

ADDIS ABABA UNIVERSITY

SCHOOL OF VETERINARY MEDICINE

**PREVALENCE OF BOVINE TUBERCULOSIS AND MOLECULAR
CHARACTERIZATION OF ISOLATES IN BARKA BREED CATTLE, IN HUMERA,
TIGRAY REGIONAL STATE, ETHIOPIA**

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GEBREMEDHIN ROMHA

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**A Thesis Submitted to the School of Graduate Studies of Addis Ababa
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ABBREVIATIONS

A	Adenine
AFB	Acid Fast Bacillus
AIDS	Acquired Immunodeficiency Syndrome
ALIPB	Aklilu Lemma Institute of Pathobiology
APC	Antigen Presenting Cells
BCG	Bacillus Calmette Guerin
BCS	Body Condition Score
bp	Base Pair
BTB	Bovine Tuberculosis
cAMP	Cyclic Adenosine Monophosphate
CD	Cluster of Diffrentiation
CFP	Culture Filtrate Protein
CFU	Colony forming units
CIDT	Comparative Intradermal Tuberculin Test
cm	Centimeter
CMI	Cell Mediated Immunity
C	Cytocine
DNA	Deoxyribonucleic Acid
DR	Direct Repeat
EDTA	EthyleneDiamine Tetraceticacid
EPTB	Extra Pulmonary Tuberculosis
ESAT	Early Secretory Antigenic Target
F	Forward
G	Guanine
HF	Holestein Fresian
HIV	Human Immunodeficiency Virus
IFN	Interferon
IL	Interleukin

IU	International Unit
IS	Insertion Sequence
Km	Kilo meter
LJ	Löwenstein Jensen
m	Meter
ml	Mili liter
MHC	Major Histocompatibility Complex
μl	Micro litere
μm	Micro meter
mm	Mili meter
m-PCR	Multiplex Polymerase Chain Reaction
MS	Microsoft
MTB	<i>Mycobacterium Tuberculosis</i>
MYCAV	<i>Mycobacterium Avium</i>
MYCGEN	<i>Mycobacterium Genus</i>
MYCINT	<i>Mycobacterium Intracellularae</i>
ng	Nano gram
OR	Odd Ratio
OIE	Office International des Epizooties
PCR	Polymerase Chain Reaction
PPD	Purified Protein Derivative
R	Reverse
RD	Region of Difference
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolution per minute
rRNA	Ribosomal RiboNucleic Acid
T	Thymine
TAE	Tris-Acetate EDTA
TB	Tuberculosis
Th	T-helper
TNF	Tumor Necrosis Factor

UK	United Kingdom
US	United State
USD	United State Dollar
UV	Ultra Violet
w/v	Weight/volume

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ABSTRACT

Bovine tuberculosis (BTB) is a chronic infectious disease of animals and humans characterized by the formation of granulomas in tissues and organs, principally caused by *Mycobacterium Bovis* (*M. bovis*). A cross-sectional study was conducted from September 2010 to May 2011 with the objectives to determine the prevalence of BTB, to characterize the isolates of *Mycobacterium tuberculosis* complex, and to assess zoonotic importance of BTB based on questionnaire survey in Setit and Kafta Humera districts, Western Zone of Tigray Region. Thirty one herds comprising 484 cattle were subjected to CIDT test. Accordingly, the individual animal prevalences were 6.6% (95% CI, 4.4–8.8) and 8.7% (95% CI, 6.2–11.2) at cut-offs greater than 4 mm and 2 mm, respectively while the herd prevalences were 61.3 % (95% CI, 43.1–79.5) at cut-off >4 mm and 77.4% (95% CI, 61.8–93.0) at cut-off >2 mm. One of the 7 milk samples collected from tuberculin-positive milking cows was culture positive. Bovine tuberculin positivity was significantly associated with body condition score ($\chi^2=13.4$, $P=0.001$ at cut-off >4 mm; $\chi^2=6.8$ $P=0.033$ at cut-off >2 mm), while the other assessed potential risk factors were not statistically significant. Thus, animals with medium body condition score (OR = 5.5 [95% CI, 1.9–15.9] at cut-off >4 mm; OR = 2.7 [95% CI, 1.2–5.8] at cut-off >2 mm) had a higher odds for tuberculin reactivity. On abattoir survey, a total of 582 cattle were subjected to postmortem examination, of which 5.8% were found to be positive for gross tuberculous lesions. Larger proportion (61.8%) of TB suggestive lesion was recorded in the respiratory pathway followed by digestive system and prescapular lymph nodes with 23.5% for each. Among the risk factors assessed in abattoir survey, age ($\chi^2=6.8$, $P=0.009$) and body condition ($\chi^2=74.5$, $P=0.000$) were significantly associated with the presence of TB suggestive lesion. Thus, animals older than 6 years (OR = 3.7 [95% CI, 1.3–10.7]) and with poor body condition score (OR = 53.4 [95% CI, 7.1–402.4]) had a higher odds for tuberculin reactivity. Of 34 tissue specimens collected from the gross lesions 35.3% (12/34) were positive on primary culture, of which four showed a positive signal for the genus *Mycobacterium* using multiplex PCR and none of them were in *Mycobacterium tuberculosis* complex. The result suggests the importance of non-tuberculosis mycobacteria in cattle of the study area and recommended further study to reveal the specific species of mycobacteria and their public health significance in human.

Key words: Bovine tuberculosis, cattle, Humera, *Mycobacterium tuberculosis* complex

1. INTRODUCTION

Globally human population is estimated to be above 6 billion and increased by 74.6 million (1.13%), and it is projected to fall steadily to about 41 million per annum in 2050, at which time the population will have increased to about 9.2 billion. Each region of the globe has seen great reductions in growth rate in recent decades, though growth rates remain above 2% in some countries of the Middle East and Sub-Saharan Africa, and also in South Asia, Southeast Asia, and Latin America (U.S. Census Bureau, 2010). As a result, the demand for animal products, in terms of both quantity and quality, is estimated to increase substantially, thereby requiring intensification of animal production integrated with proper feeding, animal health management, and genetic improvement. Additionally, potential domestic dairy market within growing urban demand (Hurrissa *et al*, 1994) and export potential of animal and animal products are great, but to this effect, control of diseases such as bovine tuberculosis (BTB) and other diseases is a prerequisite (Bonnet, 2000).

Bovine tuberculosis is a chronic infectious disease of animals characterised by the formation of granulomas in tissues and organs, more significantly in the lungs, lymph nodes, intestine and kidney including others. BTB is caused by slowly growing non-photochromogenic bacilli members of the *Mycobacterium tuberculosis* complex: *M. bovis* and *M. caprae* species. However, *M. bovis* is the most universal pathogen among mycobacteria and affects many vertebrate animals of all age groups including humans although, cattle, goats and pigs are found to be most susceptible, while sheep and horses are showing a high natural resistance (Radostits *et al.*, 2000). BTB has been significantly widely distributed throughout the world and it has been a cause for great economic loss in animal production. The global prevalence of human TB due to *M. bovis* has been estimated at 3.1% of all human TB cases, accounting for 2.1% and 9.4% of pulmonary and extra-pulmonary TB cases, respectively (Cosivi *et al.*, 1998).

In industrialized countries, human TB due to *M. bovis* is relatively rare as a result of milk pasteurization and animal tuberculosis control programs (Grange, 1996; Cosivi *et al.*, 1998). In developing countries, however, in 46% of African, 44% of Asian and 35% of the South American and the Caribbean countries, sporadic occurrences and (particularly in Africa 11%) enzootic occurrences of BTB have been reported (Cosivi *et al.*, 1998). There is also sufficient evidence to indicate that it is widely distributed in almost all African countries and even is found at high prevalence in some animal populations (WHO, 1994; Ayele *et al.*, 2004; Zinsstag *et al.*, 2006).

Bovine tuberculosis, apart from being the most important disease of intensification with a serious effect on animal production, also has a significant public health importance (O'Reilly and Daborn, 1995). Although, the direct correlation between *M. bovis* infection in cattle and human populations is not well known (Cosivi *et al.*, 1995), however, zoonotic BTB is present in most developing countries where surveillance and control activities are often inadequate or unavailable. The actual impact of BTB on human health is generally considered low in developed and developing countries, which may be based on the rare identification of *M. bovis* isolates from human patients (Amanfu, 2006). In addition, the occurrence of BTB due to *M. bovis* in humans is difficult to determine accurately because of technical problems in isolating the microorganism. Currently, BTB in humans is becoming increasingly important in developing countries, due to the association of mycobacteria with the *HIV/AIDS* pandemic and in view of the high prevalence of *HIV/AIDS* in the developing world and susceptibility of *AIDS* patients to tuberculosis (Amanfu, 2006). Thus BTB is great concern in many developing countries and Ethiopia is one of those where BTB is considered as prevalent disease in cattle populations. Its zoonotic implication has also significantly indicated an increasing trend to be of public health hazards (Kiros, 1998; Regassa, 2005). Even though bovine tuberculosis has been reported in the above mentioned findings in humans and animals, prevalence data on BTB infection in Africa is scarce because adequate research has not been conducted.

In Ethiopia, the endemic nature of the human tuberculosis has long been documented and has been recognized as a major problem of human health for accounting a high rate of morbidity and mortality (Ministry of Health, 1978-1980). At present, all types of tuberculoses cases are found to

be ranked 4th (4.3%) among the top 10 diseases leading causes of admission in 2003, also as the 2nd (11.5%) cause of death among the top 10 diseases in 2004 (Ministry of Health, 2004).

In Ethiopia, BTB is considered as endemic based on abattoir inspection (Bogale *et al.*, 2004) and tuberculin test survey (Ameni 1996; Bogale *et al.*, 2001). Studies have indicated that BTB is more prevalent in intensive dairy farms than in smallholder farm regions (Ameni, 1996) with a prevalence ranging from 3.4% in small holder production system to 50% in intensive production system (Ameni and Roger, 1998; Kiros, 1998; Bogale *et al.*, 2001; Ameni *et al.*, 2001). According to Shitaye *et al.* (2006), tuberculous lesions (3.5%) were found in 34 animals by the meat inspection surveillance of 984 cattle. Histopathologically, granulomatous inflammation (8.8%) was evident in 3 animals with tuberculous lesions. A highly sensitive PCR (IS6110) was positive in 4 (11.8%) of 34 animals with tuberculous lesions and in 1 (2.9%) of animal without lesions. Bovine tuberculosis is a major economic problem and a potential public health risk in Ethiopia (Ameni *et al.*, 2008).

The prevalence of BTB infection in cattle was investigated in extensive and intensive production systems in three districts of northwestern Ethiopia by comparative intradermal tuberculin (CIDT) test as 9.7% whereas the non-specific infection prevalence was 10.8% (Fetene and Kebede, 2009). BTB is widely spread throughout Ethiopia, albeit at a low prevalence (4.7%), and provides underpinning evidence for public health policy formulation (Berg *et al.*, 2009). One study by Biffa *et al.* (2009) demonstrated high prevalence of tuberculosis in Ethiopian slaughter cattle and this could infer to similar scenario in a reference cattle population in the country. Another study by the same investigator (2010b) provided the molecular evidence for the widespread distribution of *M. bovis* in the cattle population in Ethiopia with six new spoligotypes which were subsequently registered in the database with international identification codes of SB1517, SB1518, SB1519, SB1520, SB1521 and SB1522. It also demonstrated a relatively high degree of genetic polymorphism of the isolates (Biffa *et al.*, 2010b).

These findings indicated that bovine tuberculosis in cattle and human is an existing problem in Ethiopia which needs to be solved. However, there are no records on the nation-wide distribution of the disease because there are still areas where such study have not been yet conducted; for

example in the northern part of Ethiopia, Tigray, especially western zone of Tigray (Humera). There are many risk factors conducive to the spreading and persistence of bovine tuberculosis in developing countries such as demography, eating habits (drinking unpasteurized milk and raw meat), living and socio-economic status of families, illiteracy, culture and customs, the existence of HIV/AIDS, and close proximity with animals (Cosivi *et al.*, 1998; Edginton *et al.*, 2002; Ayele *et al.*, 2004). Consumption of unpasteurized milk (absence of pasteurization machine), eating raw meat and close proximity of human and animals in the area is very common (Personal observation). Morrone *et al.* (2007) showed that cutaneous tuberculosis, an important extra-pulmonary (EPTB) form, is commonly seen in Tigray in adult as well as paediatric patients. Clinical diagnosis was confirmed with fine-needle aspiration cytology (FNAC) and Ziehl Neelsen (ZN) or methylene blue staining (showing acid fast bacilli (AFB) or caseation necrosis) and histological investigations (Morrone *et al.*, 2007). In Tigray in general, and in Humera in particular, identification and characterization of *Mycobacterium tuberculosis* complex has not been yet done from human as well as from animal (Personal communication).

In addition, the area is found at the border of Sudan and Eritrea, therefore, there may be free mixing of animal with Sudan although contact of animal is decreased with Eritrea due to the Ethio-Eritrea conflict. A great concern to be given here is in the extensive animal production, animals may expose to wild animals contact because large number of different species of wild animals are available in the area. Generally, the human population of this area is rarely aware about the transmission, zoonotic and economic importance, as well as prevention and or control of the disease (Personal observation and communication). Assessing and identifying the risk factors which are important for spreading of bovine tuberculosis and the identification of the agent in the area will help to adopt the prevention and control methods in the area.

Therefore, this study was initiated with the following objectives:

- ☞ To determine the prevalence of *Mycobacterium tuberculosis* complex infection in Barka breed cattle in the western zone of Tigray (Setit and Kafta Humera) based on comparative intradermal tuberculin test and detailed post mortem inspection in municipal abattoir;

- ☞ To isolate and characterize the causative agent prevailing in the study site based mycobacteriological culture and molecular technique and;
- ☞ To assess the possible public health implication based on questionnaire survey.

2. LITERATUREREVIEW

2.1. Etiology

2.1.1. Taxonomy

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 the environment (Thoen and Bloom, 1995). *Mycobacterium* species that are pathogenic to man and animals include *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. avium* complex, *M. canettii*, *M. bovis subspecies caprae* and *M. pinnipedii*, and *M. marinum*, *M. leprae*, *M. lepraemarium*, *M. avium subspecies paratuberculosis*, *M. senegalense* and *M. farcinogenes* (Quinn *et al.*, 2002). The cause of BTB, *M. bovis*, belongs to the *Mycobacterium tuberculosis* complex, which includes *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. canettii*, *M. bovis subspecies caprae* and *M. pinnipedii* (Quinn *et al.*, 2002).

2.1.2. Morphology, staining, structure and composition

Mycobacteria are nonmotile, nonsporing, aerobic, oxidative and they appear in tissue as thin rod, which may be straight, curved, or in the form of clubs. They occur singly, in pairs or as small bundles of varying lengths (1.0-4.0 μm) and width (0.2-0.3 μm). On laboratory media, they may appear as cocci or rods measuring 6-8 μm (Quinn *et al.*, 1999). The cells are characterized by high lipid content consisting of long branched chains of mycolic acid (Thauro *et al.*, 1996).

Mycobacteria when stained are acid fast as they resist decolorizing 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). 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 stain and it is difficult to demonstrate that they are Gram positive because of their lipid contents. They can be Gram stained after partially removing the lipid layer (WHO, 1998).

The mycobacterial cell wall is triple layered comprising a basal peptidoglycan layer, an intermediate arabinogalactan mycolate complex and the outer lipid rich, comprising surface rope like structure peptidoglycolipid. The cell wall of mycobacteria contains N-glycomuramic acid in place of N-acetylmuramic acid and has high lipid content. Lipids account for acid fastness, pathogenic and immunogenic properties. The various structural components of mycobacteria include surface mycosides (mostly glycolipids and peptidoglycolipids), mycolic acids and associated esters, wax D, cord factor (dimycolil trehalose), sulfolipids (sulfatides), phosphatidyl inositol mannoside, mycobactins, carotenoid pigments (chromogens) and tuberculin (peptides liberated in to culture media during growth) (Biberstein and Hirush, 1999). The genome sequence of *M.bovis* is at least 99.9% identical to that of *M. tuberculosis* but deletion of genetic information has lead to a reduced genome size (Garnier *et al.*, 2003).

2.1.3. Growth requirements and cultural characteristics

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 back ground. Growth in LJ medium is replaced by pyruvate in Stonebrink's medium to enhance growth of *M. bovis* (WHO, 1998). *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%CO₂ (Quinn *et al.*, 1999). The optimal growth temperature is 37°C except for *M.avium* which needs a temperature of 40°C-42°C. Growth in liquid medium is diffused, on solid medium *M. bovis* is dry, sparse, delicate and non luxuriant (Quinn *et al.*, 1999). Mycobacteria grow on protein enriched media except *M. leprae*, *M. lepraemarium* and *Mycobacterium* causing bovine skin tuberculosis which do not grow on any artificial culture media (Biberstein and Hirush, 1999; Ochei and Kolhatkal, 2005).

