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**MOLECULAR EPIDEMIOLOGY OF BOVINE TUBERCULOSIS IN CATTLE
AND ITS PUBLIC HEALTH IMPLICATIONS IN GAMBELLA TOWN AND ITS
SURROUNDINGS, GAMBELLA REGIONAL STATE, ETHIOPIA**

MSc Thesis



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June, 2015

Bishoftu, Ethiopia

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A Thesis submitted to School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in veterinary public health.

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June, 2015

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DEDICATION

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

STATEMENT OF AUTHOR

First, I declare that this thesis is my *bonafide* work and that all sources of materials used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
BCS	Body Condition Scoring
BTB	Bovine Tubercle Bacilli
DNA	Deoxy Ribonucleic Acid
ELISA	Enzyme linked Immuno Sorbent Assay
HIV	Human Immune deficiency Virus
IFN- γ	Interferon Gamma
IS	Insertion Sequence
LJ	Lowenstein Jensen
MIRU-VNTR	Mycobacterium Interspersed Repetitive Unit- Variable Number Tandem Repeats
MTBC	<i>Mycobacterium tuberculosis</i> Complex
LSSNPA	Lineage-Specific Single-Nucleotide Polymorphism Analysis
OIE	Office of International des Epizooties
PBS	Phosphate Buffered Sulfate
PCR	Polymerase Chain Reaction
PPD	Purified Protein Derivatives
RDs	Regions of Differences
REA	Restriction Endonuclease Analysis
RFLP	Restriction Fragment Length Polymorphism
rRNA	ribosomal Ribo Nucleic Acid
SIT	Shared International Type
WHO	World Health Organization

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ABSTRACT

*A cross sectional study was conducted in Gambella town municipal abattoir and health centers in Gambella People Regional State of southwest Ethiopia, to investigate the prevalence of bovine tuberculosis, isolation and molecular characterization of its causative agents and to assess its public health implications from December 2013 to May 2014. Postmortem examination, bacteriological culturing, multiplex PCR, RD deletion typing and spoligotyping were used for investigation. A total of 500 Cattle and 50 AFB positive human TB patients were recruited under the study. The overall prevalence of bovine tuberculosis in cattle was 13.2% (66/500) (95%CI: 10.22-16.18) on the basis of detailed postmortem examination. Statistical significant difference was observed in the prevalence of BTB among different groups of body condition scoring ($\chi^2 = 39.105$, $P=0.000$, $df =1$), between sex ($\chi^2 = 7.661$, $P=0.006$, $df =1$) and breeds ($\chi^2 = 24.996$, $P=0.000$, $df =1$). Poor body conditioned animals were more likely to develop TB lesions (OR=12.16, 95%CI, 4.58-32.24) than good body conditioned animals. Out of 82 tissue samples cultured, 14(17.07%) were bacteriologically culture positive and all were acid fast positive. From a total of 50 sputum sample collected and cultured from human TB cases, 17 (34%) showed growth on LJ medium. Molecular characterization of 11 mycobacterial isolates from human patients using RD9 deletion typing showed that all were *M. tuberculosis* and further spoligotyping of the isolated revealed that SIT289, SIT134, SIT1634, SIT142 and one new strain not found in the spoligotype databases. Of these *M. tuberculosis* strains identified SIT 289 and SIT134 were found in cluster with 45.5% (5/11) cluster rate for SIT289 and 27.3(3/11) for SIT134 indicating the recent transmission of the stains in the community. Lineage of the human isolates indicated that 27.3% (3/11) Euro-American, 9.1% (1/11) Indo-oceanic and 63.6% (7/11) were unknown lineage family in TB-insight database. Interestingly, one isolate from animal taken from cranial mediastinal lymph node was confirmed to be *M. tuberculosis* using RD4 deletion typing and spoligotyping, in which the isolate was identified as SIT523 with indo-oceanic lineage family. This isolation of *M. tuberculosis* from cattle suggests that the presence of reverse zoonosis which needs further investigation. Awareness of cattle owners for BTB*

was found to be insufficient (22%) and the result also revealed the presence of potential risk factor for zoonotic transmission. In conclusion, the present study revealed a moderately high prevalence of BTB in cattle of Gambella Region, the presence of clustering indicates the recent transmission of the stains in the community and moreover isolation of M. tuberculosis in cattle and occurrence of various strains of M. tuberculosis in the communities warrants further investigation on the transmission of the disease in Gambella Region.

Key words: *Bovine tuberculosis, Gambella, Molecular epidemiology, RD typing Spoligotyping, Public health, Zoonosis*

1. INTRODUCTION

Tuberculosis (TB) is recognized as one of the most important threats to human and animal health causing mortality, morbidity and economic losses (Smith *et al.*, 2006; Pal, 2013). It remains a major global health problem and causes ill-health among millions of people each year and ranks as the second leading cause of death from an infectious disease worldwide after the human immunodeficiency virus (HIV) (WHO, 2013; WHO, 2014; Birhanu *et al.*, 2015).

Tuberculosis is communicable Mycobacterial disease caused by members of *Mycobacterium tuberculosis* complex (MTBC) (Pal, 2007; Malama *et al.*, 2013; Tamiru *et al.*, 2013). Although, recent studies indicated that *M. tuberculosis* has been isolated from cattle (Ameni *et al.*, 2011) and *M. bovis* from humans infected with bovine tuberculosis (Zeweld, 2014), *M. tuberculosis* is specifically adapted to humans while *M. bovis* is most frequently isolated from domesticated cattle (Smith *et al.*, 2006). In spite of variation in host specificity, the members of MTBC are characterized by 99.9% or greater similarity at nucleotide level and are virtually identical at 16s rRNA sequence (Brosch *et al.*, 2002).

Bovine tuberculosis is a chronic bacterial disease characterized by progressive development of tubercles in any tissue/organ of the body (Hlokwe *et al.*, 2013; Pal *et al.*, 2014; Terefe, 2014). It has been recognized from 176 countries as one of the important bovine diseases causing great economic loss (Awah-Ndukum *et al.*, 2013).

Bovine tuberculosis is a contagious disease, which can affect most warm blooded animals, including human being (Radostits *et al.*, 2007). Organisms are excreted in the exhaled air, in sputum, feaces (from both intestinal lesions and swallowed sputum from pulmonary lesions), milk, urine, vaginal and uterine discharges, and discharges from open peripheral lymph nodes of infected animals (Radostits *et al.*, 2007).

In cattle, exposure to this organism can result in a chronic disease that jeopardizes animal welfare and productivity and in some countries leads to significant economic losses by causing ill health and mortality (Ewnetu *et al.*, 2012). Moreover, human TB of animal origin caused by *M. bovis* is becoming increasingly evident in developing countries (Russel, 2003; Mamo *et al.*, 2013).

Bovine tuberculosis diseased animal loses 10 to 25% of their productive efficiency; direct losses due to the infection become evident by decrease in 10 to 18% milk and 15% reduction in meat production (Radostits *et al.*, 1994). Apart from effects on animal production, it has also a significant public health importance (Müller *et al.*, 2013). Currently, the disease in human is becoming increasingly important in developing countries, as humans and animals are sharing the same micro environment and dwelling premises, especially in rural areas, and susceptibility of AIDS patients to tuberculosis (Shitaye *et al.*, 2007). It is estimated that *M. bovis* causes 10 to 15% human cases of tuberculosis in countries where pasteurization of milk is rare and bovine tuberculosis is common (Ashford *et al.*, 2001; Berg *et al.*, 2015).

In developing countries like Ethiopia, the socio economic situation and low standard living area for both animals and humans are more contributing in TB transmission between human to human and human to cattle or vice versa (Ameni *et al.*, 2010a; Ejeh *et al.*, 2013). Human infection due to *M. bovis* is thought to be mainly through drinking of contaminated or unpasteurized raw milk and under cooked meat. The high prevalence of TB in cattle, close contact of cattle and humans, the habit of raw milk and meat consumption, and the increasing prevalence of HIV may all increase the potential for transmission of *M. bovis* and other *Mycobacteria* between cattle and humans (Shitaye *et al.* 2007).

Bovine tuberculosis is an endemic disease of cattle in Ethiopia, with a reported prevalence of 3.5–5.2 % in abattoir (mostly zebu) and 3.5–50% in crossbreed farms (Shitaye *et al.*, 2007; Demelash *et al.* 2009; Regassa *et al.*, 2010; Berg *et al.*, 2011). Nevertheless, the available information is limited due to inadequate disease surveillance

and lack of better diagnostic facilities (Cosivi *et al.*, 1998; Asseged *et al.*, 2000). In particular, information on genotypic characteristics of *M. bovis*, a strain affecting the cattle population in Ethiopia, is limited (Biffa *et al.*, 2010a). Such information is critical to monitor transmission and spread of the disease among cattle (Berg *et al.*, 2011).

The World Health Organization 2009 report indicated that the status of TB in Gambella Region was the highest from all the Ethiopian Regions, with the notification rate (new and relapse) 261- 421/100, 000 (WHO, 2009). This was one of the bases of the present study.

Gambella regional state has large livestock populations. Despite, the large number of livestock population in the region, there is no information on BTB. Despite the fact that bovine tuberculosis is a public health threat and also leads to economic losses, in Ethiopia research on and control of animal tuberculosis has not received much attention like human tuberculosis (Chukwu *et al.*, 2013). Thus the present study was designed with the following objectives:

- To determine the prevalence of bovine tuberculosis in Gambella town municipal abattoir and identifying risk factors associated with bovine tuberculosis.
- To isolate and molecular characterization of *Mycobacterial* isolates from slaughtered cattle and from human pulmonary TB patients.
- To investigate the potential risk factors for zoonotic transmission of mycobacterial infections.

2. LITERATURE REVIEW

2.1. Etiology

2.1.1. Taxonomy of Mycobacteria

The genus *Mycobacterium* is classified under the Order Actinomycetales and Family *Mycobacteriaceae* (Quinn *et al.*, 1999). The genus includes a number of species, some being pathogenic to man and animals, some are opportunistic while others are essentially saprophytic (Thoen, 1984). *Mycobacterium tuberculosis* complex (MTBC) has seven approved members and these are *M. tuberculosis*, *M. bovis*, *M. bovis BCG*, *M. africanum*, *M. microti*, *M. tuberculosis sbsp. canetti* and *M. bovis sbsp. caprae* (Corner *et al.*, 1990; Hermans *et al.*, 1991). Four members of this group cause human tuberculosis i.e. *M. bovis*, *M. tuberculosis*, *M. africanum* and *M. canetti* (Higgins *et al.*, 2011; Bilal *et al.*, 2010). Members of the MTBC are extremely similar genetically having at least 99.9% similarity on the nucleotide level and an identical 16rRNA sequence (Quinn *et al.*, 1999; Brosch *et al.*, 2002; Marie-France *et al.*, 2009).

