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**ADDIS ABABA UNIVERSITY
FACULTY OF VETERINARY MEDICINE**

**THE STATUS OF BOVINE TUBERCULOSIS IN SELECTED AREAS OF NORTH
GONDAR ADMINISTRATIVE ZONE, ETHIOPIA**

By

MOHAMMED ALI HUSSIEN

June 2006

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 to the Degree of Master of Science in Tropical
Veterinary Epidemiology

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ABBREVIATIONS

AEDC	Austrian Embassy Development Cooperation
AFB	Acid Fast Bacteria
AIDS	Acquired Immunodeficiency Disease Syndrome
BCG	Bacillus Calmette-Guerin
BTB	Bovine Tuberculosis
CFT	Complement Fixation Test
CI	Confidence Interval
CIDT	Comparative Intradermal Tuberculin Test
CMI	Cell Mediated Immunity
DTH	Delayed Type Hypersensitivity
EHNRI	Ethiopian Health and Nutrition Research Institute
ELISA	Enzyme Linked Immuno sorbent Assay
EP TB	Extra pulmonary Tuberculosis
FAO	Food and Agricultural Organization of the United Nations
FNA	Fine Needle Aspiration
FVM	Faculty of Veterinary Medicine
HIV	Human Immunodeficiency Virus
HTB	Human Tuberculosis
IFA	Immunofluorescence Antibody Test
IFN- γ	Interferon Gamma
IL	Inter leukin
ILDLP	Integrated Livestock Development Project
ILRI	International Livestock Research Institute
IU	International Unit
LJ	Lowenstein-Jenson media
MDRMB	Multidrug Resistant <i>M. bovis</i>
MHC	Major Histocompatibility Complex
MoA	Ministry of Agriculture
MoH	Ministry of Health
NALC	N-Acetyl-L-cysteine
NVL	Non Visible Lesion
OIE	Office International des Epizooties
OR	Odds Ratio
PA	Peasant Association
PCR	merase Chain Reaction
PPD	Purified Protein Derivative
SID	Single Intradermal tuberculin test
ST-CF	Short Term Culture Filtrate
TB	Tuberculosis
TCH	Thiophene-2- Carboxylic acid Hydrozide
Th	T-helper
TNF	Tumor Necrosis Factor
UK	United Kingdom
WHO	World Health Organization
γ -INF	Gamma Interferon



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ABSTRACT

Mycobacterium bovis (*M. bovis*) is one of the members of *Mycobacterium tuberculosis* complex, which infects both animals and man causing mammalian tuberculosis. A cross sectional and case control studies were conducted in Dembia and Gondar districts of North Gondar from September 2005 to April 2006 to determine the prevalence of bovine tuberculosis (BTB) and associated risk factors and assess the sources of infection of human tuberculosis (HTB). In the cross sectional study, cattle from extensive (310) and semi intensive (315) production systems were tested with the comparative intradermal tuberculin test (CID). In the case control study design, 50 human tuberculous patients (cases) and a similar number of patients visiting the hospital for some other health problems other than tuberculosis were interviewed about their hygienic practices, feeding habits and cattle management practices that would contribute to the transmission and maintenance of the disease. From each of the cases and control groups, 200 cattle owned or attended by them were tested with the CID test. Of all 1025 animals examined, 21% were positive. In the cross sectional study, assuming doubtful reactors as negative, prevalence was higher (16.8%) in semi-intensive production system than extensive (11.6%) and this difference was significant ($p < 0.05$). Herd level prevalence was 37.7% and 56.3% in extensive and semi intensive production systems, respectively. It was also significant ($p < 0.001$) between poor and good cattle management practices; cattle under poor management were 3.4 times more likely to be infected with BTB than those under good management. Other factors which were significantly ($p < 0.05$) associated with prevalence were production system, breed, age, sex and body condition. In the multivariate logistic regression analysis, production system and management were significant ($p < 0.05$). In the case-control study, out of the 200 animals tested that are owned by tuberculous human patients, 40 (20%) and from the same number of animals owned by non-tuberculous control groups, 22 (11%) were positive for the tuberculin test. The differences in prevalence of BTB in cattle owned by human TB cases and control group were significant ($\chi^2 = 98.90$, $p < 0.001$). Of 50 TB cases, 66% and 34% were diagnosed as pulmonary and extra pulmonary tuberculosis, respectively. About 86% of the patients consume raw milk and milk products. The majority (58%) of HTB patients are between the ages 15 and 45 while 24% and 18% were below 15 and above 45 years, respectively. Cattle owners who consumed raw milk were at higher risk (OR = 14.33) of being infected with tuberculosis than those who consumed boiled milk. From the questionnaire survey, significant associations ($p < 0.05$) with

cases were found when patients had physical contact with other clinical cases, drinks raw milk ($\chi^2 = 32.18$; $P < 0.001$) and are farmers compared to similar variables in the control group ($\chi^2 = 25.01$; $P < 0.001$). In the bacteriological examination, *Mycobacterium* species were isolated from 26 (47.3%) sputum, 2 (15.4%) fine-needle aspirates (FNA) and 1 (14.3%) peritoneal fluid of humans and 7 (7.1%) of 99 milk samples. The majority (88.5%) of sputum isolates were *M. tuberculosis*, and only one isolate (3.8%) was *M. bovis* while one isolate from milk was *M. tuberculosis*. Atypical mycobacteria were isolated from sputum (2), FNA (1) and milk (2). Among the isolates, 79.4% (23/29) and 10.3% (3/29) of the human isolates were *M. tuberculosis* and *M. bovis*, respectively. The rest 10.3% (3/29) were other mycobacterial species. On the other hand, 14.3% (1/7) and 57.1% (4/7) of the cattle isolates indicates were *M. tuberculosis* and *M. bovis*, respectively. The rest 28.6% (2/7) were other mycobacterial species. The findings in this study demonstrate the importance of BTB as a major health risk and cattle being one of the most important sources of infection. The findings of the present study using comparative intradermal tuberculin test and cultural examination of samples from humans and animals have shown that cattle may be important sources of infection to humans particularly of *M. bovis* infection.

Keywords: *Mycobacterium bovis*; Bovine TB; Cattle; Prevalence; North Gondar; Cross-sectional; Case-control.

1. INTRODUCTION

The world human population is growing at a rate much faster than food production that may reach 7.2 billion at the end of 2010. This increase will be mainly in developing countries, which are unable to assure adequate food for their people. Developing countries have nearly 2/3 of the world livestock population, but produce less than a third of world's meat and a fifth of its milk requirement (ILRI/FAO, 1995). The major biological constraints contributing to low productivity include low genetic potential of the animals, poor nutrition and the prevailing animal diseases (Bogale, 1999).

Programs in many developing countries, are currently, aimed at encouraging milk production through smallholder and other dairy schemes to answer the increased demand for milk and milk products. This increased demand will be partly met by an intensification of animal production. Unfortunately, Bovine tuberculosis (BTB) has shown close links with intensive management system, and can spread rapidly when there is inadequate veterinary supervision (Alhaji, 1976; Barwineck and Taylor, 1996). BTB becomes a serious problem in cattle when intensive dairying is established, particularly when European breeds are introduced (Bogale *et al.*, 2001; Kleeberg, 1984; Radostits *et al.*, 1994).

BTB caused by *Mycobacterium bovis* has been eliminated as an animal health problem, in most of the developed countries but still remains as one of the most prevalent and devastating diseases of cattle in developing countries throughout most of the world. Jointly with other diseases, it seriously affects the productivity of the livestock industry in these countries (Bogale *et al.*, 2001; Pritchard 1988). While these bottlenecks relate to the development of the dairy industry throughout the world, the disease attains much of its importance from being zoonotic, causing human tuberculosis (HTB) (Daborn *et al.*, 1996).

In developed countries the prevalence and the annual risk of human tubercular infection was shown to be closely associated with the prevalence of tuberculin positive cattle (Cook *et al.*, 1996), and was successfully controlled by tuberculin test and slaughter schemes (Pritchard,

1988). Because of the chronic nature of the disease and the multiplicity of signs caused by the variable localization of the infection, tuberculosis is difficult to diagnose on clinical examination (Radostits et al., 1994). Although other tests such as the gamma interferon assay and lymphocyte transformation test are available (Barwineck and Taylor, 1996), the intra dermal tuberculin test (IDT) with PPD is the principal diagnostic test for bovine tuberculosis in animals. The etiological agents of mammalian tuberculosis, classified as members of the *Mycobacterium tuberculosis* complex, are *M. tuberculosis*, *M. bovis*, *M. microti* and *M. africanum*; the latter consisting of a rather heterogeneous group of strains isolated from man in equatorial Africa (O'Reilly and Daborn, 1995).

Mycobacterium bovis, otherwise known as the bovine tubercle bacillus, is the cause of BTB and has exceptionally broadest host ranges of all known pathogens (Grange and Collins, 1987). Susceptible species include cattle, humans, non-human primates, goats, cats, dogs, pigs, buffalo, badgers, possums, deer, bison, sheep, horse, ferret, hare, wild and feral pig, antelope Arabian Oryx, camel, llama, and alpaca (Pritchard, 1988; Thoen and Chiodini, 1993).

With respect to the economic importance, apart from actual deaths, infected animals lose 10-25% of their productive efficiency. Direct losses due to the infection become evident by a decrease in beef production and additional processing costs for tuberculous animals and condemnation at slaughterhouses. Among dairy cattle, there is also a decrease in milk (10-18%) and in meat production (roughly, 15%). The culling loss is estimated to be 30-50% of the difference between the value of a dairy or beef breeding cow and its value at slaughter (Daborn *et al.*, 1996; Radostits *et al.*, 1994).

Human and animal tuberculosis is widespread in Africa with very close genetic and antigenic similarity between the causative organisms, *M. tuberculosis* and *M. bovis*. Both cause identical and clinically indistinguishable disease in humans. These days, Human Immunodeficiency Virus (HIV) is a potent risk factor for tuberculosis, by both reactivating latent infection and increasing rapid progression soon after infection or re-infection. It also increases the life-time risk of developing active TB (Corbett *et al.*, 2003; Cosivi *et al.*, 1998). Worldwide, 10 million cases of Human Tuberculosis (HTB) are recorded annually and 3 million people die of TB every year

(Bedard *et al.*, 1993). WHO estimates of HTB for years 1990-1999 was 88 million cases and 30 million deaths, most of which are from developing countries (Cosivi *et al.*, 1998). The situation is worsened by the fact that HIV-AIDS pandemic is associated with mycobacterial infections. An additional matter for concern is that *M. bovis* is naturally resistant to pyrazinamide, one of the principal components of modern short-course chemotherapy for tuberculosis in Africa (Daborn *et al.*, 1996).

HTB of animal origin, particularly *M. bovis* is becoming increasingly important in developing countries. In sub-Saharan Africa, humans and animals share the same microenvironment and waterholes, especially during draught and dry season. According to Cosivi *et al.* (1998), 60% of the African, 47% of the Asian and 38% of the Latin American and Caribbean countries reported the occurrence of BTB from sporadic to enzootic. The presence of *M. bovis* in cattle can pose a serious health risk for man since close contact between people and animals, and consumption of raw milk form part of the society characteristics. In such countries, where BTB is still common and pasteurization of milk is not practiced, an estimated 10-15% of TB is caused by *M. bovis* (Ashford *et al.*, 2001).

In developed countries, before the control and elimination of BTB and the wide introduction of milk pasteurization, *M. bovis* was responsible for more than 50% of the cases of the cervical lymphadenitis in children (Cosivi *et al.*, 1995) and the proportion of HTB cases due to *M. bovis* was between 5-20% (Grange, 1995). TB had at times present one of the deadly threats to human. Between 1750 and 1900, almost every one from the temperate region was infected with TB; in certain regions every fourth death was due to TB (Kleeberg, 1984). Because of the effective control measures applied, the disease has been controlled from most of the developed countries, remaining an increasingly serious problem of the developing world. It is still the greatest single cause of human morbidity and mortality in many developing countries.

Approximately 85% of the cattle and 82% of the human population of Africa live in areas where BTB is either partly controlled or not controlled at all. Of all nations in Africa, only 7 apply disease control measure as part of a test-and-slaughter Polycy and consider TB a notifiable



disease; the remaining 48 countries control the disease inadequately or not at all (Cosivi *et al.*, 1998).

TB is present in animals in many developing countries where surveillance and control activities are often inadequate or unavailable (WHO, 1993; OIE, 1996). The human disease is responsible for approximately three million deaths annually while TB in cattle is a major cause of economic loss and represents a significant cause of zoonotic infection (Pallock and Neill, 2002). Man acquires TB of bovine origin directly by the aerogenous route and indirectly by the consumption of milk and rarely meat of tuberculous cattle (Kleeberg, 1984; Cosivi *et al.*, 1998). TB can also be transmitted from man to cattle mainly through aerogenous route (Collins and Grange, 1987) and through oral route as a result of contamination of feed by workers urinating in the cow shed. Transmission of TB due to *M. bovis* or *M. tuberculosis* from HTB patients to cattle is also possible mainly due to contamination of pasture from urogenital TB cases in man (O'Reilly and Daborn, 1995). Even though man-to-man transmission of *M. bovis* is very rare, there are reports from Paris hospital in which HIV patients with open pulmonary TB due to multi drug resistant *M. bovis* acted as source of infection for other five HIV patients in the hospital (Bouvet *et al.*, 1993).

Bovine tuberculosis is an endemic disease of cattle in Ethiopia. It has been reported from different regions of the country based on tuberculin tests, ranging from 3.4% in smallholder production system to 50% in peri-urban (intensive) dairy production system (Bogale *et al.*, 2001; Ameni, 1996; Kiros, 1998) and abattoir inspection (Regassa, 1999; Bogale *et al.*, 2004; Solomon, 1975). However, the prevalence of the disease has not been well established because of inadequate disease surveillance and lack of better diagnostic facilities. Hence economic losses associated with bovine tuberculosis have not been determined fully (Bogale *et al.*, 2004; Cosivi *et al.*, 1998). In addition to this, people in rural areas live in close contact with cattle which if infected with TB are an important source of infection to man. Exposure is also great where children herd cattle, people buy milk directly from farmers and milk is consumed raw (Kleeberg, 1984).

The high prevalence of TB in cattle, close contact of cattle and human in rural areas, habit of the community to drink raw milk and increasing HIV epidemics suggest the significant role of *M.*

bovis in HTB. However the role of *M. bovis* in HTB has been undermined in Ethiopia because of the type of sampling and laboratory confirmation of HTB cases is based mainly on smear microscopy of specimens from patients rather than on culture and failure to set up appropriate cultures for bacterial growth and perform biochemical tests to differentiate *M. bovis* and *M. tuberculosis* often due to lack of facilities and logistics.

Although bovine tuberculosis is one of the major constraints for intensification of dairy production, data on the distribution of the disease in the country in general and Amhara regional state in particular, are lacking. On the other hand, various dairy development programmes / projects are being implemented in different parts of the country by private, government and non-governmental organizations including the Integrated Livestock Development Project (ILDP) in North Gondar administrative zone. Therefore, the present survey was the first attempt to address the in this continuously growing dairy development programmes body of privately, the government and non-governmental organizations including the Integrated Livestock Development Project (ILDP) in North Gondar administrative zone of Amhara regional state the problem of BTB by gathering preliminary information on the epidemiology of BTB both in local and cross-breeds of cattle and investigate the role of *M. bovis* in human tuberculosis cases. In developing countries such as Ethiopia, where *M. bovis* infection appears to be prevalent in a number of animal species, the knowledge of the distribution, epidemiological patterns and zoonotic implication of this important disease is crucial to devise effective control strategies.

