, 2006). It survives in acid milk for 15 days and in milk products such as cheese and butter for weeks but it gets destroyed when milk is heated for 30 minutes at 65 °C, and cream is heated at 85 °C for 2 minutes. The organism can also survive for over 2 years in frozen carcasses. Natural exposure to *M. bovis* or environmental *Mycobacteria* may assist in the development of specific immunity (Foster *et al.*, 2002).

2.5. Importance of bovine tuberculosis

2.5.1. Public health importance

Bovine tuberculosis is a significant zoonosis that can spread to human. Epidemiologic investigations reveal that the organism may be ingested or inhaled. Extra pulmonary lesions may occur associated to the consumption of contaminated milk, even though with the practice of boiling milk, and the growth of milk pasteurization plants all over the world, the digestive route

of infection became less important. On the other hand, airborne infection continues to occur among meat industry and slaughterhouse workers, in regions where the infection is still prevalent in cattle. In developed countries, eradication efforts have significantly reduced the prevalence of this disease, but reservoirs in wildlife make complete eradication difficult (OIE, 2005). However, bovine tuberculosis is an increasing threat to human health in many developing countries due to the endemic nature of the disease in domestic stock or wildlife and the likely contact with humans, particularly those infected with HIV (Neil *et al.*, 2002).

The paucity of information on the prevalence of *M. bovis* infection in human makes the precise identification and quantification of the threat of bovine tuberculosis to human populations difficult, especially in developing countries. In Latin America and the Caribbean countries, *M. bovis* is estimated to cause 2% of all human cases of tuberculosis. Slaughterhouse and dairy farm workers are most-frequently infected, with infection occurring via the respiratory tract (Isable and Viviana, 2006). Although other infectious diseases that are more fatal for animals are prevalent in Africa, bovine TB is a significant zoonotic human pathogen that aggravates the 'triple trouble' of HIV/AIDS, TB infection and malnutrition (Ayele *et al.*, 2004). Prior to the epidemic of HIV infection, approximately 15% of newly reported cases of TB involved only extra pulmonary sites. For reasons that are not understood, as rates of pulmonary TB decreased, rates of extra pulmonary TB remained constant, resulting in an increasing proportion of patients being extra pulmonary cases. With the onset of HIV epidemic both absolute and relative rates of extra pulmonary involvement have increased (Stewart *et al.*, 2005).

In Ethiopia, case- control studies have shown that prevalence of tuberculosis was high in cattle owned by tuberculous human patients than those owned by non-tuberculous patients indicated that reactor cattle were risk factors for human tuberculosis. Questionnaire surveys have revealed that cattle owners who consumed raw milk were at higher risk of acquiring TB than those who consumed boiled milk (Regassaa, 2005; Ali, 2006). The proportion of patients with extra pulmonary tuberculosis was high in younger age groups, in farmers, in patients with close contact to cattle, in those who frequently drink raw milk and in people from rural areas (Kiros, 1998; Regassa, 2005). Kidane *et al.* (2002) has indicated that among 35 PCR positive human patients of tuberculous lymphadenitis from Southern Ethiopia, 29 (82.9%) were caused by *M. tuberculosis*.

and 6 (17.1%) were *M. bovis*. This indicates that bovine tuberculosis could contribute to the increasing human tuberculous lymphadenitis cases of Ethiopia.

2.5.2. Economic importance

Bovine tuberculosis is important not only because it is a source of human infection, but also because of the economic losses it causes. Losses in meat inspection due to organ or whole carcass condemnation are considerably high particularly in countries with high prevalence of BTB with strict law of meat hygiene (Acha and Szyfer, 2001).

In Ethiopia the estimated cost of organs and carcasses condemned during meat inspection of 1.2 million cattle slaughtered in six export abattoirs was 600,842 Ethiopian birr equivalent to US \$300,000 at that time (Gezahegn, 1991). Recently, the condemnation rate of carcasses and organs due to tuberculosis infection in cattle slaughtered in Adama municipality abattoir was showed to be increasing from 0.38% to 1.9% from 1999 to 2003 (Ameni and Wudie, 2003). In addition, Asseged *et al.*, 2004 reported that the annual rate of whole- carcass condemnation due to generalized tuberculosis in Addis Ababa abattoir was 0.024% and it has increased annually by 0.34% over the past decade.

Loss due to milk production is also when infected cattle develop tuberculosis mastitis; 1-2% of infected cows may develop mastitis. In Hungary there was a report that ,infected cows produce 10-12% less milk than healthy once, similarly in Poland the milk production of was calculated to be 30% bellow the normal production (Morris *et al.*, 1994).

In Madagascar losses in weight gain of the Zebu cattle was found to be 12% at intensive fattening of 100 days and 28.7% in pasture fattening of 200 days. Infertility is another problem in tuberculous cows; 5-10% of the infected cows may develop tuberculous metritis out of which 5% may become infertile. It is also reported that 1% of the calves from tuberculous dams infected congenitally leading to death or retarded growth. Such cows may cull before they finish their production life that incurs additional loss (Radostits *et al.*, 1994). Costs associated with human tuberculosis due to *M. bovis* include cost of treatment, hospitalization, loss of working days and

payment for sick leave. Even though difficult to quantify, it was estimated to be 6.2 billion Lira in Italy between 1965 and 1978 (Barwinek and Taylor, 1996).

2.6. Diagnosis

2.6.1. Clinical examination

Because of chronic nature of the disease and multiplicity of signs caused by the variable localization of the infection, bovine tuberculosis is difficult to diagnose based on clinical examination. However, the disease has chronic debilitating nature, but can occasionally be acute and rapidly progressive. Early infections are often asymptomatic. In the late stages, common symptoms include progressive emaciation, a low-grade fluctuating fever, weakness, and inappetence. Animals with pulmonary involvement usually have a moist cough that is worse in the morning, during cold weather, or exercise, and may have dyspnea or tachypnea (OIE, 2005).

2.6.2. Immunological diagnosis

Tuberculin skin test

In live cattle, tuberculosis is usually diagnosed in the field with the tuberculin skin test. The tuberculin tests are the internationally accepted standard and the most robust tool currently available for the diagnosis of infection by the bovine TB organism. The test detects a delayed type hypersensitivity reaction to the intradermal injection of tuberculin (Richard, 2006).

The intradermal tuberculin test using purified protein derivative (PPD) prepared from the culture filtrate of *M. bovis* by precipitation with ammonium sulfate or trichloroacetic acid is widely used to identify tuberculous cattle and other animals in countries throughout the world. The protein content of the PPD is determined and biologic activity evaluated in sensitized guinea pigs. The single caudal fold intradermal test, the single cervical intradermal test, and the comparative

cervical test are the tuberculin tests used in most bovine tuberculosis control or eradication programs (Kaneene *et al.*, 2004). The injection site is visually observed and palpated at 72 hours following injection of *M. bovis* PPD, and any inflammatory response is classified as suspect. Cattle with suspicious reactions to the caudal fold test are subsequently subjected to a comparative cervical test to determine their relative responsiveness to biologically balanced *M. bovis* and *M. avium ss avium* PPD (Adams, 2001).

Cattle infected with *M. bovis* will develop more induration (measured as an increase in skin thickness at the site of injection) in response to the *M. bovis* PPD than to the *M. avium* PPD, except in animals with advanced disease that have become non responsive to tuberculin. The increase in skin thickness at each injection site is plotted on a scattergram. The results are classified as negative, suspect, or reactor (positive) (Theon and Barletta, 2004). The specificity and sensitivity of a diagnostic test should be determined in naturally sensitized *M. bovis* infected cattle and in cattle in *M. bovis* free herds in the geographical region under conditions in which the test will be used (Adams, 2001). This is important because nonspecific sensitization caused by other organisms that share antigenic determinants may vary for different regions and countries. For example, other *Mycobacteria*, including *Mycobacterium* subsp. *Avium* *Mycobacterium avium* subsp. *paratuberculosis*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum*, *Mycobacterium kansasii*, *Mycobacterium fortuitum*, and *M. tuberculosis*, may induce tuberculin skin sensitivity, but they do not produce progressive pulmonary disease in cattle (Ryan *et al* 2000)

The gamma-interferon assay

In vitro cell-mediated assays that monitor cell responses to PPD by incorporation of tritiated thymidine or the production of gamma interferon have been developed and evaluated in cattle and other animals in several countries. In addition, *in vitro* assays have been developed to detect antibodies in *M. bovis* infected cattle (Theon and Barletta, 2004). The tuberculin skin test, which involves the injection of 0.1 ml PPD intradermally and observation of the injection site at 72 hours in cattle, requires the handling of cattle twice. An *in vitro* blood test would provide a

distinct advantage because animals would be restrained only once; this is especially important when working with wild animals (Adams, 2001; Kaneene *et al.*, 2004).

In order to enhance the success rate of diagnosis of *M. bovis* infection, an assay system for gamma-interferon has been developed to detect and quantify release of the cytokine, when whole blood is cultured with bovine and avian tuberculin. The strategic use of the IFN gamma assay can provide a means for the early identification of *mycobacterium bovis* infected cattle, thus ensuring their removal from an infected herd. When used in parallel with the tuberculin test, it is capable of identifying infected cattle, which might otherwise not be detected until later, if at all (Gormley *et al.*, 2006). It has been reported that performance of the test can be influenced by various factors including a recent tuberculin skin test and the length of delay between collection and processing of blood samples. Gormley *et al.*, 2004 has demonstrated that a delay in processing of the blood samples from cattle subjected to routine surveillance could have significant impact on the outcome of the IFN gamma assay resulting in a change of gamma-interferon status of the animals.