2.2. Pathogenesis

The methods by which tubercle bacilli gain entrance to the animal body include: the respiratory, alimentary, genital, cutaneous and congenital routes (Menzes and Neill, 2000), the first two being the most commonly observed routes of infection resulting in pulmonary and extra-pulmonary forms of the disease, respectively. Calves are usually infected by suckling milk from cows with tuberculous mastitis (Barwinek and Taylor, 1996). 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 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 (Anderson, 1997). Humans most commonly acquire TB infection by inhaling aerosolized bacteria as droplet nuclei (Anderson, 1997).

Cattle can be infected orally with as few as 5000 colony forming units (CFU) of *M. bovis* administered in the feed (Palmer *et al.*, 2004). Conversely, mathematical modeling of *M. bovis* excretion by tuberculous cattle suggests that a single bacillus may be sufficient to cause infection by the aerosol route (Neill *et al.*, 1991). Thoen and Bloom (1995) also reported that the infectious dose is very low; 1-3 viable bacteria are considered sufficient as infectious inoculum.

A primary lesion or focus of infection is established following the interaction of the host and the agent at the site of entry within eight weeks of bacterial entrance (Radostits *et al.*, 2000). The mycobacteria are then taken by the alveolar macrophages to the circulation and establishes in lymph nodes. Cellular responses attempting to control the disease results in the accumulation of large number of phagocytes and lead to the formation of a macroscopic lesion referred as tubercle (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 epitheloid 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).

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, cyclic adenosine monophosphate (cAMP) and sulfo-lipids. *M. avium* survives within the fused phago-lysosome by a virtue of their capsule like coating material of mycosides. *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 (Quinn *et al.*, 2002).

2.3. Pathology of bovine tuberculosis

The chronic, macrophage-rich nature of the inflammatory response to mycobacteria and the formation of the typical tuberculous granuloma or tubercle are driven by factors such as: the favoured intracellular location of these organisms within macrophages; and by their poorly digestible, waxy cell wall. Granulomas represent focal expression of granulomatous inflammation that can restrict or prevent mycobacterial growth through juxtaposing infected macrophages and T cells. In this context, T cells can both upregulate macrophage microbicidal activity or lyse heavily infected macrophages as required. The architecture of the granuloma also prevents mycobacterial spread by surrounding the infected lesion core with multiple concentric layers of uninfected macrophages and fibrous tissue (Saunders and Cooper, 2000).

Solid or non-necrotic lesions occur as an early manifestation of *M. tuberculosis* infection in all species. Solid lesions represent the initial non-suppurative or granulomatous inflammatory

response that precedes organization into the classical granulomas typical of human tuberculosis and experimental infections (Elwood *et al.*, 2007). Caseous necrosis, as much as the granuloma itself, defines the response to *M. tuberculosis* infection in humans and experimental infections in some animals. Caseation describes the macroscopic and microscopic appearance of inspissated (cheese-like) exudate associated with lesion necrosis. Lesion necrosis represents irreversible tissue damage that undergoes healing in some species by calcification, fibrosis and sometimes ossification. However, incomplete healing creates a microenvironment that harbors bacilli that are visible by acid-fast staining, some of which are confirmed viable by culture (Lenaerts *et al.*, 2007).

Liquefaction leading to cavity formation within tuberculous lesions is considered a significant contributor to the perpetuation of tuberculosis as this feature enhances both patient infectivity and the development of multidrug resistant strains. Dystrophic mineralization or calcification is a pathologic process associated with intra- and extra-cellular deposition of mixed calcium salts at sites of tissue necrosis (Giachelli, 2003).

2. 4. Immune responses to mycobacteria

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 interaction of mycobacteria with antigen presenting cells (APC) is a key feature in the pathogenesis of TB and the outcome of this interaction is pivotal in determining whether immunity or disease ensues. Both macrophage and dendritic cells have been shown to become infected with mycobacteria and to produce a response to infection that reflects their suggested role in immunity. Thus, macrophage elicits anti-microbial mechanisms for elimination of mycobacteria and dendritic cells upregulate expression of molecules that aid stimulation of T lymphocytes (Hope and Villarreal-Ramos, 2008).

2.4.1. T-cell responses to mycobacteria

Inoculation of mice, humans and cattle with mycobacteria induces antigen specific CD4+, CD8+ and $\gamma\delta$ T-cell responses (Flynn and Chan, 2001; Pollock *et al.*, 1996). Inoculation of cattle with *M. bovis* induced changes in the peripheral blood lymphocyte (PBL) composition, with an early increase in the percentage of $\gamma\delta$ -WC1+ T-cells, indicating that this T-cell subtype may play a role in the early control of mycobacteria growth (Pollock *et al.*, 1996). CD4+ and CD8+ T-cells are essential for immunity to mycobacteria, as individuals deficient in either T-cell subtype are more susceptible to infection (Ehlers *et al.*, 2001).

Both T-cell subsets contribute to immunity to mycobacteria by production of IFN- γ and activation or killing of infected cells. However, killing the infected cells can result in either the release of intracellular bacteria or the killing of both the infected cell and the bacteria. The release of mycobacteria from infected APC can in turn result in spread of infection or uptake of released mycobacteria by activated macrophage able to kill the pathogen. Thus, the most efficient way of clearing the infection would be the killing of intracellular mycobacteria at the same time as the death of the infected host cell (Flynn and Chan, 2001). Once the infection has been established, if it is controlled but not all the mycobacteria have been killed, their subsequent growth is governed by CD4+ and CD8+ T-cells. Both T-cell types have been shown necessary for the maintenance of a latent state of infection. Thus, CD4+ and CD8+ T-cells contribute to the formation of the TB granuloma and to the arrest of mycobacterial growth mainly by the expression of a T_H -1 type response. This response may be sufficient to contain the growth of mycobacteria and to account for the absence of disease (Scanga *et al.*, 2000). TNF- α and IFN- γ are essential cytokines for resistance to mycobacteria, which are produced by components of both the innate and adaptive immune response (Bopst *et al.*, 2001).

2.4.2. Type IV hypersensitivity

Type IV hypersensitivity is also known as cell mediated or delayed type hypersensitivity. The classical example of this hypersensitivity is tuberculin (Montoux) reaction which peaks 48–72 hours after the injection of antigen (PPD). The lesion is characterized by induration and

erythema. Type IV hypersensitivity is involved in the pathogenesis of many autoimmune and infectious diseases, and granulomas due to infections and foreign antigens. Another form of delayed hypersensitivity is contact dermatitis (poison ivy, chemicals, heavy metals, *etc.*) in which the lesions are more papular. Mechanisms of damage in delayed hypersensitivity include T lymphocytes and monocytes and/or macrophages. Cytotoxic T cells (T_c) cause direct damage whereas helper T (T_h-1) cells secrete cytokines which activate cytotoxic T cells and recruit and activate monocytes and macrophages, which cause the bulk of the damage. The delayed hypersensitivity lesions mainly contain monocytes and T cells. Major lymphokines involved in delayed hypersensitivity reaction include monocyte chemotactic factor, interleukin-2, IFN- γ and TNF α/β (Male *et al.*, 2006).

2.5. Epidemiology of bovine tuberculosis

Mycobacterium bovis has wide host ranges with complex epidemiological patterns which involve interactions of infections among and between human beings, domestic and wild animals. However, only little is done particularly in developing countries on the epidemiology of this organism and the epidemiology requirements for its control (O'Reilly and Daborn, 1995).

2.5.1. Distribution

Out of 209 countries reported to OIE, 49.3 % (103/209) reported the detection of BTB, 37.8 % (79/209) noted its absence and the remainder didn't report any information about the disease (Amanfu, 2006).

Table 2: Distribution of bovine tuberculosis in animals and number of countries reported

Continent	Detection of BTB	Absence of BTB	No report
Africa	26(51%)	7(13.7%)	18(35.3%)
Americas	21(48.8%)	19(44.2%)	3(7%)
Asia	20(44.4%)	20(44.4%)	5(11.2%)
Europe	35(68.6%)	15(29.4%)	1(2%)
Oceania	1(5.3%)	18(94.5%)	-
Total	103(49.3%)	79(37.8%)	27(12.9%)

Note = Americas: North and South America and the Caribbean Islands

Source: Amanfu (2006)

Industrialized countries have eradicated or drastically reduced the prevalence of *M. bovis* infection in both animals and humans through animal tuberculosis control and elimination programmes, improvements in milk hygiene and pasteurization and awareness creation on the zoonotic and economic significance of *M. bovis* infection. In developing countries, however, animal TB is widely distributed but control measures are not applied or are applied sporadically and milk pasteurization is rarely practiced (Amanfu, 2006). Data on the occurrence of animal tuberculosis are generally scarce. It is difficult to measure the precise morbidity of the disease in some countries and only qualitative assessment often not supported by laboratory or field investigations, are submitted to OIE (Amanfu, 2006).

2.5.2. Risk factors

Risk factor assessment and identification of *M. bovis* in both humans and animals would be the first step towards adopting dependable preventive, therapeutic and control measures (Ayele *et al.*, 2004). The epidemiology of *M. bovis* and other mycobacterial species infection in animals is complex, with dynamic triad of interactions between “host-agent -environment” determinants (Ayele *et al.*, 2004).

Host risk factors

Genetic resistance of hosts to *M. bovis* infection has never been conclusively demonstrated, but zebu (Brahman) types of cattle are thought to be resistant to tuberculosis than the European breed (Biberstein and Hirsh, 1999). The higher prevalence of BTB in dairy than beef cattle may reflect close confinement, longer life spans, and greater productivity stress in dairy cows. Risk of a positive reaction varies with an animal's age and body condition (Biberstein and Hirsh, 1999). Herds with the following risk factors had a significantly greater prevalence of intradermal test positives: >50 cattle in the herd; herds housed inside at night and herds in contact with wildlife. Furthermore, villages that experienced annual flooding had a higher prevalence of infection (Biberstein and Hirsh, 1999).

On the abattoir report in India, tuberculosis lesions were found in 25.97 % of the pure exotic breeds, 9.7 % in the cross breeds and in only 7.1 % of the Zebu animals (O'Reilly and Daborn, 1995). In contrast, Seifert (1996) reported that cattle of the traditional pastoralists in Madagascar were affected up to 60 %.

Agent factors

The tubercle bacilli of various types are quite sensitive to heat and are killed at 60°C in 15 minutes. They are also susceptible to ionic detergents, and are killed by sunlight, ultraviolet irradiation, and pasteurization (Biberstein and Hirsh, 1999). However, tubercle bacilli are usually resistant to drying, most disinfectants, acids and alkali. This resistance is due to hydrophobic lipid surface of the agent. They also remain viable for long period in the dark, putrefying material, faeces, sputum and soil, (Biberstein and Hirsh, 1999).

M. bovis is an obligate pathogen and is highly susceptible to sunlight (UV radiation), but it can survive for several months in the environment, in moist soil and in darkness and remains viable for long periods (>6 months) in frozen tissues (Jahans and Worth, 2006). It survives in acid milk for 15 days and in milk products such as cheese and butter for weeks but it gets destroyed upon

heating of 30 minutes at 65⁰C, and cream at 85⁰C for 2 minutes. The organism can also survive for over 2 years in frozen carcasses (Seifert, 1996).

Environmental and management factors

Housing and feeding conditions are important risk factors. Housing of animals predisposes them to disease infection because of closeness of animals. For example, the disease incidence is high in intensive dairy farms than beef ranches. Factors that play role in predisposing animals to infection include type of production (dairy cattle are at risk), suckling (calves become infected by ingesting contaminated milk), and presence of wild reservoirs of *M. bovis* infection for grazing cattle (Quinn *et al.*, 1999). Cattle movement, purchase, grazing field boundaries or short swards, spreading cattle slurry on the fields are a potential risk for transmission of the disease between cattle (Theo *et al.*, 2006). Circumstantial evidence suggests that an adequate intake of mineral, vitamin and protein reduces the susceptibility of cattle to BTB infection (Theo *et al.*, 2006). Although weather patterns have been implicated in the susceptibility of herds, it is concluded that some reduction in the susceptibility of cattle to *M. bovis* infection can be achieved by modifications of the management practices to minimize the weather risk factors (Theo *et al.*, 2006).

2.5.3. The disease in animals (domestic and wildlife)

Bovine tuberculosis is caused by members of the *Mycobacterium tuberculosis* complex: *M. bovis* and *M. caprae* species. However, *M. bovis* is the most universal pathogen among mycobacteria and affects many vertebrate animals of all age groups including humans although, cattle, goats and pigs are found to be most susceptible, while sheep and horses are showing a high natural resistance (Radostits *et al.*, 2000).

Bovine tuberculosis in cattle is an infectious, chronic but progressive disease characterised by the formation of typical granulomatous lesions with varying degrees of necrosis, calcification and encapsulation. Transmission between animals is thought to occur mainly by inhalation of contaminated aerosol and therefore, affects the lungs primarily (Kaneene and Pfeiffer, 2006).

However, infection can also occur via the gastro-intestinal tract or become systemic and affect other organs, such as the urinary tract or the mammary lymph nodes (Cousins *et al.*, 2004). The number of severe cases of animals with clinical manifestation may be limited or absent in countries where active control measures are applied. Advanced disease and generalizations are usually more common in countries with insufficient or no control, adding to an increased risk for transmission to humans (Cosivi *et al.*, 1998).

At present, cases of *M. bovis* infection have been reported in more than 40 free-ranging wild animal species. Despite significant variations in size, appearance and distribution of the tuberculous lesions in different species, in the majority of affected wildlife species lesions closely resemble those in cattle (Zanella *et al.*, 2008; Drewe *et al.*, 2009). A consistently different pattern of pathological changes has, however, been described in lions where no histological evidence of necrosis was found. TB in wildlife can pose serious difficulties for BTB control and eradication. Particularly noteworthy is the case of the British Isles, where the European badger represents an important and well documented disease reservoir (Smith *et al.*, 2006).

2.5.4. Risk factors conducive to the spreading of bovine tuberculosis in human in developing countries

Culture and customs

In developing countries, particularly in Africa, patients' beliefs and cultural traditions are major obstacles to implement the designed TB control strategies. TB is stigmatized in many cultures/traditions and it remains as powerful as that of HIV/AIDS, which further complicates the process of investigation by patients hiding their TB status due to discriminatory views about TB patients. Social discrimination based on TB status is thus more a matter of stigma than of appropriate public health precautions (Shitaye *et al.*, 2007). A study in a rural area of South Africa reported the strong local community belief that TB results from breaking cultural rules and that the disease can be treated by traditional healers (Edginton *et al.*, 2002).

Illiteracy

In most African countries, illiteracy is yet another unsolved problem in most rural communities in particular. Inability to read and write, and failure to utilize modern methods of communication, and the limited knowledge of the community related to the epidemiology of BTB infection, makes prevention and control programmes difficult and often impossible to apply (Ayele *et al.*, 2004).

Demography, eating habits, living and socio-economic status of families

Furthermore, demographic factors, such as income, education, age, number of family, number of individuals dwelling per meter square and sanitation etc., are also contributing to the epidemiology of BTB (Ayele *et al.*, 2004). Moreover, reports indicated that infants are more vulnerable to food-borne *M. bovis* infection, where as older individuals averting BTB may occur as a result of endogenous reactivation (Pavlik *et al.*, 2002).

For most urban dwellers, milk is considered the main vector for infection by BTB, while farmers and abattoir workers are mostly exposed to aerosol infection by close contact with infected animals. For most rural African populations, consumption of raw milk and milk products and close association between animals and farmers are common, and encourage exposure to both alimentary and respiratory infection by *M. bovis* (Ayele *et al.*, 2004). The major factors among which contribute to the acquisition of the infection in both urban and rural populations are family ownership of cattle, previous livestock ownership, sharing of the house with animals, consumption of non-pasteurised milk (raw milk) or poorly cooked meat (Pavlik *et al.*, 2003; Ayele *et al.*, 2004). All these causalities and/or habits are the daily practices most notably of rural communities in developing countries. In particular, milk borne infection is the main cause of EPTB in areas where BTB is common and uncontrolled (Daborn *et al.*, 1996; Kiros, 1998). Professional occupation or workers such as abattoir workers, veterinarians and laboratory technicians, animal care taker in zoos and those who are working in animals reservations and at national parks can also acquire the infection in due course of regular work (Pavlik *et al.*, 2002). Families with low income often face malnutrition which, when associated with the burden of

HIV/AIDS infection, increases susceptibility to various infectious diseases such as TB by the impaired immune system, in particular the lymphocyte function which plays an important role in containing mycobacterial infections (Macallan, 1999).