Therefore, the objectives of the present investigation were:

- To determine the prevalence of BTB in Gondar and Dembia weredas of North Gondar Zone based on CIDT
- To identify risk factors and quantify their degree of association between HTB cases and tuberculin reactor cattle
- To isolate and identify mycobacteria from raw milk of cattle and human TB case
- To generate some baseline data that could be useful for the control of bovine TB in cattle and the prevention of its zoonotic transmission

2. LITERATURE REVIEW

2.1. Etiology

2.1.1 Taxonomy of Mycobacteria

The genus *Mycobacterium* is classified under the Order Actinomycetales and Family Mycobacteriaceae (Quinn *et al.*, 1999). The Genus, *Mycobacterium* includes a number of species, some being pathogenic to man and animals, some are opportunistically pathogenic while others are essentially saprophytic living in water and soil (Thoen, 1984). The classic species of *Mycobacterium* that cause disease in man and animals include: *M. bovis*, *M. tuberculosis*, *M. paratuberculosis*, *M. avium*, *M. leprae* and *M. lepraemurium*. TB in mammals is caused by *M. tuberculosis* complex (*M. bovis*, *M. tuberculosis*, *M. microti*, *M. africanum*) and by *M. avium* in birds (Bhata an Ichpujani, 1994). *Mycobacterium* species other than the *M. tuberculosis* complex that cause TB like diseases in man and animals are commonly called 'atypical mycobacteria' (Quinn *et al.*, 1999). They have been classified into four groups by Runyon in 1959 as, Photochromogenic, Scotochromogenic, Nonchromogenic and rapid growers based on growth rate and formation of pigments (Carter and Chengappa, 1991). Atypical mycobacteria are not pathogenic to man and animals except in certain situations such as direct inoculation into wounds or introduction into immunocompromised hosts due to immunosuppressive therapy or due to HIV (Thoen, 1984); however, they are very important during diagnosis as they sensitize man/animals to tuberculin test (Carter, 1986).

2.1.2. Physical and Biochemical Characteristics

2.1.2.1. Morphology and staining

Mycobacteria are non-motile, non-spore forming, pleomorphic bacilli or coccobacilli. In tissues they appear as rods, which may be straight, curved, or in the forms of clubs, measuring 1.0-4.0 μm in length and 0.2-0.3 μm in width. They occur singly, in pairs or as small bundles. On



laboratory media they may appear as cocci or rods measuring 6-8 μm (Quinn *et al.*, 1999). The distinguishing features of pathogenic mycobacteria are the formation of characteristic cords (Gillespie and Timoney, 1981).

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 (Barksdal and Kim, 1977). Mycobacteria when stained are acid fast as they resist decolorising with strong acid/alcohol solutions. This is due to a stable mole to mole binding of free mycolic acid residues of peptidoglycolipids in the outer cell wall (Quinn *et al.*, 1999) and depends on the amount and spatial arrangement of mycolic acids. Once the mycolic acid has formed a complex with the dye, the cell surface becomes extremely hydrophobic. Mycobacteria are not stained by aniline dyes such as Gram stains and it is difficult to demonstrate that they are Gram positive (WHO, 1998a) because of their lipid content. They can be Gram stained after partially removing the lipid layer. Steaming carbol fuchsin for some minutes stains them and if once stained, they resist decolorization with acid, hence, they are known as acid-fast bacteria.

2.1.2.2. Growth requirement and cultural characteristics

Mycobacterium species grow on 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 the 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). *M. bovis* grows more slowly than *M. tuberculosis*, which 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 except for *M. avium*, which needs a temperature of 40-42°C. Growth in liquid medium is diffused, but on a solid medium *M. bovis* is dry, sparse, delicate and non luxuriant.

2.2 Pathogenesis

2.2.1. Infection

The methods by which tubercle bacilli gain entrance to the animal body include: the respiratory, alimentary, genital, cutaneous and congenital routes (Menzies 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. Calves are usually infected by suckling milk from cows with tuberculous mastitis (Barwinek and Taylor, 1996). Humans most commonly acquire TB infection by inhaling aerosolized bacteria as droplet nuclei (Andersen, 1997). The infectious dose is very low; 1-3 viable bacteria are considered sufficient as infectious inoculum (Thoen and Bloom, 1995).

After infection the bacteria may localize in tissues related to the route of infection and associated lymph nodes. The disease may be self-limiting to fulminating disease with extensive tissue destruction. Miliary TB represents the most severe course of the disease with haematogenous spreading as a result of lysis of macrophages that release bacteria into the blood from the primary foci and secondary seeding to various tissues (Andersen, 1997).

2.2.2. Lesions

A primary lesion or focus of infection is established following the interaction of the host and the agent at the site of entry within 8 weeks of bacterial entrance (Radostits *et al.*, 1994). The mycobacterium is then taken by the alveolar macrophages to the circulation and establishes in lymph nodes. Cellular responses attempting to control the disease results in the accumulation of large number of phagocytes and lead to the formation of a macroscopic lesion referred as tubercle (Thoen and Bloom, 1995).

The cell mediated immunity (CMI) emerges 10-14 days after infection and triggers the release of cytokines from T-lymphocytes that activate the bacteriostatic effect of macrophages and accelerate the recruitment of additional blood-borne mononuclear cells into the site resulting in delayed type of hypersensitivity reaction. As the process progresses, monocytes mature into

epithelioid cells and multi-nucleated giant cells of the Langhan's type (Anderson, 1997) that form the center of the young tubercle which will be surrounded by lymphocytes, plasma cells, monocytes and an outer boundary of fibrous connective tissue. Caseous necrosis results from the delayed type hypersensitivity reaction. The gross appearance of the tubercle is usually firm yellow and on section a yellowish caseous necrotic material or calcified tissue is observed (Neill *et al.*, 1994; Quinn *et al.*, 1999).

2.2.3. Virulence

Mycobacteria are intra-cellular organisms and their virulence appears to be related to their ability to survive and multiply within the macrophages. The mechanism for such survival is poorly understood and may vary from species to species. For example, *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 as sulfatides play the major role in inhibiting phagolysosome formation. *M. avium* survives within the fused phago-lysosome by a virtue of their capsule like coating material of mycosides (Quinn *et al.*, 2002). *M. bovis* eludes the bacteriocidal activities of macrophages by escaping from fused phagolysosomes into non fused vacuoles in the cytoplasm. In addition to these survival mechanisms, an important aspect of pathogenicity of mycobacteria is their ability to subvert the protective immune response (Grange, 1995).

A characteristic feature of virulent strains of mycobacteria is that they form cords when they grow in a liquid culture media whereas the 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 and Bloom, 1995); the cord factor is a glycolipid that inhibits leukocytic migration and has also toxic effect on leukocytes (Gillespie and Timoney, 1981).

2.3 Immunity against Mycobacterial Infection

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

The bactericidal or bacteriostatic effect of macrophages is mediated by cytokines (*INF- γ* , TNF) that trigger the production of reactive oxygen radicals and nitrogen intermediates (Thoen and Bloom, 1995; Andersen, 1997). T-helper (T_h) lymphocytes may respond to infection by supporting cellular immune response such as delayed-type hypersensitivity in T_h1 -type response or by helping B lymphocytes to produce antibodies in T_h2 -type response. The T_h1 cells produce *INF- γ* , TNF- α and IL-2 which are directly involved in macrophage activation, but the T_h2 cells promote infection with intracellular pathogens by releasing IL-4 and IL-10 which have down-regulator effect on the T_h1 -type response (Andersen, 1997).

The T-cell sub-population changes following mycobacterial infection whereby the $\gamma\delta$ and T-cells increase in the first few hours of infection acting as first line of defense followed by an increase in CD4 T cells characterized by a significant increase in CD4 to CD8 ratio. In the advanced stage of infection CD4 to CD8 ratio decreases due to increased production of CD8 that may have a vital protective role when cells that do not express MHC class II molecules become infected. As in case of the beneficial cell mediated immunity, delayed type hypersensitivity (DTH) may also inhibit tubercle bacilli (Pritchard, 1988); but in this case macrophages containing replicating organisms are destroyed releasing the organisms from their protective intracellular environment and exposing it to phagocytosis by a new, activated mononuclear phagocytes (Neill *et al.*, 1994).

Protective immunogens released by actively growing tubercle bacilli give rise to a protective cell mediated rather than the humoral immunity. These immunogens are termed as short-term culture filtrate (ST-CF) as they can be easily filtered from a culture media containing actively growing mycobacteria (Andersen, 2001). The ST-CF is a mixture of released and secreted proteins which

contain several antigens that constitute important targets for the protective immune response and stimulate the release of *IFN- γ* as well as the CD4+ mediated antigen specific cytotoxicity.

2.4 Epidemiology of *M. bovis* Infections

Mycobacterium bovis combines one of the widest host ranges of all pathogens with a complex epidemiological pattern, which involves interaction of infection among human beings, domestic animals and wild animals (Grange and Collins, 1987). However, only little is done particularly in developing countries on the epidemiology of this organism and the epidemiological requirement for its control.

2.4.1 Risk factors

2.4.1.1. Agent risk factor

The causative organism, *Mycobacterium* is moderately resistant to heat, desiccation and many disinfectants; this is partly due to the presence of lipid in their cell wall. It is readily destroyed by direct sunlight unless it is in a moist environment. In warm moist, protected positions, it may remain viable for weeks or even months in a dark and moist environment (Table 1), (Cosivi *et al.*, 1995). Freezing temperature has little if any effect; they are also fairly resistant to acids and alkalis (Radostits *et al.*, 1994; Morris *et al.*, 1994).

Table 1: Survival time of *M. bovis* under different environmental conditions

Contaminated material	Condition	Survival time
Purulent emulsion	Direct sunlight	>10 h but <12 h
	Diffuse sunlight	at least 30 days
Cattle dung	Direct sunlight	> 6 h but <37 h
	Diffuse sunlight	15-150 days
	Covered	365-730 days
Pasture	Temperate climate	7-63 days
Water	Experimentally contaminated	28 days

Source: Cosivi *et al.* (1995)

Carter and Chengappa (1991) showed that *M. bovis* can survive for four weeks in non sterile dry and moist soils under 80% shed, in darkness and they can also retain their viability in putrefying carcasses and in moist soil for 1-4 years. Farm management practice such as feeding and nutrition, standard of fencing at farm boundaries, slurry disposal methods, cattle trading practices, presence of wild life are also significant risk factors for the occurrence and spread of TB in a given farm. Animals under good nutritional and management conditions do not excrete sufficient *M. bovis* organisms to infect others.

2.4.1.2 Host risk factor

Genetic resistance of hosts to *M. bovis* infection has never been conclusively demonstrated (Morris *et al.*, 1994), but zebu (Brahman) types of cattle are thought to be much more resistant to TB than the European cattle. The effects on these cattle are much less severe but under intensive feedlot conditions a morbidity rate of 60% and a depression of weight gain can be experienced in tuberculous zebu cattle (Radostits *et al.*, 1994). On an abattoir survey in India, TB lesions were found in 25.97% of the pure exotic breeds, 9.7% of the crossbreeds and in only 7.1% of the zebu animals (O'Reilly and Daborn, 1995). In contrary to this, Seifert (1996) reported that cattle of the traditional pastoralists in Madagascar were affected up to 60%.

2.4.1.3 Environment and management factors

Housing and feeding conditions are important risk factors. Housing predisposes to the disease because animals are closer; the greater is the chance that the disease will be transmitted. For example, the disease incidence is high in intensive dairy farms than in beef ranches (Morris *et al.*, 1994) whereas in beef cattle the degree of infection is usually low. Feeding and housing conditions are important risk factors for BTB. O'Reilly and Costello (1988) have demonstrated that cattle kept under good nutritional and husbandry conditions may not, irrespective of the nature and extent of the lung lesions, excrete *M. bovis* in sufficient number to infect contact cattle in the open grazing for a period of 4 to 9 months. Farm management practices such as feeding and nutrition, standards of fencing at farm boundaries, slurry disposal methods, cattle trading



practices, presence of wild life are also significant risk factors for the occurrence and spread of TB in a given farm (Pallock and Neill, 2002).

The ubiquitous distribution of *M. bovis* in farmed and to a large extent in the wild animal population, the trend towards intensification of animal production to meet the increase in demand for milk and meat, gathering of animals at watering points, markets and in corrals over night and lack of control measures in most African countries are some factors which are likely to facilitate *M. bovis* infection in the African animal population (WHO, 1993). The evidence suggests that even when heifers are pastured with heavily infected cows the incidence remains low until they enter the cow shed (Morris *et al.* 1994). Malnutrition, pregnancy and concurrent infections may depress the immune responsiveness in some cases and they may be important factors in cattle herds (Collins, 1994).

2.4.2 Hosts range

All species and age groups are susceptible to *M. bovis*, with cattle, goats and pigs most susceptible and sheep and horses showing a high natural resistance (Radostits *et al.*, 1994). *M. bovis* has an exceptionally wide host range but under natural conditions it infects mainly cattle (Quinn *et al.*, 1999). An extensive range of mammals including various domesticated and feral hoofed animals, primates and a wide variety of exotic species both of captive and free-living has been implicated (Thoen *et al.*, 1995b). Following eradication of *M. bovis* in cattle, re-infection of cattle herds have occurred from man, imported cattle, and wild life reservoirs. The importance of each reservoir depends on the degree of contact with cattle and the extent to which tubercle bacilli are excreted by these free-living species.

2.4.3 Transmission

The possible routes of infection in cattle include respiratory, alimentary, congenital, cutaneous, veneral and the teat canal but the major route of infection, 80-90%, is the aerogenous route. Inhalation is the almost invariable portal of entry in housed cattle, and even in cattle pastured it is considered to be the principal mode of transmission (Thoen and Bloom, 1995). Infection by

ingestion is when feces contaminate the feed and common drinking water and feed troughs. Under natural conditions, stagnant drinking water may cause infection up to 18 days after its last use by a tuberculous animal (Radostits *et al.*, 1994). TB transmission between cattle via the respiratory route occurs at with high stocking densities and cattle movement (Berrada and Barajas-Rojas, 1995). Calves can be infected orally by drinking milk from the tuberculous udder (Neill *et al.*, 1994).

Less common routes of transmission include cutaneous infection via lesion, congenital infection via the umbilical vessels, genital transmission the male or female sexual organs is tuberculous and via intramammary infusion (Neill *et al.*, 1994). Congenital transmission may occur if a calf is born from a cow with metritis originating from peritonitis, external genitalia or most commonly from haematogenous spread of the bacilli (Morris *et al.*, 1994). Udder infection due to haematogenous spread occurs only in 1-2% of the tuberculous cows and results in tuberculous mastitis. Calves can be infected orally by drinking milk from the tuberculous udder (Neill *et al.*, 1994).

Transmission from wild animals to cattle occurs when there are interactions between excreting wild life host and domestic animals. Transmission from man to animals is probably mainly airborne, but spread via urine, which contaminates the cow shed, is also mentioned to be important (Cosivi *et al.*, 1998).

2.4.4 Distribution

The global distribution of *M. bovis* infection in animals and humans varies widely. Out of 1 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 (Steele, 1995).

In the developed countries such as North America and Western Europe, BTB has been eradicated from their livestock population by the test and slaughter Policy. In others such as Australia, New Zealand, Eastern Europe, Israel, Japan eradication programs are also in progress and resulting

low or sporadic occurrence of the disease in the livestock population (WHO, 1993). Countries in South America, Asia and Africa, which probably acquired the disease by way of Europe, are today the problem areas (Steele, 1995).