2.1.2. Physical and biochemical characteristics

2.1.2.1. Morphology and staining

Mycobacteria are non-motile, non-spore forming, pleomorphic bacilli or coccobacilli. In tissues they appear as rods, which may be straight, curved or in the form of clubs, measuring 1.0-4.0 μm in length and 0.2-0.3 μm in width. They occur singly, in pairs or as small bundles. On laboratory media they may appear as cocci or rods measuring 6-8 μm (Quinn *et al.*, 1999). The distinguishing features of pathogenic mycobacteria are the formation of characteristic cords (Grange, 1995).

Mycobacteria are similar to other bacteria in many respects except for a unique cell wall that is made up of four parts. The first part is a peptidoglycan layer that is similar to that found on other bacterial species. The second layer contains arabinogalactan, which is made up of branched macromolecules of arabinose and galactose (Jovan *et al*, 2011). The third layer, which contributes to the thickness of mycobacterial cell walls, is made of mycolic acids that are long branched chains of fatty acids with differing 50 and 30 carbon atom lengths. The mycolic acids are responsible for the acid fast staining reaction of mycobacteria cells (Thoen and Bloom, 1995). The fourth, outer, layer consists of a mixture of lipids and related compounds such as trehalose-containing glycolipids and peptidoglycolipids called mycosides. Trehalose-containing glycolipids include a “cord-factor” compound that was previously believed to be responsible for the “serpentine cords” of virulent MTBC but this was later proved to be inaccurate (Chukwu *et al.*, 2013). *Mycobacteria* when stained are acid fast as they resist decolorizing with strong acid alcohol solutions (Quinn *et al.*, 1999).

2.1.2.2. Growth requirement and cultural characteristics

Mycobacterium species grows on medium containing serum, potato and egg. The most commonly used media are Lowenstein-Jensen (LJ) that contains egg, glycerol, asparagines, mineral salt and malachite green and stone brink’s medium. *M. bovis* grows more slowly than *M. tuberculosis*, which needs more than 8 weeks to appear on primary culture. The optimal growth temperature is 37⁰c (Quinn *et al.*, 1999; Simons *et al.*, 2011).

2.2. Pathogenesis

2.2.1. Infection

The method by which tubercle bacilli gain entrance to the animal body include: the respiratory, alimentary, genital, cutaneous and congenital routs (Neill *et al.*, 1994; Thoen and Bloom, 1995). After infection the bacteria may localize in tissues related to the route of infection and associated lymph nodes. Miliary TB represents the most sever course of

the disease with haematogenous spreading as a result of lysis of macrophages that release bacteria in to the blood from the primary foci and secondary seeding to various tissues (Andersen, 1997; Laisse *et al.*, 2011).

2.2.2. Lesion

A primary lesion or focus of infection is established following the interaction of the host and the agent at the site of entry within 8 weeks of bacterial entrance (Radostits *et al.*, 1994). The *mycobacterium* is then taken by the alveolar macrophages to the circulation and establishes in the lymph nodes (Taylor *et al.*, 2007). 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).

2.2.3. Virulence

Mycobacteria are intracellular organisms and their virulence appears to be related to their ability to survive and multiply with in the macrophages (Hermans *et al.*, 1991). *M. bovis* eludes the bacteriocidal activities of macrophages by escaping from fused phagolysosomes into non-fused vacuoles in the cytoplasm. In addition to these survival mechanisms, an important aspect of pathogenicity of mycobacteria is their ability to subvert the protective immune response (Grange, 1995). A characteristic feature of virulent strains of mycobacteria is that they form cords when they grow in a liquid culture media whereas the virulent strains develop as clumps (Thoen and Bloom, 1995; Ereqat *et al.*, 2013).

2.3. Immunity against Mycobacterial Infection

Both humoral and cell mediated immune responses can be induced to mycobacterial infection, but the cell mediated immunity is generally accepted to have the most significant role in protection (Neill *et al.*, 1994). The macrophages have a central role in

processing and subsequent presenting of mycobacterial antigens to antigen specific T-lymphocytes (Ali, 2006; Fentahun and Luke, 2012).

2.4. Epidemiology of *Mycobacterium bovis* Infections

The disease is found in cattle throughout the globe, but some countries have been able to reduce or limit the incidence of the disease through process of 'test and cull' of the cattle stock. Most of Europe and several Caribbean countries (including Cuba) are virtually free of *M. bovis*. Bovine tuberculosis is endemic to many developing countries particularly African countries (Abubakar *et al.*, 2011). *Mycobacterium bovis* combines one of the widest host ranges of all pathogens with a complex epidemiological pattern, which involves interaction of infection among human beings, domestic animals and wild animals (Grange, 1995; Mamo *et al.*, 2012; Gemechu *et al.*, 2013). However, only little is done particularly in developing countries on the epidemiology of this organism and the epidemiological requirements for its control (Ali, 2006).

2.4.1. Source of infection and mode of transmission

The main reservoir of *M. bovis* is cattle, which can transmit the infection to many mammalian species including man (Acha and Szytre, 2001; Tadayon *et al.*, 2013). Organisms leave the host in respiratory discharges, faeces, milk, urine, semen and genital discharges. These body excretions may contaminate grazing pasture, drinking water, feed, water and feed troughs or fomites, which may act as source of infection to other animals (Ameni *et al.*, 2001; Russel, 2003).

Inhalation of *M. bovis* bacilli is the most common route of infection with only a small number of mycobacteria required to cause an infection and spread of the infection can happen between animals when that are confined together in the same air space, such as during housing over the winter period (Neill *et al.*, 1994; Sakamoto, 2012). A secondary source of infection is the ingestion of contaminated milk or contaminated pasture and water, though environmental contamination is not believed to be a significant source of

infection for bovine TB. Infection of the reproductive system can lead to genital transmission of the bacilli but this is a particularly rare event as is congenital infection (Mathema *et al.*, 2006; Nahr *et al.*, 2011).

2.4.2. Risk factors: Animal population

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

2.4.2.1. Environment

Housing predisposes to the disease, as does high stocking intensity and a large number of animals on a farm so that the disease is more common and serious where these forms of husbandry are practiced. The closer the animals are in contact the greater is the chance that the disease will be transmitted (Panarella and Bimes, 2010). In spite of the low overall incidence in countries where cattle are at pasture all the year round, individual herds with 60-70% morbidity may be encountered (Regassa, 2005; Katale *et al.*, 2013).

2.4.2.2. Agent

The causative organism is moderately resistant to heat, desiccation, and many disinfectants. It is readily destroyed by direct sunlight unless it is in a moist environment. In warm, moist, protected positions, it may remain viable for weeks (Corner *et al.*, 1990; Srivastava *et al.*, 2008).

2.4.2.3. Host range

Cattle are the primary hosts for *M. bovis*, but other domesticated and wild mammals can also be infected (Tschopp *et al.*, 2010). Known maintenance hosts include brush tailed opossums (and possibly ferrets), badgers, bison and elk, and kudu and African buffalo (Radostits *et al.*, 1994; Regassa, 2005).

Species reported to be spillover hosts include sheep, goats (Mamo *et al.*, 2012), horses, pigs, dogs, cats, ferrets, camels, llamas, many species of wild ruminants including deer and elk; elephants, rhinoceroses, foxes, coyotes, mink, primates, opossums, otters, seals, sea lions, hares, raccoons, bears, warhogs, large cats (including lions, tigers, leopards, cheetahs and lynx) and several species of rodents (Radostits *et al.*, 2007; Tschop *et al.*, 2010). Most mammals may be susceptible, little is known about the susceptibility of birds to *M. bovis*, although they are generally thought to be resistant (Quinn *et al.*, 1999; Tschop *et al.*, 2011). Experimental infections have recently been reported in pigeons after oral or intra tracheal inoculation and in crows after intra peritoneal inoculation (Admasu *et al.*, 2014). Some avian species, including mallard ducks, appear to be resistant to experimental infection (Thoen, 1984; Demelash *et al.*, 2009; Silaigwana *et al.*, 2012; Tadayon *et al.*, 2013).

Zebu (*Bos indicus*) type cattle are thought to be much more resistant to tuberculosis than European cattle, and the effects on these cattle are much less severe but under intensive feedlot conditions a morbidity rate of 60% and a depression of weight gain can be experienced in tuberculous Zebu cattle (Regassa, 2005).

2.4.3. Risk factors: Human Population

2.4.3.1. Close physical contact

Close physical contact between humans and potentially infected animals is present in some communities, especially in developing region (Ameni *et al.*, 2007; Samuel, 2010). For example, in many African countries cattle are an integral part of human social life; they represent wealth and are at the center of many events and, therefore gatherings. In addition, with 65% of Africa, 70% of Asian, and 26% of Latin America and Caribbean population working in agriculture, a significant proportion of the population of these regions may be at risk for bovine TB (Cosivi *et al.*, 1998).

2.4.3.2. The increase in the demand for milk

The demand for milk was increasing at estimated rate of 2.5% per year over the period of 1970-1988 in sub Saharan Africa (Nwanta *et al.*, 2010). This rise demand for milk consumption will be met by increasing number of productive animals and intensifying animal production (Regassa, 2005).

2.4.3.3. Feeding habit

Consumption of raw or soured milk is mainly practiced in some parts of the world. Approximately 90% of the total volume of milk produced in sub Saharan Africa is consumed fresh or soured and only a very small proportion follows official marketing channels (Tamiru *et al.*, 2013). It is known that consumption of milk contaminated by *M. bovis* is regarded as the principal mode of TB transmission from animals to humans (Acha and Szytres, 2001).

2.4.3.4. HIV infection

In many developing countries, TB is the most frequent opportunistic disease associated with HIV infection (Cosivi *et al.*, 1998). HIV Seroprevalence rates greater than 60% have been found in TB patients in various African countries (WHO, 1997; WHO, 2005). Persons infected with both pathogens have annual risk of progression to active TB of 5 to 15% depending on their level of immune suppression; approximately 10% of non HIV infected persons newly infected with TB become ill at some time during their live (Sisay *et al.*, 2014). In the remaining 90% effective host defense prevent progression from infection to disease (Cosivi *et al.*, 1998).

