ADDIS ABABA UNIVERSITY
COLLEGE OF HEALTH SCIENCES
SCHOOL OF VETERINARY MEDICINE

ISOLATION AND MOLECULAR CHARACTERIZATION OF MYCOBACTERIA FROM INFECTED CATTLE IN AND AROUND BAKO, WESTERN OROMIA, ETHIOPIA

BY
TEMESGEN AYANA DAFA

JUNE 2010
DEBRE ZEIT, ETHIOPIA

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A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the Degree of Master of Veterinary Science in Tropical Veterinary Microbiology

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LIST OF ABBREVIATIONS

A	Adenine
AAU	Addis Ababa University
AFB	Acid fast bacilli
AHRI	Armauer Hansen Research Institute
ALIPB	Aklilu Lemma Institute of Pathobiology
AMP	Adenosine mono phosphate
BARC	Bako Agricultural Research Center
BCG	Bacillus of Calmette and Guerin
BCs	Body condition score
bp	base pair
BTB	Bovine tuberculosis
C	Cytosine
CBPP	Contagious bovine pleuropneumonia
CD	Cluster of differentiation
CFP	Crude filtrate protein
CFT	Caudal fold test
CI	Confidence interval
CIDT	Comparative intradermal skin test
CMI	Cell mediated immunity
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside Triphosphate
DTH	Delayed type hypersensitivity
DR	Direct repeat
DVR	Direct variable repeat
E	East
ECL	Enhanced chemiluminescence
EDTA	Ethyl diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
*EMbs*1	Ethiopian *M. bovis* strain1 V

ESAT	Early secretary target antigen
FAO	Food and Agriculture Organization
FMD	Foot and mouth disease
FPA	Fluorescence polarization assay
G	Guanine
gm	gram
h	hour
HCl	Hydrogen chloride
HPLC	High Performance Liquid Chromatography
H_2O	Water
iELISA	indirect enzyme linked immunosorbent assay
IFN-γ	Interfeeron-gamma
IL	Interleukin
ILRI	International Livestock Research Institute
ITS	Internal transcribed ribosomal regions
IS	Insertion sequence
IU	International unit
LJ	Lowenstein-Jenson
LSD	Lumpy skin disease
mg	Milli gram
$MgCl_2$	Magnesium chloride
min	minutes
ml	milli liter
mm	milli meter
MoA	Ministry of Agriculture
MPB	*Mycobacterium bovis* specific protein
m-PCR	Multiplex-Polymerase Chain Reaction
MTC	*Mycobacterium tuberculosis* complex
N	North
NaOH	Sodium hydroxide
ng	Nano gram

NTM	Non-tuberculous mycobacteria
NVL	Non-visible lesions
OIE	Office International des Epizooties
OR	Odds ratios
PGRS	Polymorphic Guanine Cytosine rich sequences
PIM	Phosphatidyl inositol mannoside
PPD-A	Purified protein derivative-Avian
PPD-B	Purified protein derivative-Bovine
PPD	Purified protein derivative of tuberculin
RD	Regions of difference
RFLP	Restriction fragement length polymorphism
rRNA	Ribosomal Ribonucleic acid
SIDT	Single intradermal skin test
T	Thymine
Taq	*Thermus aquaticus*
TAE	Tris Acetic acid EDTA
TB	Tuberculosis
TCH	Thiophen-2 carboxylic acid hydrazide
Th	T helper cell
UK	United Kingdom
UV	Ultra violet
VLA	Veterinary Laboratories Agency
VNTR	Variable number tandem repeat
WHO	World Health Organization
ZN	Ziehl-Neelsen
X	Times
X^2	Chi-square
µl	Micro liter
^oC	Degree Celsius
%	Percentage

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ABSTRACT

A cross-sectional study was conducted on 858 heads of cattle (371 recruited from Bako Agricultural Research Center, BARC and 487 from Bako municipal abattoir) from October 2009 to April 2010 to estimate the prevalence of bovine tuberculosis (BTB) and characterize its causative agents. To this effect, comparative intradermal tuberculin (CIDT) test, post mortem examination, bacteriological culturing, genus typing using multiplex polymerase chain reaction (PCR) were applied. At the cut-off point of ≥4mm (recommendation of the Office des International Epizooties, OIE), animal prevalence of BTB was 1.3%. Re-analysis of the same data using the newly defined cut-off point of >2mm gave a prevalence of 4.6%. There was a highly significant difference (P=0.000) in prevalence estimated using the two cut-off points in zebu cattle. The prevalence was significantly associated with age (P=0.012) and origin (P=0.04) of the animal. On the other hand, the prevalence was 9.3% (46/492) in cattle slaughtered at Bako abattoir. Lesions were more frequent and severe in the mesenteric (60.9%), retropharyngeal (17.4%), and bronchial (17.4%) lymph nodes of slaughtered cattle. Culture positivity in suspicious tissues was 32.6% (29/89) although only 69% (20/29) were confirmed to be acid-fast bacilli (AFB) by Ziehl Neelsen staining. Of the 20 AFB positive isolates, 17 were characterized by multiplex PCR and almost all (94%) of the isolates showed signal for the Genus *Mycobacterium*. Nevertheless, none of these isolates showed signal for the *Mycobacterium tuberculosis* complex as revealed by multiplex PCR. Therefore, further identification and characterization of these isolates should be done. Furthermore, the pathogenicity of these mycobacteria in livestock requires further investigation.

Key words: Bovine tuberculosis, Prevalence, Pathology, Culturing, Molecular typing, Mycobacteria, Bako, Ethiopia.

1. INTRODUCTION

BTB is a chronic infectious disease characterized by progressive development of granulomatous lesions or tubercles in the lung tissue, lymph nodes, or other organs of infected animals. *Mycobacterium bovis* (*M. bovis*), the causative agent of BTB, was discovered in 1882 by Robert Koch (1843-1910), who first showed that different organism cause TB in cattle and human. Together with other highly related bacteria; *M. bovis* forms a complex, the *M. tuberculosis* complex (MTC), a single species as defined by DNA/DNA hybridization studies (Imaeda, 1995). The MTC comprises seven members which include, *M. bovis*, which is responsible for BTB, and includes the vaccine strain *M. bovis* BCG; *M. tuberculosis*, the primary causative agent of human TB; *M. africanum*, the main causative agent of human TB in West Africa (Kallenius *et al.*, 1999); *M. microti*, which is a pathogen of voles and rarely infects humans (Rastogi *et al.*, 2001); *M. canetti*, a rare MTC strain that produces smooth and glossy colonies, with all known cases so far isolated from individuals having been to the horn of Africa (Smith *et al.*, 2006); *M. pinnipedii*, also known as the seal bacillus (Cousins *et al.*, 1993; Cousins *et al.*, 2003); and *M. caprae* primarily isolated from goats (Aranaz *et al.*, 2003). Among MTC, *M. bovis* has a wide range of both target organs (lungs, gastrointestinal tract, mammary gland, kidney and reproductive organs) and mammalian host (wild animals, domestic animals and human) (Phillips *et al.*, 2002; Rupp *et al.*, 2007).

Infection of cattle with *M. bovis* constitutes a human health hazard as well as an animal welfare problem. Furthermore, the economic implications in terms of trade restrictions and productivity losses have direct and indirect implications for human health and the food supply (Pollock and Neill, 2002; Villarreal-Ramos *et al.*, 2003). Being aware of these problems, most developed countries initiated a control strategy since the early 1920s, using wide spread application of quarantine, compulsory test and slaughter combined with management regulations (Pereira-Suárez *et al.*, 2006). In consequence, the epidemiology and public health significance of BTB was well studied and hence, BTB was controlled in farm animals. As a result, human infection with *M. bovis* has been reduced to a very low level, although a potential risk still exists in countries where wild reservoir hosts are found (Ayele *et al.*, 2004).

In low-income countries, particularly in Africa, however, the epidemiology and public health significance of BTB is largely unknown for politico-economic reasons, including the high costs of a sustainable testing programme, social unrest due to political instability and ethnic wars, resulting in the displacement of large numbers of human and animal populations, lack of veterinary expertise and communication networks. In addition, there are few or no laboratories that are capable of isolating and characterizing mycobacteria from animal and human beings (Ayele *et al.*, 2004). Nevertheless, the potential of *M. bovis* infection is straightforward in both animals and humans in many African settings where domestic animals are an integral part of human social life, poor food hygienic practices like consumption of unprocessed dairy and meat products, and HIV-AIDS infections (Oloya *et al.*, 2007).

In Ethiopia, the endemic nature of TB in cattle has long been reported (Hailemariam, 1975). Most of the BTB surveys carried out in Ethiopia have been based on tuberculin skin testing and *post mortem* inspection of carcasses of slaughter animals in a particular locality. These limited and fragmented surveys show that the prevalence of BTB ranges from 3.4% to 50% depending on the production systems under which cattle are kept (Kiros, 1998; Ameni *et al.*, 2001; Regassa, 2005; Ameni *et al.*, 2007; Berg *et al.*, 2009; Ameni *et al.*, 2010). Nevertheless, there have not been complete nationwide studies to date that can address the economic impacts and the total burden of mycobacterial infection in cattle populations managed under different systems (Tschopp *et al.*, 2009). Moreover, even though there is a direct correlation between the prevalence of human TB of bovine origin and that of TB in livestock (Cosivi *et al.*, 1998) and the elimination of TB from human society necessitates understanding and controlling *M. bovis* in animals, there is lack of attention of BTB as a public health threat in the country (Ameni *et al.*, 2010). Hence, the contribution of bovine bacillus in the overall incidence of human TB is unknown. Yet, report from the ministry of health to WHO (2005) show a significant rise in the incidence of extra-pulmonary TB in human populations of the country but did not indicate the extent attributed to *M. bovis* as the routine diagnostic procedure employed could not differentiate mycobacterium species of the *Mycobacterium tuberculosis* complex.

Studies carried out in various countries indicate the prevalence, distribution and epidemiological pattern of BTB to vary from region to region and even from farm to farm in the same region (Ayele *et al.*, 2004; Oloya *et al.*, 2006, Oloya *et al.*, 2007). However, in Ethiopia, most of the

studies on BTB were conducted in the central part of the country (Ameni *et al.*, 2003; Asseged *et al.*, 2004; Ameni *et al.*, 2007) and hence, there is still no or limited information about the actual situation of mycobacterial infections in various localities and regions of the country (Tschopp *et al.*, 2009). However, accurate baseline data for the prevalence of BTB across a range of eco-epidemiological settings is needed to ensure that public health policies and disease control strategies are based on sound scientific data (Ayele *et al.*, 2004).

The aim of this study was therefore to generate information on the epidemiology of BTB in cattle in and around Bako, Western Oromia so as to understand the disease burden and to inform public health policies. In the area, practices such as the consumption of raw milk and meat is practiced widely which means that the opportunity for zoonotic transmission of BTB is high. We therefore carried out BTB surveys in cattle kept at BARC and Bako abattoir to estimate the prevalence of BTB, and characterize mycobacteria circulating in the area. In order to achieve these objectives, both conventional and molecular diagnostic tools were used.

Objective

General objective

- To estimate the prevalence of BTB, and characterize mycobacteria circulating in and around Bako

Specific objectives

- To estimate the prevalence of BTB at Bako Agricultural Research Centre
- To estimate the prevalence of BTB in cattle slaughtered at Bako Abattoir,
- To isolate mycobacteria from tuberculous tissues and characterize the isolates using molecular typing techniques,
- To identify the risk factors associated with mycobacterial infection in animals

2. LITERATURE REVIEW

2.1. *Mycobacterium*

2.1.1. Taxonomy and nomenclature

Mycobacteria belong to the Kingdom of Bacteria; Phylum of Actinobacteria; Order of Actinomycetales; Family of *Mycobacteriaceae,* and the Genus of *Mycobacterium.* The genus *Mycobacterium* includes many pathogenic species known to cause serious diseases in mammals, and non-mammal animal species. Mycobacterial taxonomy is continuously changing since unidentified species are constantly being discovered. The species belonging to a species group (sometimes referred to as a species-complex) can be very different in virulence or pathogenesis (DSMZ, 2008).

Several previously considered species appear to consist of several closely related species, as biochemical and mainly genetic analyses have demonstrated in, for instance, the *M. tuberculosis*-complex. The complex species can be identified using differences in size of genomic sequences: Region of Difference1 (RD1), RD2, RD4, RD9 and RD12, analysed in PCR (Warren *et al.*, 2006). This complex now consists of *M. tuberculosis, M. africanum, M. microti* and *M. bovis*, the latter of which has been further differentiated into *M. bovis*, *M. caprae, M. pinnipedii* and *M. canetii,* all of which were named after their principal host (Haddad *et al.*, 2004; Pavlik *et al.*, 2005; Warren *et al.*, 2006). A similar phenomenon is found in the *M. avium*-complex. Originally, one species, but meanwhile divided into the species *M. avium*, *M. intracellulare* and *M. scrofulaceum.* Previous serovars belonging to this complex have been re-arranged (Thoel *et al.*, 1990; Butler *et al.*, 1992; Wayne *et al.*, 1993) and several subspecies of *M. avium* have been identified: *M. avium subsp. paratuberculosis, subsp. silvaticum* and *subsp. avium.* This identification was based on molecular and biochemical criteria, that is, High Performance Liquid Chromatography (HPLC) of mycolic acids and sequencing of, for instance, Internal transcribed ribosomal regions (ITS). *M. avium subsp. avium* has recently been divided into *subsp. avium* and *subsp. hominissuis* using Restriction Fragment Length Polymorphism (RFLP) of insertion sequence *IS*1245 (Mijs *et al.*, 2002).

The identification of a new species was conventionally based on the description of the Runyon classification, the biochemical properties of the strain(s) and the degree of DNA-DNA hybridization. With the growing amount of species and subspecies with various biochemical properties, new methods were developed and this created a new standard for species differentiation (Rogall *et al.*, 1990). For the acceptance of a new species, the old and new ways of identification are all included: biochemical characteristics, growth and pigmentation characteristics, HPLC analysis and a unique genetic composition determined by the sequence of genes that allow species differentiation, such as the 16S rRNA gene, the *hsp65* gene and the ITS region (Hale *et al.*, 2001; DSMZ, 2008). From 1990 to 1999, 28 new species have been recognized (Hale *et al.*, 2001) and from 2000 to September 2007, 41 more species have been identified.