2.6.3. Post mortem examination

Bovine tuberculosis is characterized by the formation of tuberculous, granulomas (tubercles) where bacteria have localized. These granulomas are usually yellowish and caseous, or calcified, and often encapsulated. Granulomas are most often found in the mediastinal, retropharyngeal, and portal lymph nodes. They are also common in the lung, Spleen, liver, and the surfaces of body cavities. In disseminated cases, multiple small granulomas may be found in numerous organs. Many infected cattle have only a few lesions at necropsy (OIE, 2005). Detailed post mortem examinations in abattoirs of Ethiopia have shown that tuberculous lesions were found in the mediastinal and head lymph nodes (Asseged *et al* 2004; Ameni, 2008).

2.6.4. Culture and direct microscopy

The egg based Lowenstein-Jensen and Stone brinks media are more commonly used in Mycobacteriology. *M. tuberculosis*, *M. avium* and many atypical *mycobacteria* require glycerol for their growth. However, glycerol is's inhibitory to *M. bovis* while sodium pyruvate (0.4%) enhances it growth. *M. bovis* has small, moist-sheen colonies that break up easily in 3-8 weeks of growth time. The Ziehl Neelsen (acid fast stain) is used to stain smears from lesions and other specimens. The *Mycobacteria* appear as slender, often beaded, red-staining rods against a blue background, if methylene blue is a counter stain (Quinn *et al.*, 2002).

2.6.5. Molecular Diagnosis

Epidemiological identification of reactor and diseased animals is a necessary part of the effective control and eradication of bovine tuberculosis in domestic livestock. By applying molecular techniques together with traditional epidemiology trace back approaches, important insights can be made regarding the sources of infection and the identification of practices or environments that may aid the spread and maintenance of tuberculosis. The basis of all molecular genotyping techniques relies on the acquisition of genetic mutations over time within specified regions of the bacterial genome, thus creating unique profiles between isolates. Although two bacterial strains of the same species have virtually identical genomes, differences will inevitably occur at a few loci. These differences may be small, such as the alteration of a single nucleotide that may either create or remove a specific restriction enzyme site, or larger changes may occur leading to the replication or deletion of DNA fragments hundreds of base pairs in size. Therefore, epidemiologically related strains will show identical or very similar genetic profiles, whereas unrelated clinical isolates will have divergent patterns (Liebana *et al.*, 2000).

The specificity and discriminatory power of any molecular epidemiology technique are dependent on the frequency at which the targeted genetic element is present in the bacterial genome. The higher this frequency, or copy number, the greater the certainty that two identical results from one method represent epidemiologically related strains. When typing methods only

detect low copy numbers of a genetic element, no conclusion about clonal identity among bacterial strains can be drawn, and other methods may be needed to verify relationships. Thus, the choice of methods for molecular epidemiology work must be selected with respect to the end goal, be it epidemiological identification of strains involved in a disease outbreak or a more global perspective of biogeographic distribution of isolates from a phylogenetic perspective (Shah *et al.*, 2002).

New molecular epidemiological tools beyond the standard fingerprinting reference technique have been described recently for *M. bovis*, expanding the repertoire of techniques available for genotyping isolates recovered from disease outbreaks. These new techniques include spacer oligotyping (spoligotyping) and variable number tandem repeat typing (Van Soolinger *et al.*, 1991).

Polymerase chain reaction (PCR)

DNA amplification by PCR provides a rapid and sensitive method for the detection of *M. tuberculosis* complex. The development of any species-specific PCR assay requires that the target gene or DNA fragment be present in all the isolates from the particular species of interest and be absent from all other unrelated species (Bakshi *et al.*, 2005). The PCR technique is much faster than culture, provides detection of *M. bovis* when rapidly growing *Mycobacterium* species are present in the sample and may be able to detect the presence of *M. bovis* in samples even when organisms have become nonviable. It could be used as a rapid screening technique which would be complementary to culture of tissue specimens for the routine diagnosis of bovine tuberculosis (Liebana *et al.*, 2000). Generally, a sample found to be positive by either culture or PCR is regarded as a true positive. However, a negative result by either method means that *M. bovis* was not detected in the sample of tissue that was tested. Other samples of tissue from the same animal could harbour *M. bovis*. Therefore, a negative test result from PCR does not provide conclusive proof that an animal is not infected with *M. bovis*, especially if the animal originated from an infected or suspect herd. PCR requires sophisticated laboratory equipment, facilities, and advanced technical skills (Haddad *et al.*, 2004; Shah *et al.*, 2002).

A conventional PCR generally only uses one set of primers. Several genes and insertion sequences have been targeted in attempts to develop PCR assays to differentiate between *Mycobacteria* in general and between members of the *M. tuberculosis* complex in particular (Jahans and Worth, 2006). PCR amplification of genomes such as 16S rRNA, MTP40 gene, MPB70 gene, *pncA* gene, the *oxyR* gene and the regions of difference (RD) were used to differentiate the genus *Mycobacterium* or, *M. tuberculosis* from *M. bovis* isolates (Haddad *et al.*, 2004). The genomic regions of difference that are either present or absent in different members of the *Mycobacterium tuberculosis* complex are presented in table 6.

Multiplex Polymerase chain reaction (m-PCR) is used to test acid-fast organisms which are neither recognized by spoligotyping nor show growth characteristics typical to *M. bovis* (Jahans and Worth, 2006). A single-step m-PCR assay for distinguishing (1) between the *Mycobacterium tuberculosis* complex and *Mycobacteria* other than tuberculosis targeted the 16S and the 23S rDNA and (2) between *M. tuberculosis* and *M. bovis* species targeted the *oxyR* gene have been developed. Besides, a single tube m-PCR has been developed and said valuable where bovine and human tuberculosis co-exist, and the distinction of *M. bovis* from *M. tuberculosis* is required for monitoring the spread of *M. bovis* to humans (Shah *et al.*, 2002).

Table 5: The presence and absence of genomic regions of difference in MTBC members.

RD	<i>M.</i>	<i>M.</i>	<i>M.</i>	<i>M.</i>	<i>M.</i>	<i>M.</i>	<i>M.</i>
Locus	<i>tuberculosis</i>	<i>Canettii</i>	<i>africanui</i>	<i>microti</i>	<i>pinnipedii</i>	<i>caprae</i>	<i>bovis</i>
			<i>m</i>				
1 TbD1	+/_	—	—	—	—	—	—
2 RD9	+	+	—	—	—	—	—
3 RD10	+	—	—	—	—	—	—
4 RD1 _{Mic}			+	—	+		
5 RD12	+	—	+	+	+	—	—
6 RD4	+	+	+	+	+	+	—
7 RD1	+	+	+	+	+	+	+
8 RD2 _{Seal}			—	—	—		
9 RD _{Can}	—	+	—	—	—	—	—

Source: Adapted from Fabre *et al.*, (2006).

Note: (+) = Present, (–) = absent, () = Unknown

Spoligotyping

Another region of the *M. tuberculosis* complex chromosome that has been exploited recently for molecular epidemiology studies is the direct repeat (DR) locus. This single locus is composed of a series of 43 identical DR sequences. Each DR is 36 bp long, interspersed by spacer sequences varying in length from 35 to 41 bp. The DR region is also a preferred “hot spot” for IS6110 insertions. For *M. bovis* strains carrying only one copy of this transposable element, IS6110 is inserted into the DR located between spacer sequences 24 and 25. As a consequence, *M. bovis* genotypic variations in the DR region can be directly correlated with polymorphisms in IS6110 RFLP types (Costellow, 1999).

DR region is the basis of a novel Mycobacterial strain-typing technique called “reverse line blot hybridization,” or spoligotyping. In spoligotyping, polymerase chain reaction (PCR) products are

amplified using primers specific for the DR sequences. Thus, products of multiple sizes are generated, representing all the spacer sequences present in an isolate's genome. Using a specialized dot-blot apparatus, this product mixture is then hybridized against a membrane to which the 43 individual spacer sequences have been covalently linked, generating a specific pattern (spoligotype) of positive and negative hybridization signals. The 43 spacers are then divided into a set of 14 triplets with one extra, and each set is then converted to a 15-digit octal code. Differences between spoligotype patterns are mainly the result of deletions of spacer sequence regions. Interestingly, the absence of spacers 3, 9, 16, and 39-43 is a characteristic of all *M. bovis* strains and is thought to be a result of the deletion of this region by homologous recombination instead of genetic divergence from the *M. tuberculosis* spacer sequence (Dale, 2001).

Spoligotyping has the advantage of being significantly less technically demanding than RFLP fingerprinting, with a much shorter turnaround time. In addition, the degree of differentiation achieved by spoligotyping is higher than that of IS6110- RFLP for strain with one or two copies of IS6110 like *M. bovis*. One of the major drawbacks to spoligotyping is that it can only identify polymorphisms within the DR cluster, whereas RFLP typing can detect genetic differences arising from multiple loci. Despite this limitation, spoligotyping is capable of providing satisfactory sensitivity for routine molecular epidemiologic analyses at both regional and global levels (Aranaz *et al.*, 1996; Cadmus *et al.*, 2006).

Variable-Number Tandem Repeat Typing

A more recent PCR-based typing method for the *M. tuberculosis* complex was first described by Frothingham and Meeker-O'Connell. This method is based on the variable-number tandem repeats of Mycobacterial interspersed repetitive units (MIRU-VNTR). In *Mycobacteria*, isolates are typed by determining the number of copies of specific repeat units at various MIRU-VNTR loci scattered throughout the genome. These short-sequence repeat units are 52–77 nucleotides in length, thus classifying these loci as minisatellite regions. In MIRU-VNTR genotyping, the number of repeat units within a specific locus is determined by PCR amplification of the entire

locus, followed by gel electrophoresis, where the number of repeat is determined by the size of the PCR amplicon (Sola *et al.*, 2003).