2.4.3.5. Absence of control mechanism

Bovine tuberculosis can be controlled/ eliminated from a country or region by implementing the test and slaughter policy (Kremer, 2005). However , because of financial constraints, scarcity of trained man power, lack of political will, as well as the

under estimation of the importance of BTB by national governments and donor agencies, control measures are not applied or are applied inadequately in most developing countries (Cosivi *et al.*, 1998).

2.4.4. Distribution

Although bovine tuberculosis was once found worldwide, control programs have eliminated or nearly eliminated this disease from domesticated animals in many countries (Regassa, 2005; Mahmood *et al.*, 2013). Nations currently classified as tuberculosis free include Australia, Iceland, Denmark, Sweden, Norway, Finland, Austria, Switzerland, Luxembourg, Latvia, Slovakia, Lithuania, Estonia, the Czech Republic, Canada, Singapore, Jamaica, Barbados and Israel (Ali, 2006; Shimeles, 2008; Shitaye *et al.*, 2009).

Eradication programs are in progress in other European countries, Japan, New Zealand, the United States, Mexico, and some countries of Central and South America. Bovine tuberculosis is still widespread in Africa, parts of Asia and some Middle Eastern countries (Ashford *et al.*, 2001).

2.4.4.1. Status of bovine tuberculosis in Ethiopia

Studies in Ethiopia revealed a higher prevalence of BTB in cattle kept indoors compared to free grazing animals and a higher susceptibility to *M. bovis* infection of exotic Holstein Bos Taurus cattle compared to local zebu cattle (Bilal *et al.*, 2010; Nemomsa *et al.*, 2014).

Moreover, a study by Ameni *et al.* (2010b) revealed a better performance of SICCT in Ethiopia if the cut-off value for positive test interpretation was lowered from > 4 mm (OIE standard cut-off) to > 2 mm. The spoligotyping pattern of 17 strains of *M. bovis*, isolated from a herd with a high prevalence of BTB was identical for all animals and recently published (Firdessa *et al.*, 2013). Berg *et al.* (2011), provided a comprehensive

investigation on BTB in Ethiopia and showed a wide spread distribution of the disease at an average prevalence of approximately 5% (Mekibeb *et al.*, 2013).

2.5. Diagnosis

A presumptive diagnosis of TB in cattle and other susceptible species is often made on history, clinical findings, tuberculin skin tests, necropsy findings and other methods (OIE, 2009; Tessema *et al.*, 2011). In vitro lymphocyte assays, including an interferon gamma assay and enzyme linked immunosorbent assays have been developed for the detection of the disease in cattle and so other animals exposed to *M. bovis* (Samuel, 2010; Nahar *et al.*, 2011; Špičić *et al.*, 2012; Sharifipour *et al.*, 2014).

2.5.1. Clinical examination

Clinical diagnosis of TB infection in cattle is difficult due to the chronic nature of the disease and the wide variety of symptoms depending on the location of the infection (Radostits *et al.*, 2007). In cattle, infection with *M. bovis* is a progressive, two stages disease. The initial phase involves TB granuloma formation, which occurs at the site of infection and the local lymph node. This phase is usually asymptomatic and further progression of the disease varies considerably in rate and route (Radostits *et al.*, 1994; Thoen and Bloom, 1995). Because of the chronic nature of the disease and the multiplicity of signs caused by the variable localization of the infection, TB is difficult to diagnose on clinical examination (Radostits *et al.*, 1994; Tsegaye *et al.*, 2010). Enlarged superficial lymph nodes provide a useful diagnostic sign when lungs are extensively involved; there is commonly an intermittent cough. The principal sign of TB is commonly chronic wasting or emaciation that occurs despite good nutrition and care (Thoen and bloom, 1995; Smith *et al.*, 2006).

2.5.2. Tuberculin skin test

The standard method for detection of bovine tuberculosis is the tuberculin test, which involves the intradermal injection of bovine tuberculin purified protein derivative (PPD) and the subsequent detection of swelling (delayed hypersensitivity) at the site of injection 72 hours later (Ameni and Erkihun, 2007). This may be performed using bovine tuberculin alone or as a comparative test using avian and bovine tuberculins (Radostits *et al.*, 1994; OIE, 2009).

2.5.3. Postmortem examination

Postmortem examinations should be supported by a histological examination of samples stained with haematoxylin and eosin (OIE, 2009). Typically, lesions caused by *M. bovis* in cattle are described as having a center of caseous necrosis with some calcification and a boundary of lymphocytes, neutrophils and epitheloid cells (Bekele and Belay, 2011; Ewnetu *et al.*, 2012; Ayana *et al.*, 2013). Some of epitheloid cells may fused together and form multinucleated giant cells (Quinn *et al.*, 1999). An outer border fibrous of connective tissue is usually present, giving the lesion a focal appearance and providing encapsulation to some extent, which may limit the spread of infection. Since the lesions are not conclusive, it is necessary to demonstrate the etiological agent using Ziehl-Neelsen stain (Thoen and Bloom, 1995).

2.5.4. Microscopic examination

Mycobacterium bovis can be demonstrated microscopically on direct smears from clinical samples and on prepared tissue materials (Quinn *et al.*, 1999). The acid fastness of *M. bovis* is normally demonstrated with the classic Ziehl Nielsen stain, but a fluorescent acid fast stain may also be used. Immunoperoxidase techniques may also give satisfactory results (Regassa, 2005; Viedma *et al.*, 2011).

The presumptive diagnosis of Mycobacteriosis can be made if the tissue has characteristic histological lesions (caseous necrosis, mineralization, epithelioid cells, multinucleated giant cells and macrophages). As lesions are often paucibacillary, the presence of acid fast organisms in histological sections may not be detected, although *M. bovis* can be isolated in culture (Shitaye *et al.*, 2006; OIE, 2009; Jovan *et al.*, 2011).

2.5.5. Differential staining

Final confirmatory diagnosis of BTB depends on isolation and identification of the bacteria, but preliminary examination of stained smears from lesions, sputum, milk, urine, pleural and peritoneal fluids, uterine discharges and feces is very important (Radostits *et al.*, 2007).

In the smear, the organism appear red rods against a blue background (Ziehl Nielsen staining), while in the fluorochrome procedures, the acid fast organisms appear as fluorescent rods, yellow to orange (WHO, 1997; OIE, 2009).

2.5.6. Culture

To process specimens for culture, the tissue is first homogenized using a mortar and pestle (Woyessa *et al.*, 2014), stomacher or blender, followed by decontamination with either detergent (such as 0.375 – 0.75% hexadecylpyridiniumchloride [HPC]), an alkali (2–4% sodium hydroxide) or an acid (5% oxalic acid) (OIE, 2009). The alkali or acid mixture is shaken for 10–15 minutes at room temperature and then neutralized (Regassa, 2005).

Neutralization is not required when using HPC. The suspension is centrifuged, the supernatant is discarded, and the sediment is used for culture and microscopic examination (Quinn *et al.*, 1999). For primary isolation, the sediment is usually inoculated on to a set of solid egg based media, such as Lowenstein Jensen, Coletsos base or Stone brinks; these media should contain either pyruvate or pyruvate and glycerol. An

agar based medium such as Middle brook 7H10 or 7H11 or blood based agar medium may also be used (Ali, 2006; Mengistu *et al.*, 2015).

Cultures are incubated for a minimum of 8 weeks (and preferably for 10–12 weeks) at 37°C with or without CO₂. The media should be in tightly closed tubes to avoid desiccation. Slopes are examined for macroscopic growth at intervals during the incubation period. When growth is visible, smears are prepared and stained by the Ziehl Neelsen technique. Growth of *M. bovis* generally occurs within 3–6 weeks of incubation depending on the media used (Silaigwana *et al.*, 2012).

Liquid culture systems are used routinely in some hospital and veterinary laboratories; in these systems growth is measured by radiometric or fluorometric means (Bilal *et al.*, 2010).

If gross contamination of culture media occurs, the culture process should be repeated using the retained inocula with an alternative decontaminating agent (Zerdo *et al.*, 2014). Characteristic growth patterns and colonial morphology can provide a presumptive diagnosis of *M. bovis*; however every isolate needs to be confirmed. It is necessary to distinguish *M. bovis* from the other members of the tuberculosis complex, i.e. *M. tuberculosis* (the primary cause of tuberculosis in humans), *M. africanum* (occupies an intermediate phenotypic position between *M. tuberculosis* and *M. bovis*), *M. microti* (the „vole bacillus“, a rarely encountered organism), *M. pinnipedii* and *M. caprae* (Hlokwe *et al.*, 2013).

2.5.7. Immunological/serological diagnostic methods

Besides the classical intradermal tuberculin test, a number of blood tests have been used. Due to the cost and the more complex nature of laboratory based assays, they are usually used as ancillary tests to maximize the detection of infected animals (parallel testing), or to confirm or negate the results of an intradermal skin test (serial testing). There is also evidence that when an infected animal is skin tested, an enhanced blood test can occur

during the following week. This allows for better separation of in vitro blood test responses leading to greater test accuracy. The gamma interferon assay and the lymphocyte proliferation assay measure cellular immunity, while the ELISA measures humoral immunity (Jovan *et al.*, 2011; Legesse *et al.*, 2012).

2.5.8. *Gamma interferon assay/Bovigam*

In this test, the release of a lymphokine gamma interferon (IFN- γ) is measured in a whole blood culture system. The assay is based on the release of IFN- γ from sensitized lymphocytes during a 16–24 hour incubation period with specific antigen (PPD-tuberculin). The test makes use of the comparison of IFN- γ production following stimulation with avian and bovine PPD. The detection of bovine IFN- γ is carried out with a sandwich ELISA that uses two monoclonal antibodies to bovine gamma-interferon (Asiimwe, 2008).

2.5.9. *Enzyme linked immunosorbent assay*

There have been numerous unsuccessful attempts to develop clinically useful serodiagnostic tests for tuberculosis. The ELISA appears to be the most suitable of the antibody detection tests and can be a complement, rather than an alternative, to tests based on cellular immunity. It may be helpful in anergic cattle and deer (Asiimwe, 2008). An advantage of the ELISA is its simplicity, but sensitivity is limited mostly because of the late and irregular development of the humoral immune response in cattle during the course of the disease. Specificity is also poor in cattle when complex antigens such as tuberculin or *M. bovis* culture filtrates are used. However, a comparison of antibody levels to PPD-B and PPD-A has been shown to be useful in increasing specificity in the ELISA (OIE, 2009).