2.1.2. 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 a high lipid content (60%) (Brennan, 2003) (Figure 1). Lipids account for acid-fastness and pathogenic and immunogenic properties. Various structural components that are found in mycobacteria include surface mycosides (mostly glycolipids and peptidoglycolipids), mycolic acids and their esters, wax D, cord factor (dimycolil trehalose), sulfolipids (or sulfatides), phosphatidyl inositol mannoside (PIM), mycobactins, carotenoid pigments (in chromogens) and tuberculins (peptides liberated into culture media during growth) (Biberstein and Hirsh, 1999). The genome of mycobacteria has a high GC content (GC% ~65%), and its polymorphism is very limited compared to its genome size (4.4 Mbp). But some regions are highly polymorphic, either by a variation in number and/or position or by a variation in primary structure (Haddad *et al.*, 2004). Strikingly, the genome sequence of *M. bovis* is >99.95% identical to that of *M. tuberculosis*, but deletion of genetic information has led to a reduced genome size (Garnier *et al.*, 2003).

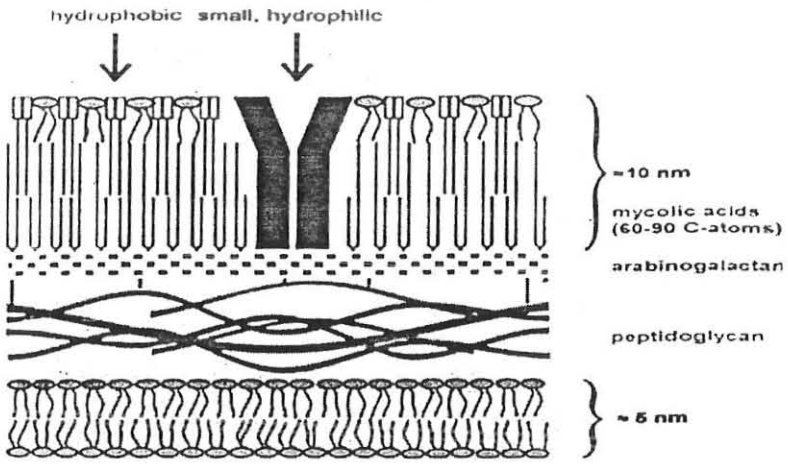

Figure 1: Schematic diagram of mycobacterial cell wall (Brennan, 2003).

2.1.3. Physical and biochemical characteristics

Morphology

The mycobacteria are thin (slender) rods of varying lengths (0.2-0.6 by 1.0-10.0 μm) (Quinn *et al.*, 1994). 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, conidia or capsules (Tauro *et al.*, 1996; Quinn *et al.*, 1999). The distinguishing features of pathogenic mycobacteria are the formation of characteristic cords (Gillespie and Timoney, 1981) and *M. tuberculosis* is straight or slightly curved rod, whereas *M. bovis* is usually straighter, stouter and shorter (Gupte, 2006). The mycobacterial cell wall is triple layered comprising a basal peptidoglycan layer and an intermediate arabinogalactan mycolate complex. The outer layer is lipid rich, comprising surface rope-like structure of peptidoglycolipid (Brennan, 2003) (Figure 1).

Staining

Mycobacteria are cytochemically gram positive, but, they often resist staining with gram stain (Biberstein and Hirsh, 1999). Thus, they cannot be classified as either gram-positive or gram-negative bacteria (Brooks *et al.*, 2004). 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 (Biberstein and Hirsh, 1999) that depends on the integrity of the waxy envelope and is related to the resistance of *Mycobacteria* to the decolorizing effect of strong acid/alcohol solutions unlike other bacteria which quickly get decoulorized. Usually the Ziehl-Neelsen technique of staining is employed for identification of acid-fast bacteria including mycobacteria (WHO, 1998a; Brooks *et al.*, 2004). Moreover, mycobacteria can also be stained with fluorescent dyes like auramine rhodamine (Biberstein and Hirsh, 1999).

Growth requirement 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, viz egg-based media, agar-based media and liquid media (WHO, 1998b). 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, 1998b). To date, the most frequently used media for isolation of *M. bovis* are Löwenstein-Jensen (LJ) and Ogawa-medium (both containing eggs phosphate and magnesium) and the former contains asparagine (Seifert, 1996).

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.*, 1994). All the members of the mycobacteria complex are slow growers (Seifert, 1996). Therefore, the inoculated media may have to be incubated at 37°C up to 8 to 12 weeks (Quinn *et al.*, 1994). The growth requirements and cultural characteristics of mycobacteria are summarized in table 1. On solid media *M. tuberculosis* appears as dry, rough, raised and irregular colony. It is creamy white first and becomes yellowish or buff coloured 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).

Table 1: Growth requirements and cultural characteristics of pathogenic mycobacteria

Growth characteristics and requirements	*M. tuberculosis*	*M. bovis*	*M. avium* complex	*M. avium paratuberculosi*
Growth rate	Slow (3-8 weeks)	Slow (3-8weeks)	Slow (2-6 weeks)	Very slow (up to 16 weeks)
Atmospheric requirements	Aerobic	Aerobic	Aerobic	Aerobic
Colonial features	Rough, buff, difficult to break apart	Cream colored raised with central roughness, break apart easily	Sticky, off white, break apart easily	Small, hemispherical, some pigmented
Essential growth supplement	None	None	None	Mycobactin
Effect of added glycerol	Enhanced growth (Eugonic)	Growth inhibited (dysgonic)	Enhanced growth (Eugonic)	

Source: Quinn *et al.* (1999)

2.2. Pathology

2.2.1. Infection

The routes through which tubercle bacilli gain entrance into the animal body include: the respiratory, alimentary, genital, cutaneous, congenital and venereal routes (Menzes and Neill, 2000); the first two being the most commonly observed routes of infection resulting in pulmonary and extra-pulmonary forms of the disease, respectively. Furthermore, infection can occur via the teat canal (Seifert, 1996). However, infection in animals is usually via the respiratory and intestinal tracts (Quinn *et al.*, 1994). In intensive animal production systems, the infection is

acquired aerogenously by the inhalation of infected droplets from a coughing or sneezing animal with open tuberculosis or infected dust particles from the floor of the paddock or cowshed; the primary focus of infection is localized in the lung. For animals in the extensive production system, the infection is acquired through the oral route; in this case, bacteria excreted with faeces, urine, milk, lochia (from cows with endometritis i.e. as high as 5%) and mycobacteria-laden exudates from the lung are ingested with contaminated feed and water. The primary focus with this route of infection is in the lymph nodes of the intestinal system (Seifert, 1996). The infectious dose is very low; 1-3 viable bacteria are considered sufficient as infectious inoculum (Thoen *et al.*, 2006).

2.2.2. Virulence factors

Since mycobacteria are intra-cellular organisms, their virulence appears to be related to their ability to survive and multiply within the macrophages. The mechanism for such survival is poorly understood and may vary from species to species (Quinn *et al.*, 2002). For instance, *M. tuberculosis* inhibits the fusion of lysosomes with the phagosome due to factors such as polyglutamic acid, ammonia, and cyclic AMP and sulpho-lipids. The sulfur-containing glycolipid (sulpholipids) commonly called sulfatides play the major role in inhibiting phagolysosome formation. 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 (Collins, 2001). *M. avium* survives within the fused phago-lysosome by a virtue of their capsule like coating material of mycosides. In addition to these survival mechanisms, an important aspect of pathogenecity of mycobacteria is their ability to subvert the protective immune response (Grange, 1995). Wax D and various tuberculo-proteins induce a delayed hypersensitivity reaction detected in the tuberculin test (Quinn *et al.*, 1994).

A characteristic feature of virulent strains of mycobacteria is that they form cords when they grow in a liquid culture media whereas the avirulent strains develop as clumps. Lipids present in the cell wall of virulent tubercle bacilli appear to contribute to the formation of "rope-like" cords in parallel form (Thoen *et al.*, 2006) the cord factor is a glycolipid that inhibits leukocytic migration and has also toxic effect on leukocytes (Gillespie and Timoney, 1981).

2.2.3. Pathogenesis

Mycobacterium bovis is an intracellular pathogen of macrophages and other cells of the monocytic type (de la Rua-Domenech *et al.*, 2006). In previously unexposed animals, following infection local multiplication of the mycobacteria occurs and the resistance to phagocytic killing allows continued intracellular and extracellular multiplication. Before cell mediated immunity is established lymphatic spread occurs with general dissemination. At the site of a lesion, aggregation of macrophages contributes to the formation of tubercle, and a fibrous layer may encompass the lesion. Caseous necrosis occurs at the centre of the lesion and this may proceed to calcification or liquefaction. After cell mediated immunity (CMI) is established spread occurs by contiguous extension or via the erosion of bronchi, blood vessels or viscera to new areas (Quinn *et al.*, 1994).

2.2.4. Lesion distribution

A primary lesion or focus of infection is established following the interaction of the host and the agent at the site of entry within 8 weeks of bacterial entrance (Radostits *et al.*, 2000). The mycobacteria are then taken by the alveolar macrophages to the circulation and establishes in lymph nodes. Cellular responses attempting to control the disease results in the accumulation of large number of phagocytes and lead to the formation of a macroscopic lesion referred as tubercle (Theon *et al.*, 2006). Tubercles commonly occur in the thoracic cavity (chest), though they may be found in other major organs such as the liver. In cattle, BTB most commonly causes 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).

2.3. Immunity

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). Immunity to mycobacteria is characterized by a CMI response where infected macrophages become surrounded by a zone of lymphocytes to form a granuloma (Wedlock *et al.*, 2002). 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)-12, and tumour necrosis factor (TNF)-α production to pathogen-associated antigen (Flynn, 2004). All of the main T-cell subsets (γδ T-cells, CD4 and CD8 αβ T-cells) have been shown to be involved in the anti-mycobacterial immune response in cattle (Pollock *et al.*, 2005). The humoral immune response to *M. bovis* infection in cattle is characterized by highly heterogeneous antigen recognition. Six proteins, early secretory target antigen-6 (ESAT-6), 14-kDa protein, *Mycobacterium* target protein (MPT63), MPT70, MPT51, and MPT32, were identified as major sero-reactive antigens in bovine tuberculosis (Lyashchenko *et al.*, 1998).

2.4. Epidemiology

The epidemiology of the *M. bovis* infection has historically been of great interest owing to the public health and economic consequences of the infection. BTB was first recognized in domesticated animals, although its host range is broad and includes most mammalian species (Theon *et al.*, 2006). *M. bovis* infection is recognized worldwide and has no geographical boundaries (Kazwala *et al.*, 2001). The epidemiology in developed and developing countries differ, owing to differences in the implementation of preventive measures (WHO, 1999). Bovine tuberculosis has been on the increase in developing countries and continues to occur in developed countries (Cosivi *et al.*, 1998; Grange, 2001; Gilbert *et al.*, 2005). Infections occur in diverse groups of mammals; including goats (Bernabe *et al.*, 1991; Garcia *et al.*, 1992), deer (Griffin and Buchan, 1994), buffaloes (Hein and Tomasovic, 1981), humans (Collins and Grange, 1987; Cotter *et al.*, 1996; Moda *et al.*, 1996) and wildlife (Grange and Yates, 1990; Grange, 2001; Pavlik *et al.* 2005). However, many epidemiologic and public health aspects of infection remain largely unknown in the world (Cosivi *et al.*, 1998). Similarly, the epidemiology and public health significance of BTB in Africa remains largely unknown (Ayele *et al.*, 2004).

2.4.1. Distribution

Bovine tuberculosis is a worldwide disease (Fra´guas *et al.*, 2006). Out of one billion cattle population of the world one third are found in areas where BTB is under control. Another third are in areas where the disease is widespread but the incidence unknown, and the remaining third are in regions where the prevalence of BTB is high (Amanfu, 2006).

Industrialized countries have eradicated or drastically reduced the prevalence of *M. bovis* infection in both animals and humans through animal tuberculosis control and elimination programmes, improvements in milk hygiene and pasteurization and awareness creation on the zoonotic and economic significance of *M. bovis* infection (Amanfu, 2006).

In developing countries, animal TB is widely distributed, control measures are not applied or are applied sporadically, and pasteurization is rarely practiced (Bakshi *et al.*, 2005). Out of 55 nations of Africa, 25 reported sporadic/low occurrence of BTB, 6 reported enzootic disease pattern, 2 were described as having a high occurrence, 4 did not report the disease and the remaining 18 countries did not have the data. Of all nations in Africa, only 7 apply disease control measures as part of a test and slaughter policy and consider BTB as notifiable disease; the remaining 48 countries control the disease inadequately or not at all. This approximates 85% of the cattle and 82% of the human population of Africa are in areas where BTB is either partly controlled or not controlled at all (WHO, 1993; Cosivi *et al.*, 1998; Ayele *et al.*, 2004 citing OIE 1998-2001). 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* (Ashford *et al.*, 2001; Ayele *et al.*, 2004).

2.5. Bovine tuberculosis in Ethiopia

In Ethiopia, there are sparse anecdotal data on the prevalence of TB in cattle. In addition, there is scarce information about the strains of *M. bovis* that are present in Ethiopia. Furthermore, the contribution of *M. bovis* to human TB, particularly to the high incidence of extra pulmonary disease is not clearly known (Young, 2008; Ameni *et al.*, 2010). Most of the studies on BTB in Ethiopia have been carried out on the basis of tuberculin skin testing of animals (Table 2), abattoir meat inspections (Table 3) and rarely on bacteriological and molecular techniques. There

are indications from these limited studies that prevalence is related to farming conditions and to cattle breed (Alemu, 1992; Ameni *et al.*, 2001; Regassa, 2005; Ameni *et al.*, 2006; Young, 2008).

The first survey on the prevalence of BTB using intradermal tuberculin skin testing was done by Shola zonal veterinary laboratory from 1984-1986 where 4,838 cows in 12 dairy farms were tested that showed an average prevalence of 16.8% with the highest prevalence (77.7%) recorded in Modjo state dairy farm (Yehualashet, 1993). A year later, a total of 3,352 dairy animals were tested by comparative tuberculin test and an average prevalence of 24.7% was reported in 10 dairy farms with Modjo still showing the highest prevalence of 92.3% (Shitaye *et al.*, 2007 citing MoA, 1987). In other studies conducted later, a prevalence as high as 50% (n=486) has been reported in dairy cattle in Eastern Shoa (Ameni and Roger, 1998) and 38.5% (n=441) in goats in Adami Tulu district (Tafesse, 2006) using the single intradermal tuberculin (SIDT) test. Furthermore, studies made using the recommended comparative intradermal tuberculin (CIDT) test showed a higher individual animal prevalence of BTB. Ameni *et al.* (2003b) reported a prevalence of 46.8% (n=1171) in cattle from 12 selected dairy farms of Ethiopia. The study showed that except one private farm with small number of animals, the rest 11 dairy farms contained the infection (i.e. a herd prevalence of 91.7%) ranging from 10.81% (n=37) in one private farm in Sebeta to 87.1% (n=140) in Debre Zeit state farm. Among the recently undertaken studies using comparative intradermal tuberculin (CIDT) test, the prevalence of BTB ranges from 3.4% in a smallholder production system to 50% in intensive dairy productions in the country (Kiros, 1998; Ameni and Roger, 1998; Asseged *et al.*, 2001; Regassa, 2001; Ameni *et al.*, 2003a; Ameni *et al.*, 2003b; Regassa, 2005; Ameni and Erkihun 2007; Shimelis, 2009).