Bacteria belonging to the *M. tuberculosis* complex contain 41 different MIRUs randomly located throughout their genomes. Within the genome, these MIRUs generally contain an open reading frame that overlaps the termination and initiation codons of their flanking genes. However, some are also found entirely within predicted coding regions (Supply *et al.*, 2000). The purpose of these minisatellite regions in the bacterial chromosome is uncertain, but some evidence indicates that similar to the PE-PGRS genes, they may code repeating amino acid motifs, thus providing another source of antigenic variation (Sola *et al.*, 2003).

Alternatively, MIRU-VNTR regions may function in transcriptional or translational regulation. Several early studies identified small sets of these MIRU loci that had sufficient allelic variation to differentiate non related bacterial strains, demonstrating the utility of these genetic markers for molecular epidemiology studies. A systematic analysis of the *M. tuberculosis* type strain H37Rv identified 11 tandem repeat loci. These 11 loci were PCR amplified and could be reproducibly found in all strains of *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, and *M. africanum* tested. Only five of these loci (designated ETR-A, ETR-B, ETR-C, ETR-D, and ETR-E) were suitable for epidemiological studies, as the remainder did not demonstrate sufficient allelic diversity to allow discrimination between non related strains (Dale *et al.*, 2001). Subsequent analyses of both the *M. tuberculosis* and *M. bovis* genomes have identified other novel randomly dispersed loci containing MIRUs suitable for strain discrimination as well. Interestingly, one locus from the *M. tuberculosis* genome, MIRU 21, is absent from all *M. bovis* strains tested to date.

A recent comprehensive analysis of all published VNTR-MIRUs compared the ability of 30 minisatellite sets to differentiate strains of *M. bovis* and found that individual loci differed significantly in their discriminatory capacity (Supply *et al.*, 2000). Of the 30 loci evaluated, 12 had no allelic diversity among the 47 different *M. bovis* isolates, and an additional five had diversity indices of 0.2. However, some of the loci with low discriminatory power in this study demonstrated significantly higher diversity in a different study (Sola *et al.*, 2003). It is possible that these differences may be attributed in part to the phylogenetic relationship of the bacterial

strains used. The study by Roring *et al.*, 2004, used field isolates from Northern Ireland and the Republic of Ireland, which may be an over representation of phylogenetically similar strains, whereas the previous research focused on more genetically and geographically diverse groups of isolates. Thus, it appears that the degree of discrimination provided by VNTR typing is dependent on both the individual VNTR loci used and on the test panel of bacterial isolates chosen for analysis. As a consequence, certain MIRU-VNTR markers may be useful for larger population-based studies, whereas others may be more appropriate for outbreak analyses.

Restriction Fragment Length Polymorphism (RFLP)

RFLP is a technique involves digestion of the DNA using restriction enzyme (either PvuII or AluI) and separation of the fragments using agarose electrophoresis. This is followed by southern blotting of the DNA on to nitrocellulose or nylon filter and then adding probes bearing labels or markers. These probes are selected to be complementary to only a fraction of the DNA of the isolate and therefore after hybridization, only some of the fragments will be visualized. The crucial first step for this technique is the selection of a DNA element, which, when used as a probe, will reveal polymorphisms or DNA difference between isolates. Probes based upon repetitive DNA element are preferred because these bind to a greater number of fragments and this can increase the potential to reveal polymorphisms. Those repetitive elements that have been utilized for RFLP include IS, polymorphic G-C rich repeat sequences(PGRS) and direct repeat (DR) sequence. The most important IS identified in *M. tuberculosis* complex is IS6110, a 1361bp fragment (Durr *et al.*, 2000).

IS6110 Restriction Fragment Length Polymorphism

The insertion sequence IS6110 is a transposable (mobile) element belonging to the IS3 family of insertion elements, and it is found only in members of the *Mycobacterium tuberculosis* complex. However, the total number of IS6110 copies found in the genome differs widely between the various *M. tuberculosis* complex species. For example, the *M. tuberculosis* genome may harbor up to 19 copies of IS6110, whereas *M. bovis* only contains one to five copies. The foundation of any RFLP technique relies on digestion of genomic DNA followed by probing with a specific

marker using Southern blotting techniques. Thus, the combination of the restriction enzyme used and the DNA sequence of the probe allows for polymorphisms in the genomic DNA to be visualized. For IS6110-RFLP, the variation in the copy number of IS6110 is a major source of genetic polymorphisms between bacterial isolates. This variation has significantly contributed to IS6110-RFLP becoming the standard reference typing technique for *M. tuberculosis*. Unfortunately, IS6110-RFLP is less useful for differentiating *M. bovis* isolates because strains of *M. bovis* only harbor one to five copies of IS6110.

Furthermore, this element is routinely present in the same 1.9-kb *Pvu*II fragment of many *M. bovis* strains (Van Soolingen *et al.*, 1991), limiting the differential capacity of this technique. However, utilization of different DNA probes against the same IS6110 target increases the resolution of this method. For example, Whipple *et al.*, 1997, used two different probes against IS6110 when analyzing 16 *M. bovis* strains from wildlife and domestic cattle. When a fragment internal to IS6110 was used as a probe, only a single band for each insertion element present in the genomic DNA could be visualized. Reprobing the same strains with a fragment of the inverted repeat region of the insertion element produced two bands per IS6110 copy, allowing a single-band cluster of isolates to be differentiated into two distinct groups. Interestingly, the copy number of IS6110 in clinical strains of *M. bovis* appears to be somewhat correlated with animal species. *M. bovis* isolates recovered from cattle generally carry one copy of IS6110, whereas strains harboring multiple copies are more likely to have originated from wildlife or zoo animals (Cousins *et al.*, 1998).

Polymorphic G-C-Rich Sequence RFLP

The polymorphic G-C-rich sequence (PGRS) loci are microsatellite regions in the Mycobacterial genome consisting of multiple short tandem repeat units that are less than 10 bp in length. The DNA residues in these PGRS locus occur within structural genes as repetitive tri nucleotide sequences and frequently contain the motif CGG. These genes are associated with a unique gene family found only in *Mycobacteria*, in which the encoded proteins contain a signature proline-glutamic acid (PE) amino acid sequence near their amino terminus (Cousins *et al.*, 1998).

Although the function of the encoded proteins has yet to be determined, current hypotheses suggest they may be cell surface constituents, presumably involved in antigenic variation designed to evade host immune responses. These PE-PGRS genes are randomly dispersed throughout the genome in all *M. tuberculosis* complex bacteria, as well as several other Mycobacterial species, including *Mycobacterium gastri*, *Mycobacterium gordonae*, *Mycobacterium kansasii*, *Mycobacterium szulgai*, *Mycobacterium malmoeense*, and *Mycobacterium phlei* (Brennan *et al.*, 2001). Thus, by combining multiple genetic targets with coverage throughout the entire genome, these PE-PGRS regions provide greater sensitivity for epidemiological studies and molecular fingerprinting of clinical *M. bovis* isolates than does IS6110-RFLP. However, because of the complexity of the fingerprint pattern obtained with PGRS-RFLP and the poor resolution of bands below approximately 1.5–2 kb in size, most studies limit the analysis of PGRS-RFLP patterns to fragments above this range (Cousins *et al.*, 1998).

2.7. Control of bovine tuberculosis

BTB is a difficult disease to control because of the large number of susceptible species, the variation in pathogenesis, and the limited effectiveness of currently available control methods for wild animals. Effective control requires an understanding of the epidemiology of infection within the ecological system that can include domestic as well as wild animal species. It is also affected by management practices within the livestock production system as well as societal considerations toward the application of control in wild animal reservoir species. The reasons for eradicating bovine TB include the risk of infections to the human population, loss in productivity due to infected animals, and animal market restrictions set by countries with advanced eradication programme (Cousins, 2001).

Developing countries such as those in Africa need to control BTB, as agriculture remains the back bone of many of these nations. In developed countries bovine TB control and elimination programs together with milk pasteurization have drastically reduced the incidence of disease in both animal and humans. However in developing countries, it is widely distributed because of control measures are not applied or are applied sporadically and pasteurization is rarely practiced. This is due to financial constraint, scarcity of trained professionals, lack of political will as well

as under estimation of the importance of TB by national government and other agencies (Ayele *et al.*, 2004).

2.7.1. Test and slaughter policy

The test and slaughter policy is the only one assured of eradicating TB and relies on the slaughter of reactors to the tuberculin skin test. In an affected herd, testing every three-month is recommended to rid the herd of individuals that can disseminate infection. Varying forms of test and segregation have been used, with test and slaughter used only in the final stages of eradication (Aiello *et al.*, 1998).

2.7.2. Hygienic measures

Routine hygienic measures aimed at cleaning and disinfection of contaminated premises, food and water troughs are useful (Aiello *et al.*, 1998). Kiros (1998) and Ali (2006) have mentioned that cattle under poor management (overcrowding and lack of ventilation) were more likely to develop tuberculosis than cattle under good management system. Feed troughs should be cleaned and thoroughly disinfected with hot 5% phenol or equivalent cresol as disinfectants. Appropriate disinfectants suitable for use in TB laboratories are those containing phenols (2.5%), hypochlorites (1-5%), alcohol (usually 70% ethanol), formaldehydes and iodophors (3-5%) and glutaraldehyde (WHO1998).