Of the 55 nations of Africa, 25 reported sporadic/low occurrence of BTB, 6 reported enzootic disease, 2 (Malawi and Mali) were described as having a high occurrence, 4 not reported 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 of 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; OIE, 1996; Cosivi *et al.*, 1998).

2.5 Status of bovine tuberculosis in Ethiopia

Most of the surveys carried out in Ethiopia have been based on abattoir reports (Table 2) and tuberculin testing of animals in different parts of Ethiopia, (Table 3).

Table 2: Prevalence of BTB based on carcass and organ condemnation in Ethiopia

Place	Year	Animals Examined	No./ % of organs affected		
			Lung	Others organs	Whole carcass
Asmara	1972-73	43753	328(0.75%)		
	1985-90	124160	782(0.63%)		
Mekele	1972-73	65544	1549(2.36%)		
	1985-90				
Gondar	1972-73	12525	3(0.024%)		
	1985-90	20496	21(0.1%)	66(0.32%)	7(0.03%)
Drie Dawa	1972-73	28926	194(0.67%)		
	1985-90	35731	241(0.67%)	163(0.46%)	9(0.025%)
Kombolcha	1972-73	47077	211(0.45%)		
	1985-90	42570	352(0.83%)	947(2.2%)	63(0.15%)
Wondo	1972-73	58239	255(0.44%)		
	1985-90	88941	337(0.38%)	1105(1.24%)	102(0.11%)
Addis Ababa	1972-73	94032	217(0.23%)		
	1985-90	854170	3417(0.4%)		
	1992-2001	1189469	3-284		
Debre Zeit	1972-73	3934	7(0.18%)		
	1985-90	64504	1907(2.96%)	631(0.98%)	746(1.16%)
Nazareth	1999-2003	43461	270(0.62%)	453(1.04%)	8(0.02%)

Adapted from Yehualashet (1993); Ameni (1996); Kiros (1998); Woldeesenbet (2002); Wudie (2003).

Table 3: Prevalence of BTB based on tuberculin test in Ethiopia

Year	Place	Animals examined	Positive reactors %
1984	Around Addis Ababa	4838	16.8
1987	Around Addis Ababa	3352	24.4
1996	Debre Zeit	486	87
1998	East Showa	788	23.9
1999	Around Addis Ababa	1241	10.3
2000	Around Addis Ababa	2953	24
2002	Wuchale-jida		7.86
2002	Shewa, Wello,	2250	24
2003	Nazeret	1125	5.16

Adapted: from Yehualashet, 1993; Ameni, 1996; Kiros, 1998; Bogale, 1999; Wudie 2003.

The endemic nature of BTB has long been recorded in Ethiopia. The first survey on the prevalence of BTB was made by Shola zonal veterinary laboratory (1984-1986) where 4838 cows in 12 dairy farms were tested by tuberculin test and showed an average prevalence of 16.8% with the highest prevalence (77.7%) recorded in Mojo State dairy farm (Yehualashet, 1993). One year later, a total of 3352 dairy animals were tested by comparative tuberculin test and an average prevalence of 24.7% was reported in 10 dairy farms with Mojo still the highest prevalence 92.3% (MoA, 1987). Other studies carried out by different researcher using tuberculin test results range 5.2% in Nazeret (Wudie, 2003) to 23% - 87% in IAR and State Dairy Farm respectively, Debre Zeit (Ameni, 1996).

2.6 Diagnosis

A presumptive diagnosis of TB in cattle and other susceptible species is often made on history, clinical findings, tuberculin skin tests, and/or necropsy findings. *In-vitro* lymphocyte assays, including an interferon gamma assay and enzyme linked immunosorbant assays have been developed for detection of the disease in cattle and so other animals exposed to *M. bovis* (Thoen *et al.*, 1995a).

Data on BTB from developing countries is always underestimated mainly due to lack of diagnostic facilities and epidemiological investigation (WHO, 1993). There are several diagnostic techniques used to diagnose BTB both on live and dead animals as described below.

2.6.1 Clinical examination

Because of the chronic nature of the disease and the multiplicity of signs caused by the variable localization of the infection, TB is difficult to diagnose on clinical examination (Radostits *et al.*, 1994). However, the general signs are weakness, erratic appetite, dyspnea, emaciation, and low grade fluctuating fever. Enlarged superficial lymph nodes provide a useful diagnostic sign. When lungs are extensively involved, there is commonly an intermittent cough. The principal



sign of TB commonly is chronic wasting or emaciation that occur despite good nutrition and care (Thoen and Bloom, 1995; Thoen *et al.*, 1995a).

2.6.2 Tuberculin skin tests

For more than 100 years, tuberculin test has been successfully used worldwide for the diagnosis of TB in cattle (O'Relly and Daborn, 1995). The standard test recommended by Office International des Epizooties (OIE) to diagnose BTB in cattle is the allergic skin test where purified protein derivative (PPD) is injected intradermally. A significant measure in size at the site of injection shows previous exposure to homologous mycobacterium. PPD is produced from *Mycobacterium* that grows in liquid media as a culture filtrate, which is further processed by chemical fractionation, is used to produce PPD. Two strains namely *M. bovis*, AN5 and vallee strains and the avian PPD is produced from *M. avium*, D4ER or TB 56 strains (Monaghan *et al.*, 1994). PPD could also produce from BCG. For screening purpose the single intradermal test (SIDT) has been widely used at a dose of 0.1 or 0.2 ml of bovine or human PPD in the cervical or caudal area (O'Relly, 1995).

Stormont test is used in problem herds where inconclusive reactions are obtained. In this method, 0.1 ml PPD (2 mg/ml concentration) is injected intradermally on the cervical region and the dose repeated on the same site 7 days later. An increase of 5 mm or more in thickness of the skin after 24 hours is a positive reaction (O'Relly, 1995). In order to distinguish animals infected with *M. bovis* from those sensitized by other atypical mycobacteria, particularly *M. avium*, the intradermal comparative tuberculin test (IDCT) has been developed whereby 0.1 ml of bovine and avian PPD are inoculated simultaneously on the same side of the neck 12-15 cm apart, and cattle infected with bovine bacilli will show greater allergic reaction to the homologous PPD.

2.6.2.1 Problems associated with tuberculin test

Sensitivity and specificity of the test have been determined in various studies that have reported different values. Data summarized by Monaghan *et al.* (1994) suggested that the sensitivity varies from 68-95% while specificity is as high as 96-99% (WHO, 1994).

Anergy is a condition in which infected animals give false negative results. Radostits *et al.* (1994) stated that recently infected animals until 6 weeks after infection and advanced cases of tuberculosis show this condition. Desensitization resulting from injection with PPD during the preceding 60 days (Radostits *et al.*, 1994) and immunosuppression during the early postpartum period is also an additional reason. The reason for this may be due to depletion of T-lymphocytes from the skin into the circulation and then into colostrum (Radostits *et al.*, 1994). Contrary to this, few animals, which are positive to tuberculin test, fail to show evidences of infection during post-mortem, which are known as non-visible lesion (NVL) reactors (Monaghan *et al.*, 1994). Calves born from infected cows may have acquired colostral immunity for about 4 weeks and react false positive to tuberculin test (Barwinek and Taylor, 1996).

2.6.3 Post-mortem Examination

Caseous or calcified foci at necropsy are indicative of BTB, but this is difficult in the initial stages of the disease or when the changes are not specific (OIE, 1992). Studies show that in cattle 86% of the cases with single lesions can be identified by examination of mediastinal, medial retropharyngeal, bronchial, and lymph nodes together with lung. Additional examination of parotid, caudal cervical and superficial inguinal as well as the mesenteric lymph nodes enabled the detection of 95% of tubercular lesions (Corner, 1994).

The distribution of the lesions in different body systems showed that 53%, 29.5% and 17.5% were found in the respiratory, digestive and billiary system respectively (Regassa and Ameni, 2001). This shows that inhalation was the most important route of infection due to congregation of livestock at watering points or grazing areas and enclosures overnight that facilitate respiratory transmission of infection (WHO, 1993). Post-mortem examination should be supported by histopathology and bacteriological examination of lesions (OIE, 1992) for definitive diagnosis of BTB.

2.6.4 Differential staining

Final confirmatory diagnosis of BTB depends on isolation and identification of the bacteria, but preliminary examination of stained smears from lesions, sputum, milk, urine, pleural and

peritoneal fluids, uterine discharge and feces is very important (Thoen *et al.*, 1995a). In the smear, the organisms appear red rods against a blue background (Ziehl-Neelsen staining), while in the fluorochrome procedures, the acid-fast organisms appear as fluorescent rods, yellow to orange (WHO, 1998b). Direct microscopic examinations of nasal discharge and tissue sections however have limited diagnostic value.

2.6.5 Culture Media

The definitive diagnosis of BTB depends on the isolation and identification of the *Mycobacterium* (Pritchard, 1988). The success of *M. bovis* isolation depends on the type of media used; procedures applied for decontamination and concentration of the specimen as well as the incubation conditions (Corner, 1994). The most commonly used media are either the egg based (Löwenstein-Jensen and Stone Brink's media) or agar-based medium enriched with blood and/or serum such as the modified Middle Brook 7H11 (WHO 1998b) and the tuberculosis blood agar medium called B83 (Cousins *et al.*, 1989). Corner (1994) indicated that growth on the agar media is much faster than on the egg based media with mean time to the first appearance of colonies being 27 days and 28 days on B83 and 7H11 respectively compared to 36 days on Stone Brink's medium. However, the agar medium is highly liable to contamination even after decontamination of the specimen. In a study with 362 clinical specimens, 3.9% of the specimens on the agar medium were contaminated compared to only 0.3% on the egg-based medium (Corner, 1994). The procedure of cultural examination includes digestion-decontamination to release the organism from tissues and to isolate from contaminated material. This is based on the relative resistance of *M. bovis* to mild acids, alkalis and to certain disinfectants (WHO, 1998b; Thoen *et al.*, 1995b). The ideal decontaminant should be toxic to other bacteria but less toxic to *Mycobacterium*. The commonly used chemicals for digestion-decontamination purpose in most laboratories are the N-Acetyl-L-cysteine (NALC) and 2-4% NaOH (WHO, 1998b).

2.6.6 Differentiation of *Mycobacterium* species

Differentiation of the pathogenic tubercle bacilli mainly *M. bovis* and *M. tuberculosis* is done based on the stimulation of growth on a medium containing pyruvic acid or glycerol. Glycerol inhibits the growth of some *M. bovis* strains; but pyruvate enhances growth of this organism in

glycerol-free media (WHO, 1994; WHO, 1998b; Quinn *et al.*, 1999). In addition to this, biochemical tests such as niacin production, nitrate reduction, urease and pyrazinamidase activity and drug sensitivity tests are also commonly used in the identification of mycobacterium. Other methods of diagnosis include immunoassays and molecular techniques (Carter and Chengappa, 1991).

2.6.7 Animal inoculation

Laboratory animals such as rabbits, guinea pigs and chicken are used for diagnosis and identification of mycobacteria (Radostits *et al.*, 1994; Thoen *et al.*, 1995a; and Quinn *et al.*, 1999). The reaction in these laboratory animals is given in Table 4.

Table 4: Inoculation of laboratory animals with mycobacteria of the tuberculosis group

Species	<i>M. tuberculosis</i>	<i>M. bovis</i>	<i>M. avium</i>
Rabbits (IV)	± (pulmonary only)	++ (miliary)	++ (generalized)
Guinea-pigs (SC)	++ (generalized)	++ (generalized)	- (+) (Focal)
Chicken (IV)	-	-	++ (generalized)

++ = systemic infection, ± comparatively mild infection, - (+) = localized infection, - = no infection, IV = intravenously, SC = subcutaneously.

Source: Quinn *et al.* (1999)

2.6.8 In-vitro cellular assay

In vitro lymphocyte assays, including interferon gamma assay and ELISA, using specific *Mycobacterium* antigens have been developed for detection of infection in cattle and some other animals exposed to *M. bovis* (Wood and Rothel, 1994).

2.6.8.1 Lymphocyte proliferation assay

In-vitro methods to detect cellular reactivity to tuberculin antigen in whole blood samples were attempted. To measure the T-cell reactivity of cells from *M. bovis* infected cattle focused on the

lymphocyte transformation tests (LTT). The method involves incubating lymphocytes in the whole blood diluted with tissue culture media for 3-5 days, and then using radioactive nucleosides to detect the level of cell proliferation (WHO, 1994; Wood and Rothel, 1994; OIE, 1996). The assay has scientific value, but is not used for routine diagnosis because the test is time consuming, logistic and laboratory executions is complicated, costly and slow for use in routine diagnosis.

2.6.8.2 Gamma interferon (γ -IFN) assay

The γ -IFN assay is an *in vitro* cellular assay that measures the release of lymphokine (γ -IFN) from the sensitized T-cells in response to specific antigen. A sandwich ELISA which utilizes two monoclonal antibodies to bovine γ -IFN is used for the detection of the γ -IFN released by sensitized T-lymphocytes (Russell, 2003). Wood and Rothel (1994) claimed γ -IFN assay as a simple and rapid *in-vitro* cellular assay for the diagnosis of BTB, and may be a practical replacement for the single intradermal tuberculin test. The major advantages of the γ -IFN assay would be that it does not compromise the immune status of the animal thus making immediate retesting of suspected animals possible, and the test results are available within as short as 24 hours. The sensitivity and specificity varies from 76.85 to 93.6% depending on interpretation method. The disadvantages of γ -IFN assay include the relatively high costs, the examination of blood samples has to be started within 8 hours after collection and only works with the Family Bovidae (Barwinek and Taylor, 1996).

2.6.8.3 Enzyme-Linked Immuno-Sorbent Assay (ELISA)

The ELISA test can be used to complement cellular immunity, because humoral and cellular immunity to *Mycobacterium* infection occurs interchangeably. The respective sensitivity and specificity of ELISA was reported to be 98% and 65.5% (Anderson, 1997; Ayancuale, 1987). Zerihun (1991) used ELISA to discriminate *M. bovis* from other mycobacteria obtained from artificially infected mice. Various workers used different antigens mainly PPD and phosphatid (Hanna *et al.*, 1992; Neill *et al.*, 1994) for serological tests; they found a sensitivity of 4.85% and 27% and specificity of 97% and 88.5%, respectively.

The role of serological tests in routine diagnosis of BTB is limited because of the low sensitivity they have, due to the large number of protein antigens present in mycobacterium and due to the variable response to mycobacterial infections (Wood and Rothel, 1994). They are only used as a supplement to pick out some of the anergic cattle which failed to respond to the single intradermal test (Radostitis *et al.*, 1994; OIE, 1996) or to complement the *in-vitro* cellular assay.

2.6.8.4 Other methods

Several new laboratory procedures have been introduced as aids in the diagnosis of mycobacterium infections. The use of monoclonal antibodies, restriction endonuclease analyses, gas-liquid chromatography, radiometric detection of growth, gene probes and polymerase chain reaction (PCR) have been evaluated (Thoen *et al.*, 1995a). However, because of technical problems and cost they have not come into widespread use in veterinary diagnostic laboratories. PCR is simple and rapid test however, the reliability and reproducibility is limited. Reference laboratories report false-positive rates varying from 3 to 77 percent (Thoen *et al.*, 1995a).