Abattoir inspection of carcasses also showed the widespread presence of BTB in Ethiopia. A prevalence of 44.27% (n=1441), 22% (n=531) and 5% (n=1138) in cattle slaughtered at Bahir Dar municipality abattoir (Negash, 2006), Debre Brehan municipality abattoir (Shihun, 2008) and Kombolcha ELFORA meat processing plant (Ameni *et al.*, 2010) respectively were recorded. In addition, bovine tuberculosis was reported in 4.34% (n=1,152) of goats and 2.86% (n=384) of sheep slaughtered at Helmix abattoir, in Debre Zeit (Nigussie, 2006), and 5.07% (n=276) of camels slaughtered at Dire Dawa municipality abattoir (Kassaye, 2007). Moreover, a ten year (1996-2005) retrospective analysis of inspection of 2,455,289 slaughtered animals at Addis Ababa municipality abattoir showed that 0.052% (n=1,336,266) of cattle, 0.001% (n=534,436) of

sheep, 0.001% (n=573,767) of goats and 0.009% (n=10,820) of pigs were found harbor tuberculous lesions in parenchymatous organs (Shitaye *et al.*, 2006).

Table 2: Prevalence of BTB in different parts of Ethiopia using intradermal tuberculin test

Location	Type of test	Species	Number examined	Number positive (%)	References
Eastern Shoa	SIDT	Cattle	486	243(50%)	Ameni and Roger, 1998
N. Shoa & S.Wollo	SIDT	Cattle	538	132(24.5%)	Dejenu, 1998
Eastern shoa	CIDT	Cattle	788	234(29.7%)	Kiros, 1998
Wolayita Soddo	SIDT	Cattle	416	149(35.8%)	Regassa, 1999
Addis Ababa	CIDT	Cattle	241	128(10.31)	Asseged *et al.* 2000
Fitche town	SIDT	Cattle	735	31(4.25%)	Gemta, 2000
Dairy Farms of Et	CIDT	Cattle	1171	548(46.8%).	Ameni *et al.*, 2003b
Wuchale Jidda	CIDT	Cattle	763	60(7.9%)	Ameni *et al.*,2003a
Assela	CIDT	Cattle	514	18(3.5%)	Redi, 2003
Bodji	CIDT	Cattle	320	12(3.8%)	Laval and Ameni, 2004
Dire Dawa	CIDT	Cattle	301	93(31%)	Adugna, 2005
Adama	CIDT	Cattle	524	59(11.3%)	Aklilu, 2005
Addis Ababa	CIDT	Cattle	1,071	197(18.4%)	Kebede, 2005
North Gonder	CIDT	Cattle	1,025	215(21%)	Ali, 2006a
Holetta	CIDT	Cattle	705	41(5.82%)	Dabese, 2006
Sululta	CIDT	Cattle	786	73 (9.29%)	Firdessa, 2006
Addis Ababa	CIDT	Cattle	798	246(32.2%)	Hussien, 2006a
Holetta DF	CIDT	Cattle	243	54(22%)	Lambert *et al.*, 2006
Addis Ababa	CIDT	Cattle	2,098	392(19%)	Shitaye *et al.*, 2006
ATARC	SIDT	Goats	191	50(26.18%)	Tafesse, 2006
Adami Tulu	SIDT	Goats	441	170(38.5%)	Tafesse, 2006
Adama	CIDT	Cattle	524	58(11%)	Ameni and Erkihun, 2007
North W. Shoa	CIDT	Cattle	1,041	259(24.9%)	Regassa *et al.*, 2007
Debre Brehan	CIDT	Cattle	521	14(2.7%)	Shihun, 2008
Central Ethiopia	CIDT	Cattle	5,424	732(13.5%)	Ameni, 2008
ADF, South Et.	CIDT	Cattle	355	101(28.45%)	Shimelis, 2009

ADF: Alage dairy farm; ATARC: Adami Tulu Agricultural Research Center; DF: Dairy farm; Et.: Ethiopia; N. Shoa: North Shoa: S. Wollo: South Wollo

Table 3: Prevalence of BTB detected by abattoir meat inspection in cattle at different slaughterhouses of Ethiopia

Location	Species	Number examined	Number positive (%)	References
Nazereth	Cattle	1,125	58(5.15%)	Ameni and Wudie, 2003
Addis Ababa	Cattle	1,350	21(1.5%)	Asseged *et al.*, 2004
Hossana	Cattle	751	34(4.5%)	Tekelu *et al.*, 2004
Modjo	Goats	1,536	65(4.2%)	Hiko, 2005
Hawassa	Cattle	469	11(2.34%)	Jemale, 2005
Nazareth	Cattle	1,601	112(7.0%)	Molla, 2005
Gonder	Cattle	402	64(15.9%)	Worku, 2005
Woldiya	Cattle	605	33(5.5%)	Ali, 2006b
Adama	Cattle	1,018	48(4.7%)	Anebo, 2006
Dessie	Cattle	805	28(3.4%)	Ayanaw, 2006
Buta Jira	Cattle	321	40(12.5%)	Hussien, 2006b
Gimbi	Cattle	420	60(14.3%)	Mulu, 2006
Bahir Dar	Cattle	1,441	638(44.27%)	Negash, 2006
HEA, DZ	Goats	1,152	50(4.34%)	Nigussie, 2006
HEA, DZ	Sheep	384	11(2.86%)	Nigussie, 2006
Addis Ababa	Cattle	984)	34(3.5%)	Shitaye *et al.*, 2006
Jinka	Cattle	337	52(15.4%)	Taddele, 2006
Yabello	Cattle	433	18(4.2%)	Adane, 2007
Woldiya	Cattle	500	30(6.0%)	Hassen, 2007
Dire Dawa	Camel	276	14(5.07%)	Kassaye, 2007
Adama	Cattle	522	129(24.7%)	Mamo, 2007
Buta Jira	Cattle	353	74(20.96%)	Mekonnen, 2007
Jinka	Cattle	317	47(14.8%)	Milkessa, 2007
Addis Ababa	Cattle	600	92(15.36%)	Seid, 2007
North Gonder	Cattle	350	54(15.4%)	Terefe, 2007
Gimbi	Cattle	758	116(15.3%)	Woyessa, 2007
Debre Brehan	Cattle	531	117(22.0%)	Shihun, 2008
Kombolcha	Cattle	1138	57(5.0%)	Ameni *et al.* 2010

HEA: Helmix abattoir; DZ: Debre Zeit; EMPP: ELFORA Meat Processing Plant

There are reports indicating the isolation of the human and bovine tubercle bacilli from clinical and tissue samples (in animals only) obtained from humans and animals in different parts of Ethiopia. Isolation of the agents was made through culture on the Lowenstein-Jensen media and

confirmed using acid-fast staining, colony morphology, biochemical tests (Ameni *et al.*, 2003b; Ameni and Wudie, 2003; Ali, 2006a; Regassa *et al.*, 2007; Shihun, 2008; Ameni *et al.*, 2010). Very recently, Ameni *et al.* (2007), Hassen (2007), Mamo (2007), Mekonnen, (2007), Seid, (2007), Berg *et al.* (2009) and Ameni *et al.* (2010) identified *M. bovis* from isolates obtained from cattle tissue using PCR. Furthermore, Ameni *et al.* (2007) reported the results of strain typing of isolates for the first time in the country from tissue samples of animals in Holetta dairy farm. Spoligotyping of 41 isolates from 17 cows gave an identical and a unique spoligotype pattern that was represented by the binary number 11000001011111101111111000100000000000100000, where 1 indicates the presence of a spacer and 0 represents a loss. This spoligotype pattern had not been reported to the *M. bovis* database (www.mbovis.org), and it was designated as *EMbs*1 (Ethiopian *M. bovis* strain 1). The variable tandem repeat (VNTR) profile of the new strain was 5254*33.1, which differed from the VNTR profile of strains reported from UK. In addition, Berg *et al.* (2009), Tsegaye *et al.* (2009) and Ameni *et al.* (2010) reported a number of strain types of *M. bovis* identified from cattle tissue in different localities of Ethiopia (Table 4).

Table 4: Strain types of *M. bovis* identified from tuberculous tissues of cattle in different localities of Ethiopia using spoligotyping

Strain type	Species of animal	Location	Author
SB1176	Cattle	Kombolcha	Ameni *et al.*, 2010
		Holetta	Ameni *et al.*, 2007
		Woldiya	Berg *et al.*, 2009
		Addis Ababa	Tsegaye *et al.*, 2009
			Berg *et al.*, 2009
SB1190, SB1191, SB1192	Cattle	Kombolcha	Ameni *et al.*, 2010
SB0133	Cattle	Kombolcha,	Ameni *et al.*, 2010
		Woldiya, Jinka	Berg *et al.*, 2009
		Addis Ababa	Tsegaye *et al.*, 2009
SB0912	Cattle	Kombolcha	Ameni *et al.*, 2010
SB0134	Cattle	Gonder	Berg *et al.*, 2009
SB1490	Cattle	Kombolcha	Ameni *et al.*, 2010
SB1491	Cattle	Kombolcha	Ameni *et al.*, 2010
SB1492	Cattle	Kombolcha	Ameni *et al.*, 2010

2.6. Diagnosis

Various types of diagnostic techniques have been developed for the detection of BTB. However, because of the dynamics of *M. bovis* transmission, the microscopic size of early lesions and the time it takes for an animal to mount a detectable immune response, no single ante mortem or post mortem test for TB can be expected, on its own, to detect every infected herd and every infected animal in such herds (de la Rua-Domenech *et al.*, 2006). The various diagnostic methods used may be well suited for one stage of the disease progression but not necessarily others. Each diagnostic method or point of surveillance offers some advantage but has associated disadvantages (Jolley *et al.*, 2007). The diagnosis of BTB in live animals mainly depends on clinical manifestations of the disease, skin testing, and subsequent identification of the pathogen

by biochemical testing (Vitale *et al.,* 1998). After death, it is diagnosed by post mortem examination and then confirmed in the laboratory by histopathological and bacteriological techniques (Jahans and Worth, 2006).

2.6.1. Clinical examination

Bovine tuberculosis may be diagnosed by its clinical symptoms (Seifert, 1996). Clinical signs such as chronic debilitation, moist cough, low-grade fever and enlargement of local lymph nodes may suggest infection with tuberculosis (OIE, 2009). Nevertheless, other techniques are required to ensure the diagnosis.

2.6.2. Post mortem inspection

A tentative diagnosis of BTB can be made following the macroscopic detection at necropsy of typical lesions (Corner, 1994; Liebana *et al.*, 2008). Therefore, during necropsy, the lymph nodes, especially those associated with the head, thorax and abdomen are closely examined (Patterson and Grooms, 2000). Careful examination of as few as 6 pairs of lymph nodes, the lungs and the mesenteric lymph nodes can result in 95% of cattle with macroscopic lesions being identified. However, this method is not useful to determine the significance of cattle that give a positive reaction in diagnostic tests but do not have visible lesions. Non visible lesion reactors (NVL) may be due to early infection, poor necropsy technique or infection with mycobacteria other than *M. bovis* (Corner, 1994). Above all, it is not always possible to differentiate tubercle lesions by gross examination from those of other infections (Liebana *et al.*, 2008).

2.6.3. Histopathological examination

Another important diagnostic method for detection of tuberculosis is histopathologic examination conducted following post mortem (Jolley *et al.*, 2007). For histopathology, fixed samples (in 10 percent formalin) of lesion are required (Quinn *et al.*, 1994) and histologic sections are stained with hematoxylin-eosin (Biberstein and Hirsh, 1999). Microscopic appearance of a typical bovine tuberculous lesion consists of a peripheral zone of mononuclear cells, fibroblasts and giant cells with central caseous necrosis (Quinn *et al.*, 1999). The presumptive diagnosis of tuberculosis can be made if the tissue has characteristic histological lesions such as caseous necrosis,

mineralization, epitheloid cells, multinucleated giant cells and macrophages. The presence of acid-fast organisms in histological sections may not be detected (OIE, 2009).

Histopathological examination requires considerable technical skill, an equipped histopathology laboratory and substantial time to perform. For these reasons, this method is not well suited to large volume surveillance testing and is limited in that definitive identification of *M. bovis* is not possible. Consequently, animals and herds may be identified incorrectly as infected with *M. bovis* if other tests are not used for diagnostic confirmation. This false diagnostic result could lead to economic losses for producers (Jolley *et al.*, 2007).

2.6.4. Immunodiagnostic methods

Intradermal tuberculin test

The tuberculin skin test has remained the primary diagnostic test for tuberculosis in both humans and cattle. They are based on eliciting a delayed-type hypersensitivity response to the intradermal injection of tuberculin, a crude protein extract from supernatants of mycobacterial cultures (de la Rua Domenech *et al.*, 2006). The tuberculin test was introduced by M'Fadyean in 1899 (Silva, 2001). It provides a measure of CMI dependent delayed-type hypersensitivity (DTH) reaction in response to tuberculin (Pollock *et al.*, 2005). Tuberculin test may be performed using bovine tuberculin (PPD-B) alone or as a comparative test (OIE, 2009). Animals, which have been sensitized by non-pathogenic environmental strains of mycobacteria, may react positively to PPD-B, due to the presence of antigens common to virulent and non-virulent mycobacterial strains. Where this occurs, discrimination between cattle infected with *M. bovis* and those exposed to environmental strains is done using the comparative intradermal skin test (Pollock *et al.*, 2005). The comparative intradermal skin test is that in which a delayed type hypersensitivity (DTH) response to purified protein derivative (PPD) of tuberculin from *M. bovis* (PPD-B) and *M. avium* (PPD-A) is assessed and compared (Thom *et al.*, 2006; OIE, 2009).

In spite of its wide use, intradermal tuberculin reactions present some important limitations, related to their sensitivity and specificity (Fra´guas *et al.*, 2006). Tuberculin skin testing lacks sensitivity, can be confounded by exposure to non-tuberculous mycobacteria, and cannot be

repeated for 60 days due to desensitization (Palmer *et al.*, 2006). When skin-test reactive animals are slaughtered and lesions characteristic for tuberculosis are not found (NVL reactors) the reliability of the test may be questioned and confirmatory tests may be required. The tuberculin PPDs are complex antigens and the presence of cross-reactive antigens between other mycobacterial species has driven research to identify *M. bovis*-specific epitopes (Pollock *et al.*, 2005).