Suspicious reactors being held for retesting should be isolated from the remainder of the herd. The usual source of *M. bovis* in milk is udder infection. It can also occur by contamination with feces, uterine discharge or air or dust borne bacilli. Such contamination can be much reduced by better dairy design and practices. Effective meat inspection and proper disposal of tuberculous lesions are also paramount important. Careful management is necessary to raise disease-free stock from an infected herd. Each calf should be taken away from its dam at birth; on no account should be dam be allowed to lick the calf. The calf should then be placed in thoroughly disinfected calf pen and be given its dam's colostrums preferably pasteurized/ boiled in clean

bucket. Farm attendants should be checked as they may produce a source of *M. tuberculosis* and also *M. bovis*, and cause transient positive reactions in cattle (WHO, 1993).

2.7.3. Vaccination

Vaccination against bovine tuberculosis is likely to become an important disease control strategy in developing countries, which cannot afford a test and slaughter control programme, or in countries which have a wildlife reservoir of *Mycobacterium bovis* infection. In the past decade, considerable progress has been made in the development and evaluation of tuberculosis vaccines for cattle and for a range of wildlife maintenance hosts including possums, badgers, deer and African buffaloes. In cattle, neonatal vaccination with BCG appeared to be more effective than vaccination of 6-month-old calves and in most situations no other vaccine has been shown to be better than BCG. However, prime-boost strategies involving combinations of BCG with a protein or DNA vaccine, to improve on BCG vaccination alone, have produced very encouraging results. BCG vaccine has been shown to reduce the spread of tuberculosis in a range of wildlife species and a prototype oral bait delivery system has been developed. Prospects for the development of improved vaccines against bovine tuberculosis are promising and vaccination approaches could become very valuable in the control and eradication of bovine tuberculosis (Edelsten, 1996; Buddle *et al.*, 2006).

3. STUDY MATERIALS AND METHODS

3.1. Study area

The study was conducted from September, 2009 to May, 2010 in East Wollega zone (at Nekemet municipality abattoir and Horro-Guduru cattle breeding and improvement center), Oromia Regional State. Nekemte is located at 331 km West of Addis Ababa. The approximate geographical location of the area is between 9° 4' 7N to 9° 11' 02N and 36° 30'E to 36° 43' 02E. The altitude is from 1500m to 2565 m above sea level and the maximum temperature is 27.4°C and the minimum temperature of the area is about 10.2 °C. The mean annual rainfall of the area ranges from 1600mm to 2000mm. The area receives long heavy rainy season from June to September and short rainy season from March to May. Among the various soil types in the area, the red brown soil with a PH ranging from 5-7 is the predominant type of soil in the Zone. The area is rich in natural vegetation that comprised of the tropical rain forest tree, all grasses and brushes. Among major crops grown in the area including; Maize, Sorghum, Teffe and Pulses are common food crops. Roots crops known by local name 'Anchote' 'Koncho' 'Godare' and other perineal crop like coffee and fruit like banana and mango are dominantly cultivated in the area.

The estimated animal population of the area is 78,178 cattle, 9,894 sheep, 6,477 goats, 3,287 donkey, 1,598 horses, 665 mules, 2,428 cats and 4572 dogs. The total land area of the region is about 769, 725 hectares, of which 336,220 hectares are used for crop production, 182,412 for animal grazing, 256,901 hectares forest covered and 20,492 hectares for other activities (EWARD, 2007).

The abattoir is the only source of inspected beef in the town. The overall hygiene of the abattoir, including the drainage, water, lighting and condemned organ and carcass disposal system is poor. Animals from different origin of the surrounding district are the source for slaughtering. There are about 30 legally registered butcheries, in the town, majority of the meat, especially of mutton are supplied by the backyard slaughter, and hence the number of animals slaughtered in the abattoir is relatively very small.

The tuberculin test was conducted in Horro-Guduru cattle breeding and improvement center, which is found in Guduru district, East Wollega zone, Oromia Regional State. It is located about 270km West of Addis Ababa. The center was established in 1990 with the aim of Horro sheep breeding and improvement, but due to high mortality with undifferentiated disease prevalence and some management problems, it is shifted to cattle rearing center in 2002. Currently the center was under the management of Wollega University for research as well as income generation to the University. There are about 1328 cattle (1178 pure Horro breed and 150 Horro-Jersey cross breed) in the center, which completely dependent on open grazing of the natural grass extensively that cover about 765 hectare of land. Except for the calves, the housing system is traditional on open air with fence only.

3. 2. Study design, sample collection and transportation

Cross-sectional study design was followed for both abattoirs based survey and skin test to determine the prevalence of BTB in the study area. In a slaughterhouse about 10 heads of cattle on average was randomly selected from the total of animals slaughtered per day. Tissue lesion samples suspected to be positive for BTB was collected aseptically from the lung lobes, lymph nodes of the head, lung, intestine and other tissue and organs. These samples collected from the abattoir were kept at 4 °C in the refrigerator for 10-15 days and transported to institute of the Akililu Lemma Patho-Biology in ice box packed with ice packs to keep the low temperature during transportation for culture.

For the comparative tuberculin test, first 500 heads of cattle were determined to be included, considering the potential of the resource used for the test in to account. Due to their relatively small proportion, all cross breed and male animals were included purposefully. The rest local female animals were randomly selected.

In both cases, sample size calculated based on 50 % prevalence assumption, 95% CI and $p < 0.05$ (Thrusfield, 2005). Therefore, the sample size calculated was 384.

$$n = Z^2 \cdot p_{\text{expe}} \cdot (1 - p_{\text{expe}});$$

d^2

Where n = required sample size

$P_{\text{expe.}}$ = expected prevalence

d = Desired absolute precision (5 %)

Z = Normal distribution constant

3.3. Study methodology

3.3.1. The comparative intra dermal tuberculin test

Two sites on the right side of the mid-neck skin of the animals, 12cm apart, were shaved and skin thickness was measured using a caliper before injection of the purified protein derivative (PPD) and recorded as A_1 for avian PPD site and B_2 for bovine. One site was injected with an aliquot of 0.1ml of 20,000 IU/ml bovine PPD (*M. bovis*, AN5 strain) in to dermis toward the shoulder of an animal and other site was similarly injected with 0.1ml of 25,000 IU/ml of avian (D4 ER strain) PPD toward the head. Then their correct administration was checked if papula (pea like swelling) was formed and detected by palpation in the site of allergen inoculation. When the tuberculin was not administered intra dermally, the administration was repeated in the site in the prescribed dosage. After 72 hours, the skin at the injection site were again measured and recorded as A_2 for avian PPD and B_2 for bovine PPD. The same person should measure the skin fold thickness before and after tuberculin injection. The results were interpreted according to the recommendation of the office of International des epizooties (OIE, 2000).

Interpretation

A reaction was considered to be positive if the difference in skin fold thickness at the bovine site of injection was 4 mm or more higher than the reaction shown at the site of the avian injection.

When the difference in the skin fold thickness at the bovine site of inoculation was greater than 2 mm but lower than 4 mm it was considered as doubtful, but if lower than or equal to 2 mm it was taken as negative (OIE, 2004).

If the increase was observed at both sites of the injection the difference was considered between the two sites of reactions. The increase in skin thickness at the injection site of avian Δ PPD is Δ PPD-A and is Δ PPD-B for the reaction at site of bovine PPD. The manipulation of the change was done as this manner, subtracting Δ PPD-A from Δ PPD-B. When the change recorded as less than 2mm negative, between 2mm and 4mm suspected and greater than or equal to 4mm positives for bovine tuberculosis (OIE, 2000).

Δ PPD-B = [Skin fold thickness after inoculation of PPD-B] – [Skin fold thickness before inoculation PPD-B].

Δ PPD-A = [Skin fold thickness after inoculation of PPD-A] – [Skin fold thickness before inoculation PPD-A].

Δ (PPD-B) – Δ (PPD-A) = > 4 mm: Positive

Δ (PPD-B) – Δ (PPD-A) = > 2 mm and < 4 mm: Doubtful

Δ (PPD-B) – Δ (PPD-A) = < 2mm: Negative

3.3.2. Ante mortem examination

Physical examination of the animals was carried out before they were slaughtered. Body temperature, pulse rate, respiratory rate, condition of superficial lymph nodes and visible mucus membranes were examined and recorded for individual animals to be slaughtered. Breed and sex was also recorded. Age was estimated as described by Amstutz, 1998 (Annex 2) and BCS was made based on the description by Nicholson and Butterworth, 1986 (Annex 1).

3.3.3. Post mortem examination in abattoir

Meat inspection for TB lesion detection was conducted in accordance with the method developed by meat inspection and quarantine division of the ministry of Agriculture (Solomon, 1975). It involves palpation and incision of the lung, liver and udder, visual inspection of the kidney,

palpation and incision of the trachobronchial, mediastinal, prefemoral and prescapular lymph nodes. If lesion is recovered in the above tissue, other lymph nodes and tissues are incised (Teklu *et al.*, 2004). Lymph nodes were sliced in to thin section of 2mm and other tissues were cut in to slices of 2cm using separate sterile surgical blades. The cut surface was examined under a bright light source for the presence of abscess and tubercules (Patterson and Grooms, 2000; Asseged *et al.*, 2004). In the presence of suspected tuberculous lesions tissue sample were collected in the universal bottles containing normal saline (0.85%) for culture. In the presence of lesions in different tissues of single animal, pooled sample for each animal collected.