2.7 Zoonotic importance of *M. bovis*

Tuberculosis caused by *M. bovis* is clinically indistinguishable from TB caused by *M. tuberculosis*. TB, one of the most widespread infectious diseases, is the leading cause of death due to a single infectious agent among adults in the world (Cosivi *et al.*, 1998). In countries where BTB is uncontrolled, most human cases occur in young persons and results from drinking or handling contaminated milk; cervical lymphadenopathy, intestinal lesions, chronic skin TB (lupus vulgaris), and other nonpulmonary forms are particularly common. Agricultural workers may acquire the disease by inhaling cough spray from infected cattle: they develop typical pulmonary TB. Such patients may infect cattle, but evidence for human-to-human transmission is limited (Cosivi *et al.*, 1998; Grange and Yates, 1994). One of the main reasons for the interest in BTB is the susceptibility of man to disease due to *M. bovis* and it is causing almost all the non-pulmonary as well as a varying proportion of pulmonary cases in human TB (Grange

and Yates 1994). *M. bovis* infection in cattle is still endemic in developing countries and some epidemiological conditions for the spread of *M. bovis* infection between animals and humans are very similar in Africa today to those in Europe in the 1930s (Cosivi *et al.*, 1998).

Information on human disease due to *M. bovis* in developed and developing countries is scarce. From a review of a number of zoonotic TB studies, published between 1954 and 1970 and carried out in various countries around the world, it was estimated that the proportion of human cases due to *M. bovis* accounted for 3.1% of all forms of TB: 2.1 of pulmonary forms and 9.4% of extra-pulmonary forms (Cosivi *et al.*, 1998). Table 5 summarizes the findings of more recent reports of TB caused by *M. bovis* in industrialized countries.

Table 5: Status of human TB due to *M. bovis* in industrialized countries

Country	Years	No	Cases	
			% of total TB	Pulmonary (% of total)
Australia	1970-94	240	0.43-3.1	71.6 ^a
England	1977-90	232	1.2	40.0
Germany	1975-80	236	4.5	73.7
Ireland				
Rural	1986-90	17	6.4	70.6
Urban	1982-85	9	0.9	88.8
New Zealand	1983-90	22	7.2	31.8
Spain	1986-90	10	0.9	50.0
Sweden	1983-92	96	2.0	-
Switzerland	1994	18	2.6	-
U.S.A	1954-68	6	0.3	33.3
U.S.A	1980-91	73	3.0	52.0 ^b , 12.0 ^c

A = over all percentage includes, 80.6% males and 51.2 females, b = adult, c = children

Source: Cosivi *et al.* (1998).

By contrast there is very little information on the prevalence of human TB caused by *M. bovis* occurring in developing countries of Africa, but a few reports available indicate that a number of countries including Morocco, Egypt, Ethiopia, Uganda, Tanzania, Zambia, and Malawi have areas with exceptionally high levels of infection (Daborn *et al.*, 1996). Conversely, the transmission of *M. bovis* from man to cattle has led to explosive herd outbreaks. Spreads of TB



from man to cattle is most frequently by urinary contamination of hay or straw bedding, but it may also occur by aerogenous route or by faecal or sewage contamination of pasture, hay or bedding (Pritchard, 1988; Grange, 1995).

The proportion in which *M. bovis* contributes to total TB cases in humans depends on the prevalence of the disease in cattle, socio-economic conditions, consumer habit, practiced food hygiene, medical prophylaxis etc. The effect of BTB on human health has also increased due to the impact of HIV/AIDS pandemic. Out of the 12 million people infected with HIV, 4 million are also concurrently infected with TB and nearly 80% of them live in sub-Saharan Africa (Daborn and Grange, 1993). In Monze district of Zambia 70% of the TB patients were also HIV positive (Cook *et al.*, 1996). According to recent WHO global estimates, of the 9.4 million people infected with both HIV and TB in mid-1996, 6.6 million (70%) live in sub-Saharan Africa (Cosivi *et al.*, 1998). The greatest impact of HIV infection on TB is in populations with high prevalence of TB infection among young adults. The occurrence of both infections in one person makes TB infection very likely to progress to active disease.

In Ethiopia, FNA specimens from 40 patients presenting at rural health center in south Ethiopia and diagnosed as positive for TBLN on the basis of clinical and cytological criteria were analyzed to mycobacterial DNA by PCR. Thirty (75%) had cervical lymphadenitis and 11 (27.5%) were sero-positive for human immunodeficiency virus (HIV). Among the forty TBLN cases, 35 (87.5%) were positive by PCR at the genus and complex levels, 29 (82.9%) of the 35 were caused by *Mycobacterium tuberculosis* and six (17.1%) of the 35 were caused by *M. bovis* (Kidane *et al.*, 2002). This indicates the importance of *M. bovis* as a cause of HTB lymphadenitis in Ethiopia.

2.8 Economic importance of bovine tuberculosis

Apart from actual death, BTB is estimated that infected animals lose 10-25% of their productive efficiency (Radostits, *et al.*, 1994). Even though the determining factor for initiating programs for control of BTB was impact on human health, the disease was also causing massive financial losses due to animal mortality and carcass and offal condemnation (Kantor and Ritacco 1994). When suitable control measures are not taken the effects on economy and health progress slowly

and steady. Direct losses due to the infection becomes evident by a decrease in productivity (milk and beef) the average age of cattle wastage of utilization food sterility losses in market value and cattle movement and additional processing costs for tuberculous animals and condemnation at slaughterhouses. Among dairy cattle there are also decreases in milk (10-18%) and meat production (15%). The culling loss is estimated to be 30-50% of the difference between the values of a dairy or beef breeding cow and its value at slaughter (Daborn *et al.*, 1996). While part of the growth retardation or weight loss seen in tuberculous cattle may be due to reduced appetite. It is certainly true that chronic disease reduces the feed conversion efficiency of animals leading to severe weight loss. In Hungary, the estimated weight loss was 15 kg in cows and 25 kg in beef cattle (Denes, 1981). Loss of milk production in cows may reach as high as 30% or more. Average infected cow give about 10% to 12% less milk than healthy ones and 5% of infected cows become infertile culling a cow early due to disease reducing her number of replacement costs per year of production. The loss in calves is much greater because of the high mortality (Barwinek and Taylor 1996; Denes, 1981).

Losses in meat inspection due to organ or whole carcass condemnation are considerably high particularly in countries with high prevalence of BTB and with strict laws of meat hygiene. In Ethiopia the estimated cost of organs and carcasses condemned during meat inspection of 1.2 million cattle slaughtered in six export abattoirs was estimated at 600, 842 Birr (Gezahegn, 1991). Infected cows may develop tuberculous metritis (Morris *et al.* 1994) out of which 5% may become infertile (Denes 1981). It is also reported that 1% of the calves from tuberculous dams infected congenitally leading to death or retarded growth (Seifert, 1996). Such cows may be culled before they finish their production life that incurs additional loss. In Hungary the production life of infected cows was reduced on an average of 1.5 lactations (Denes 1981). There are also costs associated with human TB due to *M. bovis*. This includes cost of treatment, hospitalization, loss of working days and payment for sick leave. Premature deaths in untreated cases are difficult to assess in monetary terms (WHO 1994; Barwinek and Taylor, 1996).

2.9 Control of bovine tuberculosis

In industrialized countries animal TB control and elimination programs together with milk pasteurization have drastically reduced the incidence of disease caused by *M. bovis* in both cattle and humans. In developing countries however, animal TB is widely distributed because control measures are not applied or are applied sporadically and pasteurization is rarely practiced.

The basic strategies required for control and elimination of BTB are well known and well defined. However, because of financial constraints, scarcity of trained professionals, lack of Political will as well as the under estimation of the importance of zoonotic TB in both the animal and public health sectors by national governments and other agencies, control measures are not applied or are applied inadequately in most developing countries.

2.9.1 Treatment and vaccination

Chemotherapy in man is usually required for over 6 months. In animals, chemotherapy with isoniazid has been shown to be ineffective for a variety of reasons. This is particularly because of the high cost of treatment, the frequent recurrence of the disease when the treatment is stopped and the possibility of the development of multi drug strains of *M. bovis* (WHO, 1993).

Vaccination of animals against BTB would be important in reducing the prevalence of the disease to an acceptable level before the test and slaughter Policy in domesticated animals is applied. It is also applied in wild and feral reservoirs of the disease where test and slaughter programs (stamping out) and control of animal movement have failed to achieve elimination of the disease (OIE, 1996). Many issues need to be addressed before vaccination because a realistic option for control of the disease in cattle and other domestic animals. Vaccination of human with strain Bacillus Calmette-Guerin (BCG) has been practiced in human but its efficacy in protecting the subject from infection is lower. In animals there are recent trials on the use of the vaccine in calves (Andersen, 2001). A highly effective vaccine needs to be developed. The results and efficacy have varied considerably from region to region. Vaccination with BCG may interfere

with the diagnostic test and would invalidate the key diagnostic tool used in control programs (OIE, 1996).

2.9.2 Test-and-slaughter scheme

The strategy of generally adopted worldwide for the control of BTB is diagnosis or isolation and the slaughter of infected animals to prevent transmission. Implementation of a government controlled scheme consisting of a combination of systematic testing and removal of infected animals, prevention of spread of infection avoidance of introduction of disease and if other reservoir hosts of infection do not exist are effective control measures. The tuberculin skin test is the official method for screening for TB in cattle in developing countries. However, because it is not financially practical to conduct the test on all cattle or there are no sufficiently trained personnel available to conduct the campaign the tuberculin skin test is not used effectively in most developing countries (Berrada and Barajas, 1995).

Reactors to the tuberculin skin test with no grossly visible lesions on necropsy are a problem. In developing countries, a program based on slaughterhouse surveillance and trace back of tuberculous animals to herd's origin is most appropriate since it is technically, economically and socially feasible. Test-and-slaughter Polycy is effective only in areas with relatively low BTB prevalence and effective control of animal movement (Barwinek and Taylor, 1996). In most developing and some European countries where test and slaughter could have been impractical, varying forms of test and segregation have been used with the test-and-slaughter program applied only in the final stages of eradication (WHO, 1993).

2.9.3 Sanitation

On infected properties mechanical, physical and chemical agents are used to render rooms materials, fluids and other substances non-infections. Thorough cleansing of contaminated areas is recommended. Once reactor animals to BTB test are implemented, hygienic measures to prevent the spread of infection should be instituted. Feed troughs should be cleared and

thoroughly disinfected with hot 5% phenol or equivalent cresol as disinfectants. Appropriate disinfectants suitable for use in TB laboratories are those containing phenols (2-5%), hypochlorites (1-5%), alcohol (usually 70% ethanol), formaldehydes and iodophors (3-5%) glutaraldehyde (usually supplied as 2% solution, while the activator is a bicarbonate compound) (WHO, 1998b).

Suspicious reactors being held for retesting should be isolated from the remainder of the herd. The usual source of *M. bovis* in milk is udder infection. It can also occur by contamination with feces, uterine discharge or by air or dust borne bacilli. Such contamination can be much reduced by better dairy design and practices. Effective meat inspection and proper disposal of tuberculous lesions are also paramount important (Pritchard, 1988).

Careful management is necessary to raise disease-free stock from an infected herd. Each calf should be taken away from its dam at birth; on no account should the dam be allowed to lick or suckle the calf. The calf should then be placed in a thoroughly disinfected calf pen and be given its dam's colostrum preferably pasteurized/boiled in a clean bucket. Farm attendants should be checked as they may produce a source of *M. tuberculosis* and also of *M. bovis*, and cause transient positive reactions in cattle (WHO, 1993).

3. MATERIALS AND METHODS

3.1. Study area

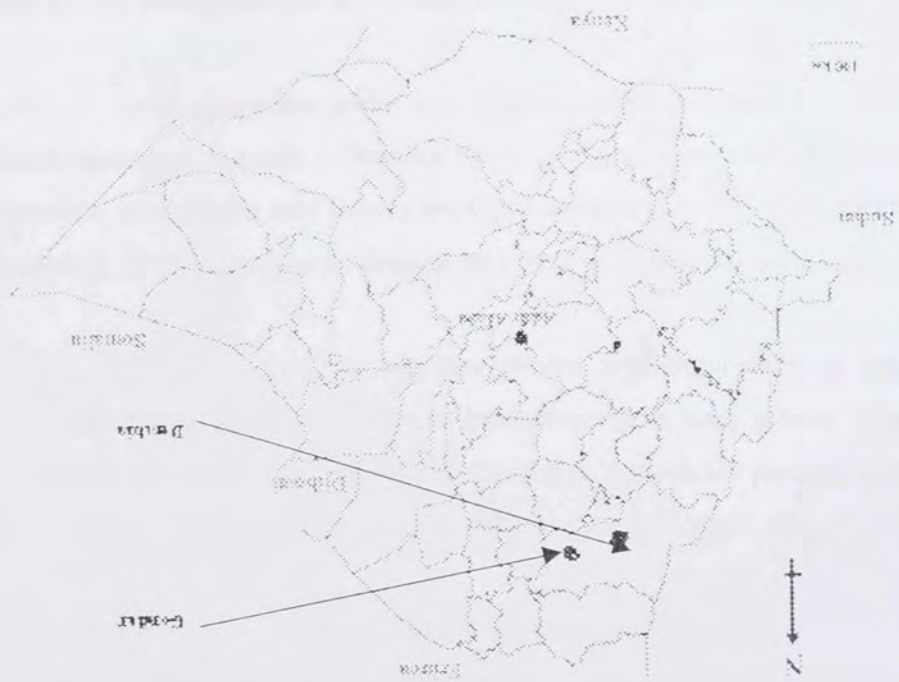
The study was conducted in Gondar hospital and the surrounding two districts of North Gondar Zone of Amhara National Regional State (ANRS) named as Gondar and Dembia districts, which found at about 750km north of Addis Ababa. The areas under the study are located in the Northwestern part of Ethiopia, bordering Lake Tana at latitude 12.4° North, longitude of 27.25° east and stands at an altitude range of 1800-2200 meters above sea level. The North Gondar has a population of 2,973,210; the district of Dembia and Gondar have also 308,988 and 201,958 respectively (CSA, 2003). The North Gondar Zone has 18 districts but these two districts were selected as the study areas based on increment of crossbred cattle, aimed at encouraging milk production through smallholder and other dairy schemes to answer the increased demand for milk and milk products and previous records showing higher number of TB patients treated in the Hospital. The laboratory work was conducted at laboratory of Gondar Hospital (direct AFB) and the Ethiopian Health and Nutrition Research Institute (EHNRI) in Addis Ababa. The ranges of maximum and minimum temperature of the area vary between $22-30.7^{\circ}$ C and $12.3-17.1^{\circ}$ C, respectively. The region receives a bimodal rainfall, the average annual precipitation rate being 1000mm. The short rains occur during the months of March, April and May while the long rains extend from June through September (CSA, 2003).

The production system observed in the selected districts of combines cereal-based agriculture and livestock farming. The farms are of small size and are characterised by a subsistence economy. The cultivation practices in the selected areas are similar to other equivalent agro-ecological zones in the Ethiopian highlands. Cattle farming play a major role in the production system. Cattle population in the North Gondar administrative zone, district of Gondar and Dembia is 1,936,543, 5144 and 166,046 respectively (CSA, 2003). The main objective of livestock farming is the use of oxen for farm works, especially ploughing activities. The other animal production purposes are milk, live animals and manure. Manure is used as an organic fertiliser and fuel. Milk is collected twice a day from lactating cows, on average 1.5 litres per day,

The study was conducted both on human and cattle, with a total of 125 human and 1025 cattle. Of the 125 human study subjects 50 were human TB patients (cases), 50 were non-TB patients as control that visited Gondar Hospital for other problems not related to TB and 25 were dairy farm workers who work in the dairy farms which were included in the study.