Blood-based laboratory tests

A number of new diagnostic blood tests have become available. However, they are usually used as ancillary tests to confirm or negate the results of the intradermal test. Of these tests, the lymphocyte proliferation assay and the gamma-interferon assay correspond to cellular immunity, while the enzyme linked immunosorbent assay (ELISA) corresponds to humoral immunity (OIE, 2009). Serological assays provide an important and needed tool for large volume testing for exposure to *M. bovis*. They offer the important advantages of ease of use, assay speed and relatively low cost (Jolley *et al.*, 2007). To overcome difficulties associated tuberculin skin testing, an effective whole blood cellular immunoassay for bovine gamma interferon (IFN-γ) has been developed. The test is commonly used in conjunction with tuberculin skin testing as a confirmatory test following a positive response to the caudal fold test (CFT) (Palmer *et al.*, 2006). IFN-γ diagnostic test is a rapid whole blood assay. The assay is based on the release of gamma-interferon from sensitized lymphocytes during an overnight incubation with a specific antigen. The detection of plasma gamma-interferon is carried out by means of a sandwich ELISA using specific monoclonal antibodies (Walravens *et al.*, 2002). This test in its current form will not generally be acceptable as a direct substitution for skin testing, but could be applied rather as an ancillary test. Benefits of this test include accelerated elimination of tuberculosis from infected herds and the possibility of the test to be performed as soon as 10 days after the application of a tuberculin skin test. Other applications of the IFN-γ test include confirmation of the immunological status of skin test reactors and the investigation of fraudulent intervention into the skin test (Vordermeier *et al.*, 2001). Combinations of specific antigens/microbial proteins such as culture filtrate protein 10kDa (CFP-10), early secreted antigen target 6-kDa protein (ESAT-6), TB27.4, TB16.2, TB15.8, and TB10.4 induce strong gamma interferon responses in cattle and

have potential as diagnostic reagents which could be used in a whole blood assay for diagnosis of tuberculosis (Aagaard *et al.*, 2003).

Different enzyme-linked immunosorbent assay (ELISA) tests have been developed recently for the detection of antibodies to *M. bovis* (Silva, 2001) and have been suggested as an alternative test for field diagnosis of tuberculosis (Fra´guas *et al.*, 2006). A study demonstrated that iELISA (indirect enzyme linked immunosorbent assay) using rM70-83-E6 antigen is simple, sensitive and easy to perform and can be used for the analysis of a large number of samples for sero-diagnosis of BTB (Liu *et al.*, 2007). However, due to its low sensitivity ELISA should not be considered as a reliable confirmatory test for BTB diagnosis at the slaughter (Fra´guas *et al.*, 2006).

2.6.5. Isolation and identification of mycobacteria

Bacteriological method

The difference among the growth rates of mycobacterial species may be justified by differences in key respiratory pathways or energy production, in the velocity of oxygen and nutrients diffusion across the cell envelope, in the rate of lipid synthesis and assimilation by cell walls, or finally in the number of rRNA operon (Goodfellow and Magee, 1977). There is clear correlation between slowly growing capacity with disease chronicity and the ability of mycobacteria to survive in a latent state in the host (Goodfellow and Magee, 1977).

The high degree of sequence conservation among members of the *Mycobacterium tuberculosis* complex makes differentiation of species in mycobacteriological laboratory a difficult task (OIE, 2009). *Mycobacterium* species grow on complex organic medium containing serum, potato and egg. The most commonly used media are Löwenstein-Jensen (LJ) that contains egg, glycerol, asparagines, mineral salt and malachite green and Stonebrink's medium. Glycerol suppresses the growth of *M. bovis* and malachite green inhibits the growth of bacterial contaminants and provides a green background. Glycerol in LJ medium is replaced by pyruvate in Stonebrink's medium to enhance growth of *M. bovis* (WHO, 1998b; OIE, 2009). *M. bovis* grows slowly with a generation time of 2-20 hours and needs more than 8 weeks to appear on primary culture. Tubercle bacilli are obligate aerobes, but growth of *M. tuberculosis* and *M. bovis* can be

enhanced at 5-10% CO_2 (Quinn *et al.*, 1999). The optimal growth temperature is 37°C . Growth in liquid medium is diffused, but on a solid medium *M. bovis* is dry, sparse, delicate and non luxuriant. Species level identification of growth of acid fast bacilli (AFB) positive mycobacterial isolates is done by standard biochemical tests including, niacin production, nitrate reduction, catalase activity, tween hydrolysis, arylsulphatase and thiophen-2 carboxylic acid hydrazide (TCH) sensitivity, and etc (WHO, 1998b; OIE, 2009).

Molecular methods

These molecular diagnostic methods are particularly useful for dysgonic slowly growing mycobacterial strains for which biochemical and growth data are difficult to obtain but for which even minimal growth can yield sufficient DNA for PCR amplification (OIE, 2009). In recent years, molecular genetic methods have been developed and widely used in the routine identification of mycobacterial species and sub-species (Wilton and Cousins, 1992; Szewzyk *et al.*, 1995). These tools can help to determine the origin of outbreaks, to understand the link between different outbreaks, to show the relationships between domestic and wild TB, and identify the source of human infection (Haddad *et al.*, 2004). In the genome of mycobacteria, polymorphism is limited compared to its genome size. But some regions are highly polymorphic, either by a variation in number and/or position or by a variation in primary structure. These areas of higher polymorphism appear to correspond essentially to segments of genes encoding proteins where variability provides a selective advantage to the bacteria, e.g., antibiotic resistance, antigens involved in escaping the immune response, or to non-coding sequences probably involved in inducing variability of neighbor genetic areas, such as insertion sequences or repeated sequences. These are the basis for molecular typing techniques (Haddad *et al.*, 2004). The various molecular techniques used in tuberculosis bacteriology includes polymerase chain reaction (PCR), variable number of tandem repeats (VNTR), restriction fragment length polymorphism (RFLP) using various genetic markers and spoligotyping (Kamerbeek *et al.*, 1997).

Polymerase chain reaction (PCR)

The principle of the PCR technique is based on the amplification of a given DNA sequence to a large number of copies that can be identified by separation on gel electrophoresis and, subsequently, either with or without probing with a labeled oligonucleotide specific for the amplified DNA fragment. PCR methods for the detection of members of the *Mycobacterium tuberculosis* complex in clinical specimens were developed in the mid-90s and provide a rapid and sensitive method for the detection of *M. tuberculosis* complex (Clarridge *et al.*, 1993; Cadmus *et al.*, 2006). 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 (Cadmus *et al.*, 2006; Liebana *et al.*, 2008). 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 (Jolley *et al.,* 2007).

A conventional PCR generally only uses one set of primers (Jahans and Worth, 2006). 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 (Kurabachew *et al.,* 2004). PCR amplification of genomes such as 16S rDNA (Wilton and Cousins, 1992), *mpt*40 gene (Parra *et al.*, 1991), *mpb*70 gene (Radford *et al.,* 1988), *pnc*A gene (Shah *et al.*, 2002), the *oxy*R gene (Espinosa de los Monteros *et al.*, 1998) and the regions of difference (RD) (Haddad *et al.,* 2004) were used to differentiate the genus *Mycobacterium* or, *M. tuberculosis* from *M. bovis* isolates.

3. MATERIALS AND METHODS

3.1. Description of the study area

The study was conducted at Bako Agricultural Research Center (BARC) and Bako abattoir (Figure 3). BARC is one of the old research centers in the country established in 1960's through a bilateral agreement between the then West Germany and the then Ethiopian government. It was established with the aim of generating and adopting improved agricultural technologies. Since its establishment, the Center has made tremendous contributions to the country's agricultural development through generating, adopting, and transferring improved agricultural technologies to the far demanding farming communities of the country in general and west Oromia in particular. Currently, the Center undertakes basic and applied research activities in areas of crop, livestock and natural resources. Consequently, the livestock farm of the center is mandated to evaluate the productive and reproductive performance of indigenous cattle breeds, mainly Horro breed, to improve the performance of the breed through selection and crossing with various exotic bloods. The improved genotypes are transferred through extension system for dissemination to livestock farmers of the area. In addition, the farm serves as a source of raw milk to residents in and around the center (Jiregna *et al.*, 2004).

Bako Town is the center of Bako-Tibe district in Western Showa administrative zone of Oromia National Regional State. It is located 250 Km in the west of Addis Ababa at an altitude of 1650 meters above sea level on 37° 09' E and 9° 06' N. The Town has hot and humid climate with average relative humidity of 60%. It gets a bimodal pattern of rainfall with the main rainy season extending from June to September and a short rainy season that extends from March to May with an average annual rainfall of 1300 mm. Mean monthly maximum and minimum temperatures are about 28°C and 14°C, respectively, with an average monthly temperature of 21°C. The mean daily minimum and maximum temperatures are 9.4°C and 31.1°C, respectively. Potential evapo-transpiration averages 62 mm per month (Gebregziabher and Mulugeta, 2006).

Post mortem examination was performed at Bako municipal abattoir. The abattoir is small and has one slaughter hall where all the procedures of stunning, skinning, evisceration, removal of rumen contents, washing of offals, etc. are usually performed. It is supplied with tap water, holding crush, examination crush and electricity. Only cattle are slaughtered in the abattoir each

day with the exception of Tuesday and Thursday. In the abattoir, on average 8 heads of cattle are slaughtered per day and the number increases to over 20 heads during the holidays. The abattoir serves 15 local butchers most of which use the abattoir regularly to supply the town residents with fresh meat.

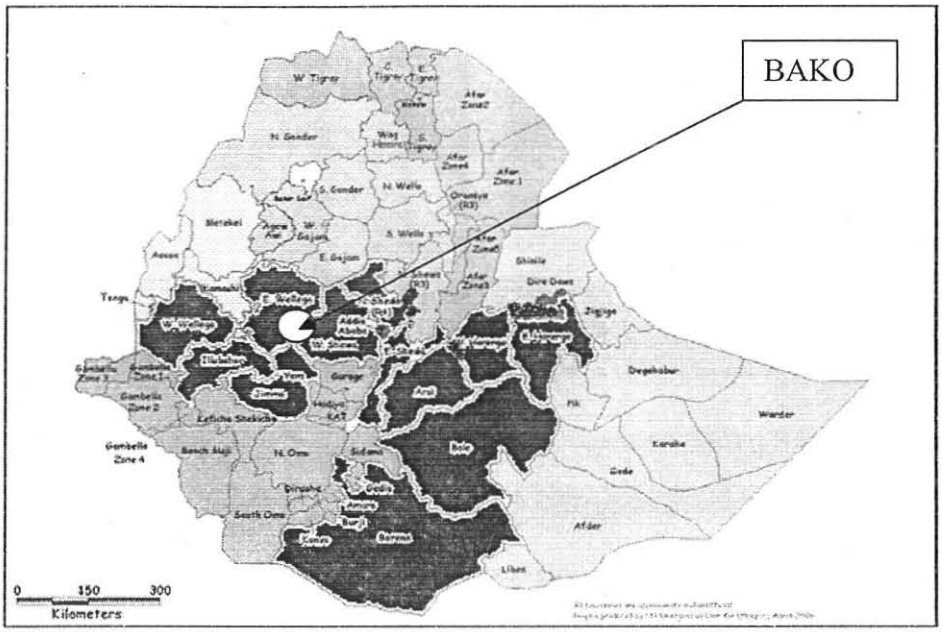

Figure 3: Map of the Federal Democratic Republic of Ethiopia showing geographic location of the study site.

3.2. Study animals and management

The study animals were cattle kept at BARC and cattle slaughtered at Bako abattoir. All the cattle kept in the farm above six months of age were tested by using CIDT test. Majority (67.7%) of the tested animals were reared at the farm since their birth, whereas, 32.3% of the tested animals were purchased from the local livestock markets and included in the main herd as replacement. The average age of the tested animals was 6.12 years with a standard deviation of 4.75 years. Among the 371 animals tested, 253 (68.2%) were indigenous Horro cattle and 118 (31.8%) were crossbred animals.. All the animals were managed by semi-intensive production system. They were allowed to graze on natural pasture (in herds of 30-40 heads) during the day for 8 hrs. Pregnant and lactating cows, breeding bulls and calves were supplemented with a concentrate mixture of maize (49%), noug cake (49%) and salt (1%). Supplementations of all animals with

hay mainly Rhodes grass *(Chloris gayana)* and natural pasture or silage (maize silage) at night is practiced depending on the availability of hay and silage. The animals are housed (in herds) in a barn with concrete floor and corrugated iron roof. The barn was cleaned daily and disinfected with formalin at monthly interval. All animals were routinely monitored for any health problem and those above six months of age were annually vaccinated against Black leg, Anthrax, Contagious Bovine Pleuro-pneuminia (CBPP), Lumpy Skin Disease (LSD) and Foot and Mouth disease (FMD). All the animals were de-wormed with anthelmintics twice a year; during the beginning of the main rainy season and end of the main rainy season. The animals were sprayed with acaricide (Diazinon 60% EC) but the frequency of spray varies depending on the degree of infestation and season of the year (Gebregziabher and Mulugeta, 2006).

Cattle slaughtered at Bako abattoir were also used for the study. Accordingly, 487 slaughter cattle were included in the study. Though the animals were purchased from local livestock markets, the geographic origins of the animals could not be traced as they were brought to the slaughterhouse by butchers or their assistants who did not have information about the origin of the animal. Nevertheless, all the slaughter animals were indigenous Horro cattle managed under integrated extensive production system where cattle are kept primarily for draught power. According to Alberro and Hailemariam (1982), Horro cattle are described as an intermediate Sanga-Zebu type and are dominant cattle breed in western Ethiopia. The cattle breed clearly shows a Sanga characteristic in terms of their horns and humps. They have uniform body conformation and are dark red to brown in color and have a medium frame size with a small to finely shaped head. These animals have medium to large horns that are generally larger than other Ethiopian zebu breeds (Annex 6).

3.3. Study design and sampling

Cross-sectional study design was used and all animals above six months of age were purposively selected and tested by CIDT test. Reactor animals were euthanized and subjected to a detailed post mortem examination. Animals were confirmed as being infected with TB on the basis of the presence of macroscopically apparent lesions followed by standard cultural isolation of mycobacteria from the affected tissues. For each animal the location and the severity of pathological lesions were registered on a preformed format (Annex 5).