3.3.4. Isolation and identification

Tissue samples were transported in the cold chain using ice box packed with ice packs to keep the low temperature. On arrival to the laboratory, tissue samples were macerated in sterile mortar and pestle using surgical blades and forceps to get fine pieces and then homogenized for 10 minutes in 5 ml of normal saline. Two ml of homogenate were transferred to centrifuge tube and decontaminated with equal volume (2ml) of 4% NaOH for 15 minutes; centrifuged at 3000rpm for 15 minutes and neutralized by 1% HCL, employing phenol red as an indicator. Neutralization was achieved when the suspension changed from purple to yellow. After neutralization, 0.1 ml of the suspension from each sample was spreaded on slants of Lowenstein-Jensen (LJ). Each sample was inoculated on to the set of Lowenstein-Jensen (LJ-pyruvate) and glycerol (standard LJ). Cultures were incubated aerobically at 37 °C for up to 12 weeks with weekly observation for growth. When visible colonies were observed, Zeil-Neelsen staining was performed to confirm the presence of acid fast bacilli (WHO, 1998; Quinn *et al.*, 2002).

3.3.5. Microscopic examination

A direct smear was prepared from pure colony grown on L-J media and stained using the Zeihl-Neelsen acid-fast staining technique; the heat fixed smear were stained with carbonfucisn, heated gently and allowed to stand for 10 minutes. The stain was then poured off and the smears washed

with tap water and then decolorized with acidic alcohol for 1 minute, each with the slides being washed under tap water between each step. The smears were then counter stained with methylene blue for 3 minutes, were dried and examined for the presence of AFB under light microscope employing a 100X oil immersion objective (WHO, 1998; Quinn *et. al.*, 2002).

3.3.6. Molecular characterization of *Mycobacteria*

Polymerase chain reaction (PCR)

Initial identification of Mycobacterial species was based on the rate of growth, pigment production and colony morphology. For further characterization of the species molecular techniques was used. The Mycobacterial cell from culture colony was killed in 80°C water bath for 1 hour and the DNA was extracted according to ALIPB-TB-Molecular biology/Immunology laboratory standard operation procedure (SOP) from the Mycobacterial culture isolates (Jordan and Victor, 2002).

Multiplex polymerase chain reaction (m-PCR)

For multiplex PCR, the procedure described by Wilton and Cousins (1992) was followed. This multiplex PCR differentiates *M. tuberculosis* complex from *M. avium*, *M. intracellulae* and other Mycobacterial species, and either heat-killed bacterial suspensions or extracted DNA was used. The PCR targets the sequence of the Genus Mycobacterium within the 16S rRNA gene (G1, G2), sequences within the hyper-variable region of 16S rRNA that is known to be specific to *M. intracellulae* (MYCINT-F) and *M. avium* (MYCAV-R), and the MTB70 gene specific for *M. tuberculosis* complex (TB-1A, TB-1B). The primers used were MYCGEN-F, 5'AGA GTT TGA TCC TGG CTC GA 3'; (35ng/μl), MYCGEN-R, 5'-TGC ACA CAG GCC ACA AGG GA 3', (35ng/μl); MYCAV-R, 5'-ACC AGA AGA CAT GCG TCT TG 3' (35ng/μl); MYCINT-F, 5'-CCT TTA GGC GCA TGT CTT TA 3' (75ng/μl); TB1-F, 5'-GAA CAA TCC GGA GTT GAC AA 3' (20ng/μl); TB1-R, 5'-AGC ACG CTG TCA ATC ATG TA 3' (20ng/μl). The reaction was carried out using Thermal Cycler. The mixture was heated for 10 min at 95 °C, further 35 cycles of 1 min at 95 °C, 1 min at 61 °C, and 1.5 min at 72 °C; and 10 min at 72 °C. Each PCR tube consisted of 5.2μl H₂O Qiagen, 8μl HotStarTaqMasterMix, 0.3μl of each of the

six primers (concentration given above), 5 µl of DNA templates of samples or controls making the total volume 20µl. *M. avium*, *M. intracellulae*, H37Rv and 2122/97 (*M. bovis* strain) were used as positive controls while H₂O Qiagen, was as a negative control. The product was electrophoresed in 2% agarose gel in TAE running buffer. SYBR Safe at a ratio of 1:10 in 2% agarose gel, 100bp DNA ladder, and orange 6x loading dye were used in gel electrophoresis. All members of the Genus Mycobacterium produce a band of 1030bp, *M. avium* or subspecies such as *M. avium* subspecies *paratuberculosis* produces a band of 180bp, *M. intracellulae* a band of 850bp while members of *M. tuberculosis* complex produce a band with 372bp.

RD4 deletion typing

The RD4 deletion typing was applied to isolates that showed band for *M. tuberculosis* complex by multiplex PCR. The primers used were RD4intF, 5'-ACA CGC TGG CGA AGT ATA GC-3'; RD4flankR, 5'-AAG GCG AAC AGA TTCAGC AT-3'; and RD4flankF, 5'-CTC GTC GAA GGC CAC TAA AG-3'. The mixture was heated in Thermal Cycler for 15 min at 95 °C, and then subjected to 35 cycles of 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C; and 10 min at 72 °C. Each PCR tube consisted of 7µl H₂O Qiagen, 10µl HotStarTaqMasterMix, 0.3µl of each of the six primers (concentration), 2µl of DNA templates of samples or controls making the total volume 20µl. H37Rv and 2122/97 (*M. bovis* strain) were used as positive controls while H₂O Qiagen, was used a negative control. The product was electrophoresed in 1.5% agarose gel in TAE running buffer 10X. SYBR Safe at a ratio of 1:10 in 2% agarose gel, 100bp DNA ladder, and orange 6x loading dye were used in gel electrophoresis. The gel was read using SYNGENE BIO IMAGING SYSTEM (A Division of Syoptics Group). The presence of RD4 (i.e. *M. tuberculosis*, *M. africanum*) gives a product size of 335bp (RD4 intF + RD4flankR) its absence (*M. bovis*) gives a product size of 446bp (RD4flankF + RD4flankR).

3.4. Data analysis

During the study, individual animal identification number, place of origin, breed, sex, age, organ or tissue affected at abattoir and cultural, staining, PCR and spoligotyping results of Laboratory Work were speeded into MS Excel data sheets. Then, coded and were analyzed using SPSS

version 15 statistical software. The prevalence rate was calculated by dividing the proportion of cattle found infected (either positive reactors or harbouring tuberculous lesions) by the total number of cattle tested or whose carcasses is inspected multiplied by 100%. The risk factors associated with *M. bovis* infection were calculated by using Chi-square (χ^2) and logistic regression. Odds Ratio (OR) was assessed to investigate the strength of association. A statistically significant association between variables was said to exist if the calculated $P < 0.05$. For the analysis of the effect of different risk factors on bovine tuberculosis status of animals, doubtful reactors were not considered as positive (Thrusfield, 2005).

4. RESULTS

4.1. Comparative intradermal tuberculin test

From the total of 500 cattle (358 local and 142 Horro-Jersey cross) tested for the presence of tuberculosis by the comparative intradermal tuberculin test 1.8%, (9/500) were positive and 15(3%) determined as doubtful; the rest 476 (95.2%) were negative. On the other hand if we consider doubtful results as positive, the overall prevalence would be 4.8 % (24/500). Out of the nine positive cattle (considering doubtful as negative) to bovine tuberculosis, 8 were female and one was male animal. Six (1.7%) were of Horro breed and 3 (2.1%) were of the Horro-Jersey cross breed (Table 6). Twenty-five (5%) of the animals in the herd reacted to both avian and bovine tuberculin.

Table 6: Host risk factors associated with *M. bovis* infection

Variables		No- of animals			χ^2	P-value
		Positive	Doubtful	Negative		
Age	≤ years	6	13	253	7.719	0.076
	5 -10 years	3	1	181		
	>10 years	-	1	42		
BCS	Lean	3	2	138	2.543	0.643
	Medium	5	11	251		
	Fat	1	2	87		
Breed	Local	6	7	345	4.746	0.079
	Cross	3	8	131		
Sex	Female	8	11	335	1.220	0.594
	Male	1	4	141		
Total		9	15	476		

4.2. Abattoir survey result

The prevalence of BTB was investigated to be 5.9% in abattoir-based surveillance. As shown below, the prevalence of BTB in different district of the study area was different. High prevalence was observed in Guto-gida (11.5%) and low prevalence was recorded in Wayu-Tuka. In the remaining part of animal origin the prevalence is almost similar (Figure 1).