Role of wild life reservoirs

Recent reports in South Africa indicated that there were four outbreaks of BTB in the buffaloes during the years 1996 to 2003. In addition to this, the disease has been prevalent in an Eastern province of South Africa in the 1930s and there are strong indications that greater kudu (*Tragelaphus strepsiceros*) act as maintenance hosts. Despite this, kudu have been the source of *M. bovis* infection in one wildlife reserve in Kwazulu-Natal in a group of BTB-free African buffaloes (Kriek, 2006). So far, to our knowledge, no BTB cases have been reported in Ethiopia in the wildlife population. However, the presence of the aforementioned animals in different wildlife reserves and even in free ranging forests may have an epidemiological role in the spread of the disease among other wild and domestic animal species (Shitaye *et al.*, 2007). Moreover, Alemu (1992) suggested that the widely distributed Rock hyrax (*Procavia capensis*) might also be a possible reservoir of BTB in Ethiopia.

Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS)

The incidence of BTB in humans has risen as a result of the impact of the HIV/AIDS epidemic (Cosivi *et al.*, 1998; Ayele *et al.*, 2004; Zinsstag *et al.*, 2006). Tuberculosis and other mycobacterial infections are major opportunistic infections in HIV/AIDS infected individuals, while HIV/AIDS is a major predisposing factor for tuberculosis including reactivation. The occurrence of both infections in one person makes TB infection very likely to progress to active disease. In many developing countries, TB is the most frequent opportunistic disease associated with HIV infection (Raviglione *et al.*, 1995).

2.5.5. Transmission

Inhalation of *M. bovis* is the most probable and principal route to bovine infection and is facilitated by close, prolonged contact between infected and healthy animals (Figure 1). Ingestion of *M. bovis* directly from infected animals or from contaminated pasture, water or utensils may also be very common in some regions. While congenital infections and vertical transmission have been recorded, these routes, like genital transmission, which occurs when reproductive organs are infected, are now rarely seen in regions that have intensive eradication programmes (Neill *et al.*, 1994).

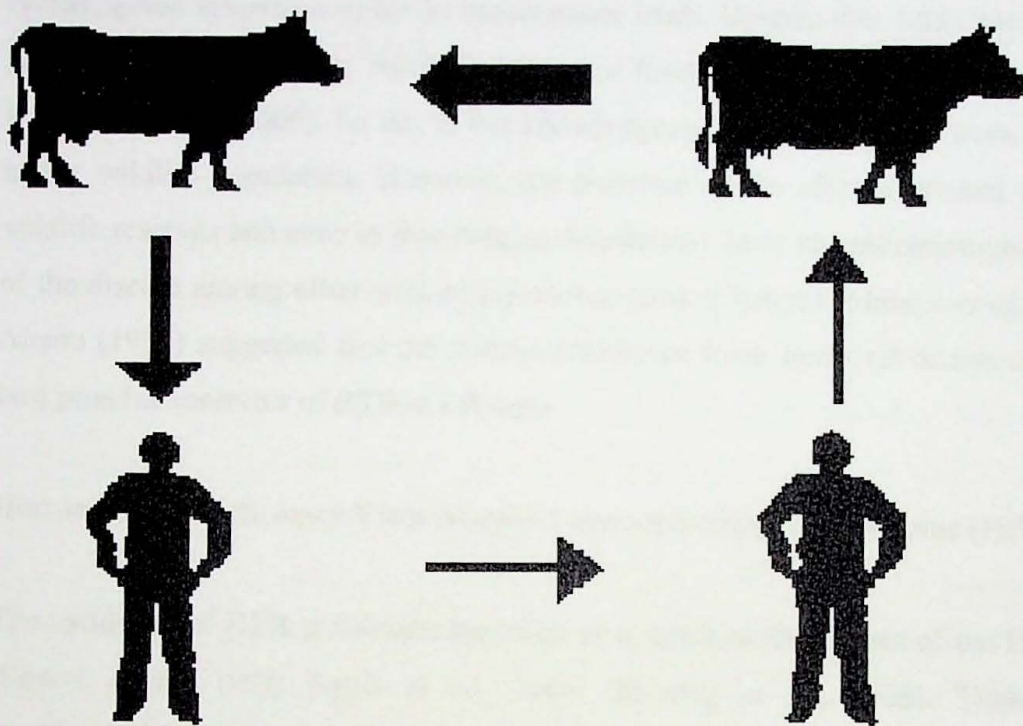


Figure 1: Cycle of *Mycobacterium bovis* transmission between cattle and humans. The thickness of the arrows suggests probability. Adapted from Collins and Grange (1987).

Animal-to-animal transmission

Infected animals may shed *M. bovis* in a number of ways: in faeces, milk, discharging lesions, saliva and urine (Neill *et al.*, 1994). Intensive livestock farming promotes close contact between

animals, favouring the spread of *M. bovis*. In Africa, grazing animals usually gather at night for protection from predators. Vaccination and artificial insemination centers, dipping tanks, auction stations, market places and transportation are the commonest animal gathering places, and again are sites where transmission of infection could easily occur. Due to the high ambient temperature in tropical zones, animals tend to concentrate under trees or other shaded areas for parts of the day, preferring to graze early in the morning and late in the afternoon. Possibly the most dangerous spots for nose-to-nose or mouth-to-mouth contact between animals are salt supplementing points. Therefore, while extensive farming is safer than zero level grazing systems to prevent disease transmission, some of the above situations simulate the dangers of intensive farming in relation to disease transmission (Ayele *et al.*, 2004).

Animal-to-human transmission

Pulmonary TB due to *M. bovis* is more common in rural dwellers, as a result of inhalation of dust particles or bacteria-containing aerosols shed by infected animals, while urban dwellers acquire the infection via the gastrointestinal route and develop EPTB (Daborn *et al.*, 1996). In countries with a relatively high prevalence of BTB in cattle, abattoir and farm workers are the groups most exposed to infection. Current economic and social globalization has created greater opportunities for the spread of zoonotic diseases such as TB. When considering the revival of TB in countries previously declared to be free of the disease, it is worth noting the statement by Grange: 'we are now learning the hard way that none are safe until all are safe' (Grange, 1996).

Human-to-human transmission

Several recent evidence based reports indicate that human-to-human transmission of *M. bovis* may be more important than originally believed. During the mid-1990s, multiple nosocomial outbreaks of multidrug-resistant *M. bovis* were documented in Europe (Charles *et al.*, 2006). All of the patients involved had HIV infected; susceptibility of HIV-infected individuals to *M. bovis* has also been documented in other settings (Lobue *et al.*, 2004). These findings are of particular concern because of the high rate of HIV infection in places like Africa, where *M. bovis* is endemic in several countries. In nations with high rates of *M. bovis* infection in cattle and high

rates of HIV infection in humans, the potential exists for transmission of *M. bovis* from cattle to immunosuppressed humans, followed by human-to-human spread among immunosuppressed (Charles *et al.*, 2006).

Human-to-animal

Grange and Yates (1994) reported that farm workers urinating in cowsheds may represent a source of infection for animals. An analogous situation is thought to occur in rural Africa, where patients with genitourinary TB may urinate on pasture: animals craving salt preferentially graze on this grass and may succumb to infection.

2.6. Diagnostic tests for *Mycobacterium bovis*

The diagnosis of BTB in live animals mainly depends on clinical manifestations of the disease and skin testing. After death, it is diagnosed by post-mortem examination and then confirmed in the laboratory by histopathological, bacteriological techniques and also molecular techniques (Jahans and Worth, 2006) (Figure 2).

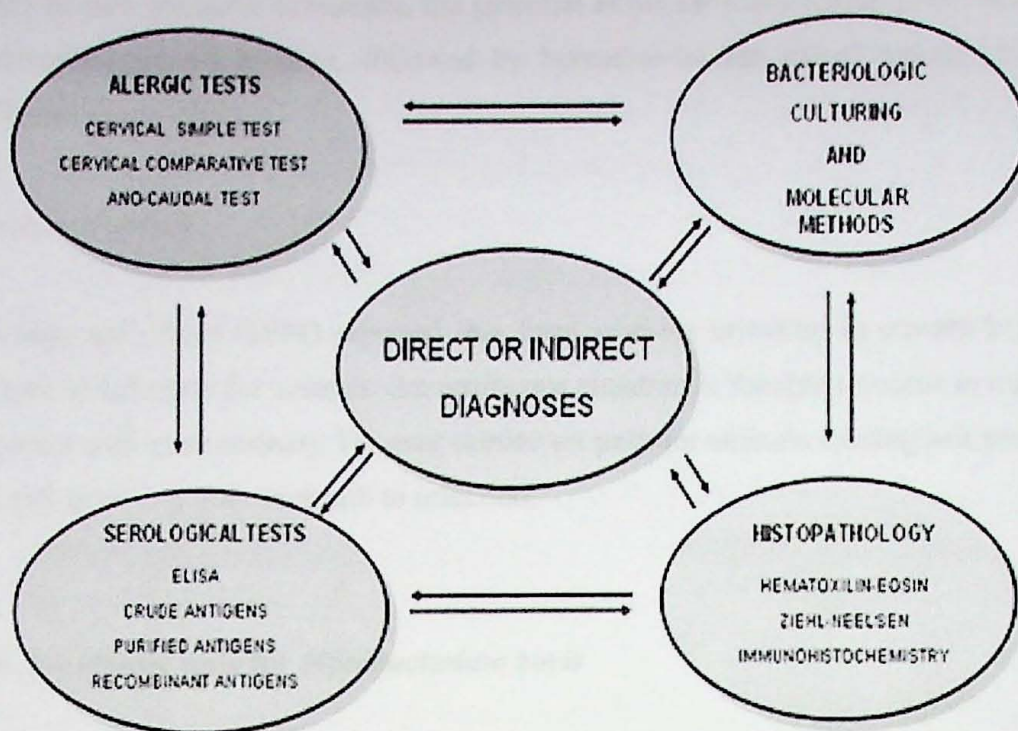


Figure 2: Interactions among various methods currently used for the diagnosis of BTB

Source: Adapted from Medeiros *et al.* (2010)

2.6.1. Clinical examination and necropsy

Bovine tuberculosis is tentatively diagnosed by its clinical symptoms (Seifert, 1996). Clinical signs such as chronic debilitation, moist cough, low-grade fever and enlargement of local lymph nodes may suggest infection with tuberculosis (OIE, 2010). Nevertheless, other techniques are required to confirm the diagnosis. During necropsy, the lymph nodes, especially those associated with the head, thorax and abdomen are closely examined. Careful examination of as few as 6 pairs of lymph nodes, the lungs and the mesenteric lymph nodes can result in 95% of cattle with macroscopic lesions being identified (Corner, 1994).

2.6.2. Tuberculin skin test

Tuberculin skin test is perhaps the first and best-known test for TB diagnosis. Although imperfect, the tuberculin test has not yet been replaced by any other more accurate or satisfactory method (de la Rua-Domenech *et al.*, 2006).

Some of the main deficiencies of the test are its inability to differentiate between distinct species of the *M. tuberculosis* complex, failure to distinguish between latent stages of infection and disease, and failure to distinguish vaccinated and infected individuals (de la Rua-Domenech *et al.*, 2006). In addition, anergy, exposure to environmental mycobacteria and operator errors can lead to false results. Two recent studies also suggested that the cut-off generally used for positive test interpretation (>4 mm) may not be applicable for at least some countries in sub-Saharan Africa and that a cut-off >2 mm could be more appropriate in some settings (Ameni *et al.*, 2008; Ngandolo *et al.*, 2009).

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, 2010). As study showed by Corner (1994) 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. 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, 1994). Post-mortem examination should be supported by histopathology and bacteriological examination of lesions (OIE, 2010) for definitive diagnosis of BTB.

2.6.4. Histopathology

Tissues are fixed in 10% neutral buffer formalin (NBF) for 7 days, dehydrated in graded ethanol solutions and embedded in paraffin wax. Histological sections (4µm) are stained with haematoxylin and eosin (HE) and by the Ziehl-Neelsen (ZN) method. Microscopically, tuberculous granulomas in LNs were graded into four categories and the number of lesions at each stage was recorded. Stage I/II granulomas are early non-necrotic lesions, stage III granulomas consisted of an infected core surrounded by multiple concentric layers of macrophages and fibrous tissue and stage IV lesions had central caseous necrosis. The microscopic area of each histological section affected by tuberculous granulomas and the relative number of acid fast bacilli (AFB) found in each ZN-stained section are estimation (Wangoo *et al.*, 2005).

2.6.5. 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. 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 (WHO, 1994; Wood and Rothel, 1994; OIE, 2010).

2.6.6. The interferon- gamma test

The more recently developed Bovigam test for cattle and its analog in humans, the QuantiFERON TB-test, detect the production of interferon-gamma (in vitro) stimulated blood samples. Applied in both standard commercial and customized test formats, this assay has contributed significantly to the improved detection of early *M. bovis* infection in cattle as well as an increasing number of wildlife species (e.g. non-human primates, cervids) (Waters *et al.*, 2008).

Recent improvements of the test include the use of two *M. tuberculosis* complex specific antigens, ESAT-6 and CFP-10, which has resulted in increased test specificity (Buddle *et al.*, 2009). In contrast to the tuberculin skin test, the interferon-gamma test could also be used to differentiate between infected and vaccinated individuals (Barth *et al.*, 2008). However, at late disease stages, the cell-mediated immune response can wane as opposed to a generally increasing humoral immune response, and the tuberculin skin test or Bovigam tests can therefore, give false negative results (de la Rua-Domenech *et al.*, 2006).

2.6.7. Serological tests

Therefore, in developing countries, serological tests, which are based on the detection of the humoral immune response, may be of particular use. Unfortunately, to date, no satisfactory serological test is available. Some of the problems related to the development of serological tests for tuberculosis diagnosis include the observed highly variable antibody responses between individuals to mycobacterial antigens and antigenic variation between mycobacterial strains. However, a recently developed lateral flow test that is based on the detection of more than one antigen has shown promising results for tuberculosis diagnosis in certain animal species (e.g. in elephant), (Greenwald *et al.*, 2009), although it may not be suitable for others, such as buffaloes (Michel and Simoes, 2009). Another recently developed serological test for animals is based on antibody detection using fluorescence polarization but has shown variable effectiveness in different settings (Ngandolo *et al.*, 2009).

2.6.8. Bacteriology

Microscopic examination

Examination of smears prepared from sputum, tissue, milk, urine and other samples taken from suspected individuals are stained with Ziehl Neelsen staining method and organisms appear as red due to their acid fast property (Quinn *et al.*, 1999).

Culture

The definitive diagnosis of TB depends on the isolation and identification of the mycobacteria in specimens taken from suspected individual. The main factors that influence the success of primary isolation of *M. bovis* from clinical specimen are the culture media, the decontamination procedure and incubation condition (Corner, 1994). Before inoculation the samples are digested, decontaminated and concentrated. The commonly used decontaminants for *M. bovis* preparation are hexadecylpyridinium chloride (HPC) at both 0.075% w/v and 0.75% w/v, benzalkonium chloride (Zephiran) 0.25% w/v, oxalic acid 5% w/v, NaOH 2% and 100% sodium hypochlorite (OIE, 2010). Corner (1994) indicated that growth on the agar base 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 stonebrink's medium. It has been demonstrated that using modified 7H11 agar medium, from infected tissue can be isolated within 3 weeks. However, the agar medium is highly liable to contamination even after decontamination of specimen, so egg base media is preferable for mycobacterial growth (Corner, 1994).

2.6.9. Molecular diagnostic techniques

Mycobacteria are present in various body fluids and mycobacterial DNA can be isolated from secretions and excretions: milk, blood, sputum and bronchoalveolar lavages, cerebrospinal fluid, semen (Ayele *et al.*, 2004) and faeces (Garrido *et al.*, 2000) during pathological processes.

The basis of all molecular genotyping techniques relies on the acquisition of genetic mutations over time within specified regions of the bacterial genome, thus creating unique profiles between isolates. Although two bacterial strains of the same species have virtually identical genomes, differences will inevitably occur at a few loci. These differences may be small, such as the alteration of a single nucleotide that may either create or remove a specific restriction enzyme site, or larger changes may occur leading to the replication or deletion of DNA fragments hundreds of base pairs in size (Figure 3). Therefore, epidemiologically related strains will show identical or very similar genetic profiles, whereas unrelated clinical isolates will have divergent patterns. Restriction fragment length polymorphism (RFLP) using the insertion sequence IS6110

is the preferred typing method for *Mycobacterium tuberculosis*. The most molecular typing method applied to *M. bovis* in developed countries is spoligotyping (Van Soolingen *et al.*, 1994).