For the abattoir-based survey, Bako abattoir was purposively selected and included in the study to get a wider picture of BTB in the area. The sample size consisted of all slaughter animals present at the 5 times weekly visits during the months of November, December, January and the first two weeks of April. During each visit, ante and post mortem data were collected from each animal. During ante mortem examination, the health status of the animal was checked; age, sex and body condition scores were recorded; and each animal was provided a unique identification number. Similarly, during post mortem examination of carcasses, distribution and severity of the lesion pathology was recorded. A total of 487 cattle were investigated during the sampling period.

3.4. Study methodology

3.4.1. Comparative intradermal tuberculin (CIDT) test

All cattle maintained in the center except the newly born calves were individually identified based on their farm records. Data on possible risk factors for mycobacterial infection were collected from the animals to look for associations between the risk factors and the infections. Potential risk factors considered were breed of the animal, age, body condition score (BCs), sex, parity, pregnancy status and origin. All the potential risk factors and skin test measurements for each animal were recorded on preformed format (Annex 4). The body condition of each of the study animal was scored using the guidelines established by Nicholson and Butterworth (1986). Accordingly, on the basis of observation of anatomical parts such as vertebral column, ribs, spines, tip of tail, etc., the study animals were classified as lean (score, 1 to 3), medium (4 to 6), or fat (7 to 9) (Annex 1).

Two sites on the right side of the mid-neck, 12 cm apart, were shaved and the skin thicknesses were measured with calipers. One site was injected with an aliquot of 0.1 ml containing 2500 IU/ml bovine purified protein derivative (PPD) (Veterinary Laboratories Agency, Addlestone, Surrey KT15 3NB, U.K.). Similarly, 0.1 ml of 2500 IU/ml avian PPD (Veterinary Laboratories Agency, Addlestone, Surrey KT15 3NB, U.K.) was injected into the second site. After 72 h, the skin thickness at the injection sites was measured and registered on a preformed format (Annex 4). The interpretation of the result was such that when reactions were observed at both injection sites, the difference between the two reaction sizes was considered. Thus, an animal was

classified as tuberculin positive if the increase in the skin thickness at the injection site for bovine PPD (B) was at least 4 mm greater than the increase in skin thickness at the injection site for the avian PPD (A) (OIE, 2009). A newly defined cut-off value of >2 mm (Ameni *et al.*, 2008) was also used alongside the OIE recommended cut-off value to determine the prevalence of BTB in local cattle kept at BARC.

3.4.2. Post mortem examination and pathology scoring

Nowadays, TB detection is often done at slaughterhouses. The discovery of characteristic granulomatous lesions in the lymph nodes or organs when inspecting animal carcasses is a sign for the presence of the disease (Artois *et al.*, 2000). Accordingly, abattoir-based surveillance of BTB was carried out on carcasses of cattle slaughtered at Bako abattoir. A total of 492 (5 skin test reactors and 487 unknown status) carcasses were examined for tubercle lesions during post mortem examination. A standard necropsy combined with routine meat inspection was done (FAO, 2000) to detect tubercle lesions in carcasses on infected animals. The lungs, lymph nodes and liver were removed for the investigation of tubercle lesions. The seven lobes of the two lungs, including the left apical, left cardiac, left diaphragmatic, right apical, right cardiac, right diaphragmatic and right accessory lobes, were inspected externally and palpated. Then, each lobe was sectioned into 2 cm thick slices to facilitate the detection of lesions. Similarly, lymph nodes, namely, the mandibular, retropharyngeal, cranial and caudal mediastinal, left and right bronchial, hepatic, and mesenteric lymph nodes, were sliced into thin sections and inspected for the presence of visible lesions. When gross lesions suggestive of BTB were found in any of the tissues, the animal was classified as having lesions. The severity of the gross lesions was scored by the semi-quantitative procedure developed by Vordermeier *et al.* (2002), with minor modifications to facilitate performance under field conditions (Ameni *et al.*, 2006). Briefly, lesions in the lobes of the lungs were scored separately as follows: 0 = no visible lesions; 1 = no gross lesions but lesions apparent on slicing of the lobe; 2 = fewer than five gross lesions; 3 = more than five gross lesions; 4 = gross coalescing lesions. The scores for the individual lobes were added up to calculate the lung score. Similarly, the severity of gross lesions in individual lymph nodes was scored as follows: 0 = no gross lesions; 1 = small lesion at one focus (just starting); 2 = small lesions at more than one focus; 3 = extensive necrosis. All the data were recorded on a preformed format (Annex 5).

Tissue showing macroscopic lesions compatible with BTB during the post mortem examination were collected and placed in a 50 ml capacity tubes with screw caps containing 5 ml of sterile 0.90% saline water. In addition, non-tuberculous tissues were collected from tuberculin skin test sensitive cattle. Overall, 89 specimens were collected from 48 animals among which five were tuberculin test reactor animals and the remaining were slaughter cattle at abattoir. Collected tissue specimens were kept frozen at -20^{O}C at BARC to retard the growth of any contaminant and to preserve the mycobacteria. The specimens were transported in ice box to Aklilu Lemma Institute of Pathobiology (ALIPB) where the specimens were stored at -80^{O}C until processing for mycobacterial culture (OIE, 2009).

3.4.3. Isolation of mycobacteria

A total of 89 tissue specimens from a total of 48 animals were collected and further processed for isolation of mycobacteria. Of the total 89 tissue specimens collected for culture and isolation, 61(68.5%) were obtained from 43 tuberculous cattle from Bako municipal abattoir and the remaining 28 (31.5%) specimens were collected from 5 tuberculin test reactor cattle of BARC. From the 28 tissue specimens collected from tuberculin test reactors, 10 (35.7%) were lesioned and the remaining 18 (64.3%) were non-lesioned. The specimens were collected and processed in accordance with OIE (2009) protocols. Briefly, tissue specimens for culture were collected in sterile universal bottles in 5 ml of 0.9% saline solution and then transported to the laboratory by maintaining a cold chain. In the laboratory, the specimens were sectioned using sterile blades and then homogenized with a mortar and pestle. The homogenate was decontaminated by adding an equal volume of 4% NaOH by centrifugation at 3,000 rpm for 15 minutes. The supernatant was discarded, and the sediment was neutralized by 1% (0.1 N) HCl using phenol red as an indicator. Neutralization was achieved when the color of the solution changed from purple to yellow (OIE, 2009). Next, 0.1 ml of suspension from each sample was spread onto a slant of Lowenstein-Jensen (LJ) medium (Annex 3). Duplicate slants were used, one enriched with sodium pyruvate and the other enriched with glycerol. Cultures were incubated aerobically at 37°C for about 10 weeks with weekly observation for growth of colonies. Cultures were considered negative if no visible growth was detected after 10 weeks incubation. Microscopic examination of cultures using the Ziehl–Neelsen (ZN) staining method was performed to select acid-fast bacilli (AFB)

positive isolates. AFB positive isolates were heat-killed by mixing approximately 2 loopfuls of cells in 200 µl dH_2O followed by incubation at 80°C for 45 minutes and stored in deep freeze at -80°C until molecular characterization was conducted.

3.4.4. Molecular characterization of mycobacterial isolates

In the present study, multiplex polymerase chain reaction (m-PCR) was used to differentiate *M. tuberculosis* complex from *M. avium*, and other mycobacterial species following the standard operating procedure (SOP) developed by VLA (2005). 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. avium* (MYCAV-R), and the MTB70 gene specific for *M. tuberculosis* complex. The reaction was carried out using Thermal Cycler (Applied Biosystems, GeneAMP 9700). Each PCR tube consisted of 5.2 µl H2O, 8 µl HotStarTaqMasterMix, 0.3 µl of each of the primers, 5 µl of DNA templates of samples or controls making the total volume 20 µl. *M. avium*, and 2122/97 (*M. bovis* strain) were used as positive controls while H2O (Qiagen), was used as a negative control. The mixture was heated for 15 min at 95°C, followed by 35 cycles of 1 min denaturation at 95°C, 1 min annealing at 61°C, and 1 min extension at 72°C; and final extension was done for 10 min at 72°C. The PCR product was electrophoresed in 1% agarose gel in 1XTAE running buffer. Ethidium bromide (DNA interacting dye) at a ratio of 1:10 in 1% agarose gel, 100bp DNA ladder, and orange 6x loading dye were used during 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, while members of *M. tuberculosis* complex produce a band with 372bp. Then, the bands on the gel were photographed with digital camera (Inotech AlphaImage) and documented for future use.

3.5. Data management and analysis

All the data obtained from the study were entered into MS Excel data sheets. Then, the data were coded and analyzed using SPSS 15.0 version programmes. The prevalence of mycobacterial infection was calculated by dividing the number of cattle found infected (either positive reactors or harboring tuberculosis lesions) by the total number of cattle tested or whose carcasses is

inspected multiplied by 100. Interval estimation was calculated using 95% confidence interval (CI) for the different prevalence values.

A total of 7 individual animal level potential risk factors were considered to analyze their association with mycobacterial infections. Due to the similarity of the age of slaughter animals at the abattoir, most of them old age as determined by teeth eruption, no statistics were computed for the variable age. However, association between age and tuberculin test positivity was assessed for animals tested with tuberculin skin test. Otherwise, the association between infection with mycobacteria and the various potential risk factors was tested. Data with discrete values were analyzed by comparing proportions using the non-parametric statistical tests including Pearson's chi-square or Fisher's exact test based on the number of observations per contingency table cells. Variables measured in scales like severity of pathology were analyzed using descriptive statistics. Logistic regression analysis was used to assess the association between prevalence and animal risk factors using SPSS 15.0 statistical package. Odds Ratio (OR) was used to assess the level of association of prevalence to the different host factors. A statistically significant association between the different variables were said to exist if the estimated P-value is less than 0.05 ($P<0.05$) and the 95% confidence interval (CI) for OR doesn't include 1.0. Microsoft office power point was used to analyze the gel images obtained from the PCR products.

4. RESULTS

4.1. Prevalence based on tuberculin skin test

Apparent prevalence of BTB at cut-off point of ≥4 mm (the recommendation of OIE) was 1.3% (5/371, 95% CI: 0.15; 2.45) (Figure 4). When doubtful reactors were considered as positive, the apparent prevalence was 8.9% (33/371, 95% CI: 6.0, 11.79). The prevalence of avian reactors was 3.5% (13/371, 95% CI: 1.6, 5.4). All the positive reactors and 78.6% (22/28) of the doubtful animals were Horro cattle while the remaining 21.4% (6/28) doubtful animals were crossbred cattle.

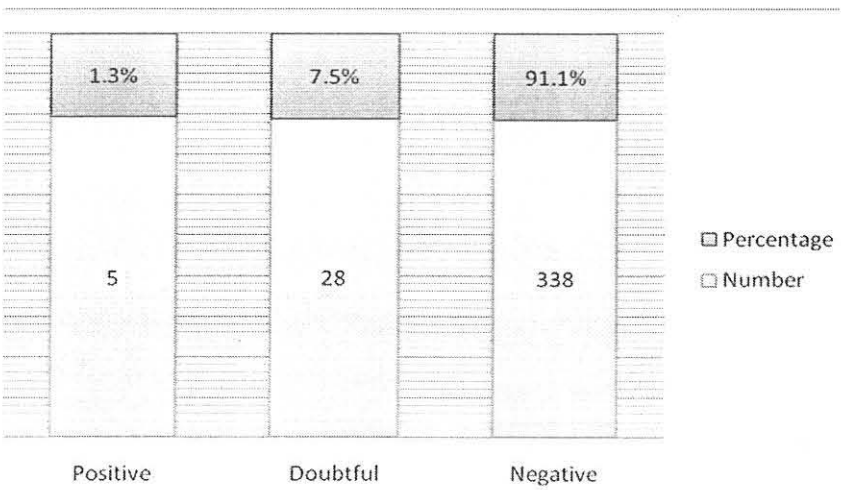

Figure 4: Responses of cattle kept at BARC to tuberculin skin test.

The apparent prevalence of BTB was re-calculated using the newly defined cut-off value of >2 mm (Ameni *et al.*, 2008) for Zebu cattle. Accordingly, the apparent prevalence was found to be 4.6% (17/371, 95% CI: 2.3, 6.9) for all tested animals. When cross breed cattle were excluded, the apparent prevalence of BTB in Horro cattle using the OIE recommended cut-off point was 2.0% (5/253, 95% CI: 0.3, 3.7) while it was 6.7 % (17/253, 95% CI: 3.6, 9.8) at a cut-off >2 mm in the same study population. There was a highly statistically significant difference (χ^2=90.543, P=0.000) in apparent prevalence estimated using the two cut-off points in local Horro cattle.

4.2. Risk factors for tuberculin test sensitive animals

In the present study, 7 individual animal characteristics were assessed to establish the association between the potential risk factors and tuberculin test positivity in cattle. The significant variables resulting from the analysis were age (χ^2=14.052, P=0.012) and animal origin (χ^2=6.082, P=0.04). Logistic regression analysis of the significant variables showed animals in the age category of 1-5 years to be at higher risk (OR=1.5, 95% CI: 0.153-14.700) of infection with BTB than the other age groups. Moreover, animals purchased from local cattle market and introduced into the farm were found to be at higher risk (OR=8.3, 95% CI: 0.916-75.057) of becoming tuberculin test reactors than animals born on farm. On the other hand, as indicated in Table 4, Fisher's exact test showed no statistical significance of breed (χ^2=3.474; P=0.147), sex (χ^2=4.796; P=0.068), body condition score (χ^2=2.270; P=0.623), pregnancy (χ^2=8.178; P=0.342) and parity (χ^2 =4.162; P=0.341) effect on apparent prevalence of BTB estimated using tuberculin skin test.

Table 5: Association of potential risk factors to the prevalence of BTB in cattle kept at BARC, Western Oromia, Ethiopia.