3.2 Study population

Figure 1. Map of study sites



and processed at farm into butter and cottage cheese. Butter can be marketed in the local market and cottage cheese, which is un-cooked, is always consumed at home. Live animals can be slaughtered at the farm for meat consumption and hides sold at the local market. One special feature of the livestock practices in Gondar and Dembia is the widespread use of animal exchanges between farmers, a possible risk factor in the spread of contagious diseases in this region.

From the total of 1025 cattle included in the study, 625 were cattle from dairy farms and individual farmers in the study sites, 200 were those cattle owned by human TB patients and 200 were those cattle owned by non-TB patients who are included in this study. Cattle owned by both groups were also investigated for reaction to the intradermal tuberculin test. Young stock less than 6 months of age, and cows in late pregnancy and those recently calved were not included in the study for fear of immune dysfunction that usually occurs in dairy cows starting around 3 weeks pre-calving to 3 weeks post-calving (Radostits *et al.*, 2001). Out of the 1025 cattle 488 (47.6 %) were local and 537(52.4 %) crossbreds. The crossbred cattle were dairy cows kept for milk production, while the local animals were mainly oxen kept for draft purpose.

Clean farms with good aeration, reasonable stocking density per unit area, with good drainage and waste removal system and with a separate feeding and watering facilities for each herd/group of animals in each barn were categorized as farms with good management system; the contrary holds true for farms under poor management system.

Human TB patients (cases): -A case was defined as patients visited Gondar hospital TB clinic showing either pulmonary or extra pulmonary signs that a physician suspected as being due to tuberculosis. Further tests including direct staining of sputum, lymphnode aspiration, chest x-ray, ultrasound and other indicative diagnostic techniques were undergone.

Control group: - In this study, control groups were selected based on the suggestion given by Martin, *et al.* (1994) that indicated the possibility of selection of controls from all non-case patients that visited the same clinics. So, the controls in this study were selected from those individuals that visited the Gondar hospital TB clinic for other diseases that clinically were excluded as not being TB patients; further tests such as sputum acid fast staining, chest x-ray, and other tests were done and were not indicative of tuberculosis.

Dairy farm workers/farmers: - In this study, dairy farm workers were defined as those individuals working in the study dairy farms cattle with tuberculin positive cows for more than six months or farmer owning tuberculin positive cow and having very close association with cattle such as owners/farmer, attendants, those involved in feeding and milking cows in the farm were included

in the study.

3.3 Study design

Two types of designs were implemented namely, cross-sectional and case-control study. In the cross-sectional survey of BTB was conducted on 625 cattle from September 2005 to April 2006 to determine the prevalence and to investigate the effect of risk factors associated with it.

In a case-control study, a total of 400 cattle having contact with human TB patients (200) and a control group of 200 cattle owned by non-tuberculous patients who were at the same visiting TB. Dairy workers associated with tuberculin positive cows were also followed to study the zoonotic importance of *M. bovis*. Based on questionnaires on human patients, the test animals were randomly selected.

3.4 Sample Size Determination

3.4.1 Cross-Sectional

Two types of cattle production systems were classified as semi-intensive and extensive. In the semi-intensive production system, the animals are mainly composed of crossbred cattle, which are kept in doors and occasionally graze in the field. They are fed with hay and supplemented with concentrates. On the other hand, the extensive production system consists of zebu cattle mainly of Fogera type and uncharacterized breeds; these animals graze at the field during the day and fed hay during the evening.

Animals were selected by one stage cluster sampling based on the sample frame provided by Zonal Veterinary department, Kebele and Peasant Association (PA). Accordingly, a total of 32 dairy farms and 53 households (HH), were first selected in semi-intensive and the extensive production systems respectively; all animals from the selected dairy farms (n=315) and HHs (310) were included in the study. Young stocks ≤ 6 months of age, and cows in late pregnancy and those recently calved were not included in the study for fear of immune dysfunction that

usually occurs in dairy cows starting around 3 weeks pre-calving to 3 weeks post-calving (Radostits *et al.*, 2001). Both dairy farms and HHs units in the two production systems were selected randomly from the lists. The sample was determined according to Thrusfield (1995).

3.4.2 Case-Control Study

Fifty human TB patients visiting TB clinics at Gondar Referral Hospital and 50 non-TB patients were included in the study. The average number of TB patients that visited the Gondar Referral Hospital was taken as the basis for sample size determination. Accordingly, the average annual number of human TB patients was 400. Assuming uniform distribution of cases during the months of the year, the number of patients that visited during the 6 months is 200. Taking into consideration the dropouts, lack of volunteers, those who do not have cattle, 25% of these patients and equal number of control groups were sampled.

Ethical consideration

Ethical issues were considered both in human and animal studies. The purpose of the study was explained to the subjects to be involved in the study and/or their guardians. A written consent was obtained from human TB patients and owners were further requested to get their animals tested. Medical personnel collected specimens as part of the routine diagnostic procedure the human TB patients. Patients benefited from the free diagnostic support for their disease. Laboratory results were reported to their physician or health care provider. Owners were informed when their animals were found TB positive either using intradermal tuberculin test or culture or advised to take some measures of disease prevention (such as isolation of positive animals, boiling of milk, etc.). Animals confirmed to have infection were communicated to their owners and advised to take some appropriate measures of disease control (such as isolation of positive animals, boiling of milk, etc.). The findings would help to increase awareness about this serious disease among the communities.

3.5 Study Methodology

3.5.1 Sampling

Specimen (Sputum and lymph node aspiration) were collected before the antimicrobial therapy commenced using sterile, leak proof, disposable plastic materials labeled with the patients code number, type of specimen and date of collection. In case of TB lymphadenitis, fine needle aspiration (FNA) procedure was used for collecting samples for isolation and identification of *Mycobacterium species*. A total of 75 specimens (55 sputum, 13 FNA and 7 ascitic fluid) from 75 human TB patients (55 pulmonary and 20 extra pulmonary tuberculosis) were collected and processed in the laboratory. The samples were transported on ice immediately after collection to the Ethiopian Health and Nutrition Research Institute (EHNRI), which is equipped with appropriate facilities for mycobacterium isolation and handling, however, if that could not be done samples were stored at 2-8 °C. The 5-10 ml morning sputum taken for three consecutive days samples included; these samples were collected if sputum samples were positive with the acid-fast stain on direct smear. Peritoneal fluid was also taken from seven extra pulmonary abdominal TB cases, which had TB peritonitis on clinical examination by doctors handling the cases. Dairy workers (owners, attendants, dairy/ feed lot workers, farmers,) associated with tuberculin positive cows were also being followed to study the zoonotic importance of *M. bovis* (Annex2).

Milk samples (50 ml) were also collected aseptically towards the end of milking from each of the 99-tuberculin positive milking cows and were processed individually for culture.

The body condition of the local zebu study animals was scored according to the guidelines established by Nicholson and Butterworth (1986), nine scores were used in which the three main scores (Fat, Medium and Lean) were divided into three categories each having F⁺, F, F⁻, M⁺, M, M⁻, L⁺, L, L⁻. Each score was given a number from 1(L⁻) up to 9 (F⁺), the former representing the most emaciated animal and the latter well-fattened one. Scoring was done by looking at the structure of the tail, head, transverse process of the lumbar vertebrae, the ribs, the hump, the hips, the brisket. Scoring of crossbreeds was done according to the guidelines forwarded by Richard (1993); all animals were graded as P, M, G. (poor, medium and good) based on the



above-mentioned anatomical structures. Each score was given a number from 0(P_1) to 5(G_2), i.e. the three main scores divided in to two sub-categories as P_1 , P_2 (0, 1), M_1 , M_2 (2, 3) and G_1 , G_2 (4, 5) (annex 4a and 4b). In addition to the body condition score other relevant data such as age, sex, breed, were also collected for each animal before tuberculin injection and sampling.

3.5.2 Tuberculin test

Animals over 6 months of age were inoculated with 20,000 IU/ml bovine purified protein (PPD) (AN_5 strain, Bovituber, Merial, France), and 25,000 IU/ml avian PPD (D4 ER strain, Avituber, Merial, France). For inoculation, two sites about 12 cm apart in the middle of the neck were disinfected and shaved and skin thickness was measured with a 0.01 mm graduated caliper. Avian and bovine PPDs were injected intradermally into each site using an automatic syringe, which constantly injects 0.1 ml tuberculin. A correct injection was confirmed by palpating a small pea-like swelling at each site of injection. The injection sites were examined for swelling, and skin thickness was measured again after 72 hours. The difference in skin thickness before and after injection at both sites was used for the interpretation of results. When these differences were greater at the site of injection for avian PPD than for bovine PPD, the animal was considered positive for *M. avium* or other atypical mycobacterium; but when the skin thickness was increased at both injection sites, the difference in thickness between the two sites was considered and the results interpreted as follows.

Interpretation: $avd > bvd$ = cattle infected with *M. avium* or other atypical mycobacteria
 $bvd - avd < 2$ mm = negative reaction for *M. bovis*
 $bvd - avd$ between 2 and 4 mm both values inclusive = doubtful reaction
 $bvd - avd > 4$ mm = positive reaction for *M. bovis*

Key: avd = skin thickness difference before and after injection of avian PPD
 bvd = skin thickness difference before and after injection of bovine PPD

3.5.3. Questionnaire

The TB patients were approached through their health personnel for their willingness to participate in this study. A total of 75 human TB patients (cases) were volunteer to participate in the study and signed consent before samples were collected by experienced health personnel. They were also interviewed using a semi-structured questionnaire (annex 1) about the degree of their association with cattle, habit of consumption of meat and milk and other relevant information related to tuberculosis in their household, which indicated in the questionnaire.

3.5.4. Direct microscopy

Microscopic examination: fifty-five sputum samples were subjected to direct microscopic examination before processing for culture and stained with Ziehl-Neelsen stain (annex 6). The stained smears were examined under oil-immersion lens.

3.5.5 Culture

All specimens collected from human TB patients and from tuberculin positive cattle were processed and prepared for mycobacterium culture at the EHNRI TB laboratory in a biological safety cabinet.

Sample processing: All samples detained for isolation were digested and decontaminated using 2-4% NaOH in order to initiate the release of mycobacterium organisms from body fluids and cells and reduce bacterial contaminants. The method used to process specimens was more or less similar to all types of samples except for some modifications.

Sputum and other fluid culture. Fifty-five sputum samples were collected from pulmonary TB patients. The samples were decontaminated by adding 4% NaOH, agitated in a vortex mixer for 15 minutes at room temperature and centrifuged at 3,000 rpm for 15 minutes at 4oC. The supernatant was taken off into another container for proper disposal and the sediment was suspended in 2ml of sterile physiological saline solution (PBS). One to two drops of 0.05%

phenol red indicator was added to indicate the PH change and then neutralized using concentrated hydrochloric acid until the colour changed to yellow. The sediment was inoculated into four slants of Löwenstein-Jensen media (LJ), two with pyruvate and the other two with glycerol. The cultured tubes were incubated at 37°C, at angle for the first week and in an upright position for up to 11 weeks; weekly visible growth was observed. Positive cultures were sub cultured onto another set of media and incubated for another 3 to 4 weeks for further identification. Thirteen FNA and seven ascitic fluid samples were collected from 13 TB lymphadenitis and 7 TB peritonitis cases, respectively and processed as in sputum samples and inoculated to the same media (Quinn *et al.*, 2002; OIE, 2000).

Milk culture: Milk samples were collected from 99 tuberculin positive milking cows and processed for culture. The procedure indicated by Kazwala *et al.*, (1998) was followed for culturing milk. Fifty ml milk was taken from the four quarters of each cow towards the end of milking and transported at 4 °C to EHNRI Laboratory and centrifuged at 3,000 rpm for 15 minutes at 4°C. The cream was removed with a sterile spatula then the supernatant was discarded and the sediment resuspended using 2ml of sterile water. In to each tube with the suspension, 2% ml of NaOH was added for decontamination (Quinn *et al.*, 2002) and left at room temperature for 15 minutes. Neutralization was effected using concentrated HCl. Adding a drop or two of 0.05% phenol red indicator until the color changed to yellow monitored the neutralization process. Then it was processed as for the sputum.

3.5.6 Identification

Slants with colonies of acid-fast bacilli were subjected to mycobacterium species identification using their growth characteristics and reaction to some biochemical tests and drug sensitivity test.

Growth intensity and colony form. Dysgonic growth, after 4-8 weeks with small, roundish, whitish and moist colonies mostly underneath the pyruvate enriched media was considered as *M. bovis*; whereas eugonic growth, which was relatively rapid, seen in 2-3 weeks with luxuriant, dry, cauliflower like, yellowish colonies were considered as the primary culture of *M. tuberculosis*. Fast growing colonies that appear in a week time and which were mostly yellow/deep orange in color were considered as atypical mycobacterium.

Differentiation of mycobacterium species: generally identification of mycobacterium isolates was based on colony morphology and colonies appeared in less than a week on primary culture; pigmented colonies were excluded and those suspected of *M. tuberculosis* complex were subjected to secondary culture in order to isolate pure colonies for further differentiation. To differentiate various species of mycobacteria isolated from our samples, we conducted standard biochemical and drug sensitivity tests such as the nitrate reduction test, sensitivity to thiophene-2-carboxylic acid hydrazide (TCH) and pyrazinamide (PZA). These tests were applied according to procedures to differentiate *M. bovis* and other species of *Mycobacterium*. The test procedures are given in appendix (annexes 7, 8 and 9).

3.6 Data Analyses

The data collected from the two study areas was entered into MS Excel spread sheets and analyzed using STATA 7.0 (Stata corporation, (2001). and Win Episcopo epidemiological software (Thrusfield *et al.*, 2001) Percentages were applied to summarize the distribution of tuberculin reactivity at herd and animal level. Multinomial logistic regression was used for univariate and multivariate analysis of risk factors for tuberculin positivity.

In modeling the associations, factors showing $P < 0.2$ in bivariate analysis were included in the final model. Beginning with the full model, the factors were dropped in a back ward elimination procedure. A statistically significant association between variables were said to exist if the computed p-value < 0.05 and the 95% confidence interval for odds ratios (OR) does not include 1.0. Odds ratio (OR) was calculated to assess strength of association of different factors to the occurrence of BTB in cattle and its potential risk to humans and to see the effects of different risk factors on human tuberculosis

4. RESULT

4.1. Cross-Sectional Study

4.1.1 Intradermal Tuberculin Test

Out of the total 1025 cattle (625 cross-sectional and 400 case control) subjected to comparative intradermal tuberculin test, 151 (14.7%) positive and 64 (6.3%) doubtful reaction; the remaining 810 (79.0%) were negative (Table 6). Considering doubtful results as positive, the overall average prevalence is 21.0% (95% CI, 8.6-32.5). In the two extensive and semi-intensive production systems, a prevalence of 11.6% (36/310) and 16.8% (53/315) individual animal prevalence and 37.7% (20/53) and 56.3% (18/32) of herd prevalence were obtained, respectively. Results of the skin test are shown on Table 6.