2.5.10. Molecular techniques for diagnosis of BTB

2.5.10.1. Multiplex PCR

Multiplex PCR as molecular technique differentiates MTBC from *M. avium*, *M. intracellulerae*, and other mycobacterial species (Mamo *et al.*, 2011; Araújo *et al.*, 2014; Ramos *et al.*, 2014). Heat killed AFB positive sample DNA was used as source of DNA template. The PCR targets the sequence of the genus *Mycobacterium*, within the 16S rRNA gene (G1,G2) sequences, within the hyper variable region of 16S rRNA, that is known to be specific to *M. intracellulerae* (MYCINT-F) and *M. avium* (MYCAV-R), and the MTB70 gene specific for MTBC (TB-A, TB-1B) (Atiadeve, 2010; Mamo *et al.*, 2013; Rekha *et al.*, 2015).

2.5.10.2. RD Deletion typing

Primers directed against the RD4, RD9, and RD10 loci are used to generate a deletion profile that would allow species identification of the isolates (Cadmus *et al.*, 2006). *Mycobacterial* genomic DNA will be obtained by heat killing, the isolates at 80 °C for 60 min and stored at -20 °C until it will be subjected to PCR. Isolates will be confirmed as *M. bovis* by deletion typing of the RD4 region according to a PCR protocol (Brosch *et al.*, 2002).

2.5.10.3. Spoligotyping

Another molecular typing method for *M. tuberculosis* complex is the PCR based spacer oligonucleotide typing (Spoligotyping) (Diriba *et al.*, 2013). This method was proposed as an alternative to hybridization based fingerprinting methods for diagnosis and epidemiology of tuberculosis (Kamerbeek *et al.*, 1997).

Spoligotyping is based on the variability of spacer sequences interspersed with repeat sequences in the polymorphic chromosomal direct repeat (DR) locus. This locus contains multiple, well conserved 36-bp long direct repeats (DR) (Hermans *et al.*, 1991).

Strains vary in the number of DRs and in the presence or absence of particular spacers and *M. bovis* characteristically lacks spacers 39 to 43 in the spoligotype system (Kamerbeek, *et al.*, 1997). Spoligotyping is thus not only useful for differentiation of *M. bovis* strains but can also be used to distinguish these from *M. tuberculosis*, a distinction which is often difficult to make by using conventional bacteriological techniques (Wayne, 1984).

2.5.10.4. MIRU-VNTR

This is a PCR based method that analyses multiple independent loci containing variable numbers of tandem repeats (VNTR) of different families of interspersed genetic elements collectively called Mycobacterial interspersed repetitive units (MIRU) (Supply *et al.*, 2000). In its original format, the PCR primers are each run in separate reactions and the sizes of the products are analyzed by gel electrophoresis. Currently, the most widely used version of MIRU-VNTR analysis includes 12 tandem repeat loci (Supply *et al.*, 2000; Supply *et al.*, 2006). A set of 24 MIRU-VNTR loci was standardized to increase the discrimination power (Supply *et al.*, 2006).

The advantages of MIRU-VNTR analyses are that the results are intrinsically digital and analysis can be applied directly to culture without the need for DNA purification (Supply, 2001). The discriminatory power of MIRU-VNTR analysis is typically proportional to the number of loci evaluated; in general, when only the 12 loci are used, it is less discriminating relative to IS6110 RFLP genotyping for isolates with high-copy-number IS6110 insertions but more discriminating than IS6110 RFLP genotyping for isolates with low copy number IS6110. When more than 12 loci are used, or MIRU analysis is combined with spoligotyping, the discriminatory power approximates that of IS6110 RFLP analysis. A comparative study of genotyping methods aimed at evaluating novel PCR-based typing techniques found VNTR analysis to have the greatest discriminatory power among amplification based approaches (Kremer *et al.*, 2005).

MIRU-VNTR genotyping has been used in a number of molecular epidemiologic studies, as well as to elucidate the phylogenetic relationships of clinical isolates (Kremer *et al.*, 2005; Warren *et al.*, 2004). VNTR analysis has also been used to evaluate *M. bovis* transmission (Roring *et al.*, 2004) In addition to their use for tracing TB transmission at the strain level, MIRU-VNTR markers can also provide useful predictions for classifying strains into genetic lineages (Allix *et al.*, 2004; Huang *et al.*, 2013).

2.6. Zoonotic Importance of Bovine Tuberculosis

Human tuberculosis of animal origin is an important public health concern in developing countries. Due to poor experimental controls, *M. bovis* was initially believed not to be a disease of man but this was later proved to be inaccurate (Thoen and Bloom, 1995; Rodwell *et al.*, 2008). The consumption of unpasteurized milk from infected cattle is the primary route of *M. bovis* infection in man and is associated with non-pulmonary TB, particularly in children (Radostits *et al.*, 2007). Cervical lymphadenitis and lupus vulgaris (chronic skin TB) are the most common presentations of a non-pulmonary *M. bovis* infection (Romha *et al.*, 2013). Pulmonary TB due to *M. bovis* is clinically, radiologically and pathologically identical to one caused by *M. tuberculosis* but it is uncommon and usually associated with animal handlers and abattoir workers (Elias *et al.*, 2008; Tigre *et al.*, 2011).

Human TB caused by *M. bovis* is unusual in countries in the developed world, due to the implementation of eradication programs for domesticated animals, accounting for <1% of TB infections (Pal *et al.*, 2014; Romha *et al.*, 2014). In the developing world, *M. bovis* is responsible for 5-10% of human TB cases but this varies between countries (Nwanta *et al.*, 2010; Parmar *et al.*, 2014). Limited laboratory facilities, in most developing countries, means that bacteriological diagnosis of a TB infection tends to be carried out by acid fast bacillus smear examination only, so under diagnosis of *M. bovis* infection may be occurring (Mihret *et al.*, 2012).

Due to the effectiveness of the WHO recommended TB treatment regime against human *M. bovis* infections, an argument can be made that diagnosis is not necessary from a case management and therapy viewpoint (Michel *et al.*, 2014; Sisay *et al.*, 2014).

The current increasing incidence of tuberculosis in humans, particularly in immunocompromized persons, has given a renewed interest in the zoonotic importance of *Mycobacterium bovis*, especially in developing countries (Pal, 2007). Due to the grave consequences of *M. bovis* infection on animal and human health, it is necessary to introduce rigorous control measures to reduce the risk of the disease in human and animal populations (Saidu *et al.*, 2015). The institution of proper food hygiene practices and stronger inter sectoral collaboration between the medical and veterinary professions is vital to the control of the disease (Nwanta *et al.*, 2010; Tamiru *et al.*, 2013; Zeweld, 2014).

Animal and human health is inextricably interwoven and food animals, especially cattle serve as a reservoir of diseases of public health importance (Tschopp *et al.*, 2011; Müller *et al.*, 2013; Pal *et al.*, 2014). The safety of food of animal origin with regard to infection by *M. bovis* is worth giving consideration, taking into cognizance the current tuberculosis crisis ravaging the world. Though animals with tuberculosis pose some risk to humans, this risk is extremely remote in developed countries due to introduction of milk pasteurization and effective bovine tuberculosis control programmes (Shitaye *et al.*, 2006; Munyeme *et al.*, 2010).

In contrast, spread from animals to humans in developing countries remains a very real danger, mostly from infected milk. This seems to be a danger, which is being entirely ignored (Gemechu *et al.*, 2013; Michel, 2014). The animal and public health consequences of *M. bovis* are grave. Disease surveillance programmes in animals and humans should be considered a priority, especially in areas where risk factors are present (Biru *et al.*, 2014). Other recommendations made by the WHO (2005) in its memorandum on zoonotic tuberculosis include: Training of personnel at all levels of control programmes and the urgent need for further research on the diagnosis and control,

immunological, epidemiological and socioeconomic aspects of the disease. International cooperation in all aspects of zoonotic tuberculosis remains essential in the fight against this disease (Nwanta *et al.*, 2010).

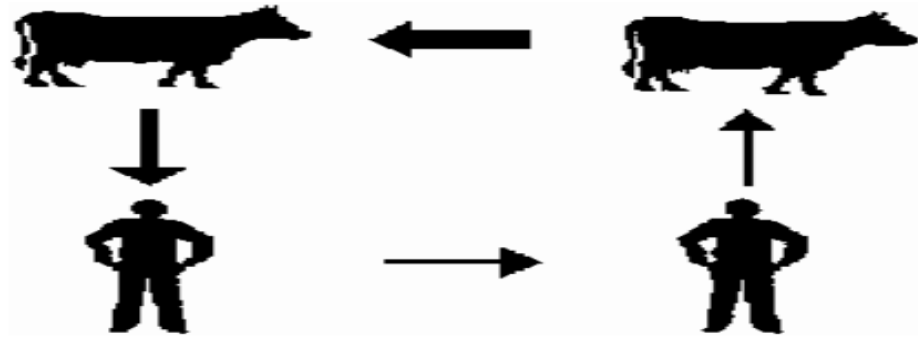


Figure 1. Cycle of *M. bovis* transmission between cattle and human (Grange and Collins, 1987)



Figure 2. A farmer in central Ethiopia discharging tobacco juice directly into the oral cavity of his cattle, a common practice in this region and a possible route of transmission of *Mycobacterium tuberculosis* from humans to cattle (Ameni *et al.*, 2011).

2.7. Molecular Epidemiology of BTB in Ethiopia

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

With respect to molecular epidemiology of bovine tuberculosis in livestock of Ethiopia, isolation and molecular characterization of the causative agent of BTB has been carried out in the last decade mainly in cattle and a number of isolates has been reported from different regions of the country and the first study was carried out by Ameni *et al.* (2007).

Human infection due to *M. bovis* is thought to be mainly through drinking of contaminated or unpasteurized raw milk. The high prevalence of TB in cattle, close contact of cattle and humans, the habit of raw milk and meat consumption, and the increasing prevalence of HIV may all increase the potential for transmission of *M. bovis* and other *Mycobacteria* between cattle and humans (Ameni *et al.*, 2013; Zeweld, 2013). The indicated table (Table 1) shows some studies on molecular basis of tuberculosis in Ethiopia.