Factor	Category	Number examined	Number (%) positive	χ^2 test	P-value
Sex	Male	83	1(1.2)	4.796	0.68
	Female	288	4(1.4)		
Breed	Horro	253	5(2.0)	3.474	0.147
	Cross	118	0(.0)		
Age (year)	½-1]	39	0(.0)	14.052	0.012
	1-5]	156	3(1.9)		
	5-10]	106	1(.9)		
	>10	70	1(1.4)		
Body condition score	Thin	36	0(.0)	2.270	0.623
	Medium	299	4(1.3)		
	Fat	36	1(2.8)		
Pregnancy	Late	57	0(.0)	8.178	0.342
	Mid	12	0(.0)		
	Early	7	0(.0)		
	Non-pregnant	55	1(1.8)		
	Heifer	157	3(1.9)		
Parity	Nullipara	162	3(1.9)	4.162	0.341
	Unipara	16	0(.0)		
	Pleuripara	110	1(.9)		
Origin	Born on farm	251	1(.4)	6.082	0.04
	Purchased	120	4(3.3)		

4.3. Prevalence based on post mortem examination

Gross TB lesions were detected in 46 of the 492 study cattle examined during post mortem examination. Lesion prevalence was 9.3% (95% CI: 6.3, 11.8). Characteristic TB lesions were

observed in different tissues of the study animals (Table 5). Lesions were most often detected in mesenteric lymph node (60.9%, 28/46), followed by retropharyngeal (17.4%, 8/46), and left bronchial (17.4%, 8/46) lymph nodes. On the other hand, lung lesions were detected in 6 (13.0%) of the 46 lesioned cattle among which 2 (4.3%) animals had lesions only in lung lobes and the remaining 4 (8.7%) animals had lesions both in lung lobes and lymph nodes (Figure 5). There was a significant difference (χ^2=91.174; P=0.000) in occurrence of lesions in different tissues. However, there was no significant association (P>0.05) in tuberculous lesion prevalence between different sex and body condition scores of the animals.

Of the five skin test positive cattle examined at post mortem, tubercle lesions were detected in 3 (60%, 3/5) of the animals for which lesion distribution and severity was established. Lung lesion was observed in 1 (33.3%, 1/3) of the lesioned reactor cattle. In contrast, all the 3 (100%, 3/3) lesioned tuberculin reactor animals had lesions in mesenteric lymph node (Figure 6) and 2 (66.7%, 2/3) had lesions in retropharyngeal and cranial mediastinal lymph nodes.

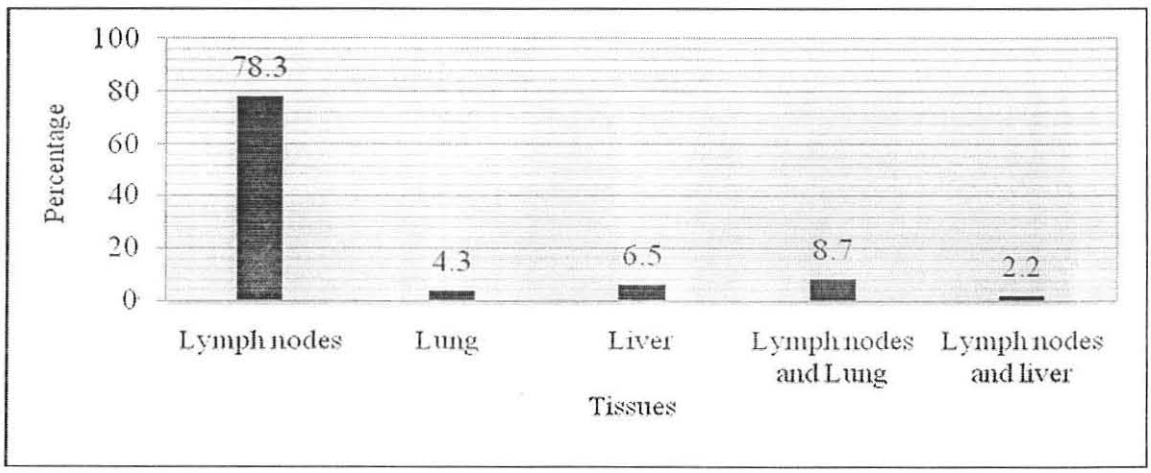

Figure 5: Distribution of tuberculous lesions in different tissues of cattle with tuberculosis lesion in at least one tissue

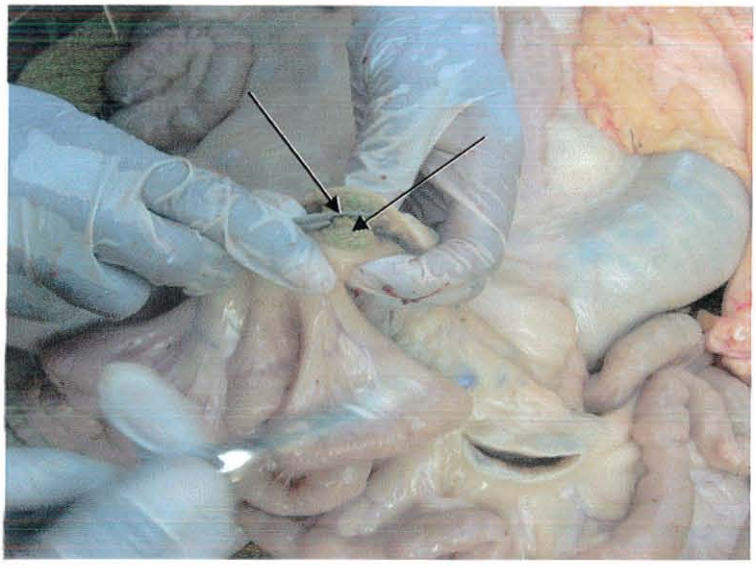

Figure 6: Tuberculous lesion detected in mesenteric lymph node of one of the tuberculin skin test positive cattle at BARC.

4.4. Pathology scoring

In our study, investigation was made to determine the severity of pathology of BTB by scoring the disease severity using a semi-quantitative pathology scoring system (Vordermeier *et al.*, 2002). Accordingly, mesenteric lymph node was found to be the most severely affected tissue (mean pathology score±SEM, 1.11±0.15) followed by retropharyngeal (0.33±0.117) and left bronchial (0.28±0.102) lymph nodes (Table 5). On the other hand, mandibular lymph node was found to be the least severely affected tissue (0.02±0.022). The present study also indicated severe lung lesions in right diaphragmatic (0.17±0.122) and left diaphragmatic (0.15±0.108) lobes of both lungs than the other lung lobes. Moreover, lesions were frequent and severe in mesenteric (mean pathology score±SEM: 1.67±0.333), retropharyngeal (1.0±0.577) and cranial mediastinal (0.67±0.333) lymph nodes of tuberculin reactor cattle.

Table 6: Proportion and severity of lesioned tissues of bovine tuberculosis in cattle slaughtered at Bako municipal abattoir, western Oromia, Ethiopia.

Tissue	Number examined	Number (%) Positive	Lesion severity (Mean±Sem)
Lung lobes			
Left apical	46	1(2.2)	0.04±0.043
Left cardiac	46	2(4.3)	0.13±0.096
Left diaphragmatic	46	2(4.3)	0.15±0.108
Right cardiac	46	1(2.2)	0.04±0.043
Right accessory	46	1(2.3)	0.04±0.043
Right diaphragmatic	46	2(4.3)	0.17±0.122
Lymph nodes			
Mandibular	46	1(2.2)	0.02±0.022
Retropharyngeal	46	8(17.4)	0.33±0.117
Left bronchial	46	8(17.4)	0.28±0.102
Cranial mediastinal	46	3(6.5)	0.07±0.037
Caudal mediastinal	46	4(8.7)	0.13±0.067
Hepatic	46	6(13.0)	0.22±0.093
Mesenteric	46	28(60.9)	1.11±0.15
Liver	46	4(8.7)	0.15±0.082

4.5. Isolation of mycobacteria

The presence of mycobacteria in post mortem specimens may be confirmed by cultivation of the organism on primary isolation medium (OIE, 2009). Accordingly, 89 tissue specimens collected from 48 cattle were cultured on primary culture media for mycobacterial isolation. Consequently, specimens from 22 (46%) animals had shown growth of mycobacteria on primary culture media in at least one tissue (Figure 7). On the other hand, from the total 89 specimens, 29 culture specimens showed growth of mycobacteria on primary culture media constituting a culture positivity of 32.6%. Out of the total culture positive specimens, 9 (31%) were from tuberculin test reactor animals among which, 6 culture positive specimens were from tissues that had no

gross lesion of TB. Most of the isolates produced white, moist, slightly rough and friable colonies with no pigmentation while some of the isolates showed slightly yellow colonies. The earliest growth was observed after 3 weeks of culture while the latest growth recorded was after 10 weeks of culture.

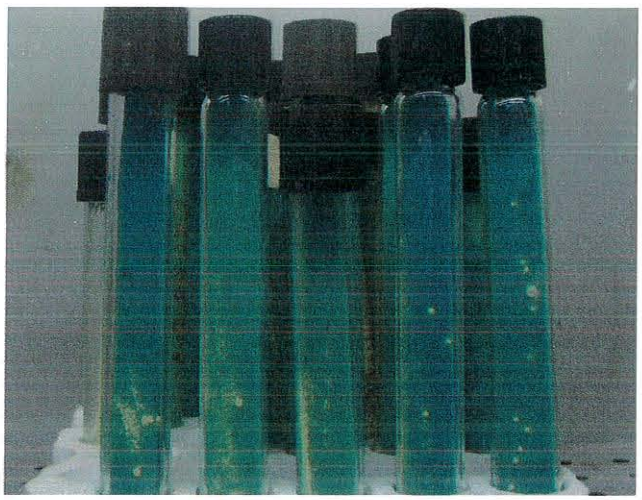

Figure 7: Some of the mycobacterial culture isolates of tissues and organs obtained from suspicious tubercle lesions.

In the present study, the highest proportion of culture positivity (72.7%) was observed in left bronchial lymph node followed by right bronchial lymph node (50%), while the lowest percentage of positive cultures were observed in caudal mediastinal (16.7%) and hepatic (12.5%) lymph nodes (Figure 8). On the other hand, mycobacterial growth was not observed from mandibular lymph node culture. Furthermore, out of the 10 lung specimens cultured for mycobacterial isolation, growth was observed on left diaphragmatic (50%) and right diaphragmatic (50%) lobes of lungs (Figure 8). In contrast, no growth was observed from left apical, left cardiac, right cardiac and right accessory lung lobes. Culture positivity was confirmed by the demonstration of acid-fast bacilli in the colonies. Accordingly, 69% (20 of 29) isolates showed acid-fast bacilli on ZN staining.

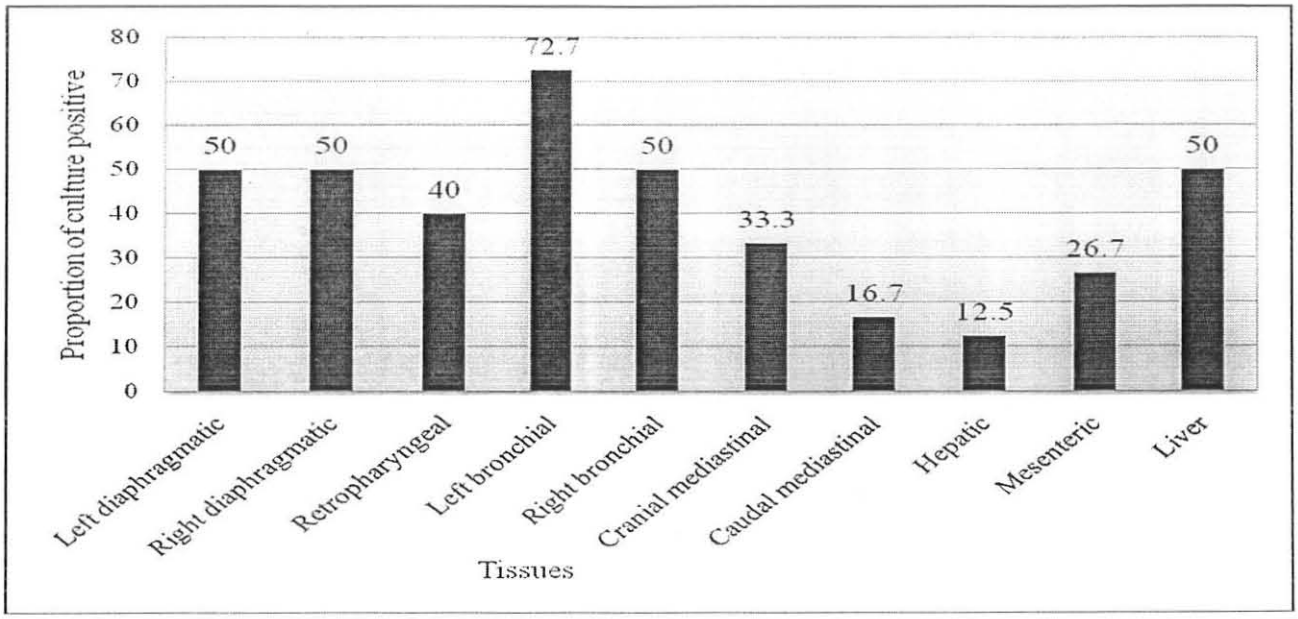

Figure 8: Proportion of culture positivity of tissues and organs obtained from suspicious tissues.

4.6. Molecular characterization of mycobacterial isolates

Characterization of mycobacteria was performed by genus typing (VLA, 2005). Accordingly, multiplex polymerase chain reaction (m-PCR) was used to differentiate species of the *Mycobacterium tuberculosis* complex (MTC) organisms from other *Mycobacterium* species. Consequently, 94% (16 of 17) isolates were positive for the 16S rRNA genus specific locus and thus identified as mycobacteria (Table 7). However, as indicated in Figure 9, none of the isolates showed signals for the presence of *Mycobacterium tuberculosis* complex and *Mycobacterium avium* complex organisms. Furthermore, one isolate did not show amplification signal for the genus *Mycobacterium*. Although complete identification of these mycobacteria to the species and subspecies level is required to have complete information about the organisms, we could not characterize the isolates further due to lack of necessary materials.

Table 7: Summary of the result of m-PCR products of mycobacterial isolates obtained from cattle tissues.