Table 6. Results of comparative intradermal tuberculin test results in different type of studies

Study type	Tuberculin test reaction (n, %)				95% CI for positive	Total (%)	n
	Negative	Doubtful	Positive	Positive + doubtful			
Cross section							
Extensive	247 (79.7%)	27 (8.7%)	36 (11.6%)	63 (20.3%)	8.5 – 15.8	310 (30.2%)	
Semi-intensive	255 (81.0%)	7 (2.2%)	53 (16.8%)	60 (17.5%)	12.9 – 21.2	315 (30.8%)	
Case control							
Cases	144 (72%)	16 (8%)	40 (20%)	56 (28%)	15.2 – 26.4	200 (19.5%)	
Controls	164 (82%)	14 (7%)	22 (11%)	36 (18%)	7.4 – 16.3	200 (19.5%)	
Total	810 (79.0%)	64 (6.3%)	151 (14.7%)	215 (24.5%)	12.6 – 17.0	1025	

* Doubtful reactors were added to the positive reactors in calculating the overall prevalence due to the fact that sensitivity of tuberculin test is low which may miss some infected animals and under estimate the actual prevalence of BTB.

4.1.2 Risk factors for Bovine Tuberculosis

The farms in the semi-intensive production system were classified into two management categories as good and poor based on farm cleanness, stocking rate, waste disposal, aeration, feeding and watering facilities. In relation to the extensive production system classification of the management was not carried out due to similarity of the open grazing system of the animal in the production system.

Table 7. Relationship between management and tuberculin test positivity

Management	Tuberculin test Result		95% CI for positive	Total examined n (%)
	Positive n (%)	Negative n (%)		
Good	7(6.9)	95 (93.1)	3.4 - 14.0	102(32.4)
Poor	46 (21.6)	167 (78.4)	16.7 - 27.9	213(67.6)
Total	53(16.8)	262(83.2)	13.2-21.5	315

The effect of management on the prevalence of BTB is shown in Table 7. The prevalence of BTB in the two categories of management was significantly different ($\chi^2 = 39.15$, $df = 2$, $p < 0.001$). The strength of association was high (OR=3.42, 95% CI 1.55 – 7.53); cattle under poor management system were 3.42 times more likely to develop tuberculosis than cattle under good management system.

Out of the 625 animals included in the cross-sectional study 52.2% were local, 47.8% cross breed animals. Prevalence was high in cross breeds (17.4% with 95% CI=13.6 - 22.3); relatively lower prevalence rates were found in local breeds, (11.35% with 95% CI=8.4 - 15.4). Breed did exert a highly significant effect on TB prevalence ($\chi^2 = 24.86$, $df = 2$, $p < 0.001$). The strength of association (OR) was calculated considering breed as risk factor where crosses were compared against the local breeds, accordingly crosses were 1.17 times more likely to develop tuberculosis than local breeds.

Animals were grouped into 4 age groups as calf (1/2-1 yr.), heifer/bull (>1- 4 yr.), adult (>4-7 yr.) and old animal >7 yr.). The distribution of tuberculous cattle among different age groups is shown on Table 8. Prevalence increases with age being maximum in adult (>4-7) then decreased slightly when animals were older than 7 years and the association between age and prevalence was highly significant ($\chi^2=28.65$, $df = 6$, $P<0.001$). Group 2, 3, and 4 were 1.56, 2.74 and 1.54 times more likely when compared to group 1(calves).

Table 8.. Age distribution and prevalence of tuberculosis in cattle

Age group	Tuberculin test result			95% CI for positive
	Positive n (%)	Negative n (%)	Total n (%)	
½ -1	13 (10.1%)	116 (89.1%)	129 (20.7%)	6.0 - 16.9
>1 - 4	19 (12.8%)	130 (87.2%)	149 (23.8%)	8.4 - 19.4
>4 - 7	43 (24.0%)	136 (76.0%)	179 (28.6%)	18.5 - 31.2
>7	14 (8.3%)	154 (91.7%)	168 (26.9%)	5.0 - 13.8
Total	89 (14.2%)	536(85.8%)	625 (100%)	11.7-17.3

The method used to rank body condition of local and crossbred animals was different; however, all were grouped into three main categories as poor, medium and good body condition. Accordingly, 88 (14.1%) animals were graded as poor with a prevalence of 8.0 % (95% CI=3.9 - 16.2), 444 (71.0%) medium with a prevalence of 16.2% (95% CI=13.1 - 20.0) and the remaining

93 (14.9%) were under good body condition with a prevalence of 10.8 % (95% CI=6.0 - 19.3). The association between body condition and prevalence was analyzed and found to be significant ($\chi^2 = 9.90$, $df = 4$, $P < 0.05$). The OR calculation for degree of association indicated that animals with medium body condition were more likely to react to bovine PPD (OR=1.27, 95% CI=0.71-2.24)

Table 9..Summary univariate analysis of the risk factors on BTB prevalence

Risk factors	No. tested	Prevalence (%)	OR(95% CI)	χ^2 test	P-value
Breed				24.86	0.000
Local	326	11.35	1		
Cross	299	17.40	1.17 (0.79-1.74)		
Management				39.15	0.000
Good	102	6.90	1		
Poor	213	21.60	3.42 (1.55-7.53)		
Age group (yr)				28.65	0.000
0.5 -1	129	10.1	1		
>1-4	149	12.8	1.56 (0.80-3.05)		
>4-7	179	24.0	2.74 (1.48-5.07)		
>7	168	8.3	1.54 (0.80-2.96)		
Body condition				9.9	0.042
Good	93	10.8	1		
Medium	444	16.2	1.27 (0.71-2.24)		
Poor	88	8.0	0.45 (0.18-1.20)		
Sex				8.05	0.018
Male	196	8.7	1		
Female	429	16.8	1.91 (1.20-3.06)		
Production system				26.78	0.000
Extensive	310	11.6	1		
Semi-intensive	315	16.8	1.28 (0.86-1.90)		

The multinomial logistic regression was applied for multivariate analysis of risk factors of BTB in the cross sectional study design. In the final model, significant risk factors included were production system ($P < 0.05$) and status of management ($P < 0.05$).

4.2 Case-Control Study

4.2.1 Individual animal prevalence

The result of the intradermal tuberculin test in cattle owned by human TB patients (case group) and non-tuberculous patients (control group) human patients is given in Table 10. The overall prevalence, considering doubtful reactors as negative was 15.5% (62/400). When doubtful reactors were considered as positive, the overall prevalence was 23% (92/400).

In the case group, which comprised of 200 animals owned by tuberculous human patients, 40 (20%) and 16(8%) were positive and doubtful for the intradermal tuberculin test, respectively. When doubtful reactors were added up to the positive group, the prevalence of tuberculin reactivity in this group was 28%.

The prevalence in the control group, which comprised of 200 cattle owned by non tuberculous patients visiting Gondar hospital, only 22 (11%) and 14 (7%) of the animals were positive and doubtful with the intradermal tuberculin test, respectively.

4.2.2 Risk factors for bovine tuberculosis

The univariate analysis of the different likely risk factors considered for tuberculin positivity in both cases and control group of cattle are given in Table 10. Most of the presumed risk factors (sex, breed, body condition, and production system) did not differ significantly ($P > 0.05$) between the cases and controls. However significant risk factors included were, age ($\chi^2 = 25.82$, $df = 6$, $P < 0.001$), status of management ($\chi^2 = 9.81$, $df = 4$, $P < 0.045$) and between animals owned by tuberculous patients compared to cattle owned by non-tuberculous patients ($\chi^2 = 98.9$, $df = 2$, $P < 0.013$). The tuberculin positivity was more likely to occur in cattle owned by TB patients (OR = 2.10) compared to control.

Table 10. Summary univariate analysis of the effect of risk factors on BTB prevalence of case-control study.

Risk factors	No. tested	Prevalence (%)	OR (95% CI)	χ^2 test	P-value
Sex				1.22	0.543
Male	118	13.6	1		
Female	282	16.3	1.34 (0.79-2.27)		
Age				25.82	0.000
0.5 -1	82	3.7	1		
> 1 - 4	65	16.9	1.94 (0.82-4.57)		
> 4 - 7	124	25.8	3.68 (1.77-7.65)		
> 7	129	12.4	1.26 (0.57-2.76)		
Breed				2.51	0.285
Cross	189	18.5	1		
Local	211	12.8	0.73 (0.46-1.17)		
Body condition				4.67	0.323
Good	52	21.2	1		
Medium	300	14.0	0.97 (0.49-1.97)		
Poor	48	18.8	1.11 (0.44-2.78)		
Production system				1.99	0.38
Semi-intensive	200	18.0	1		
Extensive	200	13.0	0.75 (0.47-1.20)		
Management				9.81	0.044
Good	79	11.4	1		
Poor	121	22.3	2.66 (1.29-5.47)		
Owner status				98.9	0.000
Control	200	11.0	1		
Case	200	20.0	2.10 (1.18-3.65)		

* Only positive reactors were considered as positive and doubtful considered as negative

4.2.3 Multivariate analysis of effect of risk factors on BTB prevalence in cattle

Table 11 shows the multivariate analysis of risk factors of BTB in the case-control study design. The multinomial logistic regression was applied for this analysis. In the final model, significant risk factors included were production system ($P < 0.05$), age ($P < 0.05$) and management ($P < 0.05$).

Table 11. Result of the multiple logistic regression analysis of the risk factors on BTB prevalence

Risk factor	OR	P-value	95% CI
Production system	0.03	0.013	0.002 - 0.48
Status of management	2.40	0.037	1.05 - 5.48
Age	1.34	0.041	1.01 - 1.76

4.2.4 Herd prevalence

In each of the case and control groups, 50 herds were tested. Herd prevalence in cattle owned by TB patients 64% (32/50) and 10% (5/50) for positive and doubtful reactors respectively. The number of positive and doubtful herds was 22 and 10 respectively (i.e. 44% and 20%) in those owned by TB free individuals (table 12). In both cases the herd prevalence was significantly ($P < 0.05$) higher in herds owned by TB patients than those owned by non-TB individuals. The tuberculin positivity was 2.1 more likely to occur in herds owned by TB patients compared to cattle herds owned by non-TB patients.

Table 12. Prevalence of intradermal tuberculin reactor herds owned by human tuberculous and non-TB patients in Gondar.

Household	Tuberculin reactivity in herds			Total herds
	Positive	Doubtful	Negative	
TB positive	32 (64%)	5 (10%)	13 (26%)	50
TB negative	22 (44%)	10 (20%)	18 (36%)	50
Total	54 (54%)	15 (15%)	31 (31%)	100

4.2.5. Tuberculosis in cattle owners and associated risk factors

Summary of the questionnaire survey are shown in Table 13. Of 50 TB cases, 33 (66%) and 17 (34%) were diagnosed as pulmonary and extra pulmonary tuberculosis, respectively. About 86% (43/50) of the patients were consume raw milk and milk products, while only 14% (7/50) were consuming boiled milk. The majority of (58%) human TB patients are between the ages 15 and 45 while 24% and 18% were below 15 and above 45 years, respectively. Classification of the patients on the basis of residence showed that 74% of them were rural dwellers among which 46% of had pulmonary tuberculosis was also from rural areas.

None of the presumed risk factors (sex, age, and the origin of cattle owners) did not differ significantly ($P>0.05$) between cases and controls. However, a significant difference ($P<0.05$) was observed when patients had physical contact with other clinical cases, drinks raw milk ($\chi^2=32.18$; $P<0.001$) and are farmers compared to the control groups ($\chi^2=25.01$; $P<0.001$). Cattle owners who consumed raw milk were at higher risk (OR = 14.33) of infection with tuberculosis than those who consumed boiled milk (Table 13).



Table 13. Univariate analysis of risk factors with tuberculosis status in humans treated at Gondar Hospital

Risk factors		Case		Control	χ^2	P-value	Odds Ratio
		Pulmonary TB	Extra-Pulmonary TB				
Sex	Male	18	10	31	0.37	0.54	1.28
	Female	15	7	19			
Age	<15	4	8	8	0.59	0.74	1.38
	15-45	23	6	33			0.94
	>45	6	3	9			0.89
Contact	No	9	4	35	27.21	0.000	10.29
	Yes	24	13	15			
Origin	Rural	23	14	37	0.05	0.82	0.90
	Town	10	3	13			
Milk consumption behavior	Boiled	4	3	41	32.18	0.000	14.33
	Rarely	13	4	4			
	Frequently	16	10	5			
Occupation	Farmer	23	13	12	25.01	0.000	9.0
	Others	10	4	38			

4.3 Bacteriological findings

On primary tests, 37 mycobacteria were growth from specimens from human TB patients while 10.1%(10/99) of the milk sample from tuberculin positive were positive on primary culture. But after sub-culturing 36 (29 human and 7 cattle) isolates were able to be recovered from the specimens (Table 14).



Table 14. Culture results of the specimens on Lowenstein Jenson media

Type of sample	Growth on L-J media		Total
	Positive	Negative	
Sputum	26 (47.3%)	29 (52.7%)	55
FNA	2 (15.4%)	11(84.6%)	13
Peritoneal fluid	1(14.3%)	6(85.7%)	7
Milk	7 (7.1%)	92 (92.9%)	99
Total	36 (20.7%)	138 (79.3%)	174

Table 15 shows summary of mycobacterium species isolated from the sputum of human TB patients and milk samples of tuberculin positive cows. Subjecting both human and cattle isolates to biochemical and drug sensitivity tests, human isolates indicated that 79.4% (23/29) and 10.3% (3/29) of the human isolates were *M. tuberculosis* and *M. bovis*, respectively. The rest 10.3% (3/29) were other mycobacterial species. On the other hand, 14.3% (1/7) and 57.1% (4/7) of the cattle isolates indicates were *M. tuberculosis* and *M. bovis*, respectively. The rest 28.6% (2/7) were other mycobacterial species.

Table 15. Species of mycobacteria isolated from sputum of human tuberculous patients and interadermal tuberculin test positive animals in Gondar, (2005/06)

Source of sample	Type of sample	<i>M. tuberculosis</i>	<i>M. bovis</i>	Other	Total
Human	Sputum	23 (88.5%)	1 (3.8%)	2 (7.7%)	26
	FNA	0	1 (50%)	1 (50%)	2
	Peritoneal fluid	0	1	0	1
Bovine	Milk	1 (14.3%)	4 (57.1%)	2 (28.6%)	7
Total		24 (66.7%)	7 (19.4%)	5 (13.9%)	36



5. DISCUSSION

Bovine tuberculosis is one of the major constraints for intensification of dairy production, however, data on the distribution of the disease in the country in general and Amhara regional state in particular, are lacking. On the other hand, various dairy development projects are being implemented in different parts of the country. Therefore, the present survey was the first attempt to study the role of BTB in the developing livestock industry initiated by private, government and non-governmental organizations including the Integrated Livestock Development Project (ILDP) in North Gondar administrative zone of Amhara regional state and preliminary information were gathered on the epidemiology of BTB both in local and cross-red cattle and the role of *M. bovis* in human tuberculosis cases. To this effect crossbred cattle in semi-intensive and local Fogera type zebu in extensive production system were included in the study, besides human TB patients from Gondar hospital and dairy farm workers were also sampled.

Tuberculosis accounts for about 25% of all avoidable adult deaths in developing countries (Murray *et al.*, 1990). The epidemiology of TB has been affected in recent decades by the upsurge in HIV/AIDS pandemic. As many HIV- infected individuals are co-infected with TB, the incidence of the disease may rise in the coming years (Zumla, *et al.*, 1999). The correlation of *M. bovis* infection in humans and that in local cattle populations highlights the potential threat of bovine tuberculosis for humans (Daborn *et al.* 1996). The global prevalence of human TB due to *M. bovis* has been estimated at 3.1% of all human TB cases, accounting for respectively, 2.1% and 9.4% of pulmonary (PTB) and extrapulmonary (EPTB) cases (Cosivi, *et al.* 1998).