Table 1: Molecular epidemiology of bovine tuberculosis in Ethiopia

Title of the study	Molecular techniques used	Authors
Molecular typing of <i>Mycobacterium bovis</i> isolated from tuberculosis lesions of cattle in north eastern Ethiopia	RD4; PCR; Spoligotyping	Ameni <i>et al.</i> (2010)
Prevalence of tuberculosis in pigs slaughtered at two abattoirs in Ethiopia and molecular characterization of <i>Mycobacterium tuberculosis</i> isolated from tuberculous like lesions in pigs.	RD10; mPCR; Spoligotyping	Arega <i>et al.</i> (2013)
Molecular characterization of <i>Mycobacterium bovis</i> isolates from Ethiopian cattle	Deletion typing; Accuprobe gene probe method; PCR; Spoligotyping	Biffa <i>et al.</i> (2010a;2010b)
Mycobacteria and zoonoses among pastoralists and their livestock in South-East Ethiopia	RD4; RD9 deletion typing; Genus typing ;16S rDNA sequencing; Spoligotyping of human isolates	Gumi <i>et al.</i> (2012)
Tuberculosis in Goats and Sheep in Afar Pastoral Region of Ethiopia and Isolation of <i>Mycobacterium tuberculosis</i> from Goat	<i>Mycobacterium</i> genus typing; Spoligotyping	Mamo <i>et al.</i> (2012)
Epidemiology and Molecular Characterization of Causative Agents of Bovine Tuberculosis in Ruminants	mPCR	Ashenafi <i>et al.</i> (2013)
Prevalence study on bovine tuberculosis and molecular characterization of its causative agents in cattle slaughtered at Addis Ababa municipal abattoir, Central Ethiopia	PCR;RD4 deletion typing; Spoligotyping	Mekibeb <i>et al.</i> (2013)
Epidemiology of bovine tuberculosis in Butajira southern Ethiopia: A	mPCR	Nemomsa <i>et al.</i> (2014)

cross sectional abattoir based study		
Epidemiology of mycobacterial infections in cattle in two districts of Western Tigray Zone, northern Ethiopia	mPCR	Romha <i>et al.</i> (2013)
A prevalence study of bovine tuberculosis by using abattoir meat inspection and tuberculin skin testing data, histopathological and IS6110 PCR examination of tissues with tuberculous lesions in cattle in Ethiopia	mPCR system	Shitaye <i>et al.</i> (2006)
Conventional and Molecular Epidemiology of Bovine Tuberculosis in Dairy Farms in Addis Ababa City, the Capital of Ethiopia.	RD; m-PCR; spoligotyping; VNTR analysis	Tsegaye <i>et al.</i> (2010)
Molecular Epidemiology of <i>Mycobacterium Tuberculosis Complex</i> at Nekemte Municipality Abattoir, Western Ethiopia	RD deletion Typing; m-PCR	Woyessa <i>et al.</i> (2014)
Gross and Molecular Characterization of <i>Mycobacterium tuberculosis Complex</i> in Mekelle Town Municipal Abattoir, Northern Ethiopia	mPCR; spoligotyping	Zeru <i>et al.</i> (2013)
Cultural and molecular detection of zoonotic tuberculosis and its public health impacts in selected districts of Tigray region, Ethiopia	m-PCR; Deletion typing; Spoligotyping	Zeweld, 2014
Strain Diversity of <i>Mycobacterium tuberculosis</i> Isolates from Pulmonary Tuberculosis Patients in Afar Pastoral Region of Ethiopia	m-PCR; Deletion typing; Spoligotyping	Belay <i>et al.</i> (2014)
Identification and Characterization of <i>Mycobacterium Tuberculosis</i> Isolates from Cattle Owners in North Western and North Eastern Parts of Rural Ethiopia	Deletion typing; Spoligotyping; SNPs typing	Mengistu <i>et al.</i> (2015)

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted in Gambella town municipal abattoir and Gambella hospital of Gambella regional state, southwest Ethiopia from December 2014 to May 2015.

The Gambella People's Regional State is located south west Ethiopia between the geographical coordinates of 6° 28'38" to 8° 34' North Latitude and 33° to 35° 11'11" East Longitude, 766 km far from Addis Ababa which covers an area of about 34,063 km². The Region is bounded to the North, North East and East by Oromya National Regional State, to the South and Southeast by the Southern Nations and Nationalities People's Regional State and to the Southwest, West and Northwest by the Republic of south Sudan (Behailu *et al.*, 2011).

Most of Gambella region is flat and its climate is hot and humid. The mean annual temperature of the Region varies from 17.3⁰C to 28.3⁰C and absolute maximum temperature occurs in mid-March and is about 45⁰C and the absolute minimum temperature occurs in December and is 10.3⁰C. The annual rainfall of the Region in the lower altitudes varies from 900-1,500mm; at higher altitudes it ranges from 1,900-2,100mm. The annual evapotranspiration in the Gambella reaches about 1,612mm and the maximum value occurs in March and is about 212 mm (Tilahune, 2012).

Based on the 2013/2014 Census conducted by the Central Statistical Agency of Ethiopia (CSA), the Gambela Region has total population estimation of 406,000 (CSA, 2013) and livestock population of Gambella 253,389 cattle, 39,564 sheep and 83,897 goat (CSA, 2010/2011).

The Gambella town municipal abattoir: - The abattoir which is administered under Gambella town municipality is the only source of inspected beef for all inhabitants of the

town. The average number of animals slaughtered per day during the study period was about 25 with all 100 % of the slaughtered animals being cattle. The overall abattoir sanitary environment is below the requirements of good hygiene practices (GHP) in slaughterhouses. The internal and external facilities and sanitary conditions of the slaughter house were very poor. Neither place for disposal of condemned carcasses nor facilities for wastewater treatment exist and it is not friendly with the environment. The abattoir workers had no clothing, boot, apron and other accessories. Three assistant meat inspectors were delivered services only during ante mortem and no one was carried out post mortem examination during the study period in such a ways the population is endanger of meat born zoonosis and sanitation problems.

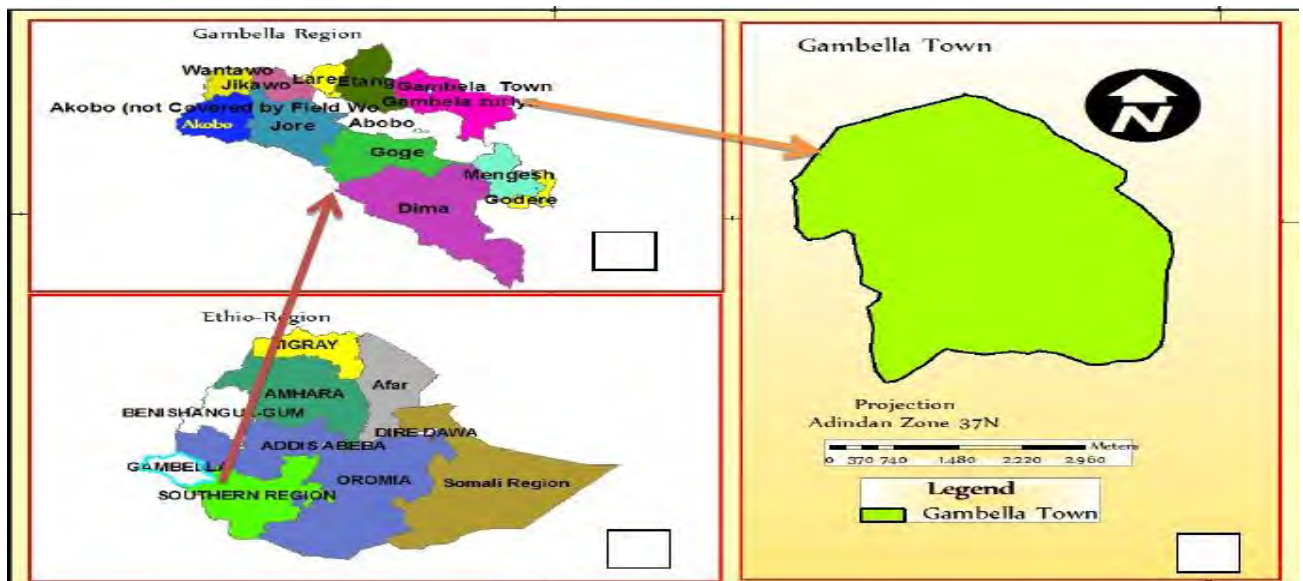


Figure 3. Map of the study area (CSA, 2007)

3.2. Study Population

According to the available logistics and time a total of 500 apparently healthy animals slaughtered in the abattoir of Local Nuer, Horro and Felata breed cattle were included as study population for the stated objectives and the major sources of cattle for this abattoir were Gambella town and its surroundings, Mettu, Gore, Bure, Sibbo and Gumero. In

addition, samples from human TB patients attending the health facilities in Gambella town were included.

3.3. Study Design

A cross sectional study with systematic random sampling was carried out in abattoir to examine the carcass and sample suspected TB lesions from slaughtered cattle at Gambella town municipal abattoir. Briefly daily 25 animals were slaughtered and it was decided five animals per day were supervised. Similar cross sectional study and purposive sampling was carried out to collect samples from all AFB positive TB patients attending Gambella Hospital. Both sputum and extra pulmonary TB samples mainly fine needle aspirate from suspected human case was taken in the course of the study period for isolation and molecular characterization of the causative agents.

3.4. Sample Size Determination and Study Methodologies

All animals coming to the slaughter house from different areas during the study period were considered for sampling. The sample size calculation was based on 50% prevalence assumption (since there was no study on bovine tuberculosis in the area), 95% CI and $d=0.05$ (Thrusfeild, 2005).

$$n = \frac{Z^2 \times p_{\text{expe}} (1-p_{\text{expe}})}{d^2}$$

Where n = required sample size

P_{expe} =expected prevalence

d =Desired absolute precision (5 %)

Z = Normal distribution constant

Therefore, the sample size calculated was 384, but to increase the precision using thumb rule by 20% and the total animals supervised were 500. Animals were selected using systematic random sampling method. For human study, all smear positive patients were requested to submit on spot and an early morning sputum sample in a 50 ml screw cap

centrifuge tube before they started TB treatment (50 AFB patients) were included under the sampling protocol.