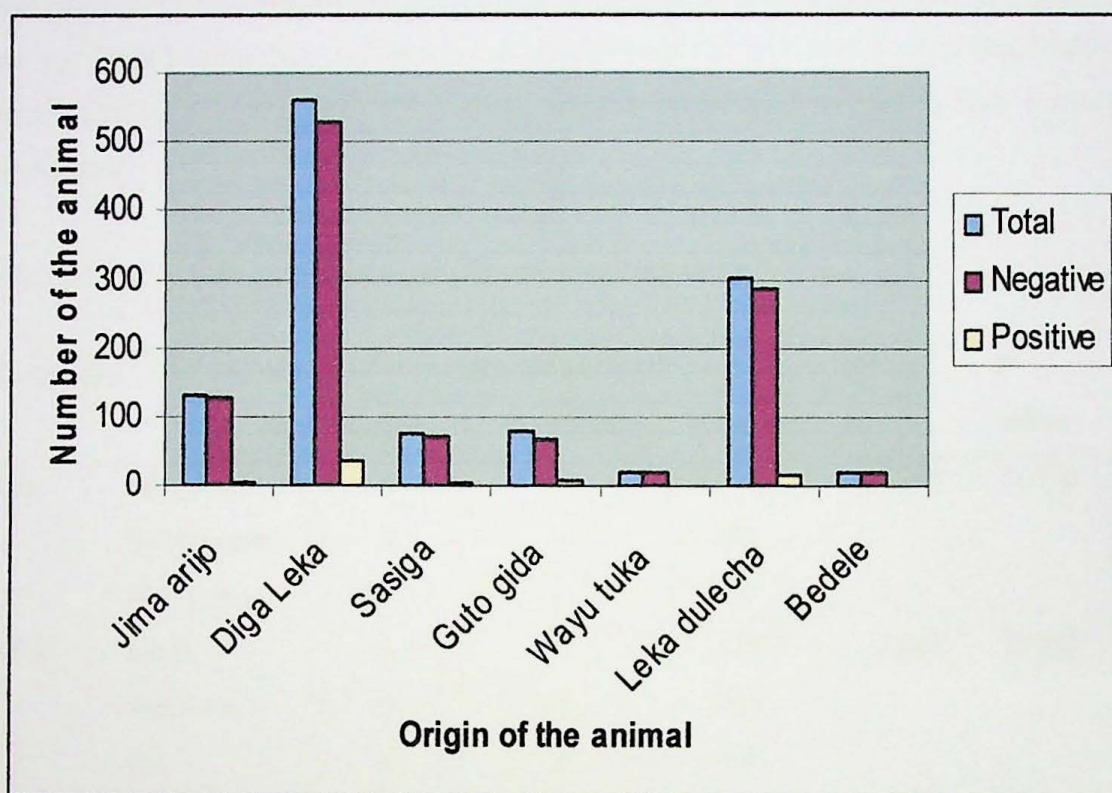


Figure 1. Prevalence of bovine tuberculosis in different district of animal origin

From the risk factors considered (Table 7), only breed was found significantly associated with BTB infection ($P < 0.05$) and the rest were not found significantly associated with the presence of gross tuberculous lesions ($P > 0.05$).

Table 7: Level of association of various host and environmental risk factors with *M. bovis* infection.

Variables		No. of animals						
		Posi tive	Negati ve	Total (% of positive)	χ^2	OR	OR 95% CI	p- value
1.Age	1-5 yrs	2	26	28(7.1)	1.560	1		0.456
	5-10 yrs	57	960	1017(5.6)		.899	.187-4.336	
	>10yrs	11	130	141(7.8)		.684	.348-1.345	
2.BCS	Poor	11	133	144(7.6)	2.662	0.742	0.254-1.993	0.262
	Medium	52	909	961(5.4)		0.598	0.562-1.366	
	Good	7	74	81(8.6)				
3.Breed	Local	68	1115	1183(5.7)	19.995	1		0.010
	Cross	2	1	3(6.7)		.053	.004-.723	
4.Sex	Female	10	134	144(6.9)	0.321	1.127	0.554-2.292	0.571
	Male	60	982	1042(5.8)		1		
5.Origin	High Land	15	234	249(6)	0.008	1		0.881
	Low Land	55	882	937(5.9)		.899	.482-1.676	
Total		70	1116	1186(5.9)				

Note: χ^2 = chi square

OR = Odd's ratio

CI = confidence intervals

The distribution of tuberculous lesions in tissues of positive animals is presented in (Table 8). The entire lesion observed were localized lesions involving frequently a single organ. A high proportion (70%) of the lesions was located on the thoracic cavity lymph nodes, while 25.7% and 4.3% of the lesions were found in the abdominal cavity and head regions respectively.

Table 8: The distribution of tuberculous lesion in the tissues of infected animals

Region of the body	Anatomic site	No. of infected tissue	% of positive
Head			
	Mandibular LN	2	2.9
	Retropharyngeal LN	1	1.4
	Total	3	4.3
Thoracic cavity			
	Cranial mediastinal LN	21	30.0
	Caudal mediastinal LN	14	20.0
	Left bronchial LN	11	15.7
	Right bronchial LN	3	4.3
	Total	49	70
Abdominal cavity			
	Mesenteric LN	17	24.3
	Hepatic LN	1	1.4
	Total	18	25.7

Note: LN= Lymph node

4.3. Bacteriology

From 70 tuberculous suspected tissue samples on postmortem inspection, that transported ALIPB, at different laboratory entry batch, showed 31.4 % (22/70) growth on primary culture media. The outcome of the culturing activity is indicated in table 11. Among those cultures which showed visible grows, only 31.8 % (7/22) were on L-J media enriched with pyruvate and the rest 68.2 % (15/22) were on L-J media enriched with glycerol (Table 9).

Table 9: Cultural result of tuberculous tissues from slaughtered cattle

Sample type	Growth on			
	L-J media with		L-J media with	
	Glycerol		Pyruvate	
	Total	Positive (%)	Total	Positive (%)
Bronchial LN	14	2(14.3)	14	1(7.1)
Mediastinal LN	35	8(22.9)	35	5(14.3)
Mesenteric LN	17	5(29.4)	17	-
Mandibular LN	2	-	2	1(50)
Retropharyngeal LN	1	-	1	-
Hepatic LN	1	-	1	-
Total	70	15(68.2)	70	7(31.8)

The total 22 grown cultures were subjected to Zeihl- Neelsen staining technique in the laboratory in order to check for the presence of acid fast bacilli organisms. Out of these, only 13.6% (3/22) were acid fast negative. The remaining 19 grown culture media were confirmed for the presence of acid fast bacilli (Figure 2).



Figure 2. Acid fast bacilli by using Zeihl Neelsen stainingh technique

4.5. Retrospective data of human tuberculosis from the study area.

Reviewing the annual quarter report of the Nekemte hospital, a total of 30,903 patients were admitted and diagnosed as TB infected in a five year time period (2005-2009 G.C.). From these 61.7% (19,068) were pulmonary TB and 38.3% (11,835) were registered as positive to extra-pulmonary TB (Figure 5).

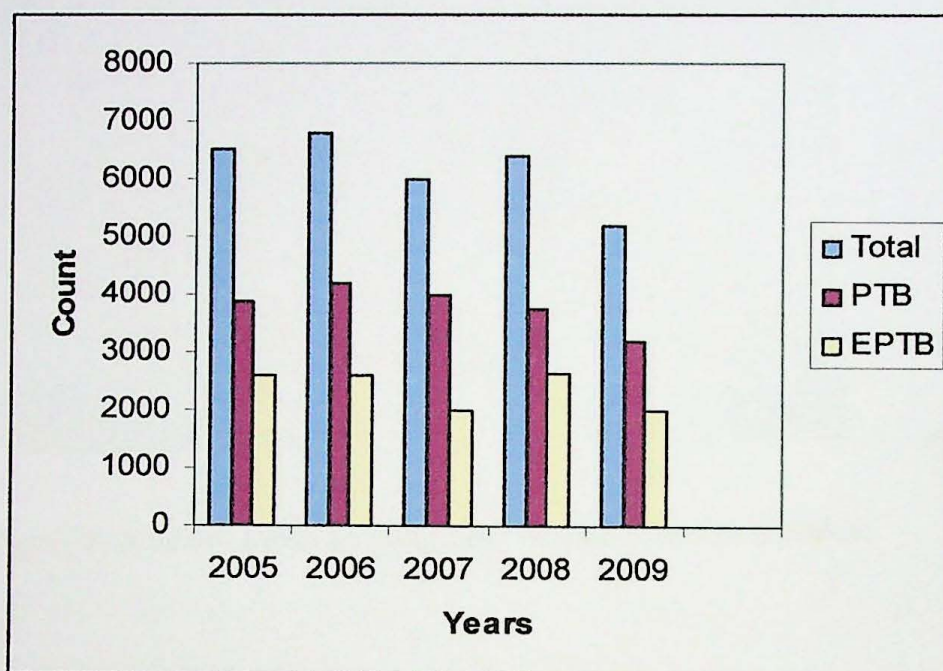


Figure 5. Retrospective data on human tuberculosis

5. DISCUSSION

In the current study, the comparative intradermal tuberculin test (CIDT) showed the prevalence of BTB in Horro-Gudurru cattle breeding and improvement center was 1.8%. This is moderately lower than the previous studies conducted by Ragassa, 2001 (3.4%) in West Wollega and Redi, 2003(3.5%) at Assella in similar extensive management system. But there is a great difference when compared with tests conducted on intensive management (Kiros, 1998; Anonymous, 1999; Ameni *et al*, 2003b). This variation might be due to the difference in the study animals used, study design and type of farming system. The disease is said to be more prevalent in dairy cattle kept under intensive management than others due to closer confinement, longer life spans and greater productivity stress (Biberstein and Hirsh, 1999; Ayele *et al.*, 2004).

The present study site was established on the area which was previously used by near by farmers as a communal grazing land. There is an opportunity of contamination of the grass by parasitic egg and larvae from animal faces. Additionally there is swampy area in the middle of the ranch and the water used for the animals is stagnant damped for this purpose. According to Ameni and Medhin, 2000, infection with different gastrointestinal parasites namely, *fasciola*, *paraphistomium*, *schistosoma* and *strongyle* has an effect on the immune response to tuberculin test. This is also supported by previous studies as *fasciola* and *strongyle* possess an external plasma membrane and particularly in their migratory larval stage, they are in close contact with the immune component such as antibody and the complement which act directly against them. Detailed study on the infection with *fasciola* showed that, antibody mediated cytotoxicity response are capable of killing the larval stage and young flukes as they migrate across the body cavity and through liver (Wakelin, 1994). Similarly, nematodes such as *strongyle* release a wide variety of excretory or secretory antigens that are powerfully immunogenic. Therefore, infection with *fasciola* or *strongyle* has induced strong Th2 cell response there by inhibiting Th1 cell response to tuberculin injection (Tizard, 1996; Unger *et al.*, 2003).