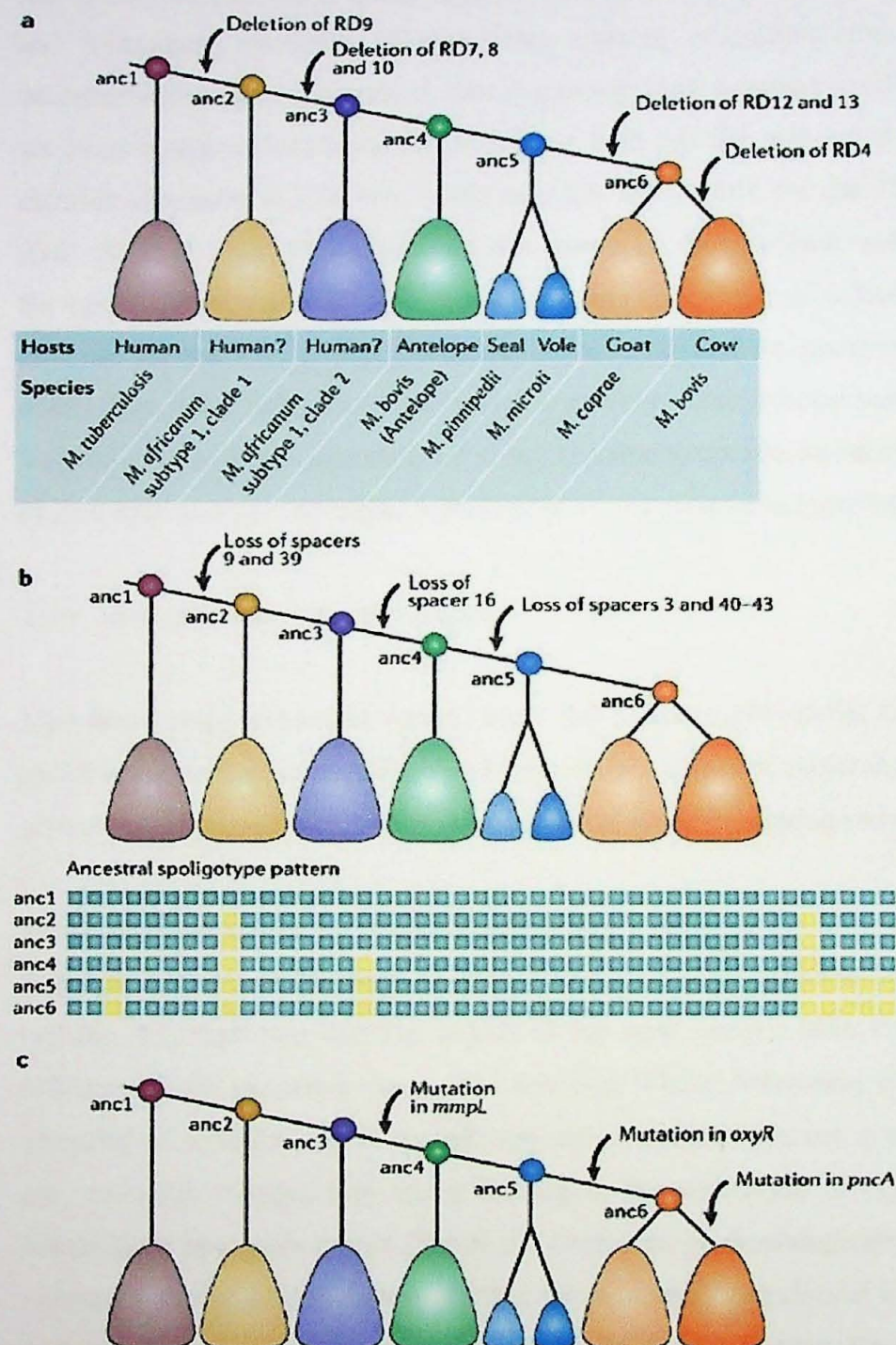


Figure 3: The phylogenetically informative mutations in the lineage leading to *Mycobacterium bovis*
Source: (Smith *et al.*, 2006)

2.7. Public health importance of bovine tuberculosis

Tuberculosis, 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). Bovine tuberculosis is an infectious disease caused by *M. bovis*, a member of the *Mycobacterium tuberculosis* complex, which also comprises the closely related *M. tuberculosis*, the major causative agent of human TB (Van Soolingen *et al.*, 1994). Around 9 million new cases of TB and 2 million deaths are reported worldwide annually (CDC, 2007), with sub-Saharan Africa displaying the highest annual risk of infection with TB, probably catalysed by the HIV/AIDS pandemic (Corbett *et al.*, 2003). It has been estimated that *M. bovis* accounts globally for 3.1% of all human TB cases (2.1% of all pulmonary and 9.4% of all EPTB cases) (Cosivi *et al.*, 1998). However, the extent of *M. bovis* involvement in the global TB burden in Africa is still largely unknown. This can be partly explained by the fact that in humans, TB due to *M. bovis* is indistinguishable from that due to *M. tuberculosis* in terms of clinical signs, radiological and pathological features (Grange, 2001). In addition, most laboratories in sub-Saharan Africa do not have the capability to differentiate *M. bovis* from *M. tuberculosis* (Ayele *et al.*, 2004).

More detailed data including strain characterization have recently been presented and confirm that the occurrence of *M. bovis* in the human population is a persistent, though insufficiently quantified feature in developing countries. The isolation rate of *M. bovis* from symptomatic human patients in specific studies was 13.8% in Mexico (Pe' rez-Guerrero *et al.*, 2008), 6.9% in Uganda (Oloya *et al.*, 2008), 5% in Nigeria (Cadmus *et al.*, 2006), 0.5% in Taiwan (Jou *et al.*, 2008) and between 0 and 2.5% in ten Latin American countries (de Kantor *et al.*, 2008).

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 (Grange and Yates, 1994; Cosivi *et al.*, 1998). 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).

According to Hassanain *et al.* (2009), 30.5% and 40% of the examined dairy cattle and farm workers respectively in Egypt, were infected with BTB and they indicated that presence of *M. bovis* in milk represented a major source of infection to human and other animals.

A case control study was conducted to determine the prevalence of BTB in cattle in the central Ethiopia relative to the TB status of their owners. The prevalence of TB was higher in cattle owned by farmers with active TB (24.3%) than in cattle owned by farmers who did not have active TB (8.6%), which could suggest possible transmission of *Mycobacterium* species between cattle and their owners. Furthermore, cattle owners who consumed raw milk were at greater risk of having active tuberculosis than those who consumed boiled milk (Regassa *et al.*, 2008). Defar *et al.* (2009) indicated that a rampant occurrence of BTB and its potential risk to owners and consumers of raw animal products from infected animals.

2.8. Bovine tuberculosis in Ethiopia

In Ethiopia, BTB is considered as endemic based on abattoir inspection (Bogale *et al.*, 2004) and tuberculin test survey (Ameni, 1996; Bogale *et al.*, 2001). Studies have indicated that BTB is more prevalent in intensive dairy farms than in smallholder farm regions (Ameni, 1996) with a prevalence ranging from 3.4% in small holder production system to 50% in intensive production system (Kiros, 1998; Bogale *et al.*, 2001; Ameni *et al.*, 2001).

According to Shitaye *et al.* (2007), tuberculous lesions were found in 34 (3.5%) animals by the meat inspection surveillance of 984 cattle. Histopathologically, granulomatous inflammation was evident in 3 (8.8%) animals with tuberculous lesions. A highly sensitive PCR insertion sequence 6110 (IS6110) was positive in 4 of 34 (11.8%) animals with tuberculous lesions and in 1 (2.9%)

of animal without lesions. BTB is a major economic problem and a potential public health risk in Ethiopian (Ameni *et al.*, 2008).

The prevalence of BTB infection in cattle was investigated in extensive and intensive production systems in three districts of northwestern Ethiopia by comparative intradermal tuberculin test as 9.7% whereas the non-specific infection prevalence was 10.8% (Fetene and Kebede, 2009). BTB is widely spread throughout Ethiopia, albeit at a low prevalence (4.7%), and provides underpinning evidence for public health policy formulation (Berg *et al.*, 2009). One study by Biffa *et al.* (2009), demonstrated that high prevalence of TB in Ethiopian slaughter cattle and this could infer to similar scenario in a reference cattle population in the country. Another study by the same investigator (2010b) provided that molecular evidence for the wide spread distribution of *M. bovis* in the cattle population in Ethiopia with six new spoligotypes which were subsequently registered in the database with international identification codes of SB1517, SB1518, SB1519, SB1520, SB1521 and SB1522. It also demonstrated a relatively high degree of genetic polymorphism of the isolates (Biffa *et al.*, 2010b). The individual overall prevalence of cattle BTB in rural livestock production systems, from four regions in Ethiopia was 3%; Family members diagnosed with TB or showing clinical signs of EPTB were (19%) (Tschopp *et al.*, 2009). High prevalence of BTB in Addis Ababa City dairy farms and the identification of three *M. bovis* spoligotype pattern suggest a possible presence of more strains in the region (Tsegaye *et al.*, 2010).

2.9. 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.*, 2000). 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).

In Argentina, the annual loss due to BTB is approximately US\$ 63 million (Cosivi *et al.*, 1998). The socio-economic impact of BTB to the agriculture and health sector in Turkey has been estimated at between 15 and 59 million US\$ per year (Barwinek and Taylor, 1996). In the UK, where badger and other wildlife such as deer remain an important source of infection for livestock, approximately £100 million is spent annually in efforts to control the disease. However, in Africa, the economic losses associated with livestock infected with BTB have not been examined sufficiently or have not been studied at all (Ayele *et al.*, 2004).

Moreover, in Ethiopia, the economic impact of BTB on cattle productivity, BTB control programmes and other related economic effects of the disease are not yet well documented or studied. Few abattoir meat inspection surveillances have shown the condemnation rate of the total or partial carcass and organs (Shitaye *et al.*, 2007). With this respect, Abel (1989) reported that out of 29 956 slaughtered cattle in Dire-Dawa city abattoir, a total of 31.2% and partial of 16.4% condemnation rates that may result in economic losses significantly. Gezahegne (1991) demonstrated that from 1.2 million slaughtered cattle in eight export abattoirs had an estimated cost of more than 600 000 ETB (300 000 USD) during a respective time, resulted due to condemned carcasses and organs. Asseged *et al.* (2004) demonstrated that, based on the ten years retrospective analysis of the detection of BTB lesions in the Addis Ababa abattoir, there was a cause of 0.024% for whole carcass condemnation. Recently, Shitaye *et al.* (2006) indicated that, in both Addis Ababa and Debre-Zeit abattoirs tuberculous lesions that, causes condemnation of carcasses and/or organs have also been found to be highly significant economically.

2.10. Prevention and control measures

Bovine tuberculosis can be eliminated from a country or region by implementing a test-and-slaughter policy, if no other reservoir host of infection exists. While the test-and-slaughter policy

is likely to remain the backbone of national elimination BTB programs, the policy has numerous constraints in developing countries. Alternative strategies (e.g., programs based on slaughterhouse surveillance and traceback of tuberculous animals to herds of origin) may be technically and economically more appropriate in these countries (Good, 2006). Measures to prevent transmission of infection should be the primary objective to be achieved with trained public health personnel, public education, and proper hygienic practices. Test-and-slaughter programs may be feasible and appropriate in areas with low BTB prevalence, and effective control of animal movement. Although not usually considered relevant to elimination programs in livestock, vaccination of animals against TB would be a viable strategy in two disease control situations: in domesticated animals in developing countries and in wildlife and feral reservoirs of disease in industrialized countries where test-and-slaughter programs have failed to achieve elimination of the disease (Cosivi *et al.*, 1998).

3. MATERIALS AND METHODS

3.1. Description of the study site and population

3.1.1. Study site

The study was conducted in Setit and Kafta Humera districts, Western Zone of Tigray region, North-west of Ethiopia (Figure 4). The western zone is one of the seven zones in the Tigray regional state. It has four districts, namely; Setit Humera, Kafta Humera, Welkait and Tsegede. The study area (Setit and Kafta Humera) is located 650 kms far away from Mekelle, the capital city of Tigray, 1433 kms from Addis Ababa. Throughout the zone, livestock agriculture is the predominant economic activity with about 95% of the total population engaged directly or indirectly on it. The geographical location of the research site is $13^{\circ}42'$ to $14^{\circ}28'$ North latitude and $36^{\circ}23'$ to $37^{\circ}31'$ East longitude. Its elevation is 560 to 2800 m above sea level (a.s.l). The study area is bordered north by Eritrea, South by Welkait district and Amhara regional state, East by Tahtai Adiabo district, west by Sudan. It is the transit point for cross border trade and traffic as well as live animals export to Sudan and Egypt.

The study area has agro-ecological zones that are hot to warm semiarid lowland. The rainfall in the area is monomodal falling between the months May and September. Over 65% of rainfall is received in the months of July and August. The mean monthly minimum and maximum rainfall ranges from 0 mm in January, February and March to 245.4 mm in August. The mean monthly maximum temperature remains high throughout the year ranging from 18.5°C in January to 42.4°C in March, in the semiarid lowlands. The relative humidity is higher during the rainy months and lowest at the tail end of the dry season in April (NMA, 2008). The study site has an estimated total human population of 71,415, of whom 33,282 were men and 38,133 were women; 28,900 or 40.47% of its population are urban dwellers. With an estimated area of 6,196.56 square kilometers, the study site has an estimated population density of 11.5 people per square kilometer. The total cattle populations in the western zone are estimated as 702,119. The main cattle breed raised are local Arado and Barka (Begait) cattle; the later is the dominant breed of cattle in this zone (BOANRD, 2003).

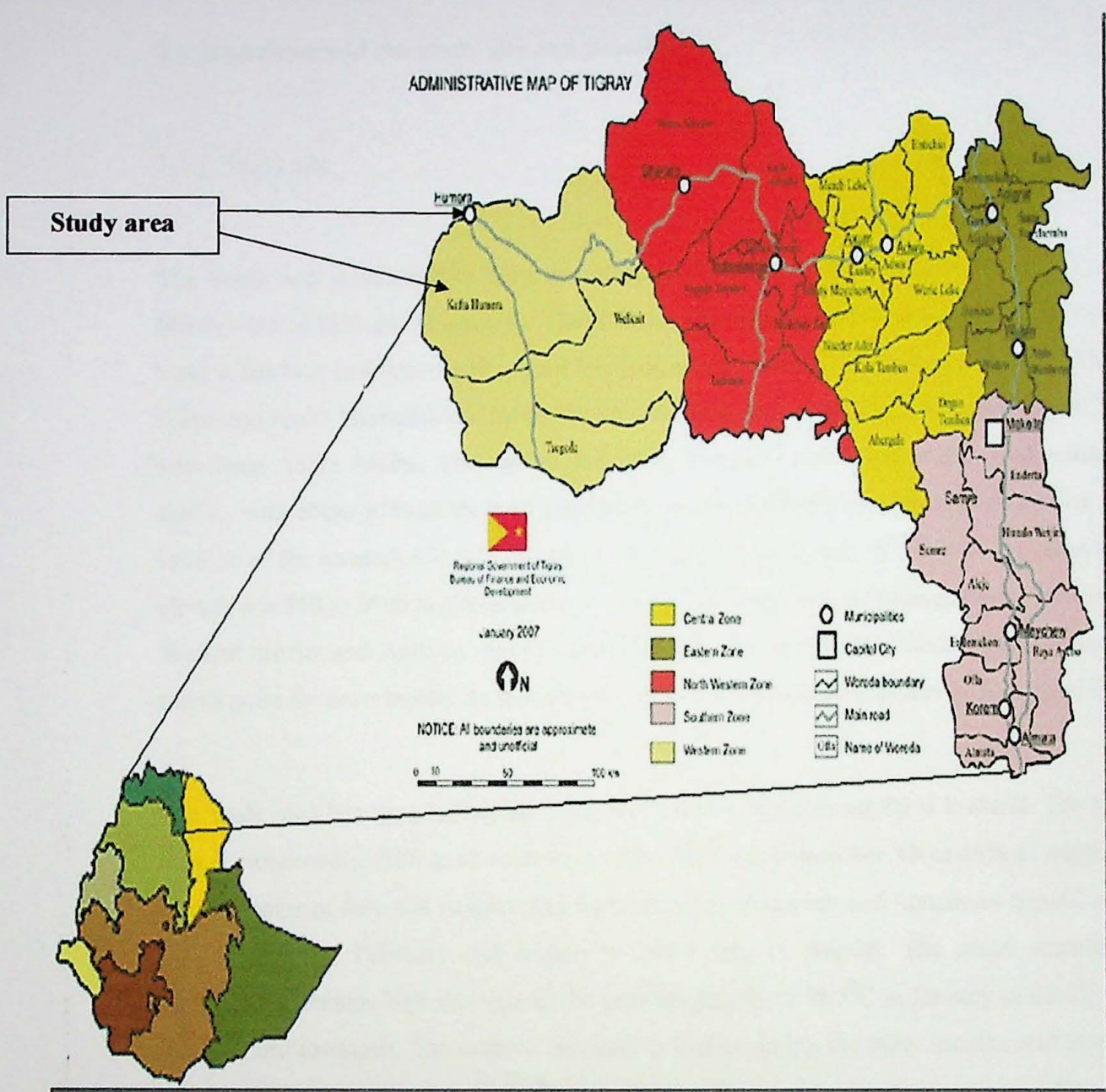


Figure 4: Location of the study area

3.1.2. Study population

The study animals for skin test were those cattle which were originated from Setit and Kafta Humera woredas (western zone of Tigray). Extensive type of cattle production system was dominated in the study area. But a practice, called “stall feeding” was started in a few house holds; Animals were tied and kept indoors in an open-air barn with only over head shelter and supplemented with feed and water without contact to other animals. In this study; however, animals managed in this way were grouped as “semi intensive” production. Animals are mainly composed of Barka breed cattle which depend for feed on grazing at the field.