The sample size for the questionnaire survey used was 100 for livestock owners, and abattoir workers. The questions included during the interview are shown in appendix 2.

For human case, a total of 50 acid fast positive patients were interviewed about their association with cattle, habit of consumption of meat and milk and other relevant information related to tuberculosis and the questionnaire is indicated at appendix 3.

3.4.1. Ante and postmortem examination

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

Detailed postmortem examination (inspection, palpation and incision) of the carcass, lungs, liver and kidneys together with mesenteric, hepatic lymph nodes and lymph nodes of the head was undertaken in accordance with the method developed by Ethiopian meat inspection and quarantine division of the Ministry of Agriculture (Hailemariam, 1975; Ameni *et al.*, 2007).

Lymph nodes were incised into a size of 2 mm to facilitate the detection of tuberculous lesions from each animal. These include Mandibular, Retropharyngeal, Bronchial, Mediastinal, and Mesenteric lymph nodes. The animal was classified as lesioned (infected) when tuberculous lesion was found, and if not as non lesioned (not infected).

The severity of gross lesions in individual lymph nodes and other organs were scored as follows; 0= no gross lesion, 1= small lesion at one focus, 2= small lesions at more than one focus and 3= extensive necrosis as developed by Ameni *et al.* (2006). The cut surfaces were examined under bright light for the presence of abscess, cheesy mass, and tubercles (Corner *et al.*, 1990). In the presence of suspected tuberculous lesion, tissue samples were collected in sterile universal bottles containing 0.85% normal saline for culture kept at -20⁰c refrigerator. The samples were transported under cold chain by ice box with packed ice to the Akililu Lema Institute of Pathobiology for culture and further processing in three week basis.

3.4.2. Isolation of mycobacteria

Tissues with suspected lesions were collected and subjected to bacteriological culture examination. The tissue specimen or sputum collected from AFB and gene expert positive patients for culture were collected individually in to sterile universal bottles in normal saline and then labeled and kept frozen (-20 °C) at Gambella regional hospital before being transported to Aklilu Lema institute of Pathobiology laboratory Addis Ababa.

The specimens were labeled and pooled together, kept in universal bottle containers, and then transported in ice pack box to Aklilu Lemma Institute of Pathobiology, Addis Ababa Ethiopia, within three week basis by airplane. There the samples were processed for isolation of *M. tuberculosis* complex according to the standard methods (Ameni *et al.*, 2007).

In brief, the sputum samples were decontaminated by shaking in an equal volume of 4% NaOH for 15 minutes and concentrated by centrifugation at 3000 rpm for 15 minutes. The sediment was neutralized with (1%) 2NHCl, using phenol red as an indicator. Bacteriological culturing of sputum samples were performed using the conventional Lowenstein-Jensen (LJ) egg slant medium, containing 0.6% sodium pyruvate and glycerol media, for the recommended time. Then the cultures were incubated at 37 °C for

4 to 8 weeks and examined on a weekly base for the presence of any mycobacterial colonies. Microscopic examinations of the cultures using the Ziehl Neelsen staining method performed to select AFB positive isolates.

Tissue sample from animals were transported in cold chain by using icebox packed with ice packs. On arrival at laboratory, tissue samples were macerated in sterile mortar and pestle by using surgical blades and forceps to get fine pieces and then homogenized with pestle for 10 min in 5 ml of PBS. The cultures were incubated at slant position aerobically at 37°C for 1 week and in upright position for the test for 7 weeks, with weekly observation for growth.

3.4.3. Identification and characterization of mycobacteria

Initial identification of mycobacterial species from animal tissue was based on the rate of growth, pigment production, and colony morphology as described in OIE (2009). When visible colonies were observed, Ziehl Neelsen staining was performed to confirm the presence of acid-fast bacilli. AFB positive isolates were prepared by mixing two loops full of colonies in 200 mL distilled water, heat-killed at 80°C for 1 hour using water bath, and stored at -20°C until molecular characterization was performed and were subjected to PCR based on amplification of a multi copy DNA target sequence for identification of *M. bovis* and *M. tuberculosis* (Debebe *et al.*, 2013).

3.4.4. RD deletion typing

For RD9 deletion typing of culture positives of sputum; RD9 intR: CTG GAC CTC GAT GAC CAC TC, RD9 flankF: GTG TAG GTC AGC CCC ATCC and RD9 flankR: GCC CAA CAG CTC GAC ATC primers to check for the presence of RD9 locus was used; The HotStarTaq Master Mix system from Qiagen was used for PCR, with primers described previously (Ameni *et al.*, 2013).

Interpretation of the result was based on the detection of bands of different sizes. For a band size of 396 bp, the isolate was considered as *M. tuberculosis*, whereas a band size of 575 bp was considered to correspond to either *M. bovis* or *M. africanum*. *M. tuberculosis* H37Rv and *M. bovis* BCG were used as a positive control; whereas Qiagen distilled water was used as a negative control.

For tissue sample cases, the isolates were harvested for RD4 deletion typing by scraping the growth from the slopes. Next, these shavings were poured into 200 ml of sterile distilled water, the mix was heated to reach a maximum temperature of 80°C and it was maintained at this temperature for one hour (Brosch *et al.*, 2002).

The primers used were RD4flank int: 5'-ACACGCTGGCGAAGTATAGC-3'; RD4flankR: 5'-AAGGCGAACAGATTCAGAT-3' and RD4flankF: 5'-CTCGTCGAAGGCCACTAAG-3'. The mixture was heated in a Thermal Cycler (Applied Bio-systems; Gene AMP 9700) for 15 minutes at 95°C and then subjected to 35 cycles of one minute duration at 95°C, one minute at 55°C, one minute at 72°C and 10 minutes at 72°C. Each PCR consisted of 7 µl distilled water (Qiagen), 10 µl Hot Star Taq Master Mix, 0.3 µl of each of the three primers (1.5µM final concentration) and 2 µl of DNA templates of samples or controls, thus making the total volume of primers 20 µl. *M. tuberculosis* H37Rv and *M. bovis* SB0933 was used as positive control, and distilled water was used as a negative control. The product was electrophoresed in 1.5 percent agarose gel in 1 x TAE running buffer. SYBR Safe at a ratio of 1:10000, 100 base pair (bp) DNA ladder and orange 6 x loading dye were used in gel electrophoresis. The gel was visualized using Syngene Bio Imaging System (Syoptics Group). The presence of RD4 (RD4 is intact in *M. tuberculosis*, *M. africanum*) gives a product size of 335 bp (RD4 intF + RD4flankR), and its absence (*M. bovis*) gives a product size of 446 bp (RD4flankF + RD4flankR).

3.4.5. Spoligotyping

Spoligotyping was carried out using the commercially available kit according to the manufacturer's instructions and as previously described by Kamerbeek *et al.* (1997).

Briefly, the direct repeat (DR) region was amplified with primers DRa: (5'-GGT TTT GGG TCT GAC GAC -3' biotinylated at the 5' end) and DRb: (5'-CCG AGA GGG GAC GGA AAC -3'). PCR amplification was done for 30 cycles with denaturation and annealing for 1 min at 95°C and 1 min at 55°C, respectively, and extension for 30s at 72°C in each cycle. The amplified DNA was hybridized to inter-DR spacer oligonucleotides covalently bound to a membrane for 1 hour at 60°C. Reference strains of *M. tuberculosis* H37Rv and *M. bovis* SB0933 were used as positive control whereas sterile water was used as a negative control. The amplified DNA was subsequently hybridized to a set of 43 oligonucleotide probes by reverse line blotting. The hybridized PCR products were incubated with streptavidin-peroxidase conjugate, and signal detection was obtained with an enhanced chemiluminescence detection system followed by exposure to an X-ray film according to the manufacturer's instructions. The X-ray film was developed and washed using standard photochemical procedures.

3.4.6. Questionnaire survey

The roles of various risk factors in the occurrence and spread of bovine TB among cattle, and between cattle and people, were assessed by a questionnaire. Structured questionnaire was distributed to TB patients, cattle owners, and abattoir workers to assess the perception of stakeholders on the occurrence of bovine tuberculosis, livestock constraints, socioeconomic status, herd composition, awareness on the potential risk of zoonotic transmission of bovine tuberculosis.

3.5. Data Management and Analysis

Prevalence was calculated as the proportion of suspected lesion positive animals from the total number of animals visited (Thrusfield, 2005). Data related with age, sex, breed, origin and body condition of each animal was recorded on a data sheet during antemortem examination. Presence or absence of TB like lesions and affected tissues were recorded during postmortem examination. The recorded data was entered and stored in Microsoft Excel computer program and analyzed by STATA version 11 (STATA

Corp. College station, TX). The variations between different factors were also analyzed using multi variable logistic regression and chi-square (χ^2) was used for association of different risk factors. A p-value <0.05 was considered statistically significant, 95% confidence interval was considered and Odds ratio analysis was used.

In molecular epidemiology study of isolates from human pulmonary tuberculosis patients and animals tissue, the spoligotype patterns were converted in to binary and octal formats and entered to the online spoligotype database, http://www.pasteur.guadeloupe.fr:8081/SITVIT_Demo/index.jsp to determine the shared international spoligotype (SIT) number and the results were compared with already existing designations in the international spoligotyping database (SpolDB4.0 database). Those isolates with no designated SIT number were considered as new to the database. Two or more isolates with identical spoligotype pattern were considered as clustered while those with single SIT were considered as non-clustered isolates. TB-lineage and family were determined using SPOTCLUST database, http://tbinsight.cs.rpi.edu/about_spotclust.html

3.6. Ethical Considerations

Ethical clearance was obtained from the Ethical Committee of Gambella regional health office (Ref. number of 16/3776/7) indicated in appendix 6 and working permission was gotten from the hospital higher managers and the municipality. The study subjects were informed about the objective of the study and all the reasons why participants were chosen and why the research wants to be done. The participants were allowed to consider their participation and given the opportunity not to participate in the study if they wished to do so. Finally patient results were reported to the appropriate physician at Gambella hospital in order to manage and treat patients appropriately. Additionally confidentiality of all the information was assured.

4. RESULTS

4.1. Prevalence of Bovine Tuberculosis

The overall prevalence of bovine tuberculosis in slaughtered cattle of Gambella municipal abattoir was 13.2% (95% CI, 10.22-16.18) based on the occurrence of gross tuberculous lesions. Associations of the putative risk factors, As indicated in Table 2, the difference in prevalence of BTB in different breeds and body condition scorings were statistically significant ($\chi^2=24.996$, $P=0.000$, $df=1$; $\chi^2=39.105$, $p=0.000$, $df=1$) respectively.