The result of comparative intradermal tuberculin test conducted on 1025 cattle has indicated an overall prevalence of 14.7% (151/1025) and if the 6.3% doubtful reactors considered as positive, the overall prevalence become 21%. This is in agreement with previous reports in Ethiopia 18.4% (Elias, 2005), 16.2% (Regassa, 2005), 10.31 % (Bogale, 1999).

The result showed a slight variation from study done by Kiros, 1998 who reported 23.9% prevalence in predominantly exotic and cross breed (61.7%) of Debre Zeit and Addis Ababa under intensive system. This variation observed in the prevalence may be related to the difference in the geographic location, exposure rate based on their management system and the relative compositions of the breeds considered in each particular study. With this respect, the present study consists of 52.4% (537/1025) local and 47.6% (488/1025) cross breed as compared to the study by Kiros, (1998) which consisted of 26.2% exotic that had a prevalence of 86.4% that increased the overall prevalence of the study. In addition the production system may have also affected the relative exposure rate of the cattle as 61% of the cattle in our study were in the extensive system with less exposure rate as compared to the intensive ones considered in the above study to intradermal tuberculin test there were relatively higher doubtful reactors that showed skin thickness which was reflected in the difference in the overall prevalence of tuberculosis between the two studies. In local cattle, even though there were more reactor animals, below the standard considered as negative. There was also relatively higher proportion of animals that had doubtful reactors than that occurred in cross breed animals. A similar result was observed by Kiros (1998). This may warrant for further investigation to set the standard of skin test result interpretation for local breeds.

The prevalence in the two production systems in the cross sectional study showed a significant difference in their prevalence, where higher positive reactors were observed in the semi-intensive (16.8%) than in the extensive production system (11.6%). This difference was significant ($\chi^2=26.78$, $p<0.001$).

The effect of management on the prevalence of BTB is shown on table 7; the results indicate that there was a significant association between management and prevalence ($\chi^2=39.15$ $df=2$, $p<0.001$). OR was calculated to measure the strength of association and was found to be 3.42 with 95% CI (1.55 - 7.53) indicating that cattle under poor management system were 3.42 times more likely to develop tuberculosis than cattle under good management system. This result was in agreement with other studies in Ethiopia (Kiros, 1998; Bogale 1999). There are numerous reports documenting that poor housing and other poor managerial inputs predispose to tubercular

infection (e.g. Barwinek and Taylor, 1996; Griffin *et al.*, 1993; Marangon *et al.*, 1998). There is also some evidence that animals resistance to tuberculosis is reduced by a shortage of feed and/or an in-balanced diet, attributable to a deficiency of protein, minerals and vitamins in the diet (Griffin *et al.*, 1993). Accordingly in the current study herds under poor management conditions had higher rates of reactors than those under good management.

There was also a significant association ($\chi^2=24.86$, $p<0.001$); between breed and prevalence of BTB where higher prevalence was observed in cross breed animals (OR=1.17) than in local zebu cattle. The result is in agreement with other studies that reported the lower reaction to bovine PPD in local Zebu breed (O'Reilly and Dabron, 1995; Yehualashet, 1995; Kiros, 1998; Ameni and Roger, 1998). This result is the first report of BTB using intradermal in the Fogera type breeds, the dominant in the study area. The result indicated similarity in their reaction to bovine PPD observed in other Zebu breed types in Ethiopia. The main reason for the difference between the cross-breed and local zebu breeds may be related to their genetic difference and other factors like malnutrition and managements in which case the high yielding cross breed are more prone to the effects of such factors as compared to local breed and these stresses may result susceptibility to infection. This result is in contrary to the result obtained by other works (Berdard, *et al.*, 1993; Marangon, *et al.*, 1998 and Bogale, 1999), who reported higher prevalence rate in local Zebu cattle and lower prevalence in cross breed than pure breeds.

The association between age and prevalence of BTB was highly significant ($\chi^2=28.65$, $df=6$, $p<0.001$). In the univariate analysis, animals older than 4 - 7 years had the highest prevalence compared to the other age groups; but decreased slightly in very old animals. This finding is in consistent with other works reported by Doherty *et al.*, (1996), Ameni (1996), Cook *et al.* (1996), O'Reilly and Dabron (1995). O'Reilly and Dabron (1995) reported that the reaction to tuberculin test in cattle increases uniformly by 7.5% for every year of life reaching 40% at 6 to 7 years old. As explained by other workers (Barwinnek and Taylor, 1996), this could be due to the fact that as the age increases the probability of acquiring tuberculosis infection also increases. On the other hand the decrease in prevalence observed in very old animals may not reflect the actual disease status of the animal. As animals become older, the response to intradermal tuberculin test becomes lower due to immunodepression (Tizard, 1996). The calves' prevalence of this study

10.1% was beyond expected, might have contracted the bacilli soon after birth through the pooled milk they were being offered.

In this study, animals with good and medium body condition were more likely to react to bovine PPD than animals with poor body condition of the animal was a significant association to the tuberculin reaction of the animal ($\chi^2=9.90$, $df=4$ $p=0.042$). This was in agreement with previous reports Kiros (1998) and Regassa (2005). This could be justified by the fact that animals under good body condition are with good immune status that can respond to any foreign protein better than those with poor body condition, which can be immunocompromised due to other disease or malnutrition

In the case-control study, out of the 200 animals owned by tuberculous human patients; 40 (20%) and from the same number of animals owned by non-tuberculous control groups, 22 (11%) were positive for the tuberculin test. The differences in prevalence of BTB in cattle owned by human TB cases and control group were significant ($\chi^2=98.90$, $p<0.001$). Tuberculin reactivity was more likely to occur in animals owned by cases (OR=2.10; $p<0.001$) compared to cattle owned by non-tuberculous control group. The herd prevalence was also significant ($\chi^2=201$; $p<0.001$), which is higher prevalence 32/50 (64%) and a more likely occurrence of tuberculin positivity in cattle herds owned by TB patients than those owned by control groups 22/50 (44%) (OR=2.26; $p<0.001$). The result is inline with the previous works in other parts of Ethiopia (Regassa, 2005) where 62.1% to 72.4% in animals of case group and a range of 28.7% - 45.9% in animals owned by control group were positive with the intradermal tuberculin test. The result of this study may suggest the possibility of transmission of mycobacterial species between man and cattle (Cook, *et al.*, 1996; Ameni, *et al.*, 2001, Kazawala, *et al.*, 2006).

The findings that raw milk consumption and the presence of close contact was significantly associated with higher TB in humans are in line with previous reports Kiros (1998) and Regassa (2005), who found a 38.5% and 31% respectively, of positive isolates from primary culture of the sputum, FNA, peritoneal fluid and milk samples. Other factors such as sex, age and origin (being rural or urban dweller) did not show difference between case and control groups. These results

were in agreement with Regassa (2005), but Kiros (1988) had reported higher prevalence of TB from hospital patients visiting Debre Zeit TB clinic.

From the milk sample 7/99 (7.1%) were culture positive. Though identification of Mycobacterium species from milk is generally lower, the result obtained in this study is comparable with Bogale (1999) who reported 8.7% culture positive from pooled milk samples. On the other hand the result is slightly different from the works of Kiros (1998) and Regassa (2005) who reported 17.8% and 18.3% respectively. This difference may be related to the fact that the samples were originated from high TB prevalent farms as compared to ours and our samples were cultured after being stored and transported over 800 kilometers to Addis Ababa. Out of those 7 bovine milk mycobacterial growth, they were 4/7 (57.1%) identified as *M. bovis* and only one 14.3% as *M. tuberculosis*; 2/7 (28.6%) were other mycobacterial species. The identification of *M. bovis* and *M. tuberculosis* in milk of positive reactors may suggest the zoonotic role of BTB in the study area. Similar results were also seen in other works (Kiros, 1998; Regassa, 2005; Boulahbal, 1978; Idrisu and Schnurrenberger, 1977). Interestingly, *M. bovis* were identified in human samples in which case 1/26 (3.8%) in sputum of culture positive case, 1 out of the 2 culture positive FNA and 1 of the culture positive of peritoneal fluid sample; this may indicate the possible role *M. bovis* in the pulmonary and extra pulmonary tuberculosis of humans in the study area in which case the organism may be acquired through consumption of unpasteurized milk and milk products and close physical contacts with animals that lead to aerosol transmission from infected animals for the case of pulmonary tuberculosis. Similarly, different studies, Kidane, *et al*, (2002) have identified *M. bovis* in FNA sample of cervical TB lymphadenitis case in Butajjara, and Regassa (2005) in Sellale and Fiche area have found *M. bovis* in human TB indicating the important of *M. bovis* in human in various parts of Ethiopia.

Human tuberculosis in Ethiopia is markedly increasing together with the HIV/AIDS pandemic. HIV/AIDS accounted for an estimated 38% or 54,000 of all TB case incidences in 2003 in Ethiopia, in the same year the prevalence of HIV infection in Gondar was 13.9% which is the second highest prevalence in Amhara region next to Bahir Dar. This proportion is expected to increase in the coming years (MOH, 2004).

Tuberculosis in cattle is endemic in the country, however the extent of BTB and its role in the increment of human tuberculosis is not yet known, as there is no nation wide epidemiological study conducted so far. But few study indicated that *M. bovis* has been identified as a cause of EPTB. In Butajira, South Ethiopia, *M. bovis* has been isolated in 17.1% of human extrapulmonary cases, based on PCR method (Kidane *et al.*, 2002). In this study, 27.5% of the study subjects were HIV seropositive. The higher probability that these extrapulmonary cases are caused by *M. bovis* may thereby indicate the role of *M. bovis* in HIV patients. In addition, according to the 2004/2005 National Tuberculosis Control program report, for the first time the EPTB cases in the country reached 34% more than PTB (33%), indicating the possible role of *M. bovis* in human TB (MOH/ TLCP, 2004).

6. CONCLUSION AND RECOMMENDATIONS

The findings of the present study using comparative intradermal tuberculin test showed *M. bovis* infection is common both in cattle and humans and indicate its transmission between the two species and intensification was found to significantly increase the prevalence of Bovine tuberculosis.

The isolation of *M. bovis* in milk together with the culture of the public to consume raw milk warrants the public health significance of Bovine tuberculosis. Many extra pulmonary tuberculosis in humans also shows that *M. bovis* is the causal agent.

The information obtained from Gondar hospital and the study districts indicated that a large proportion of the human tuberculosis cases originated in rural parts of the district might be associated with poor awareness of the transmission of BTB by the raw milk consumption behavior of people and by the close interaction between humans and cattle.

The existence of significant difference in the prevalence of tuberculosis among the cattle owned by human TB patients and TB free individuals indicate transmission of the infection between humans and cattle.

Furthermore, isolation *M. bovis* from tuberculin positive cows' milk and sputum of TB patients signifies the public health importance of *M. bovis* particularly in raw milk consumers;

In conclusion, although the biochemical and drug sensitivity tests are the definite identification tools of mycobacterial species, the isolation of both species of mycobacteria (*M. bovis* and *M. tuberculosis*) from both man and cattle suggests the existence of transmission of both species between cattle and their owners and should be seriously considered in countries like Ethiopia, where dual HIV and TB infection are likely to be more common.

Therefore on the basis of the results of the present study and the available information, the following recommendations are forwarded:

1. An integrated national survey of bovine tuberculosis in cattle should be conducted to assess the human risk from animals.
2. Regular tuberculin testing of animals under high risk area and isolation of reactors to concentrate them in a particular concentration camp until they finish their production life. Tuberculin reactor cattle should be isolated and eliminated in the most economical way.
3. Isolation of calves born in infected farms soon after birth and rearing of a replacement stock in a separate disease free farm. Workers in this farm should not come from infected farms and they have to be tested regularly for TB.
4. Stringent meat inspection in abattoirs and proper disposal of positive organs/carcass is very important to prevent spread of the disease among livestock and to man. Abattoir data should be properly used in the epidemiological investigation of the disease; slaughterhouse surveillance and trace-back of animals to herds of origin is most appropriate in the epidemiological study, as it is technically and economically feasible.
5. Public education to increase the awareness of the community about the potential risk of raw milk consumption and expansion of milk pasteurization practices should be implemented.
6. Attention should be given, by the medical profession, to the importance of *M. bovis* as a public health hazard; in addition to this strong collaboration of medical and veterinary personnel is paramount important in investigating and controlling the zoonotic importance of *M. bovis*.



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8. ANNEXES

Questionnaire format to assess risk factors of Bovine tuberculosis in the study sites.

Annex 1. Questionnaires for human TB patients (Part one)

Date..... Code/Case No.....

1. Patient's Name.....

Age in Years: - a) <15, b) 15-45, c) >45,

Sex: - a) Male, b) female

Address: a) Urban (city-----, wereda-----, kebele-----, house number-----)

b) Rural (wereda-----, PA-----, Village-----)

Occupation: a) Farmer 2) Civil servant 3) Others.....

2. The last school he/she attended:

a) No formal education c) Secondary school

b) Primary school d) College/ University graduate

3. How long have you been sick? -----Months

4. Have you ever taken any treatment? a) Yes b) No

5. If yes, what type? a) Traditional b) drug

6. Are there other members of the family with a similar disease? a) Yes b) No

7. Do you have /had any type of contact with cattle? a) Yes b) No

8. Do you drink raw milk? a) Yes b) No

9. How frequent do you drink raw milk/its products? a) Never b) Rarely c)
Frequently

10. Do you have cattle at present? a) Yes b) No

11. Do cattle live in the same house you live in? a) Yes b) No

Clinical Record

1. Type of TB suspected. a. Pulmonary b. Extra pulmonary

2. IF extra pulmonary, specify? -----

3. Sample(s) taken -----

4. Result of direct smear (AFB) a) positive b) negative

5. Result on culture (AFB) a) positive b) negative

6. If positive, the type of mycobacterium identified.

a) *M. bovis* b) *M. tuberculosis* c) Other atypical mycobacterium

Annex 2. Questionnaire for Cattle owners/attendants (Part two)

Date.....

Code No.....

1. Name of the Owner.....
2. Which species of domestic animals do you own?
a) Cattle, b) sheep c) goats d) Horses /mules / donkeys, e) Poultry
3. Which breeds of cattle do you own? a) Local b) cross c) exotic
4. Purpose of cattle keeping
a) Draft b) Milk for home use c) Milk for sale d) Other
5. How many cattle do you have? a. Less than 10 b. 10-20 c. More than 20
6. How do you manage cattle? A) Free grazing b) Stall feeding
7. Do you mix your cattle with other cattle? a) Yes b) No
8. If yes, where? a) Watering points b) Grazing fields c) Market
9. Do you use the same watering point with animals? a) Yes b) No
10. Do you share the same house with your animals? a) Yes b) No
11. Habit of drinking milk a) Raw b) boiled c) mixed
12. Do you boil milk? a) Yes b) No
13. Reason for boiling a) Fear of milk borne diseases b) due to culture
14. Habit of eating meat a) Cooked b) raw c) mixed
15. If you sell milk/ milk products, who buys it?
a) Local people
b) Milk collection Unit
c) Others (specify) -----
16. Do you have (other) tuberculosis patient in your family or farm workers? a) Yes b) No
17. If yes, how many? -----
18. If you have tuberculosis patient in your family or farm workers, indicate the type of TB?
a) Pulmonary b) extra pulmonary c) do not know
19. How long have you been sick? a). More than a year b) Less than a year c) Other
20. Have you ever taken any treatment? a) Yes b) No
21. If yes, a). Traditional b) drugs

Annex 3: - Body Condition Scoring

A) For Exotic Cattle

0. Animals are emaciated with spinous processes, hipbones, tail head and ribs projected prominently. No fatty tissue can be detected, neural spines and transverse processes feel sharp.
1. Individual spinous processes are still fairly sharp to the touch and there is no fat around tail, head, hip bones, tail head and ribs and still prominent, but appear less obvious.
2. Spinous processes can be identified individually when touched, but feel rounded rather than sharp. There is some tissue cover rounded tail, over hip bones and flank individual ribs are no longer visually obvious.
3. Spinous processes can only be felt with firm pressure. Areas on either side on tail head now have a degree of fat cover which can be easily felt.
4. Fat cover around tail head is evident as slight rounds soft to touch spinous processes can not be felt even with firm pressure and folds of fat are beginning to develop over ribs and thigh of animal.
5. Bone structure is no longer noticeable and animal presents a blocky appearance. Tail head and hip bone are almost completely buried in fatty tissue and folds of fat are apparent over ribs and thighs. Spinous processes are completely covered by fat and animal's mobility is impaired by large amounts of fat carried.
- 6.