The comparison made between crosses and local cattle supports the idea that, cross cattle are found to react more likely to tuberculin than local (1.7% in local and 2.1% in cross breed). But,

the difference is not as high as the one, seen between local and local-Holstein Friesian cross. The strength of association (OR) was calculated considering sex as a risky factor, where crosses were 5 times more likely to develop tuberculosis than local breeds. The possible explanation for this might be stress of high production.

The overall prevalence of BTB (5.9%) obtained in the current abattoir survey was high when compared with Regassa *et al*, 2009 (1.1%) at Hawassa, Asseged *et al.*, 2004 (1.48%) in Addis Ababa, Shitaye *et al.*, 2006 (3.46%) in Addis Ababa and Taklu *et al.*, 2004 (4.53%) at Hossana through similar diagnostic methods but in consistence with previous reports of Ameni and Wudie, 2003 (5.16%) from Adama Municipality abattoir, Gudeta, 2008 from Nekemte Municipality abattoir (5.1%) and Desta, 2008 (5%) at Kombolch meat processing plant, Southern Wollo, based on postmortem inspection. However, the presently recorded prevalence was low as compared to previous report by Shimels, 2008 at Debre Brehan, central Ethiopia and Regassa, 1999 (7.96%) at Wolaita Sodo. The infection rate in cattle has been found to differ greatly from place to place (Shitaye *et al.*, 2006) and the difference might be most probably linked to the type of production system (most notably in extensive), which is unlikely to favor the spread of the disease in contrast to the intensive dairy farms as cited by Ameni *et al.*, 2006 and Shitaye *et al.*, 2006).

There was statistical significant ($\chi^2=19.9$ and $P=0.010$) difference between breed of the animal and tuberculous lesion. Radostitis *et al.*, 1994 has indicated that zebu breeds are relatively resistant for BTB than exotic breeds. The remaining assumed risk factors both in abattoir survey and comparative intradermal test revealed no statistical significant difference. The possible reason might be due to in proportionality in the number of the animal compared in specific variable. For example, less number of female animals came to the study abattoir to be slaughtered and the proportion of very young animal and animal with lean body condition encountered in abattoir was low. However there is biological association as observed from the Odds' ratio value.

In the present study, gross tuberculous lesions were found most frequently in lymph nodes of the thoracic cavity (70%); followed by lymph nodes of the abdominal cavity (25.7%) and the lesser frequency was found in the lymph nodes of the head region (4.3%). The occurrence of

tuberculous lesions in thoracic cavity was lower than the results of previous studies, where greater than 90% occurrence of TB lesions in the respiratory system was reported in developed countries (Neill *et al.*, 1994; Collins, 1996; Whipple *et al.*, 1996). However it is higher than the report of Regassa *et al.*, 2009 which implies that inhalation is the most important route of infection. Husbandry factors such as enclosures of the animals overnight may facilitate respiratory transmission of the infection (WHO, 2005). The followed high proportion in lymph nodes of the abdominal cavity indicate, the other important route of transmission is ingestion of the agent which may happen during the suckling time from the infected dams or by licking, feeding or drinking contaminated materials (Hardie and Watson, 1992; Morris *et al.*, 1994; Smyth *et al.*, 2001).

In the current study, the chance of growing *Mycobacteria* was less than 50% which might be due to either losses of the agent during freezing or delayed transportation from the site of collection. WHO (1998) indicated losses of 5-10% due to contamination resulting from prolonged preservation, which in turn resulted into, overgrowth of *M. bovis* with environmental *Mycobacteria* and a loss of up to 60% due to decontamination procedure. Besides, *M. bovis* grows poorly on standard Löwenstein-Jensen medium (Cleaveland *et al.*, 2007). Therefore, the use of proper time in culturing and application of standard laboratory technique could increase the chance of recovery of acid fast bacilli. However, the result was higher than the previous report (Cristina Pires de Araujo *et al.*, 2005) where only 17 isolates obtained from 72 (23.6%) lesion positive samples and in agreement with the report of Shimels, 2008 (32%).

Molecular analysis of the isolates revealed that the lesions were caused not only by the members of *M. tuberculosis* complex but also by members of the non-*M. tuberculosis* group. Even majority of the isolates belonged to the later group. Similar results were reported by other workers in Ethiopia (Berg *et al.*, 2009). The techniques employed by the present study could not further identify these isolates to the species level.

6. CONCLUSIONS AND RECOMMENDATIONS

The result of the present study has shown that bovine tuberculosis is prevalent in cattle slaughtered at Nekemete Abattoir although it is less prevalent in cattle kept at Horro-Guduru Cattle Breeding and Improvement Center. Further, it was observed that the lesions were caused by *Mycobacteria* that belong to both *M. tuberculosis* complex and non-*M. tuberculosis* complex group. Even the majority of the isolates were members of the non-*M. tuberculosis* members. However, the prevalence of tuberculin result was low, enormous numbers of tubercle bacilli can be excreted by a cow with tuberculous mastitis. One cow can excrete enough viable bacilli to contaminate the milk of many cows, when their milk is mixed. Additionally it is much enough to contaminate an environment in which they belong (particularly pasture and water body), through faces and air droplet. In addition to this people of the area have the habit of consuming raw meat and milk and share the same microenvironment with their livestock. This further disseminates the causative agent, both through inhalation and ingestion resulting in high economic loss and public health effect. This study reveals a high proportion of tuberculous lesion in the thoracic cavity lymph nodes (70%). It implies that respiratory rout is the major means of transmission. On the basis of this conclusive remark, the following recommendations were forwarded:

- ❖ Detailed abattoir inspection should be implemented by focusing on lymph nodes of the thoracic, mesenteric and Head region.
- ❖ Further similar studies in the slaughterhouse across the country so as to estimate the national prevalence of BTB,
- ❖ Further indentification and characterization of the non-*M. tuberculosis* complex, and evaluation of their pathogenecity in bovine.
- ❖ Public education to increase the awareness of the community about the potential risk of consumption of raw animal products is necessary.

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8. LIST OF ANNEXES

Annex 1. Description of body condition scoring

1. Condition Score 1(L-)	Marked emaciation- the animal could be condemned ante-mortem
2. Condition Score 2 (L)	Transverse processes project prominently, spine appear sharply
3. Condition score 3 (L+)	Individual dorsal spines are pointed to the touch, hips, tail-head and ribs are prominent
4. Condition score 4 (M-)	Ribs hips and pins are clearly visible, muscle mass between hooks and pins are slightly concave.
5. Condition score 5 (M)	Ribs usually visible, little fat cover, dorsal spines are barely visible
6. Condition score 6 (M)	The animal is smooth, dorsal spines can not be seen, but are easily felt.
7. Condition score 7 (F-)	Animal is smooth and well covered but fat deposit are not marked
8. Condition score 8 (F)	Fat cover in critical areas can easily be seen and felt; transverse Process can be seen or felt
9. Condition score 9 (F+)	Heavy deposits of fat is clearly visible on tail-head, brisket, dorsal spines ribs and hooks

Source: (Nicholson and Butter worth, 1986)

Key: 1-3 Lean, 4-6 medium, 7-9 Fat

Annex 2. Age determination

Age (in years)	Characteristic Change
1 ½ - 2	I1 erupts
2 - 2 ½	I2 erupts
3	I3 erupts
3 ½ - 4	I4 erupts
5	All incisors and canine are in wear

- 6 I1 is level and the neck has emerged from gum
- 7 I2 is level and the neck is visible
- 8 I 3 is level and the neck is visible,
- 9 I4 may be level
- 10 I4 is level and the neck is visible
- 11 The dental star is squire in I 1 and in all teeth by 12 years
- 12 The teeth that are not fallen out are reduce to small round pegs
- * Canine of ruminant is usually combined as fourth incisor

Source: Amstutz (1998)

Annex 3. Slaughterhouse data collection sheet for bovine tuberculosis

ID	Sex	Age	Breed	Origin	BCS	MD	MR	MDF	MDR	BR	BL	HP	MES	Lung	Others

MD, mandibular (right and left) ; MR,medial retropharyngeal ; MDF, mediastinal cranial ; MDR, caudal mediastinal; BR, bronchial right ; BL, bronchial left ; HP, hepatic; MES, mesenteric

Annex 4. Preparation of Lowenstein Jensen Media

Ingredients (WHO, 1998)

Mineral salt solution

Potassium di hydrogen phosphate	2.4gm
Potassium Sulphate	0.24gm
Magnesium citrate	0.69gm
Asparagines	3.6gm
Glycerol	4ml
Sodium Pyruvate	12gm
Distilled Water	600ml

Malachite green solution 2%

Malachite green dye	2gm
Sterile water	100ml

Homogenized whole eggs. Fresh hen eggs not more than 7 days old.

For Preparation of Complete Media, the following ingredients are essentially pooled in a large sterile flask and mixed well.

Mineral salt solution 600ml

Malachite green solution 20ml

Homogenized eggs (20- 25 depending on site) 100ml

These are mixed well and added in each of the universal bottles for media preparations. Bottles are put in an autoclave in slant position at 80-85 °c for 45 minutes to make the media sterile. The slant media is solidated and cooled after which it is kept in deep freezer.

Annex 5. Procedures for tissue sample culturing

Homogenization:

- Take samples from the freezer (Field sample).
- Frozen samples must be completely defrosted prior to processing
- Samples should be properly labeled
- Homogenize the tissue with sterile mortar and pistol.
- Transfer 1ml of the homogenate into the labeled centrifuge tube

Decontamination (Petroff's method):

- Add equal volume of 4% NaOH and shake using automatic shaker for 15 min
- Centrifuge at 3000 rpm for 15 min and decant the supernatant.
- Add 1-2 drops of phenol red indication.
- Neutralize by using 2N HCl; add drop by drop and shake continuously until the color changes from red to yellow.