No.	Sample code	Sample type	Multiplex PCR result		
			Genus *Mycobacterium*	*M. tuberculosis* complex	*M. avium* complex
1	2276MES	Mesenteric LN	+	-	-
2	63207RPH	Retropharyngeal LN	+	-	-
3	52809RB	R. bronchial LN	+	-	-
4	2276LB	L. bronchial LN	+	-	-
5	63207CRMD	Cr. Mediastinal LN	-	-	-
6	52809MES	Mesenteric LN	+	-	-
7	52809RB	Retropharyngeal LN	+	-	-
8	GN1MES	Mesenteric LN	+	-	-
9	T1MES	Mesenteric LN	+	-	-
10	G1 Hepat	Hepatic LN	+	-	-
11	S1LIV	Liver	+	-	-
12	S2LB	L. bronchial LN	+	-	-
13	G12HEPAT	Hepatic LN	+	-	-
14	D16MES	Mesenteric LN	+	-	-
15	GU1RD	R. diaphragmatic LL	+	-	-
16	B27LD	L. diaphragmatic LL	+	-	-
17	G23MES	Mesenteric LN	+	-	-

LN: Lymph node, LL: Lung lobe, L: Left, R: Right, Ca: Caudal, Cr: Cranial, +: positive, -: negative

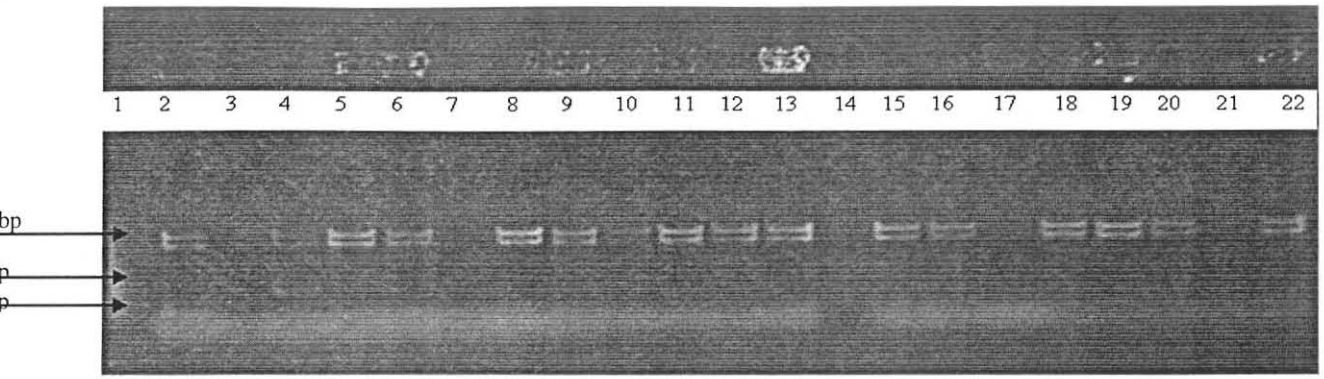

Figure 9: Electrophoretic separation of multiplex polymerase chain reaction (m-PCR) products for 16S rRNA typing of mycobacteria.

Lane 1:100bp DNA ladder; Lane 2: genus *Mycobacterium* and *Mycobacterium avium* positive control; Lane 3: Water (Qiagen) negative control; Lane 4: genus *Mycobacterium* and *M. tuberculosis complex* positive control; Lanes 5, 6, 8-13, 15-22, were positive samples (1030bp) for the genus *Mycobacterium*; Lane 7: negative sample; Lane 14: PCR product evaporated.

5. DISCUSSION

Infection of cattle with *M. bovis* constitutes a human health hazard as well as an animal welfare problem. Furthermore, the economic implications in terms of trade restrictions and productivity losses have direct and indirect implications for human health and the food supply (Villarreal-Ramos *et al.*, 2003). The present study using the CIDT test showed the prevalence of BTB in cattle kept at BARC to be 1.3%. The overall prevalence found in the current study is consistent with the results of Shihun (2008) and Tschopp *et al.* (2009), who found individual animal prevalence of 2.7% and 3.1% in Ethiopia, respectively. Moreover, the result of the present study is consistent with the results found in some African countries. In Uganda, Oloya *et al.* (2006) and Inangolet *et al.* (2008) reported a prevalence of 1.3% and 1.4%, respectively from cattle in different districts of Uganda. Similarly, Jiwa *et al.* (1997), Shirima *et al.* (2003) and Cleaveland *et al.* (2007) found an individual animal prevalence of 0.2%, 1.3% and 0.9%, respectively from different areas of Tanzania. Nevertheless, the prevalence of BTB in the present study is lower than most of the recently undertaken studies in different areas of Ethiopia, where prevalence ranges from 3.4% in a small holder production system to 50% in intensive dairy productions were reported (Kiros, 1998; Ameni and Roger, 1998; Ameni *et al.*, 2003a, b; Asseged *et al.*, 2001; Regassa, 2001, 2005; Shitaye *et al.*, 2006; Ameni *et al.*, 2008; Teshome and Nigatu, 2009; Shimelis, 2009; Regassa *et al.*, 2009; Ameni *et al.*, 2010).

The low prevalence of BTB in the present study could be due a number of factors among which are; exposure to environmental mycobacteria which lowers sensitivity, as the reaction to avian tuberculin could be high and thus interferes with the interpretation of the result (Hope *et al.*, 2005). In our study, 22.6% animals responded positively (A2-A1≥4mm) to avian PPD and hence, could have interfered with the interpretation of the result. Oloya *et al.* (2006) suggested that unusual low prevalence of BTB in some areas in endemic countries could be due to non-specific response to environmental mycobacteria In addition, adverse effects of pregnancy on cellular immunity have been reported to lower the sensitivity of cattle to tuberculin skin test (Kehrli *et al.*, 1989). Recently calved or pregnant tuberculous cattle go through a period of desensitization immediately before and after calving and as many as 30% give false negative reactions returning to a positive status 4-6 weeks later. The loss of sensitivity is probably due to the removal of fixed cell antibodies from the skin into the general circulation and subsequent drainage into the

colostrums (Radostitis *et al.*, 2000). In the present study, 15.4% of the cows tested for BTB using tuberculin skin test were at late pregnancy or early parturition.

Moreover, some of the animals may be in an advanced stage of the disease in which they do not give a positive reaction to a tuberculin test (Radostits *et al.*, 2000). Previous reports indicate that since the test-and-slaughter-based control method is not applied in Ethiopia, the disease could progress longer with a greater proportion of animals reaching a more severe disease status (Ameni *et al.*, 2006). Animals in late stage of the disease do not respond to the tuberculin test because of changes in the nature of the immune responses with disease progression, from a dominant Th1-type response at early stages of disease toward B-cell antibody response and lower T-cell responses at more progressive disease stages (Buddle *et al.*, 2005; Hope *et al.*, 2005). A consequence of lower cell-mediated immune responses in animals with severe disease could be their escape from detection by cell-mediated immune response-based tests like the tuberculin skin test (Ameni *et al.*, 2006).

The low number of tuberculin test positive animals may also reflect the low prevalence of BTB in the farm which may indicate a low transmission rate of mycobacteria in the investigated cattle farm. This low prevalence may indicate, among others, transmission might be maintained by few animals shedding mycobacteria in the milk causing early infection of young animals, of which a low proportion themselves become infectious in latter stage (Tschopp *et al.*, 2009 citing Francis, 1947).

The prevalence of BTB varied significantly among different age groups. No reactor animals were recorded in animals aged less than one year and reactivity to CIDT test increased with age, up to five years of age, after which it declined. This finding agrees with findings of other researchers (Asseged *et al.*, 2000; Ameni *et al.*, 2001; Kazwala *et al.*, 2001; Ameni *et al.*, 2002; Ameni and Erkihun, 2007; Shimelis, 2009). It is possible that the infection may not become established in very young animals but, as they get older, their chance of acquiring infection also increases, due to the increased time of exposure to the infectious agent particularly in contaminated farm.

Moreover, reactivity to tuberculin test was significantly higher in animals purchased for replacement purpose than animals born on farm. This indicates the risk of purchasing and

introducing mycobacteria infected cattle for replacement purpose. This finding suggests that avoiding purchasing replacement animals from market without testing for BTB might help in decreasing the risk of introduction of infectious animals. The prevalence of mycobacterial infection in purchased cattle directly affects the risk of buying infected cattle and the rate at which the whole herd could be infected.

Despite age, and source of the animal, no statistical association was observed between the potential risk factors and the prevalence of BTB in cattle kept at BARC. The absence of differences in prevalence between animals of different sexes was consistent with previous reports (Woldesenbet *et al.*, 2002; Asseged *et al.*, 2004; Ameni and Erkihun, 2007; Elias *et al.*, 2008; Shimelis, 2009). However, the absence of statistical association between body condition scores of the animals and tuberculin test positivity in the present study is in contrast with the report of Ameni and Erkihun (2007), Elias *et al.* (2008), Shimelis (2009). The difference could be explained by the high proportion (80.6%) of animals with medium body condition score in our study, making comparison of different sized categories difficult.

In the present study, no significant difference in prevalence of BTB between local Horro cattle and crossbred animals was observed although only local animals were positive to the tuberculin skin test. This finding is consistent with the finding of Ameni (2008) and Shihun (2008) who reported no significant difference in prevalence of BTB between local Zebu cattle and their crosses with exotic cattle breeds managed under identical condition. In contrast, very few comparative susceptibility studies between zebu and their crosses with European cattle breeds indicate that zebu type cattle are much more resistant to BTB than their crosses with exotic bloods (Radostitis *et al.*, 2000).

In our present study we found the prevalence of BTB in local Horro cattle to be 2.0%. Previous studies of BTB in Horro cattle under traditional management in the Bodji district of Western Ethiopia using tuberculin skin test revealed a prevalence of 3.5% (n= 353) (Regassa, 2001) and 4.1% (n=460) (Laval and Ameni, 2004). Moreover, Ameni and Erkihun (2007) and Shihun (2008) reported a prevalence of 2.4% (n=130) and 4.0% (n=76) in local Zebu cattle managed under smallholder system in Debre Brehan and Adama areas of Ethiopia, respectively. On the other hand, a higher individual animal prevalence of 11.6% (n=2,578) was reported in local Arsi

cattle in central Ethiopia (Ameni *et al.*, 2007; Regassa *et al.*, 2009). Comparative studies conducted in Chad (Diguimbaye-Djaibe *et al.*, 2006b) and Cameroon (Njanpop-Lafourcade *et al.*, 2001) to assess susceptibility differences between the different local breeds revealed a difference in susceptibility to BTB even within the local Zebu breeds.

The difference in apparent prevalence of BTB in local Horro cattle varied significantly at the two cut-off values. The prevalence was 2.0% when a cut-off ≥ 4mm was used while analysis of the same data using a cut-off value >2mm gave an apparent prevalence of 6.7%. Similarly, in his recent study, Ameni *et al.* (2008) reported a statistically significant difference in apparent prevalence of BTB at the two cut-off points in study cattle in central Ethiopia. Ameni and his colleagues showed that sensitivities and specificities of CIDT test were optimal at cut-off values ranging from >2mm to >4mm in Zebu cattle in central Ethiopia. According to this intensive work report, a cut-point of >2mm was more sensitive in detecting infected animals than the OIE recommended cut-off value. Furthermore, Ameni *et al.* (2008) evaluated the specificity of CIDT at the two cut-off points in local Arsi cattle breed and found no difference between the specificities at the two cut-off points, and therefore, the new cut-off point was recommended for use for testing Zebu cattle nationally. The use of appropriate cut-off point of a diagnostic test is critically important in disease surveillance and hence disease control and eradication.

In the present study, detailed post mortem examination of carcasses revealed an apparent lesion prevalence of 9.3%. This finding is in agreement with the report of Demelash *et al.* (2009) who found a prevalence of 10.2% (n=3322) from slaughter cattle at five abattoirs located in various cattle husbandry systems in Ethiopia. A relatively higher lesion prevalence of 14.8% was reported by Mohamed *et al.* (2009) from carcasses of pigs slaughtered in Egypt. In contrast, the present finding is higher than previous findings by various researchers (Ameni and Wudie, 2003; Asseged *et al.*, 2004; Shitaye *et al.*, 2006; Regassa *et al.*, 2009; Berg *et al.*, 2009; Ameni *et al.*, 2010). On the contrary, the result of the present study is lower than reports of Negash (2006), Cleaveland *et al.* (2007) and Shihun (2008).

The frequency and severity of tubercle lesion in slaughter cattle was higher in the mesenteric lymph node than the thoracic lymph nodes. A number of studies in developed countries, and in contrast to our finding, revealed that the majority of tuberculosis lesions in cattle are located in

the thoracic cavity suggesting the inhalation route being the principal route of BTB transmission (Collins, 1996; Whipple *et al.*, 1996; Radostitis *et al.*, 2000). Similarly, Ameni and Wudie (2003), Shitaye *et al.* (2006) and Ameni *et al.* (2010) reported a higher frequency of lesions in thoracic cavity than in abdominal cavity of animals in Ethiopia. Nevertheless, in our case, the result contrasts with the above reports, and agrees with the findings of Cleaveland *et al.* (2007), and Shihun (2008) who reported higher lesion prevalence in alimentary tract of animals managed under similar settings. Furthermore, in a comparative BTB study in grazing and housed cattle, Ameni *et al.* (2006) observed a significant difference in lesion distribution between animals farmed intensively and those kept on pasture. According to the report, digestive tract lesions predominated in animals kept on pasture whereas animals in intensive farms were presented with lesions predominantly in the respiratory tract. In the present study, almost all the slaughter animals, except the three tuberculin reactors, were from an extensive communal pasture grazing condition which could suggest that shedding of mycobacteria in the feces and ingestion of the bacilli with contaminated pasture/water may be the main route of transmission in cattle in the area.

For animals in the extensive production system, the infection is acquired through the oral route; in this case, bacteria excreted with faeces, urine, milk, lochia (from cows with endometritis i.e. as high as 5%) and mycobacteria-laden exudates from the lung are ingested with contaminated feed and water. The primary focus with this route of infection is in the lymph nodes of the intestinal system (Seifert, 1996). The infectious dose is very low; 1-3 viable bacteria are considered sufficient as infectious inoculum. 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 (Thoen *et al.*, 2006).

Out of the 89 tissue specimens cultured, 32.6% specimens of different kind showed growth of mycobacteria on primary culture media. A comparable culture positivity result of 32% was reported by Shihun (2008). On the other hand, a lower culture positivity of 12% (n=170) was reported by Asiimwe (2008) from Uganda. In contrast to our finding, Munyeme *et al.* (2009) and Ameni *et al.* (2010) observed a relatively higher culture positivity of 42.8% (42 of 98) and 47.4% (27 of 57) from suspicious lesions of cattle slaughtered in Zambia and northeastern Ethiopia,

respectively. Furthermore, Oloya (2007) reported a higher culture positivity of 60.6% from lesioned tissue specimens collected from cattle in Uganda. In addition, Ameni (2008) reported a culture positivity of 55.8% from tuberculous lesions of tuberculin skin test positive cattle.

A low isolation rate of mycobacteria may have resulted from reduced sensitivity of culture arising from prolonged storage prior to cultivation. WHO (1998b) indicated losses of 5-10% due to contamination resulting from prolonged preservation and a loss of up to 90% due to decontamination procedure. In addition, specimens taken from tuberculous cattle contain low numbers of bacilli that may be killed during processing, thawing and decontamination (Cleaveland *et al.*, 2007). A high amount of completely calcified lesions, without viable tubercle bacilli, could also explain the low recovery of bacteria (Teklu *et al.*, 2004). Besides, mycobacteria grow poorly on standard Löwenstein-Jensen medium (Amanfu, 2006). In consequence, Berg *et al.* (2009) collected mycobacterial colonies at a higher frequency from 7H11 media than from LJ glycerol or LJ pyruvate media indicating that LJ media is less suitable for mycobacterial isolation.