Out of 500 slaughtered animals included in the study 301 (60.2% were Nuer, 185 (37%) Horo and 14 (2.8%) Felata breeds. The prevalence of tuberculous lesion is higher in the case of Felata breed (57.14%) followed by Nuer and Horo breeds 12.96%, 10.27% respectively and this difference in prevalence was statistically significant ($\chi^2 = 24.996$, $P=0.000$, $df=1$).

The multi variable logistic regression analyses of the different risk factors considered for tubercle lesion positivity were shown in (Table 2). Poor body conditioned animals were more likely to develop TB lesions (OR=12.16, 95%CI, 4.58-32.24) than good body conditioned animals. Felata breed cattle were more likely to have tuberculous lesion (OR=6.43, 95% CI, 1.96 to 21.04) than Nuer breed cattle.

Table 2. Univariate and Multivariable logistic regression analysis of tuberculous lesion with various host related risk factors in Gambella municipal abattoir

Risk factor	Number examined	Number positive	χ^2	P-value	Crude odds ratio (95% CI)	Adjusted odds ratio (95%CI)
Age (year)						
<5	24	2			1	1
5-8	188	24	0.646	0.724	1.61(0.36-7.28)	1.26(0.25-6.43)
>8	288	40			1.77(0.40-7.84)	1.08(0.22-5.37)
Sex						
Male	346	36	7.661	0.006	1	1
Female	154	30			2.08(1.23-3.53)	1.05(0.52-2.15)
BCS						
Good	89	29			1	1
Medium	173	22	39.105	0.000	2.17(1.089-4.31)	3.54(1.45-8.64)*
Poor	238	15			7.19(3.62-14.26)	12.16(4.58-32.24)*
Breed						
Nuer	301	39			1	1
Horo	185	19	24.996	0.000	0.77(0.43-1.38)	0.67(0.09-4.97)
Felata	14	8			8.96(2.95-27.198)	6.43(1.96-21.04)*
Origin						
Gambella town & surroundings	328	47			1	1
Sibo	45	3			0.598(0.18-2.04)	3.0(0.29-30.67)
Gore	29	5	2.987	0.702	0.85(0.19-3.88)	5.88(0.49-71.13)
Mettu	49	6			0.83(0.34-2.08)	4.9(0.57-42.38)
Gumero	33	3			1.25(0.45-3.43)	6.94(0.82-59.00)
Bure	16	2			0.43(0.13-1.434)	2.01(0.22-18.47)

*Statistically significant

4.2. Gross Pathology Results

Gross lesions were observed in the lymph nodes and lung of the slaughtered cattle and the majority of the lesions were considered typical of tuberculous lesions characterized by central round, oval, or irregular, often coalescing areas of caseous necrosis and mineralization (calcification) (Figure 4). Whenever gross lesions suggestive of TB were detected in any of the tissue, the tissue was classified as having lesions.

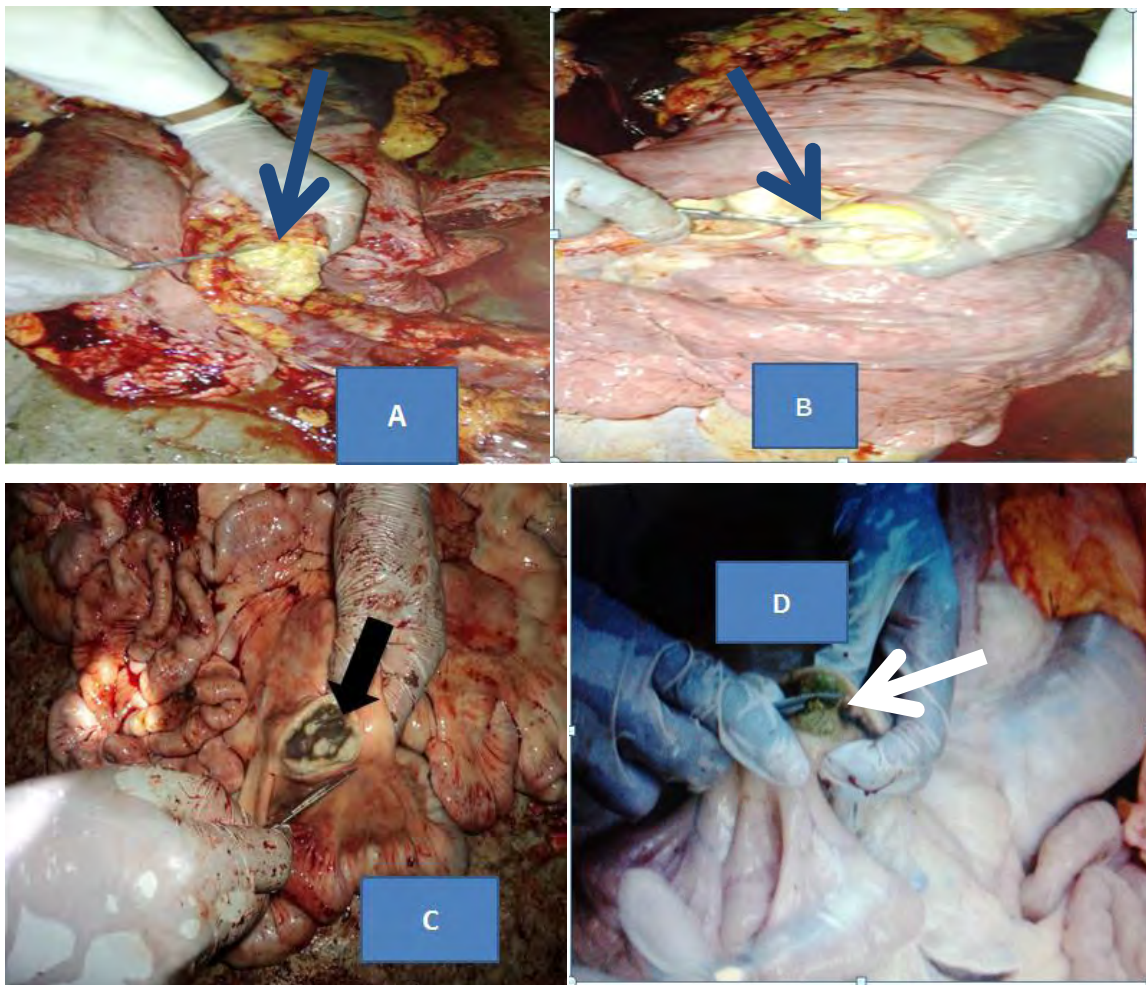


Figure 4. Typical TB lesions of cattle slaughtered in Gambella town municipal abattoir, A= granulomatous lesion from mediastinum B=Caseous and granulomatous necrosis in lung C&D= calcified and granulomatous lesion in mesenteric lymph nodes.

The frequency and distribution of lesions according to organ level and anatomical site is indicated in (Table 3) and 50% (41/82) of the gross lesions were examined from lymph nodes of thoracic cavity followed by mesenteric and head lymph nodes 25.6% (21/82) and 24.3% (20/82), respectively and based on this result respiratory route of transmission was the most important route of infection.

Table 3. Distribution of lesions in different anatomical sites with their respective frequency of occurrence

Anatomical site	Organ affected	Frequency (%)
Head	Mandibular lymph node	5(6.1%)
	Retropharyngeal lymph node	15(18.5%)
Thoracic	Bronchial lymph node	8(9.8%)
	Mediastinal lymph node	19(23.2%)
	Lung	14(17.1%)
Abdominal	Mesenteric lymph nodes	21(25.6%)
Total		82(100%)

The pathological scoring analysis of different organs revealed the mean severity of lesion was higher in mandibular lymph node (2.6 ± 0.245) followed by bronchial lymph nodes (2.25 ± 1.035), mesenteric lymph nodes (2.14 ± 0.168), retropharyngeal (1.93 ± 0.245) and lung (1.86 ± 0.231) (Table 4, Figure 5).

Table 4. Mean pathology scoring of lesion from lung and lymph node of bovine tuberculosis in cattle slaughtered at Gambella municipal abattoir

Tissue	Number examined	Number positive (%)	Mean \pmSE)
Lung	500	14(2.8)	1.86 ± 0.231
Mandibular	500	5(1)	2.6 ± 0.245
Bronchial	500	8(1.6)	2.25 ± 0.366
Mediastinal	500	19(3.8)	1.5 ± 0.159
Retropharyngeal	500	14(2.8)	1.93 ± 0.245
Mesenteric	500	22(4.4)	2.14 ± 0.168

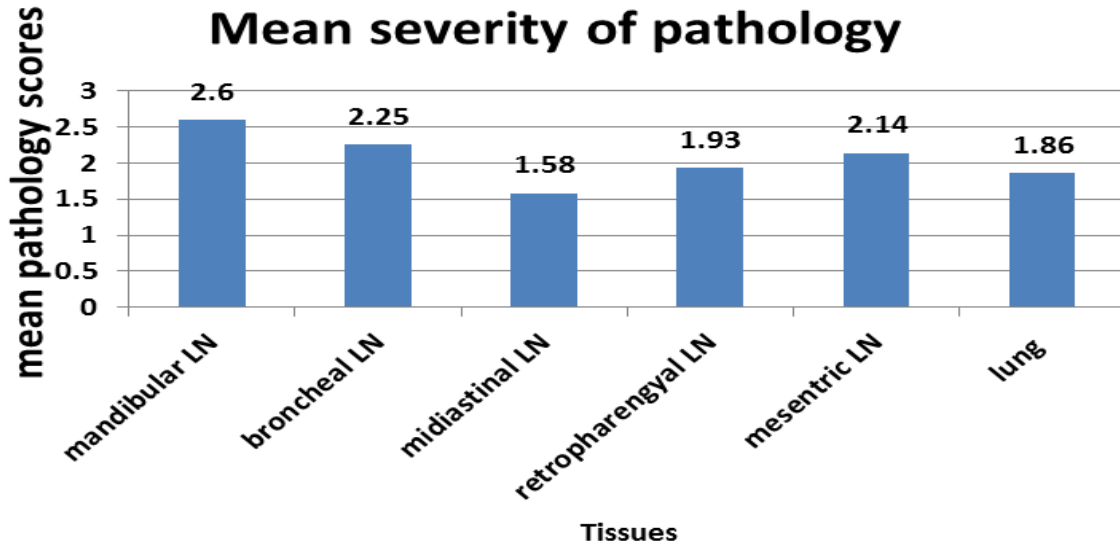


Figure 5. Mean severity of lesion in lung and different lymph nodes

4.3. Mycobacteriological Culture Result

Out of 82 tissue samples 14(17.07%) showed a growth on LJ medium and out of 50 sputum samples and one FNA sample, 17(34%) of sputum samples had showed growth on LJ media while the FNA sample did not grow (Table 6).