Cattle that are found in Setit Humera woreda were kept around the town. They traveled short distance for grazing and watering purpose; they came back to their home in the evening. There is mixing with other herds or cattle at grazing field and watering point, and with other species (goat and sheep around their home) but semi intensive production was started in a few house holds (cross bred, Barka X Holesian Fresian).

Cattle that are found in Kafta Humera woreda (rural) are slightly different in their management from cattle that are found in Setit Humera woreda (urban). Most of them are kept far from human residence. They traveled long distance and most of them did not come back to their home but some milking cows come back to their home for milking purpose. There is mixing of herds at grazing field and watering point in the day time, and a single herd uses communal barn in the night. As information was gained from the owners/herders, animals that were found in this study area had extensive contact with wild animals at grazing site and watering points.

Regrading Cattle included for abattoir survey were originated both from the Setit and Kafta Humera woredas, and also adjacent woredas of the study area including Tsegede and Sheraro. Averagically the numbers of animals slaughtered per day were 14 (8–20), and the breeds included in abattoir survey were mainly Barka and Medenes as evaluated phenotypically.

3.2. Study design

A cross-sectional study was carried out from September, 2010 to May, 2011 using comparative intradermal tuberculin test on 484 cattle, and abattoir based survey on 582 slaughtered cattle. Collection of milk samples from tuberculin test positive milking cows and suspected TB lesion tissue samples from slaughtered cattle at municipal abattoir were conducted for mycobacteriological culture to isolate the causative agent and further molecular characterization of the agent. A questionnaire survey was administered to cattle owners/herder to assess associated epidemiological factors and public health issues.

3.2.1. Sampling method and Sample size determination

Comparative intradermal tuberculin test

The Districts (Setit and Kafta Humera) were selected purposively due to Barka breed cattle concentration at the sites which were not studied before. The Districts have a total of 23 kebeles; (Kafta Humera 21 kebeles, and Setit Humera 2 kebeles). It was difficult to create sampling frame for herds and households in the study areas because reliable demographic data were not available. Therefore, by the help of the concerned bodies, like the veterinarian, and other interested farmers, some herds and house holds were recorded from the six randomly selected kebeles (25%); namely Adebay, Idris, Rawian and Maykadra from Kafta Humera district, and kebele one and two from Setit Humera district. However, animals found in the six kebeles were grouped in to two groups by location (Setit Humera and Kafta Humera) in this study because of similar management system was observed among kebeles found in each district. Kebeles were considered as primary units, while herds and individual animal were secondary and tertiary units, respectively. A herd was defined in this study as groups of large number of animals belonging to the same cattle camp/group, and those identified as communal grazing and watering points only in the day time for urban (Setit Humera) residence, and used communal camp/barn in the night also for rural (Kafta Humera) residence.

The sampling method designed was a multistage cluster sampling method. However, this was not done, because between-cluster variation in the prevalence of BTB was not determined earlier in

the vicinity. As a result, simple random sampling was assumed for calculation of sample size. If the simple random sample formula is applied, it is likely to produce conservative estimates. Applying the simple random sampling formula and then, for all types of cluster sampling, multiplying the estimated sample size by between 2 and 4 (Thrusfield, 2005). The expected prevalence of BTB was 50% as there was no study conducted on prevalence rate of BTB in the study area, with 95% confidence level, and 5% desired absolute precision (d^2). Accordingly, the sample size was determined to be 384 for simple random sampling, when converted to cluster sampling 768 (2x384) animals were assumed to be tested. But due to limitation of PPD availability, sample size was reduced to only 484 animals. Thirty one herds (20%) from the 6 kebeles were selected randomly and 15% of the individual animal was included.

Abattoir survey

In the abattoir-based survey, the sampling method involved was census type as all the animals slaughtered during the study period (from December to February) were sampled for the determination of the magnitude of BTB in the study area, and try to include enough cases since the research also aimed at characterization of isolates. Accordingly, 582 animals were inspected. The sampling procedure took on complete enumeration of all slaughtered animals and TB suspected patients in the duration of the field study on individual animals which was depending on the number of animals slaughter on each day.

3.3. Study Methodology

3.3.1. Questionnaire survey

Structured questionnaires were prepared and administered to cattle owners/herders mainly by the principal researcher who is a native speaker of the local language spoken in the study areas. One hundred and fifty seven interviewee were included, and the questionnaire involved in surveying animal tuberculosis and this included gathering cattle inventory, cattle grazing systems, herd size, livestock-wildlife contact and contact with other cattle, habit of drinking milk, species of animals owned and also to assess the previous history of human tuberculosis in their household (Annex-1).

3.3.2. Comparative intradermal tuberculin test

The comparative intradermal tuberculin test (CIDT) was conducted on 484 dairy cattle. Management and other important data were collected (Annex-2). Young stocks ≤ 6 months of age, and cows in late pregnancy and those recently calved excluded 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.*, 2000).

Body condition scoring

All animals used in the study were thoroughly observed for their body condition. The body condition of local Zebu study animal was scored using the guidelines established by Nicholson and Butterworth (1986) which is more elaborated in (Annex-3B). Accordingly, on the basis of observation of anatomical parts such as vertebral column, ribs, spines, tip of tail, etc., the study animals were classified as lean (L^- , L and L^+) (score, 1 to 3), medium (M^- , M and M^+) (4 to 6), or fat (F^- , F and F^+) (7 to 9).

Estimation of the age by examination of the teeth

Age of cattle in this study was determined based on teeth eruption and wear of the teeth. Eruption times of the permanent incisors are primarily used to estimate age up to 5 years in cattle which is more elaborated in (Annex-4). Signs of wear are much less reliable than eruption because wear is largely determined by nutrition (for instance drought or sandy grazing will increase the wear on teeth), and macroscopic age-related dental features are scarce (dental stars) or absent (cups and marks) (Kahn *et al.*, 2005).

Test procedures

At the midcervical region of a test animal, two areas (12-15 cms apart) were shaved, cleansed with a swab of 70% alcohol and dried. A fold of skin was pinched with tips of fingers; its thickness was measured with graduate caliper and recorded. A short needle, bevel edge outwards and is inserted obliquely and 0.1 ml (25,000 IU/ml, 0.5 mg / ml, *M. avium subspecies avian*, strain D4ER, Lelystand Biologicals BV, Lelystand, the Netherlands) avian tuberculin was injected into the skin intra-dermally in the upper site. Similarly, fold of skin was pinched, its thickness measured, and 0.1 ml (20,000 IU/ml, 0.5 mg / ml, *M. bovis*, strain ANS, Lelystand Biologicals BV, Lelystand, the Netherlands) bovine tuberculin was injected intradermally in the lower site (12-15 cms apart). Both injection sites were encircled with indelible ink of different colors. A correct injection is confirmed by palpating a small pea-like swelling at each site of injection. The results were recorded after 72 hours post-inoculation by measuring the thickness of skin fold. Any gross / visible changes at the site of the injection was also recorded and interpreted according to guidelines of OIE (2010).

Interpretation

The difference in skin thickness before and after injection at both sites was used for the interpretation of results. When differences in skin thickness were greater at the site of injection for avian PPD than for bovine PPD, then the animal was considered positive for *M. avium* or other atypical mycobacteria; but when the change in skin thickness was increased at both

injection sites, difference in thickness of the two sites was considered and results interpreted according to OIE (2010) as follows:

A reaction was considered to be Positive if the increase in skin thickness at the bovine site of injection was more than 4 mm greater than the avian reaction shown at the site of the avian injection. The reaction was considered to be inconclusive (doubtful) if the increase in skin thickness at the bovine site of injection was from 1 to 4 mm greater than the avian reaction. The reaction was considered to be negative if the increase in skin thickness at the bovine site of injection was less than or equal to the increase in the skin reaction at the avian site of injection. If there was diffuse or extensive oedema, exudation, necrosis, pain or inflammation of the lymphatic ducts in that region or of the lymph nodes regardless of measurement the result was considered as positive. However, a herd was considered as positive when the herd had at least one positive reactor animal to bovine tuberculin.

3.3.3. Abattoir survey

Antemortem Examination

This abattoir based survey included 582 cattle, and animal identification and epidemiological information were carried out during antemortem inspection (Annex-5). Body condition score (Annex-3B) and age determination (Annex-4) were done as for the intradermal tuberculin test.

Detailed post-mortem examination

Inspection of each of the carcass was undertaken in detail according to Ameni *et al.* (2007) and Biffa *et al.* (2010a). In the slaughterhouse, carcasses and offals were identified using the butcher number and affected organs were obtained during carcass inspection. All the lobes of the lung and associated lymph nodes were inspected, palpated and incision was made on the suspected organs. The animal was classified as lesioned (infected) when tuberculous lesion was found, and if not as non lesioned (not infected). The lymph nodes and other tissue specimens such as the lungs, liver, and kidneys were examined in detail during post-mortem in the abattoir under a

bright light source. The lobes of the two lungs, including the left apical, left cardiac, left diaphragmatic, right apical, right cardiac, right diaphragmatic and right accessory lobes, were inspected and palpated externally. Then, each lobe was sectioned into about 2-cm-thick slices to facilitate the detection of lesions. Similarly, lymph nodes (Annex-5) were sliced into thin sections (about 2 mm thick) and were inspected for the presence of visible lesions.

3.3.4. Sample collection and transportation

For laboratory investigations, samples were aseptically collected into sterile universal bottles both from tuberculin reactor animals (for milk sample) and slaughtered animals (for tissue samples). Milk samples (50 ml) were taken from all tuberculin positive milking cows aseptically at the end of milking using a sterile disposable universal bottle and kept at -20°C (frozen) until they were transported. Tissues containing tuberculous lesions were collected from slaughtered animals by carefully removed from the carcass and placed in a 50 ml capacity of universal bottle with screw caps containing 5 ml of sterile 0.85% saline water and kept at -20°C (frozen) until they were transported. During this study period, 484 cattle were tested using comparative intradermal tuberculin test, and 582 slaughtered animals were examined and inspected thoroughly; Accordingly, 7 milk samples from PPD positive milking cows and 34 suggestive TB lesion tissues samples were collected, and transported kept in icebox with solid ice packs to keep the cold chain to ALIPB, Addis Ababa for bacteriological examination and molecular typing.

3.3.5. Mycobacteriological culture

Using the procedure documented in OIE (2010), all specimens collected from slaughtered cattle and milk from bovine PPD positive reactor milking cows were processed and prepared for mycobacterial culture at the ALIPB in a biological safety cabinet.

Culture for tissue samples

Thirty four tissue samples with Suggestive TB lesions were first ground (homogenised) manually using a mortar and pestle followed by decontamination with an alkali (4% sodium hydroxide). The alkali mixture was shaken for 10 minutes and centrifuged at 1500 revolution per minute (rpm) for 15 minutes at room temperature then neutralised with an acid (10% HCl) using one drop of phenol red indicator for the sediment after the supernatant was removed. For primary isolation, about 5ml of the sediment of each sample was inoculated on to two solid egg-based Lowenstein Jensen (LJ) media with either pyruvate or glycerol. Cultures were incubated for 8–12 weeks at 37°C without CO₂. Cultures were examined for macroscopic growth at weekly intervals during the incubation period. The colonies were then collected and heat killed in water bath with a temperature of 80°C for 45 minutes and stored at -20°C until molecular typing carried out for further identification and characterization.

Culture for milk samples

About 50 ml of each of the 7 milk samples were collected from tuberculin positive milking cows. Then 7 ml of each sample was centrifuged at 1500 rpm for 15 minutes at room temperature. The cream was removed with a sterile spatula, the supernatant was taken off and the sediment was decontaminated with 4% NaOH (1:1). Again the mixture was centrifuged at 1500 rpm for 15 minutes at room temperature. Then it was processed with similar procedures described in culture for tissue samples.

3.3.6. Molecular characterization of Mycobacterial Isolates

Multiplex Polymerase chain reaction (m-PCR)

Thirteen (1 from milk and 12 from tissues samples) culture isolates from cattle were subjected to m-PCR, using the procedure described by Wilton and Cousins (1992) was followed. This analysis differentiates genus *Mycobacterium* from other bacteria and within *Mycobacterium* genus it differentiates *M. tuberculosis* complex from *M. avium* and other mycobacterial species. Heat killed samples were used as source of DNA template. DNA amplifications was done in

thermocycler with 20 µl reaction volumes consisting: 5 µl of genomic DNA as a sample template, 8 µl HotstarTaqMasterMix (MgCL₂, dNTP, Taq polymerase and PCR buffer) (Qiagen, USA) for each sample, 0.3 µl of forward and reverse primers per each sample and 5.2 µl per sample of Qiagen water. The primers used were MYCGEN-F, 5'AGA GTT TGA TCC TGG CTC AG 3', (35 ng/µl); MYCGEN-R, 5'-TGC ACA CAG GCC ACA AGG GA 3', (35 ng/µl); MYCAV-R, 5'-ACC AGA AGA CAT GCG TCT TG 3', (35 ng/µl); MYCINT-F, 5'-CCT TTA GGC GCA TGT CTT TA 3', (75 ng/µl); TB1-F, 5'-GAA CAA TCC GGA GTT GAC AA 3', (20 ng/µl); TB1-R, 5'-AGC ACG CTG TCA ATC ATG TA 3', (20 ng/µl). The PCR amplification was carried out using Thermal Cycler (VWR Thermocycler, 732-1200, Leicestershire, UK) based on the following programme: for 10 minutes at 95°C; 35 cycles of 1 minute at 95°C, 1 minute at 61°C, and 1.5 minutes at 72°C; and finally for 10 minutes at 72°C. *M. avium* and *M. bovis* strain 2122/97 were used as positive controls while H₂O Qiagen, was as a negative control. The PCR products were electrophoresed in 1% agarose gel in 10x TAE running buffer. Ethidium bromide at a concentration of 0.5 µg/ml was added to the melted agarose to stain the DNA; 100bp DNA ladder was used as a reference to DNA size determination; PCR products were mixed with orange 6x loading dye so as to load to agarose gel wells. All members of the genus *Mycobacterium* produce a band of 1030bp after gel electrophoresis. On the other hand, *M. avium* or subspecies such as *M. avium subsp. Paratuberculosis*, *M. intracellulae* and members of *M. tuberculosis* complex produce band sizes of, 180bp, 850bp and 372bp, respectively.

4. DATA ANALYSIS

The data collected from the study areas were entered into MS Excel spread sheets and analyzed using MS Excel STATA (11.0) statistical software. The prevalence of TB computed as the number of positive reactor animals to bovine tuberculin divided by the total number of animals sampled and multiplied by 100. The prevalence rate of TB was computed and analyzed at different levels such as age, groups, sex, areas, body condition score and other relevant risk factors. Categorical data analyzed first using pearson chi-square (χ^2) statistics as a screening process. For significant variables generalized linear model (logistic regression) was used to evaluate the strength of the association. A statistical significant association between variables is said to exist if the computed p-value<0.05 and the 95% confidence interval for odds ratios (OR) does not include 1.