Although culture is still internationally considered the gold standard for detection of mycobacteria; the intensity of labour required and the possible presence of viable non-cultivable mycobacteria in some clinical specimens requires more appropriate method (Ayele *et al.*, 2004).

The culture yield of acid-fast bacilli (AFB) from visible lesions in the current study was calculated to be 69% (20 of 29). This is a relatively high number when compared to other countries such as Great Britain, where typical BTB lesions collected at slaughterhouses yield AFB in approximately 50% of cases (Liebana *et al.*, 2008). The proportion of AFB positive isolates in our study extremely differs from that of Berg *et al.* (2009) who reported only 11% of AFB isolates. This may reflect subjective differences in identifying tuberculous lesions leading to misclassification of lesions during examination of carcasses with subsequent low yield of AFB of the colonies.

In the present study, of the 17 AFB isolates typed using molecular method, 94% (16 of 17) were positive for the 16S rRNA genus specific locus and thus identified as mycobacteria. However, none of the isolates showed signals for the presence of *Mycobacterium tuberculosis* complex and

Mycobacterium avium complex organisms. In a similar study, Shihun (2008) typed 9 AFB positive mycobacterial isolates from tuberculous tissues using m-PCR and revealed seven isolates belong to the genus *Mycobacterium*. However, only 28.6% (2 of 7) of them were found to be members of the *Mycobacterium tuberculosis* complex suggesting that majority of tuberculosis lesions in grazing animals are caused by mycobacteria other than the tuberculosis complex. Furthermore, Berg *et al.* (2009) showed other acid-fast bacilli (AFB), notably non-tuberculous mycobacteria (NTM), as considerable causes of infection and disease in cattle. In their recent finding, Berg *et al.* (2009) typed AFB positive isolates using molecular methods and confirmed about one third of the isolate from tuberculous lesions as NTM. Similarly, Diguimbaye-Djaïbe *et al.* (2006) and Oloya *et al.* (2007) reported the isolation of NTM strains from more than 40% of the animals with tuberculous lesions in Chad and Uganda, respectively. Furthermore, Fujimura *et al.* (2003) isolated NTM from pathologic specimens of livestock and milk in Brazil.

The isolation of mycobacteria mostly considered less virulent in conditions of disseminated tuberculosis with fully caseated and calcified lesions is documented elsewhere (Falkinham *et al.*, 2002; Biet *et al.*, 2005). Mixed infections, in caseated and/or calcified disseminated lesions were also documented in other studies (Godfroid *et al.*, 2005; Oloya *et al.*, 2007). These findings could suggest secondary mycobacterial infections or may have outgrown the slow growing *M. bovis* during culture (Biet *et al.*, 2005).

6. CONCLUSION AND RECOMMENDATIONS

Bovine TB surveillance and control contributes to poverty alleviation through direct benefits to human health and improvements in livestock production that are needed to meet an ever growing demand for meat and milk. The current study, which is the first of its kind in the area, revealed the presence of mycobacterial infections in cattle found in and around Bako Town. The identification of mycobacteria from tuberculous cattle at BARC has serious implications on the health of other animals and humans working in the farm. Similarly, the identification of mycobacteria from carcasses of slaughter cattle at Bako abattoir coupled with the very limited awareness of the meat inspector about TB of animal origin highlights the possibility of the presence of TB infected meat for sale for human consumption on market which has serious implications on the spread of tuberculosis in human population in the area. On the other hand, although *M. bovis* has been known to be the major cause of BTB, in the present study, mycobacteria other than the *M. tuberculosis* complex members were isolated from the suspicious lesions. The method used by the present study could not identify these mycobacteria at the species level. The isolation of these mycobacteria from lesions underlines their roles in causing lesions, which are similar with lesions caused by *M. bovis*. However, the magnitude of severity of the lesions is not known.

Therefore based on the above conclusive remark, the following were recommended:

- Further identification and characterization of mycobacteria that were isolated by the present study,
- Investigation of the pathogenicity of these mycobacteria in cattle as compared to the pathogenicity of *M. bovis,*
- Further similar studies in different regions of the country so that the national epidemiology of mycobacterial infections is established.

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8. 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 (Mt) the animal is smooth, dorsal spines can not be seen, but are easily felt.
7. Condition score7 (F-) animal is smooth and well covered but fat deposit are not marked
8. Condition score8 (F) fat cover in critical areas can easily be seen and felt; transverse process can be seen or felt
9. Condition score9 (F+) heavy deposits of fat is clearly visible on tail-head, brisket, dorsal spines ribs and hooks

Source: (Nicholson and Butterworth, 1986)

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

Annex 2: Age determination in cattle

Age (years)	Characteristic change
1 ½ - 2	I1 erupt
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 11 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: De Lahunta and Habel, (1986)

Annex 3: Preparation of Löwenstein-Jensen egg-based medium

1. Mineral salt solution

1.1. Ingredients

Potassium dihydrogen phosphate anhydrous (KH2PO4)	2.4 g
Magnesium sulphate (Mg SO4. 7H2O)	0.24 g
Magnesium citrate	0.6 g
Asparagine	3.6 g
Glycerol (reagent grade)	12 ml
Distilled water	600 ml

1.2. Preparation

Dissolve the ingredients *in order* in the distilled water by heating. Autoclave at 121°C for 30 minutes to sterilize. Cool to room temperature. This solution keeps indefinitely and maybe stored in suitable amounts in the refrigerator.

2. Malachite green solution, 2%

2.1. Ingredients

Malachite green dye	2.0g
Sterile distilled water	100 ml

2.2. Preparation

Using aseptic techniques dissolve the dye in sterile distilled water by placing the solution in the incubator for 1-2 hours.

3. Homogenized whole eggs

Homogenised eggs (20-25 eggs, depending on size)	1000 ml

Fresh hens' eggs, not more than seven days old, are cleaned by scrubbing thoroughly with a band brush in warm water and plain alkaline soap. Let the eggs soak for 30 minutes in the soap solution. Rinse eggs thoroughly in running water and soak them in 70% ethanol for 15 minutes. Before handling the clean dry eggs scrub the hands and wash them. Crack the eggs with a sterile knife into a sterile flask and beat with sterile egg whisk or in a sterile blender.

The ingredients are aseptically pooled in a large, sterile flask and mixed well.

Mineral salt solution	600ml
Malachite green solution	20ml
Homogenized whole eggs	1000ml

The complete egg medium is distributed in 6-8ml volumes in sterile 14ml or 28ml McCartney bottles or in 20ml volumes in 20 × 150ml screw-capped test tubes and the tops are securely fastened. Before loading, heat the inspissator to 80°C to quicken the build-up of the temperature. Place the bottles in a slanted position in the insipissator and coagulate the medium for 45 minutes at 80°C-85°C. Inspissate the medium within 15 minutes of distribution to prevent sedimentation of heavier ingredients.

Source: WHO (1998)

Annex 4: Individual animal tuberculin skin test result recording sheet

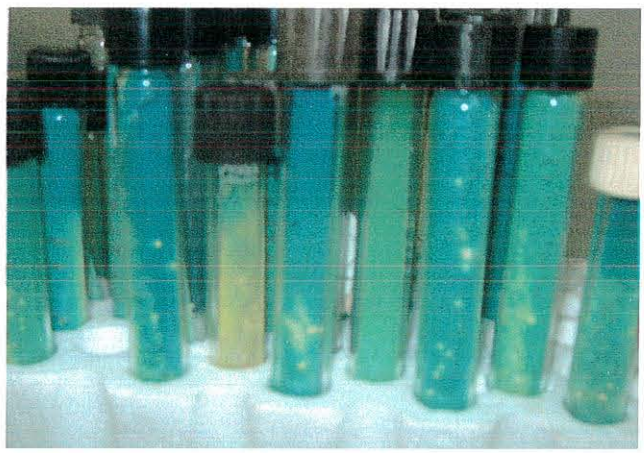
No.	ID	Breed	Sex	Age	BCs	Pregnanc	Parity	A1	A2	B1	B2	B-A	Remark

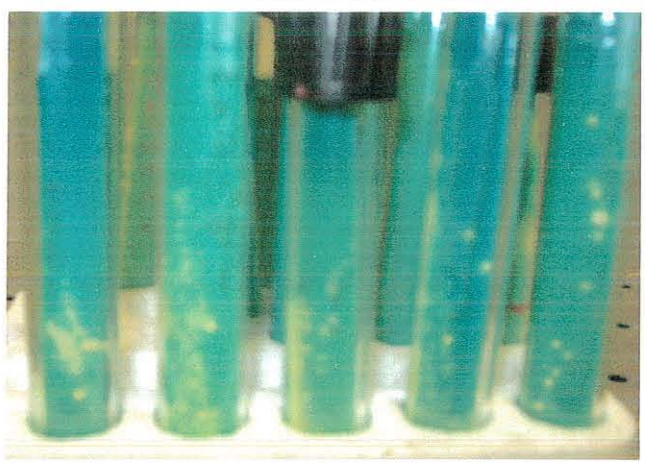
Annex 5: Bovine tuberculosis pathology scoring recording format

ID	L.api LL	L.car LL	L.dia LL	R.api LL	R.car LL	R.acc LL	R.dia LL	MB LN	RPH LN	LB LN	RB LN	CRMD	CAMD	HPAT	MES LN	Others	Remarks

MB: Mandibular; RPH: Retropharyngeal; CRMD: Cranial mediastinal; CAMD: Caudal mediastinal; RB: Right bronchial; LB: Left bronchial; HPAT: Hepatic; MES: Mesenteric; LL: Lung lobe; LN: Lymph node

Incubation of tissue specimens cultures aerobically at 37°C





Some of the mycobacterial colonies as grown on slants of LJ media

9. CURRICULUM VITAE

1. Biographical data

Full Name: Temesgen Ayana Dafa

Sex: Male

Birth Date: 01 December, 1979

Birth Place: Jimma Arjo district, East Wallaga zone, West Oromia, Ethiopia

Languages: Afaan Oromoo, English, and Amharic

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2. Educational Background

University/school	Duration	Award
Addis Ababa University	1996/97-2003	DVM degree
Mekonnen Demissew S.S.School	1994-1996	ESLCE certificate

3. Computer skill

Efficient in using Ms-word, Excel, Power point, Internet, SAS and SPSS programmes

4. Work Experiences

Assistant Researcher III: From May 2007 to date

Assistant Researcher II: From February 2006 to April 2007

Assistant Researcher I: From November 2005 to January 2006

Junior Researcher II: From July 2004 to September 2005

Bako Agricultural Research Center, Oromia Agricultural Research Institute

Instructor: From October 2003 to July 2004

MoARD, ATVET Project, Nedjo ATVET college

5. Research outputs

Ayana, T. (2003): Study on prevalence of tick-borne hemo-parasites of dairy cattle in and around Bedelle town, Western Ethiopia. DVM thesis, Addis Ababa University, Debrezeit, Ethiopia

Riedel, S., Wollny,. C. B. A, **Ayana, T.** and. Ayalew, W. (2006): Filling knowledge gap of smallholder cattle keepers on prevalence and control of bovine trypanosomosis: an Example from Dano district, Western Ethiopia. In: Proceedings of the Conference on International Agricultural Research for Development, Deutscher Tropentag, October 11-13, 2006 Bonn, Germany.

Riedel, S., Wollny, C.B. A., **Ayana, T.** and Ayalew W. (2007): Participatory assessment of incidence and perception of bovine trypanosomosis by cattle farmers in Dano district, Western Ethiopia. In: Proceedings of the Conference on International Agricultural Research for Development, Deutscher Tropentag, October 09-11, 2007, Kassel-Witzenhausen, Germany.

6. Training/Workshop/Seminar/conference attended

- Training on application of Participatory Research Approach (PRA) for agricultural research held at International Livestock Research Institute (ILRI), December 13-19, 2004, Addis Ababa, Ethiopia.
- Training on 'Communicating for Impact: Creating a strategic communication plans' conducted at International Livestock Research Institute (ILRI), April 12-20, 2006, Addis Ababa, Ethiopia
- Attended Second Annual Planning Workshop of ILRI-BMZ project entitled' Improving the livelihood of poor small scale livestock keepers in Africa through community based management (CBM) of farm animal genetic resources' conducted at International Livestock Research Institute (ILRI), Addis Ababa, Ethiopia;

- Participated on Prior Informed Consent (PIC) workshop conducted at Dano district with small scale farming community of the area, Ethiopia
- Attended Feedback workshop conducted at Dano district with selected small scale farming community of Dano district, Ethiopia
- Presented senior seminar paper to academic staff of the Faculty of Veterinary Medicine on 'The principle and role of embryo transfer in bovine reproduction'
- Participated on 'Planning workshop on next steps of cattle Trypanosomosis research in Ethiopia' held at International Livestock Research Institute (ILRI) from 20-25 Nov. 2006. Addis Ababa, Ethiopia;
- Participated on planning workshop of ICARDA-ILRI-BOKU project entitled 'Designing Community-Based breeding strategies for indigenous sheep breeds of smallholder farmers in Ethiopia' held at ILRI, November 14-16, 2007, Addis Ababa, Ethiopia.

7. Leadership role

- Head of animal science department of Nedjo ATVET College from Nov.2003-July 2004
- Head of animal health research division of Bako agricultural research center from August 2004-January 2008.

8. Membership

- Worked as member of ILRI-BMZ project research team which works with small scale farming communities of three countries in Africa (Ethiopia, Kenya and Benin) on conservation and sustainable use of indigenous farm animal genetic resources through community-based management of the animals for improvement of the livelihood of the poor livestock keepers;
- Member of Ethiopian Veterinary Association (EVA);
- Member of Ethiopian Society of Animal Production (ESAP);
- Member of dairy technology demonstration and evaluation team of Bako Agricultural research center;
- Member of Research-Extension farmer linkage advisory council of Bako Agricultural research center.

- Worked as member of ICARDA-ILRI-BOKU project entitled 'Designing community- based breeding strategies for indigenous sheep breeds of smallholders in Ethiopia'.

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10. SIGHED STATEMENT OF DECLARATION

I, the under signed, declare that this thesis is my original work and has not been presented for a degree in any other University, and that all sources of material used for the thesis have been duly acknowledged.

Name: Temesgen Ayana Dafa

Signature _____

Date of submission: Tuesday 22, 2010

This thesis has been submitted for examination with our approval as University advisors.

1. Dr. Tesfaye Sisay (DVM, MSc, PhD, Assitant Professor) _____
2. Dr. Gobena Ameni (DVM, PhD, Associate Professor) _____ Gobena Ameni, 24/06/2010