Inoculation:

- Label 2 LJP medium tube
- Inoculate 100µl to each tube.

Incubation and follow up

- Incubate the newly inoculated culture in a slant position for one week at 37 °C.
- Turn them upright in the second week and follow up for growth over a period of 12 week.

Annex 6. Acid Fast Procedures (Zeil Neelsen Procedure)

- ❖ Carbol-fuchsin: Dissolve 3gm of basic-fuchsin in 10ml of 90-95% ethanol, add 90ml of 5% solution of phenol.
- ❖ Acid- alcohol: add 3ml of concentrated HCL slowly to 97 ml of 90-95% ethanol.
- ❖ Methylene blue counter stain: dissolve 0.39ml of methylene blue chlorides in 100ml of distilled water.
- ❖ Cover a heat fixed dried smear with filter paper, apply 5-7 drops of carbol-fuchsin stain on moistened filter paper.
- ❖ Heat the stain- covered slide.
- ❖ Remove the paper with forceps, rinse and drain. Decolorize with acid alcohol (2 min).
- ❖ Rinse, drain and air dry (11-12min), Counter stain with methylene blue (30 sec- 1min).
- ❖ Examine with 100X oil immersion objective. Mycobacteriums are stained red and background is blue.

Source: Quinn *et al.*, 2002

Annex 7. DNA extraction from Mycobacterial culture isolates

Method: (CTAB/NaCl method)

- Transfer at least 2 loops of mycobacteria into a microcentrifuge tube containing 400µl of 1XTE buffer.
- Kill the cells by heating for 20min at 80 °C and cool at room temperature.
- Add 50 µl of 10mg/ml lysozyme, vortex and incubate, while shaking for at least 1hr at 37 °C
- Place the CTAB/NaCl solution at 65 °C water bath for use in the following step.
- Add 100 µl of 5M NaCl and 100 µl of prewarmed CATB/NaCl solution and vortex until the liquid content become white (milky). Incubate for 10min at 65 °C
- Add 750 µl of chloroform/Isoamyl alcohol and vortex for at least 10sec.
- Centrifuge for 5min at 13000 rpm.
- Make new eppendorf tubes (as the same as the sample).
- Take the supernatant from centrifuged sample to new eppendorfs. Taker not to take the white matter below the supernatant.
- Add 450 µl isopropanol (kept at +4 °C), then mix it well. Then keep it in freezer (+4 °C) for 2hrs or over night.
- Take out from the freezer and keep it at room temperature.
- Centrifuge it at 13000 rpm for 10min.
- Discard most of the supernatant; leave about 20 µl (3mm height) above a pellet.
- Add 900 µl cold 70% ethanol alcohols (from -20 °C freezer or from +4 °C).
- Centrifuge it for 10 minutes at 13000 rpm.
- Discard most of the supernatant; leave about 20 µl above the pellet.
- Permit the pellet to dry for 15minute at room temperature or left it to dry faster in hybridization oven at 37 °C until all the ethanol evaporated, if the ethanol is not evaporated extend the drying time.
- Add 20 µl of 1XTE buffer for dissolving the DNA.

Source: ALIPB-TB-Molecular Biology/Immunology Laboratory.

Annex 8. Polymerase chain reaction procedures

1. PCR- Mix

7 μ L H₂O Qiagen

10 μ L Mastermix

0.3x3 primers

2 μ L DNA template

2. PCR amplication Cycle

	Temperature	Time
Cycle1 /Denaturation	95 °C	60 sec
Cycle 2 /Annealing	55 °C	60 sec
Cycle2/extension	72 °C	60 sec

3. Gel-separation

A 1% agarose gel stained with ethidium bromide is used

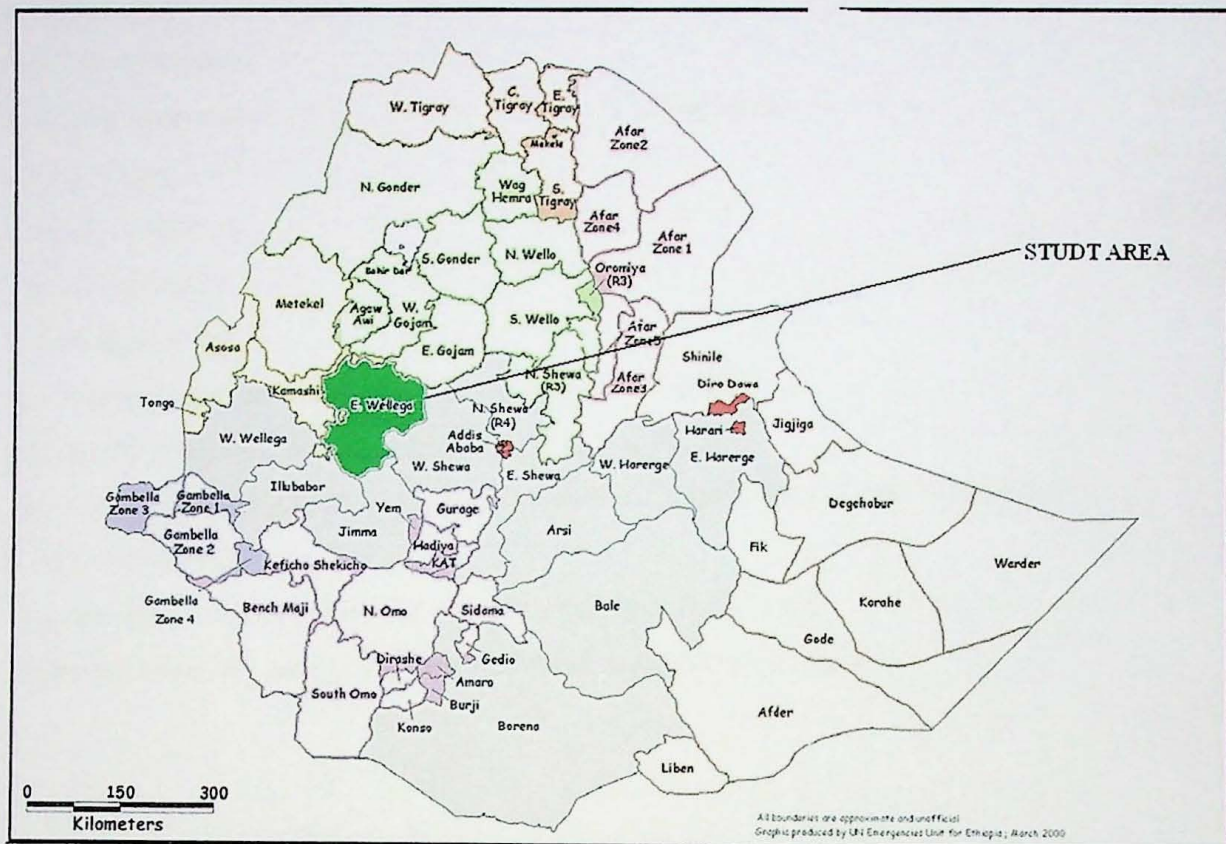
10 μ l of the product is loaded with 2 μ l loading buffer

2 μ l of a 100 bp DNA Molecular weight marker is loaded with 2 μ l loading buffer in a Single outside well

Gel electrophoresis is performed at 100 to 120 volts for 60 min

Analysis is done by using an automated photo documentation system

Annex 9. Pictures of the study area and laboratory work



Map of the study area



Figures of the study animal population

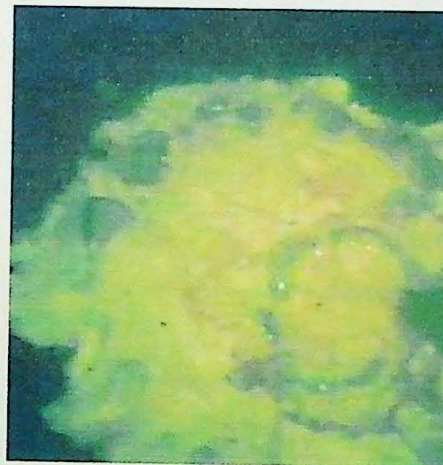
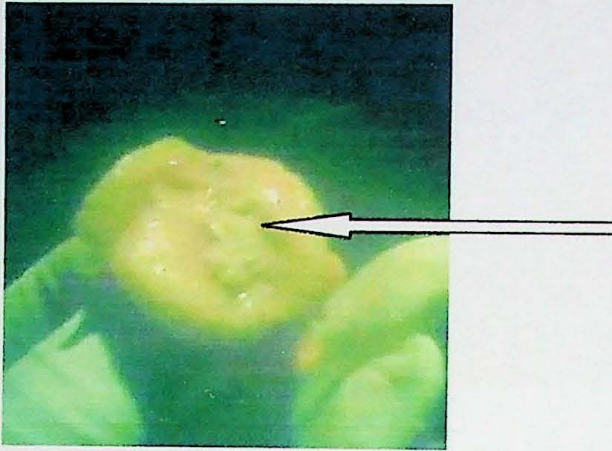




Tuberculin reaction



Age determination



Different tuberculous lesions

9. DECLARATION

I, the undersigned, declare that the thesis is my original work and has not been presented for a degree in another university, and that all sources of material used for the thesis have been duly acknowledged.

Name Mezene Woyessa Busa

Signature _____

Date of submission _____

The thesis has been submitted for examination with our approval as university advisors

Dr. Yasmin Jibril _____

Dr. Gobena Ameni _____

Dr. Reta Duguma _____