5. RESULTS

5.1. Comparative intradermal tuberculin test

Out of the total 484 cattle subjected to CIDT test for the presence of BTB, the individual animal prevalence was 6.6% (95% CI, 4.4–8.8) and 8.7% (95% CI, 6.2–11.2) at cut-offs greater than 4 mm and 2 mm, respectively. Considering doubtful reaction as a positive, the overall average prevalence is 9.7% (95% CI, 7.1–12.4). Out of 484 animals 4 animals were positive for avian PPD (0.83%). The remaining animals were negative for bovine and avian PPD positivity (Table 2). Herd prevalence was obtained as 61.3% (95% CI, 43.1–79.5) at cut-off >4 mm and 77.4% (95% CI, 61.8–93.0) at cut-off >2 mm for bovine tuberculin positivity. The association between age, sex, reproductive status, location, production system, cattle-wild animals contact, herd size, breeds and body condition score of the study site with prevalence of BTB for CIDT test positivity is presented in (Tables 3 and 4).

Table 2: Tuberculin test positivity for bovine and avian PPD in each category of animals at cut-offs > 4 mm and > 2 mm

Risk factors	>4 mm cut-off				> 2 mm cut-off			
	BTB positive	Negative	Avian positive	Total	BTB positive	Negative	Avian positive	Total
Age (in years)								
≤2	5	67	1	73	5	67	1	73
2<x<6	9	114	1	124	12	111	1	124
6<x≤10	16	156	0	172	19	153	0	172
>10	2	111	2	115	6	107	2	115
Total	32	448	4	484	42	438	4	484
Sex								
Male	5	40	1	46	6	39	1	46
Female	27	408	3	438	36	399	3	438
Total	32	448	4	484	42	438	4	484

Reproductive status								
Lactating	14	234	0	248	21	227	0	248
Pregnant	7	100	2	109	9	98	2	109
Dry	6	74	1	81	6	74	1	81
Total*	27	408	3	438	36	399	3	438
Breed								
Barka	31	420	4	455	39	412	4	455
BarkaXHF	1	28	0	29	3	29	0	29
Total	32	448	4	484	42	438	4	484
BCS								
Good	4	181	1	186	9	176	1	186
Medium Poor	26	214	3	243	29	211	3	243
Total	2	53	0	55	4	51	0	55
	32	448	4	484	42	438	4	484
Location								
Setit Humera	9	174	0	183	13	170	0	183
Kafta Humera	23	274	4	301	29	268	4	301
Total	32	448	4	484	42	438	4	484
Wild animal relation								
Yes	24	278	4	306	31	271	4	306
No	8	170	0	178	11	167	0	178
Total	32	448	4	484	42	438	4	484
Herd size								
≤15	12	175	0	187	15	172	0	187
>15	20	273	4	297	27	266	4	297
Total	32	448	4	484	42	438	4	484
Production								
Extensive	30	415	4	449	38	407	4	449
Semi intensive	2	33	0	35	4	31	0	35
Total	32	448	4	484	42	438	4	484

Table continued

*=Male animals were excluded

Prevalence of intradermal test positivity increased in the age group $6 < x \leq 10$ years, then decreased when animals were older than 10 years at four and two cut-offs but there was no significant association between age and prevalence ($\chi^2=9.2$, $P=0.162$ at cut-off >4 mm; $\chi^2=6.1$, $P=0.410$ at cut-off >2 mm) (Table 3).

The proportion of male animals in comparison to female animals included in this study was small during the study time, and 39.1% of them were less than two years old. The intradermal tuberculin test positivity was higher in male (10.9% at cut-off >4 mm and 13.0% at cut-off >2 mm) than female (6.2% at cut-off >4 mm and 8.2% at cut-off >2 mm) animals despite insignificant association between sex and tuberculin test positivity ($\chi^2=2.7$, $P=0.261$ at cut-off >4 mm; $\chi^2=2.4$, $P=0.299$ at cut-off >2 mm) was observed.

Even though cows in late pregnancy and those recently calved were excluded in this study for fear of immune dysfunction, pregnant animals earlier than eight months and late lactating animals were tested. Therefore, based on reproductive status animals were grouped into lactating, pregnant and dry cows. Accordingly prevalence of lactating animals was (5.7 %,) less than pregnant animals (6.4%) and dry cows (7.4%) for skin test positivity while dry cows were more positive reactors to bovine tuberculin test at cut-off >4 mm. But at cut-off >2 mm, dry cows were fewer positive reactors to bovine tuberculin (7.4%) than pregnant (8.3%) and lactating (8.4%) animals. Reproductive status and prevalence of BTB were not significantly associated ($\chi^2=4.6$, $P=0.334$ at cut-off >4 mm; $\chi^2=4.3$, $P=0.177$ at cut-off >2 mm) (Table 3).

The predominant breeds that are found in the study areas were Barka breeds. But in a few households the rearing of cross-bred of Holstein Friesian (HF) and Barka was started. Out of 484 cattle tested only 29 (29/484) were cross-bred of Holstein Friesian (HF) and Barka. Though they were incomparable in number with those of Barka breeds, they were tested using intradermal tuberculin test to evaluate BTB positivity in these breeds. Therefore, 3.5% and 10.3% of them were positive for tuberculin test at four and two cutoffs, respectively (Table 3).

Based on body condition, a maximum prevalence was recorded in medium body condition animals in both cutoffs (Figure 5). The association between body condition and prevalence was

analyzed and found to be significant ($\chi^2=14.70$, $P=0.001$ at cut- off >4 mm; $\chi^2=6.8$, $P=0.033$ at cut- off >2 mm) (Table 3).



Figure 5: Prevalence of tuberculin test positivity based on body condition score at cut-offs greater than 4 mm and 2 mm in Setit and Kafta Humera

Table 3: Association of host related risk factors to skin test positivity in cattle in Setit and Kafta Humera

Risk factors	No.tested	>4 mm-cutoff			>2 mm-cutoff		
		Prevalence	χ^2	P-value	Prevalence	χ^2	P-value
Age(yrs.)			9.2	0.162		6.1	0.410
≤ 2	73	5(6.9%)			5(6.9%)		
$2 < x < 6$	124	9(7.3%)			12(9.7%)		
$6 < x \leq 10$	172	16(9.3%)			19(11.1%)		
> 10	115	2(1.7%)			6(5.2%)		
Total	484	32(6.6%)			42(8.7%)		
Sex			2.7	0.261		2.4	0.299
Male	46	5(10.9%)			6(13.0%)		
Female	438	27(6.2%)			36(8.2%)		
Total	484	32(6.6%)			42(8.7%)		
Reproductive status			4.6	0.334		4.3	0.177
Lactating	248	14(5.7%)			21(8.4%)		
Pregnant	109	7(6.4%)			9(8.3%)		
Dry	81	6(7.4%)			6(7.4%)		
Total^a	438	27(6.1%)			36(8.2)		
Breed			0.8	0.679		0.4	0.840
Barka	455	31(6.8%)			39(8.6%)		
BarkaxHF	29	1(3.5%)			3(10.3%)		
Total	484	32(6.6%)			42(8.7%)		
BCS			13.40	0.001*		6.8	0.033*
Good	186	4(2.2%)			9(4.8%)		
Medium	243	26(10.7%)			29(11.9%)		
Poor	55	2(3.6%)			4(7.3%)		
Total	484	32(6.6%)			42(8.7%)		

a= Male animals were excluded

* =Statistically significant

Some of the environmental risk factors that were related to animals in the two locations were assessed and analyzed (Table 4).

The prevalence of tuberculin test positivity was higher (7.8%, 10.1% at cut-offs; >4 mm and >2 mm, respectively) in animals that had an information contact with wild animals than animals that had no contact (4.5%, 6.2% at cut-offs; >4 mm and >2 mm, respectively). But the association was not significant between the prevalence of skin test positivity and cattle-wild animal contact ($\chi^2=9.4$, $P=0.105$ at cut-off >4 mm; $\chi^2=4.7$, $P=0.096$ at cut-off >2 mm). Large number of animals per herd was observed in Kafta Humera than setit Humera. But skin test positivity was not as such different in the two groups with insignificant association between herd size and prevalence of skin test positivity ($\chi^2=2.6$, $P=0.277$ at cut-off >4 mm; $\chi^2=0.3$, $P=0.254$ at cut-off >2 mm) (Table 4).

Animals that were sampled from Kafta Humera for skin test were slightly more positive reactors to bovine tuberculin with a prevalence of 7.6% and 9.6% at cut-offs; >4 mm and >2 mm, respectively than animals that were sampled from Setit Humera with a prevalence of 4.9% (cut-off >4 mm) and 7.1% (cut-off >2 mm). However, the association between location and skin test positivity was not significant at both cut-off points ($\chi^2=3.9$, $P=0.142$ at cut-off >4 mm; $\chi^2=3.9$, $P=0.177$ at cut-off >2 mm).

Table 4: Association of environmental related risk factors to skin test positivity in cattle in Setit and Kafta Humera

Risk factors	No.tested	>4 mm cut-off			>2 mm cut-off		
		Prevalence	χ^2	P-value	Prevalence	χ^2	P-value
Location			3.9	0.142		3.5	0.177
Setit Humera	183	9(4.9%)			13(7.1%)		
Kafta Humera	301	23(7.6%)			29(9.6%)		
Total	484	32(6.6%)			42(8.7%)		
Cattle-wild animal contact			9.4	0.105		4.7	0.096
Yes	306	24(7.8)			31(10.1%)		
No	178	8(4.5%)			11(6.2%)		
Total	484	32(6.6%)			42(8.7%)		
Herd size			2.6	0.277		0.3	0.254
≤15	187	12(6.4%)			15(8.0%)		
>15	297	20(6.7%)			27(9.1%)		
Total	484	32(6.6%)			42(8.7%)		
Production			0.4	0.831		0.7	0.720
Extensive	449	30(6.7%)			38 (8.5%)		
Semi intensive	35	2(5.7%)			4(11.4%)		
Total	484	32(6.6%)			42(8.7%)		

The Generalized linear model (logistic regression) was done to evaluate the variation among the body condition scores; accordingly, the result of this model analysis showed that significant variation in the frequency of responders among body condition score for the CIDT test; and therefore, medium body condition scored animals were more at risk than good and poor body condition scored animals (Table 5).

Table 5: Univariate analysis of body condition scores in cattle to evaluate the strength of the association with skin test positivity

Risk factor	At cut-off >4 mm		At cut-off >2 mm	
	Odds ratio(OR)	P- value	Odds ratio(OR)	P- value
	(95% CI)		(95% CI)	
Body condition score				
Good *	1		1	
Medium	5.5(1.9–15.9)	0.002	2.7(1.2–5.8)	0.013
Poor	1.7(0.3–9.6)	0.539	1.5(0.5–5.2)	0.486

*=Reference category

5.2. Abattoir survey

5.2.1. . Postmortem examination

In this abattoir based study, age of animals were grouped into middle (≤ 6 yrs.) and old (> 6 yrs.) age groups because slaughtering of young animals (≤ 2 years old) was not observed during this study. Out of 582 carcasses inspected, 34 (5.8%, [95% CI, 3.9–7.8]) had lesions suggestive of tuberculosis, and showed a marked variation between categories of age and body condition. TB suspected lesions were more prevalent in > 6 years (7.6%) than ≤ 6 years (2.2%) groups, and prevalence of lesion increases as the body condition score decreases as 0.6%, 2.9% and 24.2% for good, medium and poor body condition, respectively (Figure 6). There was significant association between age and prevalence of tuberculous lesions ($\chi^2=6.8$, $P=0.009$), and body condition was highly significant to prevalence of tuberculous lesion ($\chi^2=74.5$, $P=0.000$). But there was no significant association between prevalence of lesion with breed ($\chi^2=1.7$, $P=0.280$), and origin ($\chi^2=2.3$, $P=0.129$) (Table 6).

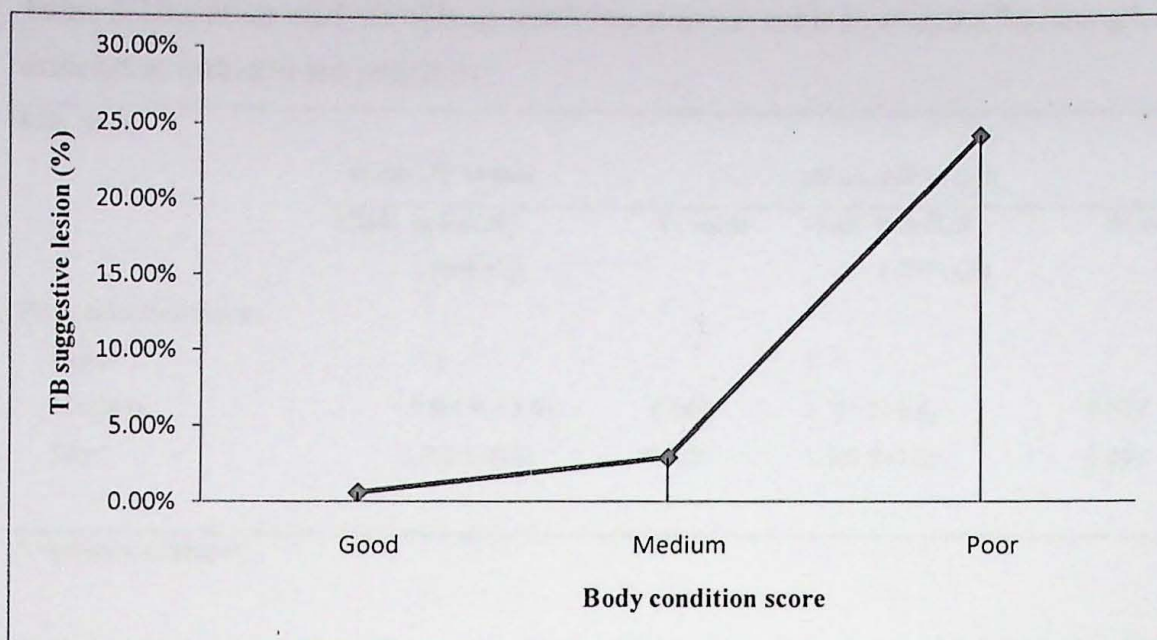


Figure 6: prevalence TB suggestive lesion in Setit Humera municipal abattoir based on body condition score in cattle

Table 6: Selected risk factors to evaluate prevalence of TB suspected lesions in Setit Humera municipal abattoir

Risk factors	No. slaughtered	Lesioned carcasses (%)	χ^2	p-value
Sex			0.19	0.665
Male	579	34(58.7)		
Female	3	0		
Total	582	34(5.8)		
Age			6.77	0.009*
≤6 years	186	4(2.2)		
>6years	396	30(7.6)		
Total	582	34(5.8)		
Breed			1.68	0.280
Barka	223	16(7.2)		
Medenes	359	18(5.0)		
Total	582	34(5.8)		
Origin			2.31	0.129
Setit and Kafta	394	19(4.8)		
Humera				
others	188	15(8.0)		
Total	582	34(5.8)		
Body condition			74.45	0.000*
Good	168	1(0.6)		
Medium	315	9(2.9)		
Poor	99	24(24.2)		
Total	582	34(5.8)		

*=Statistically significant

Generalized linear model (GLM) (logistic regression) was applied for risk factors that had significant association with lesion distribution in the chi-square analysis in (Table 6). Thus, the result of this analysis showed significant variation in the frequency of cattle with lesions among body condition score and age groups (Table 7).

Table 7: Univariate analysis of selected host related risk factors for the prevalence of TB suspected lesions in cattle in Setit Humera municipal abattoir

Risk factors	Odds ratio (OR)	95% CI	P- value
Age			
≤6 years*	1	–	–
>6years	3.7	1.3-10.7	0.015
Body condition			
Good *	1	–	–
Medium	4.9	0.62–39.1	0.133
Poor	53.4	7.1–402.4	0.000

*=Reference category

Out of 34 TB suggestive lesions, 61.8% were found in the respiratory path way (53% lung, 8.8% bronchial lymph node) followed by mesentric and prescapular lymphnodes which is the same figure (23.5%). Multiple tuberculous suggestive lesions were observed on different lymph nodes/ organs of the same carcass; for example on lung and mesentric lymph nodes or lung and prescapular lymph nodes. The topography of lesions and their frequency that were found in different lymph nodes/ organs in this study was summarized in (Table 8).

Table 8: Distribution and tropism of suggestive tuberculous lesions in the lymph nodes and lung lobes in 34 postmortem positive cattle with lesions in at least one tissue or organ

Tissue	No. (%) of carcass with suggestive tuberculous lesions	
Lung lobes	Total	Positive organ or tissue
Left apical	34	4(11.8%)
Right apical	34	5(14.7%)
Left diaphragmatic	34	4(11.8%)
Left cardiac	34	2(5.9%)
Right cardiac	34	3(8.8%)
Lymphnodes		
Mesentric	34	8(23.5%)
Prescapular	34	8(23.5%)
Mediastinal	34	2(5.9%)
Retropharyngeal	34	1(2.9%)
Thigh muscle	34	1(2.9%)
Left bronchial	34	2(5.9%)
Right bronchial	34	1(2.9%)
Prefemoral	34	4(11.8%)

5.2.2. Mycobacteriology

Of 7 milk samples collected and cultured, 1/7 (14.9%) yielded mycobacteria on primary culture. Growth of mycobacteria was observed in 12/34 (35.3%) of tissue samples with suspicious TB lesion on primary culture (Table 9). Out of 35.3% that did grow, 50% (6/12) of them were exclusively from the lung followed by prescapular lymph node 16.7% (2/12) (Figure 7).