B) For Zebu Cattle

1. Condition score 1 (L-) marked emaciation- the animal could be condemned ante mortem
2. Condition score 2 (L) transverse processes project prominently, spines appear sharply
3. Condition score 3 (L+) individual dorsal spines are pointed to the touch, hips, tail-head and ribs are prominent
4. Condition score 4 (M-) ribs, hips and pins are clearly visible, muscle mass between hooks and pins are slightly concave.
5. Condition score 5 (M) –ribs usually visible, little fat cover, dorsal spines are barely visible
6. Condition score 6 (M+) the animal is smooth, dorsal spines can not be seen, but are easily felt
7. Condition score 7 (F-) animal is smooth and well covered but fat deposits are not marked

8. Condition score 8 (F) fat cover in critical areas can easily be seen and felt; transverse processes cannot be seen or felt.

9. Condition score 9 (F+) heavy deposits of fat is clearly visible on tail-head, brisket, dorsal spines, ribs and hooks.

Annex 4: Description of Body condition scoring

Body Scoring Description

Score 1: Severe Emaciation



Individual bony plants are all prominent: the vertebrae are sharp and distinct, hooks and pins are sharp, with negligible flesh covering them, the pararectal fossa below the tail head is deeply sunken. The area over the gluteal musculature (between hooks and pins) is severely sunken. This Score is extremely rare in healthy dairy cattle. It is indicative of Severe cachexia.

Score 2: Thin condition

Bony processes are notable, but not sharp. Individual lumbar vertebrae are not distinct but are Visible. The transverse process of the lumbar is prominent and stands out sharply. They form a notable shelf. The area between the hooks and pins is more filled than with the score of 1 but is still concave. The pararectal area is still concave and depressed, but the bones have a fleshy covering.

Score 3: Moderate Condition

There is a smooth covering of flesh over all bony parts: Individual lumbar prominences are not visually discernible but form a rounded ridge. The transverse processes of the vertebrae no longer form a distinct shelf. Pins and hooks are rounded. There is only a slight concavity between them.

The pararectal fossa is smooth, with out obvious fat filling.

Score 4: Heavy condition

All bony parts are well Covered:

The lumbar area is fat, with no dorsal processes of the spine visible. The Hooks and pins are well covered and the area between them is flat. The tail head is surrounded by obvious subcutaneous fat.

Score 5: OBESE

Almost no bony structure is Visible. There is significant subcutaneous fat. A body score of 5 is rare in dairy Cattle.

Source: Radostits *et al.*, (2001).

Annex 5: List of Reagents

I. Reagents for LJ media

Lacto-asparagines	3.6g
Mono Potassium Phosphate	2.4g
Magnesium sulfate	2.4g
Magnesium citrate	0.6g
Potato flour	30.0g

Glycerol *	12.0 ml
Malachite green	0.4 g
Whole egg	1000.0ml
Distilled water	600.0ml

- Replaced by 0.4% pyruvate for *M.bovis*

II. Regents for Decontamination procedure:-

1. Sodium Hydroxide (2-4%)

➤ Sodium hydroxide	4.0g
➤ Distilled water	200.00ml

2. 0.1 N Hydrochloric acid

3. Bromo cresol solution (0.005-0.01%)

N.B. All the three are autoclaved and stored at 4 °C

III Regents for Ziehl - Neelsen Acid-Fast Stain

1. Carbol-fuchsin Stain

➤ Basic fuchsin	0.3g
➤ Ethyl alcohol (95%)	10.0 ml
➤ Phenol (melted crystal)	5.0 ml

2. Acid - Alcohol

➤ Hydrochloric acid (concentrated)	3.0 ml
➤ Ethyl alcohol (95%)	97.0 ml

3. Methylene blue (Counter stain)

➤ Methylene Blue (90% dye content)	0.3g
➤ Ethyl alcohol (95%)	30.0 ml
➤ Potassium hydroxide 80.01%)	100.0 ml

IV. Regents for Niacin production test

1. 4% aniline: 96 ml ethyl alcohol (95%)+ 4 ml colorless aniline stored in a brown bottle at 4⁰C.
2. 10% Cyanogen bromide: 5 gm Cyanogen bromide in 50ml distilled water. Stored in a brown bottle at 4⁰C.

V. Regents for Nitrate Reduction test

1. 0.01M NaNO₃ in 0.022M Phosphate buffer
 - NaNO₃=0.085g
 - KH₂PO₄=0.485g
 - Distilled H₂O=100ml.
2. concentrated HCl: 10 ml conc. HCl in 10 ml dist. water
3. 0.2% sulfanilamide 0.2 gm sulfanilamide in 100 dist. Water.
4. 0.1 % ethylene diamine dihydrochloride: 0.1 gm n-(1-naphthyl) ethylene diamine dihydrochloride in 100 ml dist. Water.

N.B. 2-4 kept in the refrigerator

Annex 6: -Test Procedure of AFB (vestal, 1981)

- ♣ Prepare smear, a 3mm loop to spread a loopful over an area of 2-3 square cm or smear a drop on the slide with a pipette.
- ♣ Fix smear using a Bunsen-flame and pass the slide 3 times slowly over its cone of heat. Do not scorch.
- ♣ Place a piece of filter paper over the smear. This holds the carbol fuchsin on the slide.
- ♣ Cover the filter paper with carbol fuchsin and heat the slide to steaming with a Bunsen flame
 - If a Bunsen-flame is used, heat gently to steaming (DO NOT BOIL) and allow standing 5 minutes without further heating. If the slide dries, keep it moist by adding more carbol fuchsin without additional heating.
- ♣ Remove paper strips and wash slides with tap water. Drain.
- ♣ Decolourize with acid alcohol until no more colour appears in the washing (about 2 minutes). Thick smears may require longer, but not over-decolourize, since some mycobacteria may lose their acid-fastness if this is done.
- ♣ Wash with distilled or tap water. Drain.
- ♣ Flood slide with methylene blue Counterstain for 1-2 minutes.
- ♣ Wash with distilled or tap water. Drain.
- ♣ Dry in air or over gentle heat. Do not blot.
- ♣ Examine smear with oil immersion lens, taking care to wipe the lens well after each examination, especially if the smear is positive. Three long lines the length of the smear or nine short lines the width of the smear are examined. The bacilli are stained red and the background material is stained blue.

Annex 7: - Nitrate Reduction Test

Mycobacterium tuberculosis produces the enzyme nitroreductase, which catalyzes the reduction of nitrate to nitrite. Some of the other atypical mycobacterial species that reduce nitrate could be differentiated from *M. tuberculosis* by the Pyrazinamidase test. The development of a red colour upon addition of the reagents indicated the presence of nitrite and a positive test result for *mycobacterium tuberculosis*. Most mycobacterial cultures to be tested for nitrate reduction were examined 3 to 4 weeks after inoculation onto the subculture medium i.e. after visible colonies appear.

A) Procedure

1. Add 0.2 ml of sterile distilled water to a 16 x 125-mm screw cap tube.
2. Use a sterile spade or applicators -----the water 2 spadesful of growth from a 4 week old culture on Lowenstein-Jenson or some other egg-base medium.
3. Add 2 ml of the NaNO_3 substrate to the tube.
4. Shake by hand and incubate upright for 2 hours in a 37°C water bath.
5. Remove from the water bath.
6. Add one drop of reagent #1
7. Add two drop of reagent #2
8. Add two drop of reagent #3
9. Examine immediately for a pink-to-red colour



B) Results and interpretation

Positive = May range from pale pink (\pm) to deep red (5+) when compared with the colour standards. Only 3+ to 5+ are considered positive.

Negative = No colour. If no colour develops, the test is either negative or the reduction has proceeded beyond nitrite. Add a small amount of powdered Zink to all negative tests.

- a) If nitrate is still present, it will be catalytically reduced by the Zink, and a red colour will develop, indicating a true negative
- b) If no colour develops when Zink dust is added, the original reaction was positive, but the nitrate was reduced beyond nitrite. Repeat the test in this case to confirm the observation.

Annex 8: - Pyrazinamidase (PZA)

One of the most useful biochemical tests in the classification scheme for a *M. bovis* is susceptibility to Pyrazinamidase. The deamination of Pyrazinamidase (PZA) to pyrazinoic acid and ammonia is helpful in separating the weakly niacin-positive strains of *M. bovis* from *M. tuberculosis* and in distinguishing *M. bovis* from members of the *M. avium* complex. This enzyme acts to split pyrazinamide to pyrazinoic acid in 4 to 7 days. *Mycobacterium bovis* was Pyrazinamidase negative even at 7 days, whereas both *M. tuberculosis* and the *M. avium* complex were positive within 4 days.

A) Procedures

1. Inoculate the surface of two tubes of medium with a heavy loopful of growth from an actively growing culture (2 to 3 weeks old). The inoculum should be heavy enough to be visible.
2. Incubate cultures and controls at 37°C.
3. After 4 days, add 1.0 ml of freshly prepared 1% ferrous ammonium sulfate solution to each unknown culture, to the colour control standard, and to one each of the positive and negative controls.
4. Leave tubes at room temperature for 30 minutes and then examine for a pink band in the agar medium.
5. Refrigerate negative tubes for an additional 4 hours to minimize growth of contaminants from nonsterile ferrous ammonium sulfate solution and examine the medium again for a pink band in the agar. The colour is easiest to detect by examining the tube against a white background and using incident room light.
6. If the four-day tube is negative or doubtful, repeat the test at 7 day using the second tube.
7. If the four-day tube is positive, the second tube may be discarded without further examination.

B) Result and interpretation

A pink band, which forms in the substrate agar and diffuses into the medium, indicates the enzymatic hydrolysis of PZA to free pyrazinoic acid.

Positive = Pink band in agar

Negative = No pink band in agar

Annex 9: - Thiophene-2- Carboxylic acid hydrozide (TCH)

Thiophene-2- carboxylic acid hydrozide sensitivity test was used to distinguish *M. bovis* from *M. tuberculosis* and other non- chromogenic slow growing mycobacteria. *M. bovis* is sensitive to low concentration of TCH. *M. tuberculosis* and other species of mycobacteria generally are resistant to this compound. The test procedure for TCH sensitivity is indicated in the annex---

A) Media and supplies

Middlebrook 7H-10 medium

Thiophen-2-carboxylic acid hydrazide (Aldrich Chemical Co.,Milwaukee,WI)

Strile screwcap tubes or four-section Petri plates (plastic quadrant plates).

B) Preparation

Prepare two batches of complete,enriched Middlebrook 7H-10 medium. One batch is poured as a drug-free control medium; to the other is added sufficient filter-sterilized TCH to make a final concentration of 2 µg/ml. Dispense each medium into sterile, screwcap tubes or, preferably, into four-sectioned Petri dishes.

C) Procedure

1. Dilute a 7-day-old liquid test culture to 10^{-3} and 10^{-5} (1:1000 and 1:100,000) in sterile saline or water.
2. Inoculate one control and one drug-containing medium with 0.1 ml of each dilution.
3. Incubate for 3 weeks at 35°C in an atmosphere of 10% carbon dioxide -90% air.

D) Result and Interpretation

Record the organisms as resistant to TCH if growth on the drug-containing medium is equal to or greater than 1% of that observed on the drug-free control medium.

9. CURRICULUM VITAE

1. Personal data

Name Mohammed Ali Hussien
Date of birth August 18, 1960 EC
Place of Birth Adi-Arkay, North Gondar
Nationality: Ethiopian
Marital status Married
Children Two
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Profession Veterinarian
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2. Educational background

Year in EC	Institution	Award
1966 - 1971	Adi-Arkay elementary and Junior School	-
1972 - 1975	Addis Ketema (Addis Ababa) and Fasiledes(Gondar)Comp.Sec. School	Ethiopian School Leaving Certificate Examination
1976 - 1981	Addis Ababa University, Faculty of Vet. Medicine	Doctor of Veterinary Medicine (DVM)
1997 - 1998	Addis Ababa University, Faculty of Vet. Medicine	MSc in Tropical Veterinary Epidemiology

3. Additional Training

1. International Technical Artificial Insemination Training, Upsala, Sweden, organized and financed by Sweden International Development Agency (SIDA) and air ticket by ILDP (Sept.-Oct.2000).
2. Computer literacy: Word processing, Microsoft excel
3. Applied sampling methods, socio-economic and agricultural research data , analysis and interpretation using SPSS and MSTATC at ILDP head quarter office (9-13 March 2002).
4. Forage and seed production, and inventory held at ILDP head quarter office, Gondar (15-20 April 2002).

5. Participatory Rural Appraisal organized by ILDP head quarter office and the Ethiopian Management Institute, held at Gorgora port, Gondar (23 Dec.2002 to 1 Jan 2003).
6. Educational tour to National Dairy Development Board of India (NDDB), Anand, Gujarat state, organized by ILDP and AEDC (1 July – 26 August 2003)

4. Work experience

Year	Institution	Responsibility
1980 - 1981	National Ticks and Tick disease Control Center and Institute of Agricultural Research Institute (IAR)	Research as undergraduate associate on host resistance to ticks in different breeds of cattle.
1982 - 1989	Ministry of Agriculture	District and Zonal Veterinary department head.
1990 - 1996	Integrated Livestock Development project (ILDP), Gondar	Veterinary department head
1998	Ethiopian Health and Nutrition Research Institute (EHNRI)	Graduate associate dealing with human and bovine TB research mainly isolation and characterization of mycobacterium.

5. Language skill

Amharic: Mother tongue
 Tigrigna: Communicate
 English: Writing and speaking

6. **Membership:** Member of the Ethiopian Veterinary Association (EVA)

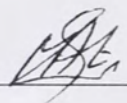
7. Research out put

- 1) Host resistance to ticks in different breeds of cattle at Bako, IAR (DVM thesis paper, 1991)
- 2) Surveillance of Bovine brucellosis in North Gondar administrative zone.
- 3) The Status of bovine tuberculosis in selected districts of N.Gondar (MSc thesis)

10. SIGNED DECLARATION SHEET

I, the undersigned, declare that the 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 Mohammed Ali Hussien

Signature 

Date of submission 23-06-06

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

Dr. Ademe Zerihun _____

Dr. Gezahagn Mamo _____

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2006

The Status Of Bovine Tubercul
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Gonder Adminstrative Zone, Ethiop

Mohammed Ali

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