Table 9: Mycobacteria growth on primary culture from milk and different tissue/organs in their respective media

Samples	No.(%)of positive culture	Löwenstein Jensen media
Milk	1(14.9%)	Glycerol
Tissues		
Lung	3(25%)	Glycerol
Lung	1(8.3%)	Pyruvate
Lung	2(16.7%)	Glycerol and pyruvate
Lung and retropharyngeal LN	1(8.3%)	Pyruvate
Lung and prescapular LN	1(8.3%)	Glycerol and pyruvate
Prescapular LN	2(16.7%)	Glycerol
Prescapular and prefemoral LN	1(8.3%)	Pyruvate
Bronchial LN	1(8.3%)	Pyruvate

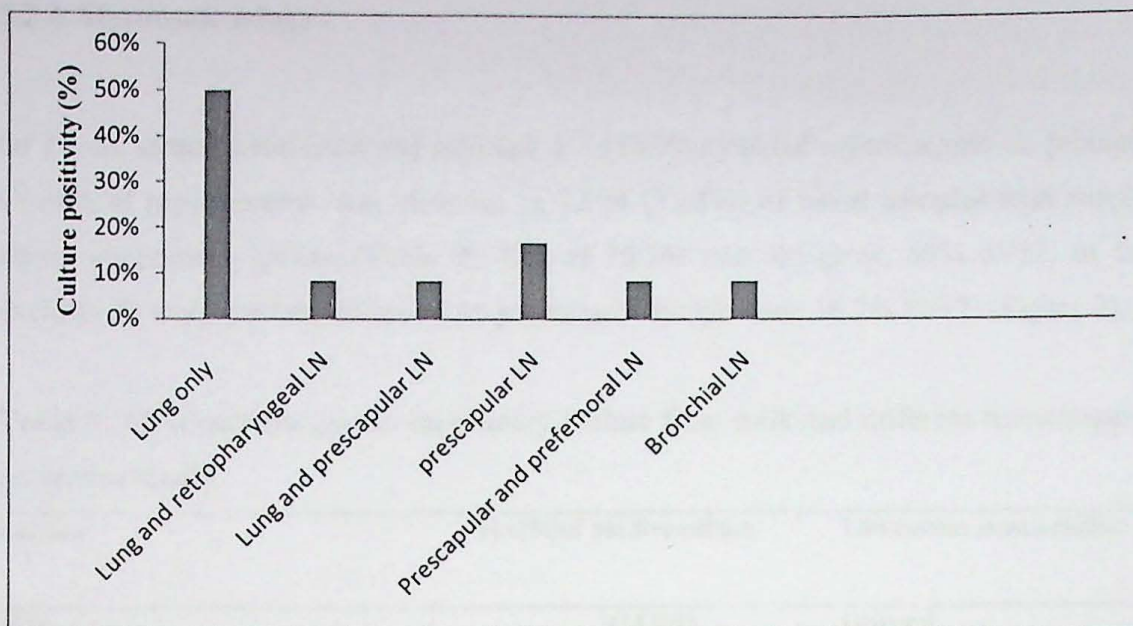


Figure 7: Proportion of mycobacterial culture positivity of the lymph nodes and lungs of TB suspected lesion in cattle collected from Setit Humera municipal abattoir

Of the 13 (1 milk and 12 tissue) culture positive samples, 23.1% (3/13) grew on both pyruvate and glycerol media while 46.2% (6/13) and 30.7% (4/13) grew on media containing glycerol and pyruvate, respectively (Figure 8)

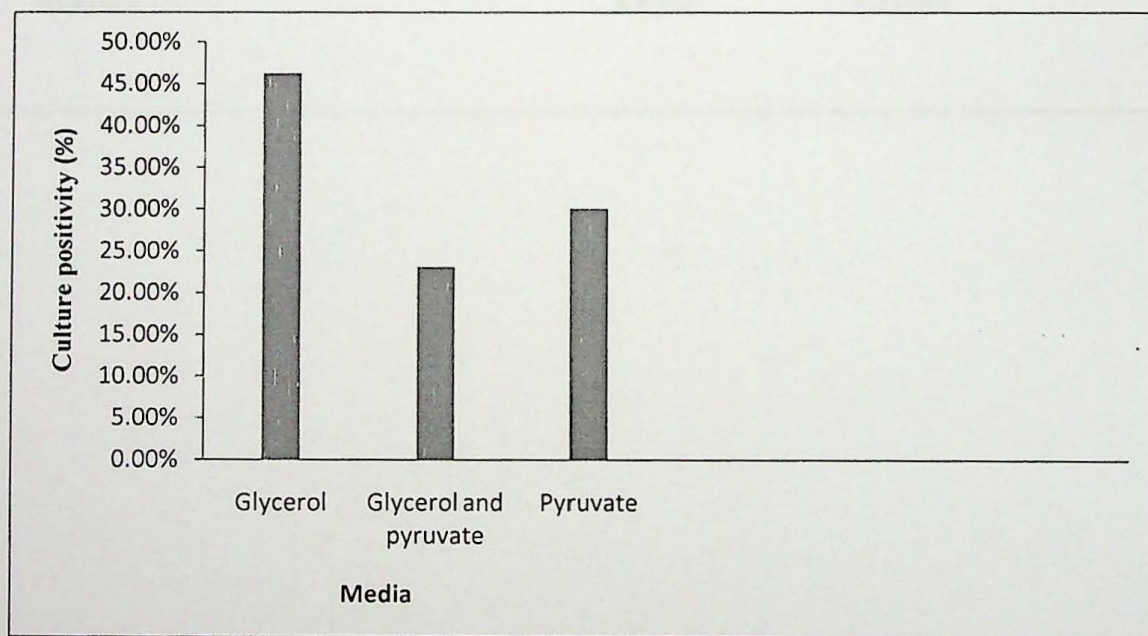


Figure 8: Mycobacterial growth in Lowenstein Jensen media containing glycerol and pyruvate differently

5.2.3. Molecular characterization of the isolates

Multiplex Polymerase Chain Reaction (m-PCR)

Further *Mycobacterium* genus typing was conducted on the 13 culture isolates from cattle (1 from milk and 12 from tissues samples). Based on multiplex PCR result, 4 isolates from tissue sample gave a signal to the genus *Mycobacterium* (Figure 9).

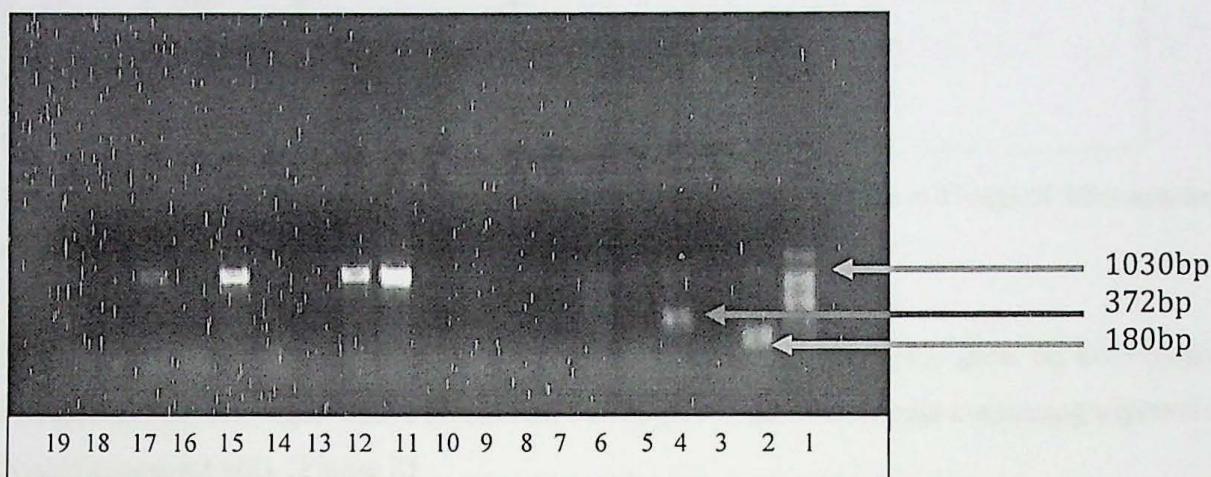


Figure 9: Gel electrophoresis separation of PCR products by multiplex PCR genus typing of mycobacteria isolated from naturally infected cattle. Lane 1 = 100bp DNA Ladder; Lane 2 = *Mycobacterium avium* complex (positive control), Lane 3 = Qiagen H₂O (negative control), Lane 4 = *Mycobacterium tuberculosis* complex (positive control), Lanes 5–19 were isolates from individual cattle with tuberculous lesions. Lane 11, 12, 15, 17 were positive for genus *Mycobacterium*, Lane 5–10, 13, 14, 16, 18, 19 were negative for genus *Mycobacterium*.

5.3. Questionnaire survey

A total of 157 cattle herders/owners were interviewed; 31 herders in each 31 herd, and from 67 households (households were traced back and recorded by the help of herders for questionnaire purpose), except for 8 households a single interviewee, in the rest 59 households two individuals (considering family size with different occupation among the members of the family) were asked for their habit of drinking milk, species of animals owned, milk selling system, and contact of their animals with man, wildlife and other cattle, the presence of tuberculosis patient and types of

tuberculosis in human in their home. Summary of the questionnaire surveys are shown in (Table 10). Accordingly, all of the respondents (100%) confirmed that milk was sold only to local people and it was observed that there was no modern milk processing method (like pasteurizer) in the study areas. Most bovine PPD positive reactor animals were found in animals which had contact with other domestic animals, with other cattle and wild animals (Table 10).

Table 10: Selected risk factors of the questionnaire survey and their relation to bovine PPD positive reactor animal in Setit and Kafta Humera

Risk factors	No.of respondents (%)	Bovine PPD positive reactor (cut-off >2 mm)
Species owned		
Cattle only	119(75.8)	15(35.7%)
Cattle+other animals	38(24.2)	27(64.3%)
Mixing with other cattle		
Yes	150(95.5)	41(97.6%)
No	7(4.5)	1(0.4%)
Cattle- wild animal contact		
Yes	66(42.1)	24(57.1%)
No	91(57.9)	18(42.9%)
Habit of drinking milk		
Boiled	129(82.2)	12(28.6%)
Boiled and raw	28(17.8)	30(71.4%)
Milk selling		
To local people	157(100)	42(100%)
Milk collection units	0(0)	0(0%)
Sharing same house		
Yes	110(70.1)	32(76.2%)
No	47(29.9)	10(23.8%)
Knowledge about TB		
Yes	87(55.4)	16(50%)
No	70(44.6)	16(50%)
Awareness about TB		
Yes	41(26.1)	10(23.8%)
No	116(73.9)	32(76.2%)

Out of 157 owners and herders, 7/157 (4.5%) respondents had TB patient in their household, and 110 (70.1%) (Table 10) of the respondents told that they shared the same house with their animals and of the 7 TB patients 4 (57.1%) of them were did share the same house with their animals with 3 (42.9%) pulmonary and 1 (14.3%) extrapulmonary TB. Of the respondents used both raw and boiled milk (Table 11), 2/7 (28.6%) were found as TB patient with 1 (14.3%) pulmonary and 1 (14.3%) extrapulmonary TB. About 57.1% (4/7) and 85.7% (6/7) of the TB patients were belong to people who did not know about TB and people who had lack of awareness about TB, respectively (Figure 10).

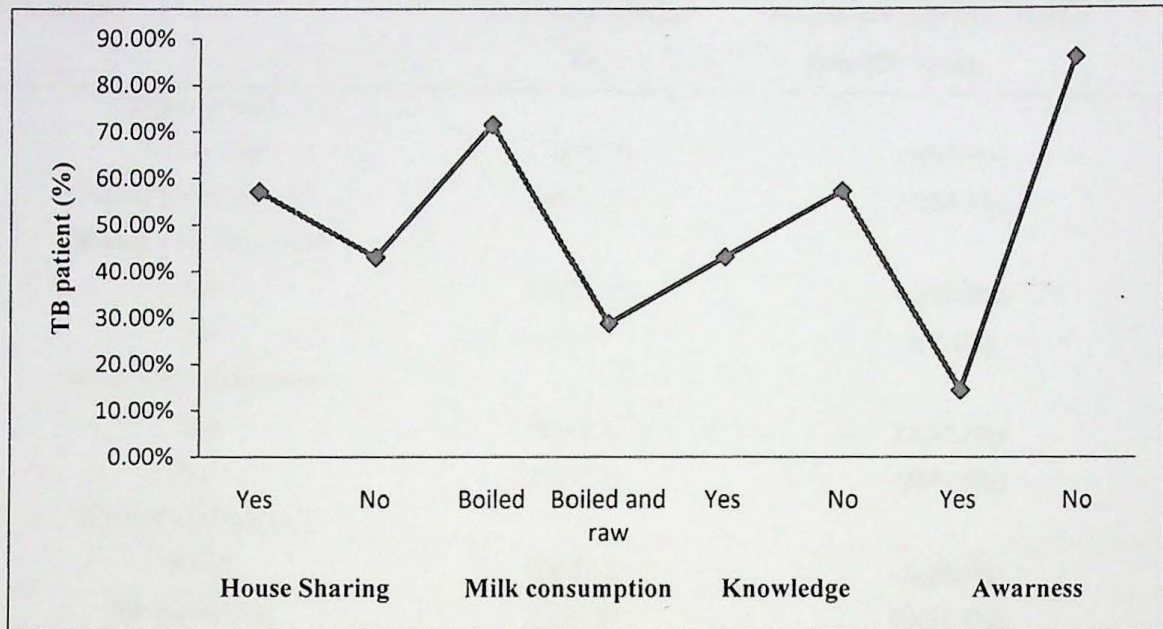


Figure 10: Risk factors assessed based on questionnaire in relation to the occurrence of TB in cattle owners in Setit and Kafta Humera

6. DISCUSSION

6.1. Intradermal tuberculin test

Multiple studies have been performed in the Ethiopian Highlands in recent years on BTB in cattle, which included urban and peri-urban areas with high concentration of dairy farms or rural areas characterized by traditional mixed crop-livestock small holder farms with small Zebu herds (Ameni *et al.*, 2007; Tschopp *et al.*, 2009, 2010). But no BTB research has been done in Humera, western part of Tigray, in northern Ethiopia, one of the settlements of the Ethiopian peripheral communities although they own high number of cattle.

The tuberculin test positivity prevalence for BTB recorded in the area was as 6.6% (at cut-off >4 mm) and 8.7% (at cut-off >2 mm) which is lower as compared to the previously reported prevalence rates 23.9 % (Kiros, 1998), 10.3 % (Bogale *et al.*, 2001), 13.5% (Ameni *et al.*, 2007), 11.6% (Regassa *et al.*, 2009) and 14.7 % (Mohammed, 2006) in different part of the country. Those studies, however, were carried out with higher numbers of exotic breeds (Holstein cows and their crossbreeds) and more intensive livestock-keeping than the present study population. In contrast, the present study included predominantly local Zebu breeds (Barka breeds) under extensive management.

The present study was consistent with the low prevalence rates of bovine tuberculosis recorded by Gumi *et al.* (2011) in pastoral cattle herds in the Oromia region and by Tschopp *et al.* (2007, 2010) in cattle kept in a traditional husbandry system in Ethiopia. This may be due to the fact that the present study was conducted on herds largely dependent on grazing in the field. Moreover, the present study supported the theories; when larger proportion of the study animals was grazing in the field, the level of confinement is reduced to a certain degree, which in turn minimizes the rate of infection in the herd (OIE, 2010; O'Reilly and Daborn, 1995; Kiros, 1998; Cook, 1997).

The prevalence of intradermal test positivity of the present study increased with age being maximum in the age group of $6 < x \leq 10$ years, then decreased when animals were older than 10

years (Table 3). O'Reilly and Daborn (1995) indicated that the chance of infection with *M. bovis* increases as the age increases. 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 TB 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 become lower due to immuno depression (Tizard, 1996), but in this study the tuberculin test positivity was not statistically significant with age.

In contrast to findings reported by Defar *et al.* (2009) in Holeta and Ameni *et al.* (2001) in Wolaita Sodo, the intradermal tuberculin test positivity of the present study was higher in male (10.9% at cut-off >4 mm and 13.0% at cut-off >2 mm) than female (6.2% at cut-off >4 mm and 8.2% at cut-off >2 mm) animals despite insignificant association between sex and tuberculin test positivity was observed. Movement of male animals was observed from herd to herd for reproduction purpose in the study area, and thus, male animals may acquire the disease more frequently than female animals.

When body condition of animals was considered large numbers of positive reactors to bovine tuberculin were observed in animals with medium body condition (Table 3). In contrast to reports by Ameni (1996), Cook (1997) and Kiros (1998) tuberculin test positivity was higher in better body condition, the present study showed tuberculin test positivity was higher in medium body condition. This may be due to more developed immunity in good body condition that can minimize rate of infection or can remove infection at early stage; on the other hand, poor body condition animals can not reflect the actual disease due to immunocompromisation.

Ameni *et al.* (2003b) indicated that late pregnancy and lactation reduce the immune reaction of female animal to tuberculin test. The result of this study; prevalence of lactating and pregnant animals was less than dry cows for skin test positivity at cut-off >4 mm. But at cut-off >2 mm, dry cows were fewer positive reactors to bovine tuberculin than pregnant and lactating animals (Table 3). This is because the reproductive stages (pregnant and lactating animals) are stressful and thus reduce the immune reaction of the female animal to tuberculin test at cut-off >4 mm but they were included at cut-off >2 mm; the probable reason could be due to the presence of slow

immune reaction to PPD antigen in pregnant and lactating animals. In the present study, few numbers of crossbred (HolesteinXBarka) breeds were tested making comparison of the two breeds difficult in this study.

In contrast to Ameni *et al.* (2003a) and Cleaveland *et al.* (2007), but in line with Tschopp *et al.*, (2009) herd size did not play a significant role as risk factor for tuberculin reactivity (Table 4). Tschopp *et al.* (2009) indicated that there seems to be very little transmission during extensive communal grazing, even on crowded pasture. In the present study the type of production system was extensive type of production and animals were largely kept grazing on the field extensively which could be reduces the transmission even in large herd sizes.

Munyeme *et al.* (2008) reported that herds which had no livestock-wildlife interaction (like antelope) were more likely to contain animals that failed to tuberculin test in comparison to livestock which had frequent contact with wildlife. In agreement to Munyeme *et al.* (2008), in the present study cattle which had contact with wildlife had higher tuberculin test positivity (7.8% at cut-off>4 mm and 10.1% at cut-off>2 mm) than those that had no contact (4.5% at cut-off>4 mm and 6.2% at cut-off>2 mm).

Culture positivity of milk on primary culture was observed only from 1 (14.9%) of the 7 bovine tuberculin positive sampled cows which is consistent with the report (13.3%) by Ameni *et al.* (2003c). This substantiates the fact that TB in cattle is principally a pulmonary disease; only approximately 1% of the tuberculous cows excrete tubercle bacilli in their milk (Pritchard, 1988).

In the present study area, many animals were observed with chronic debilitating infections, and according to local veterinarians diseases such as contagious bovine pleuropneumonia (CBPP) and trypanosomiasis are prevalent in the area. These diseases may likely to affect the CIDT test result by down-modulating the animal's immunity and thus leading to increased false negative animals in this area and thus possible underestimation of the BTB prevalence. Exact prevalence of most of these chronic diseases in Humera and their role in immuno-modulation is unknown and more research is warranted on that subject.

6.2. Abattoir survey

The proportion of carcasses with TB suggestive lesions based on the detailed inspection procedure was found to be 5.84% which is lower as compared to the previously reported prevalence rates 12.5% (Hussen, 2006), 10.2% (Biffa *et al.*, 2009). Even though the present finding is low, it is relatively higher than Teklue *et al.* (2004) and Shitaye *et al.* (2006) who reported 3% and 3.5% in Addis Ababa municipal abattoir, respectively, Mihreteab and Indris (2011) who reported 2.7% in Jimma municipal abattoir, and Regassa *et al.* (2009) who reported 1.1% in Hawassa municipal abattoir. This difference in prevalence of TB suggestive lesions could be due to the difference in origin or types of production system and breed of animals that are slaughtered in the abattoir.

This study revealed that the prevalence of tuberculous suggestive lesion increases as the age increases, animals with age group >6 years were more affected than animals ≤ 6 years old. This is consistent with the previous reports (Hussein, 2006; Biffa *et al.*, 2009; Mihreteab and Indris, 2011). It has also been reported that the duration of exposure to different stressors, malnutrition and various immunosuppressants increases with age; older animals are more likely to have been exposed than younger ones (Marie-France, 2009).

The difference in the prevalence among body condition scores was assessed. In agreement to Biffa *et al.* (2009), but in contrast to Mihreteab and Indris (2011) the result of the post mortem lesion was statistically significant to body condition score. Prevalence of TB suggestive lesion was higher in poor body condition (24.2%) than medium (2.97%) and good (0.6%) body condition. This might be due to the fact that the mycobacteria organisms invaded the tissue or organs of the immunocompromised animal than animal with good body condition, implies that large and more clearly visible lesion was seen in these classes of animals. On the other hand the majority of good body condition cattle may have a less likelihood of acquiring the infection. In support of this finding, Collins and Grange (1994) reported 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. Thus the agent could be removed before disease establishment.

Biffa *et al.* (2009) reported that cattle maintained in dairy farms had high degree of exposure to BTB infection than those maintained in other types of production system. In the present finding there was no significant association between TB suggestive lesions with origin of animals. As information was gained from the traders and personal observation in the study area, all animals that were supplied to the abattoir were managed under extensive type of production system although the origin of these animals was different. Therefore, the similarity in production system between the two groups may contribute for the insignificance.

According to Biffa *et al.* (2009) abattoir based post mortem examination and (Tschopp *et al.*, 2007; Tschopp *et al.* 2010) based on tuberculin test, exotic breeds were more susceptible to BTB infection than local Zebu. However, a different susceptibility has been observed even within the local breeds (Mbororo Zebu and the Arab Zebu cattle in Cameroon (Njanpop-Lafourcade *et al.*, 2001) and in Chad (Diguimbaye-Djaibe *et al.*, 2006). But in the present finding there was no significant association between TB suggestive lesions with breeds. In the present study, animals that were slaughtered and inspected in the municipal abattoir were 38.3% Barka and 61.7% Medenes (BarkaXArodo) which may not have basic difference in both breeds to disease susceptibility due to the fact that both breeds are local Zebu and with the same production system (extensive); animals under this production system hardly acquire the disease.

In this study, TB suggestive lesions were detected predominantly in the respiratory path way (61.8%) (Table 9) which was consistent with the previous findings (Mialiana-Suazo *et al.*, 2000; Asseged *et al.*, 2004; Shitaye *et al.*, 2006; Regassa *et al.*, 2009; Mihreteab and Indris, 2011). According to Corner (1994), between 70 and 90% of the lesions are found in either the lymphnodes of the thoracic cavity or in the head. Thus, the present finding supports the theory that most cases of BTB infections in cattle are acquired by inhalation. Although in the present study the type of production system was extensive, most of the time animals were sharing the same house with human and large number of animal congregated for sheltering and in watering points. Hence, animals could be acquired the disease during this time. However, slightly lower (61.8%) of TB suggestive lesions in the respiratory path way of the present study was recorded when compared with previous reports of 90% (Corner, 1994 and Neil *et al.*, 2001) and 72.7% (Tsegaye *et al.*, 2010). This is due to the fact that animals that are kept on pasture or extensive

production have lower exposure to respiratory route of transmission. Out of 34 TB suggestive lesions, the same frequency (23.5%) was observed in mesenteric and prescapular lymph nodes. The relatively higher prevalence of TB suggestive lesions in mesenteric lymph nodes next to lung in this study may be due to ingestion of the agent on pasture and watering points because animals were kept extensively.

Tuberculous lesions were subjected to bacteriological culture so as to identify and characterize the causative agents. However, culture positivity of suspicious tissues was 35.5% which is lower than what have been reported previously from cattle (Ameni *et al.*, 2007, 2010). The present study is consistent with culture positivity (34%) as reported by Mamo *et al.* (2011) in camel in pastoral area. The lower culture positivity might be related to the non-optimal condition of the culture for non tuberculous mycobacteria which are assumed to be the major isolates causing pathology in grazing animals. Regarding culture positivity of each organ, the highest culture positivity was recorded in the lung (Table 9) in agreement to other authors who reported that culture positivity was higher in lung tissue and thoracic lymph nodes than in the head and mesenteric lymph nodes (Corner, 1994; Ameni *et al.*, 2007; O'Reilly and Dabron, 1995).

6.3. Questionnaire

Out of 157 owners and herders, 7 (4.5%) respondents had TB patient in their household, and 110 (70.06%) of the respondents told that they shared the same house with their animals and of the 7 TB patients, 4 (57.1%) of them did share the same house with their animals with 3 (42.9%) pulmonary and 1 (14.3%) extrapulmonary TB.

Tschop *et al.* (2009) reported that the presence of other stock, purchase of cattle, and communal grazing, and all these variables showed a higher risk for having positive reactor cattle to bovine tuberculin. In this study, 64.3% and 97.6% of the bovine PPD positive reactor cattle had contact with other species of animals (like goat and sheep) and with other cattle, respectively (Table 10). Sharing of watering point between wildlife and cattle was identified as a significant factor for BTB positivity (Munyeme *et al.*, 2010) despite this may have been a bit subjective considering

that not every cattle owner may have had seen their cattle sharing watering points with wild animals simultaneously. However, the contamination of pasture and watering points could be a source of infection. In the present study, the herds which had contact with wild animals had higher bovine PPD positive reactor (57.1%) animal than which had no contact.

As stated by O'Reilly and Daborn, (1995) if all tuberculin-positive cattle are regarded as "open" cases of tuberculosis potentially capable of transmitting infection to other animals and humans, the animals and the community in the area are at risk of infection with *M. bovis*. Ameni and Erkihun (2007) also indicated that the presence of human TB patients and positive reactor cattle to bovine tuberculin in the same household may facilitate the transmission of mycobacterial species between cattle and their owners. Of 157 owners and herders, 110 (70.06%) of the respondents told that they shared the same house with their animals, during this time, the agent of animal TB could be transmitted to humans through inhalation of the cough spray from infected animals and ingestion of infected animal products. Kleeberg (1984) indicated that one cow with tuberculous mastitis can excrete enough viable tubercle bacilli to contaminate the milk of up to 100 cows when milk pooling and bulk transportation is used. The same author noted tubercle bacilli has been found in milk products such as yoghurt and cheese made from non-pasteurized milk 14 days after processing and in butter as long as 100 days after processing. In the present study, the entire respondents responded that they sold their milk to local people without treating it with heat, and therefore, risk of milk contamination with the agent of animal TB could be a potential major health hazard to consumers.

Ameni *et al.* (2003a) also reported that BTB had potential risk to public health based on food consumption, poor sanitary measures, and the lack of understanding about its zoonosis. In the present study, despite 55.4% respondents knew about animal TB, only 26.1% of them were aware of it. Of the seven TB patient, 4/7 and 6/7 were belong to people who did not know about bovine TB and people who had lack of awareness about bovine TB, respectively.

7. CONCLUSION AND RECOMMENDATIONS

The individual animal prevalence of bovine tuberculin positivity recorded by the present study in Setit and Kafta Humera woredas in grazing cattle 6.6% and 8.7% at cut-offs greater than 4 mm and 2 mm, respectively was lower, in contrast to a higher prevalence reported in central Ethiopia in intensive dairy farms but slightly higher when compared with reports from smallholder farmers or rural areas. Similarly, TB suggestive lesion prevalence recorded from Setit Humera municipal abattoir was moderate (5.84%) when compared with others that were reported by other authors in different abattoirs. The lack of awareness about animal TB, the very common close contact of animals with humans and the prevailing low standard of hygienic status may favor the spreading of BTB in the study area. During the abattoir survey also tuberculous suggestive lesions were collected, cultured and characterized using multiplex PCR, then finally resulted in non tuberculous mycobacteria which could be the cause of pathology and this need further investigation.

In line with the above conclusion the following recommendations are forwarded:

- ☞ Regular testing of herds followed by segregation of positive reactors, isolation of calves born in infected herd soon after birth and rearing of a replacement stock in disease free farms need to be applied;
- ☞ Regular public education about the zoonotic importance of BTB, its route of transmission and prevention method is mandatory in combating the disease;
- ☞ The role of non tuberculous mycobacteria species in causing pathology and disease should be investigated, and further study to reveal the specific species of these mycobacteria and their public health significance in human should be studied and;
- ☞ Government should aware to cattle owners, local leaders and concerned bodies about the importance of BTB and its diagnostic methods like CIDT test, and should provide enough logistics which are used to facilitate for fast and easy diagnosis of the disease.

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9. ANNEXES

Annex 1: Questionnaire format to assess risk factors and public health importance of Bovine tuberculosis in the study sites (Questionnaire for Cattle owners/attendants)

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 Do you mix your cattle with wild animals? a) Yes b) No

12If yes, what kind of animal?.....

13 Habit of drinking milk a) Raw b) boiled c) mixed

14. Do you boil milk? a) Yes b) No

15. Reason for boiling a) Fear of milk borne diseases b) due to culture

16 Habit of eating meat a) Cooked b) raw c) undercooked

17 If you sell milk/ milk products, who buys it?

a) Local people

b) Milk collection Unit

c) Others (specify) -----

18. Do you have (other) tuberculosis patient in your family or farm workers? a) Yes b) No

19. If yes, how many? -----

- 20 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
21. How long have you been sick? a). More than a year b) Less than a year c) Other
- 22 Have you ever taken any treatment? a) Yes b) No
23. If yes, a). Traditional b) drugs
24. Knowledge about TB a) yes b) no
25. Awareness about TB a) yes b) no

Annex 2: Bovine TB Survey, animal tuberculin test result record sheet

ID	Age	sex	Reproductive status	location	management	Cattle-wild animal contact	Herd size	Body condition	Skin fold measurement				result		
									Before injection		After 72hrs		Difference		
									A	B	A	B	ΔA	ΔB	$\Delta B - \Delta A$

A=Avian PPD

B=Bovine PPD

Annex 3: Body Condition Scoring

Annex 3A: For Exotic and crossbred 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 process 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 hips 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.

Source: Radostits *et al.*, (2000).

Annex 3B: 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: Estimation of age by examination of the teeth

Birth to 5 years (**See Table below**) and after 5 years in cattle, the following is a guide for age estimation.

5 years: All incisors in wear. Occlusal surface of I1 beginning to level.

6-7 years: I1 is leveled and neck is visible.

8 years: I2 is leveled and neck is visible.

9 years: I 3 is leveled and neck is visible. C may be leveled.

10 years: C is leveled and neck is visible.

At ten or eleven years, used surfaces of the teeth begin to bear a square mark surrounded by a white line, and this is pronounced on all the teeth by the twelfth year; between the twelfth and the fourteenth year this mark takes a round form. As cattle continue to age, the teeth wear shorter and more neck becomes visible; they loosen in the sockets and eventually drop out.

Table: Eruption of the Teeth in cattle

Type of teeth	Time of eruption
Di 1	Before birth
Di 2	Before birth
Di 3	0-1 wk
I 1	2 yr
I 2	2½ yr
I 3	3½ yr
Dc	0-2 wk
C	3½-4 yr
Dp 2	0-3 wk
Dp 3	0-3 wk
Dp 4	0-3 wk
P 1	—
P 2	2-2½ yr
P 3	2-2½ yr
P 4	2½-3 yr
M 1	5-6 mo
M 2	1-1½ yr
M 3	2-2½ yr

C=Canine

D =deciduous

I=Incisor

Mo=Month(s)

M=Molar

P= Premolar

Wk=week(s)

Yr=Year(s)

Source: Kahn *et al.* (2005)

Annex 5: Abattoir data collection sheet for bovine tuberculosis

I D	Sex	Age	Breed	Origin	Condition	MD	MR	MDF	MDR	BR	BL	HP	MES	LUNG	OTHERS

MD- Mandibular (right and left); MR- Medial Retropharyngeal; MDF- Mediastinal Cranial; MDR- Caudal Mediastinal; BR - Bronchial right; BL- Bronchial Left; HP- Hepati; MES- Mesenteric

Annex 6: List of Regents

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. Regent for Decontamination:-

1. Sodium Hydroxide (4%)

- ✓ Sodium hydroxide 4.0g
- ✓ Distilled water 200. 00ml

2. Reagent for neutralization

✓ Hydrochloric acid (10%)

3. Reagent for disinfectant

✓ Phenol (5%)

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

10. SIGNED DECLARATION SHEET

I, the undersigned, declare 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 Gebremedhin Romha

Signature _____

Date of submission _____

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

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