Table 5. Bacteriological results on LJ media

Type of Specimen	Number of sample	Growth on LJ-pyruvate	Growth on LJ-glycerol	Growth on both	Total growth (%)
Sputum	50	8	9	5	17(34)
FNA	1	-	-		0
Animal tissue	82	5	10	1	14(17.07)
Total	133	13	19	6	31(23.3%)

4.4. Molecular Characterization of Mycobacterial Isolates

Molecular analysis was performed on 11 isolates from human samples and 8 isolates from animal's tissue samples using RD9, RD4 and spoligotyping. The RD9 analysis of 11 isolates from human showed that all were positive for *M. tuberculosis* species with 396bp band size (Figure 6).

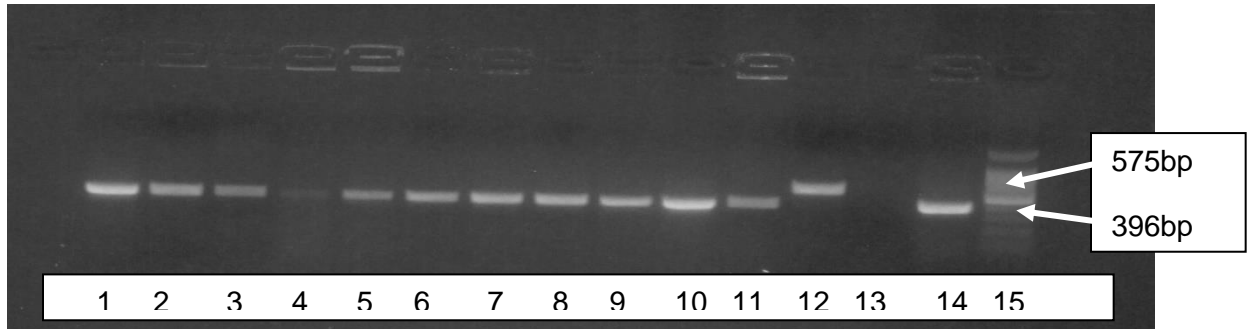


Figure 6. Electrophoretic separation of PCR products by RD9 deletion typing of 11 mycobacteria isolates from human TB patients. 15-ladder (100bp), 14-*Mycobacterium tuberculosis* control, 13-distilled water negative control, 12-*Mycobacterium bovis* control and 1 to 11 are samples from TB patients.

RD4 deletion typing of the 8 isolates from animal tissue samples; only one isolate was confirmed to be *M. tuberculosis* indicating 335bp band size while the rest isolates did not show any signal (Figure 7). This result revealed the presence of *M. tuberculosis* infection in cattle, in which the tissue was obtained from mediastinum lymph node and might suggest respiratory transmission of *M. tuberculosis* from infected humans as a case of reverse zoonosis.

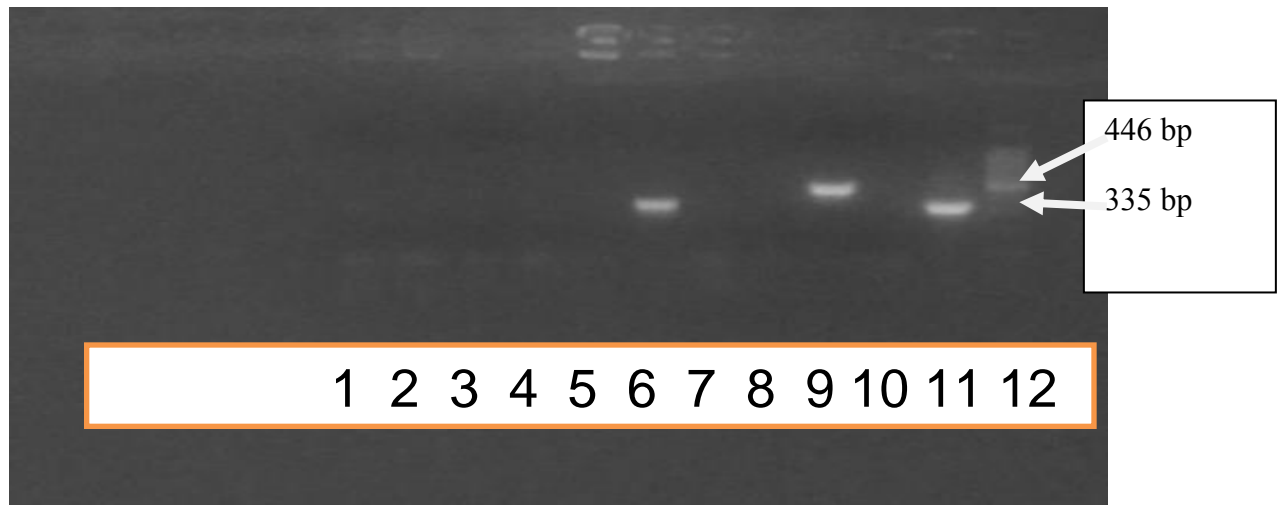


Figure 7. Electrophoretic separation of PCR products by RD4 deletion typing of 8 mycobacteria isolates from tissue sampled culture. 12- Ladder (100bp), 11- *Mycobacterium tuberculosis* control, 10-Distilled water negative control, 9- *Mycobacterium bovis* control and 1 to 8 are samples from tissue culture positives.

Spoligotyping of 12 isolates from human (n=11) and animal (n=1) which were positive on RD9 and RD4 analysis, respectively showed 6 different spoligopattern with 50% (6/12) of genetic diversity of *M. tuberculosis*. Based on web-based international spoligotype (SpolDB4.0) database analysis of spoligotype results the human isolates identified SIT289 (n=5), SIT134 (n=3), SIT1634, SIT142 (n=1) and one new strain not found in the spoligotype databases (Figure 8). The new spoligotype pattern had octal number of 503757740003571. Of these *M. tuberculosis* strains identified SIT 289 and SIT134 were the most predominant strains which were found in cluster of 45.5%(5/11) for SIT289 and 27.3(3/11) for SIT134 indicating the recent transmission of the stains in the community. Analysis of one of the *M. tuberculosis* isolate from Mediastinal lymph node of the animal tissue with spoligotyping analysis identified the isolates as SIT523 The *M. tuberculosis* strain from animal tissue sample had SIT523 could indicated reverse zoonosis of *M. tuberculosis* from human TB patient to cattle.

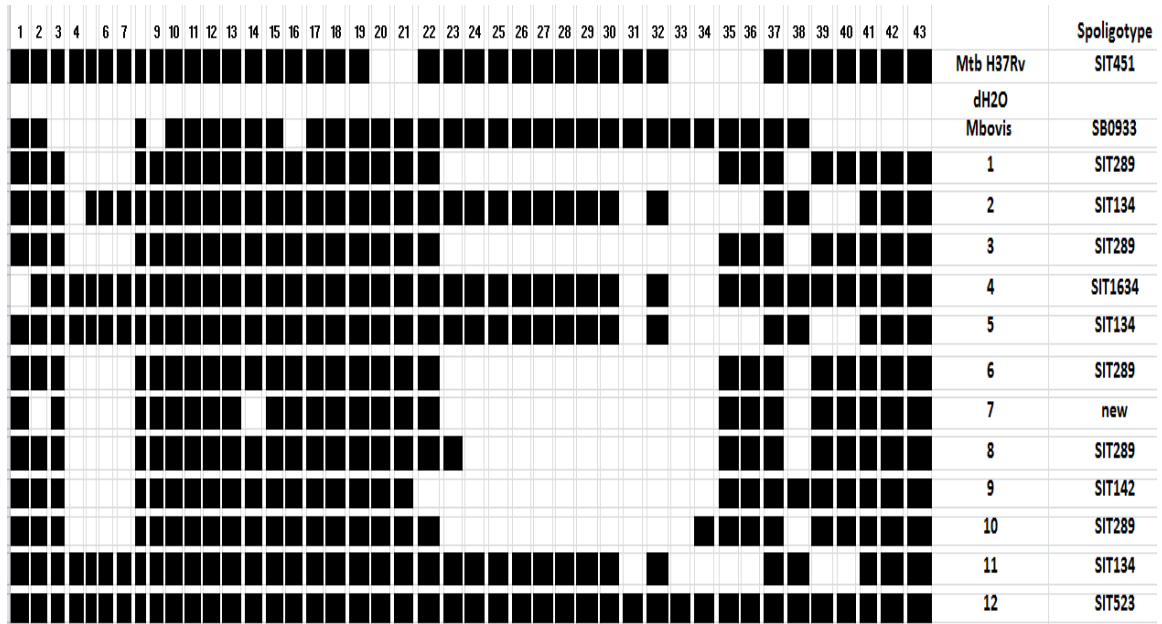


Figure 8. Spoligotype patterns of mycobacterial isolates recovered from sputum of human patient and tuberculosis lesions in cattle (1 to 12). The filled boxes (blacks) represent the presence of spacers, and the empty boxes represent the absence of spacers.

Lineage of the human isolates indicated that 27.3% (3/11) modern Euro-American, 9.1% (1/11) ancient Indo-oceanic and while 63.6% (7/11) were unknown lineage family in TB-insight database. Interestingly, the new isolate did not belong to any of the known lineage and classified as unknown lineage in the database (Table 6). The one that was found from animal was with indo-oceanic lineage family.

4.5. BTB Awareness and risk factor Assessment

From the questionnaire survey majority of the respondents (78%) had no awareness about bovine tuberculosis only 22% had knowledge regarding BTB signs. 37% and 45% of the respondents drink raw milk and eat raw meat respectively. Additionally, 48% of the respondent used the same watering point with animals and 30% of the respondents shared the same house with animals which may be a risk of zoonotic and/or reverse zoonotic tuberculosis transmission (Table 7 and 8).

Table 7. Client's (farmers, abattoir workers TB patients) awareness of bovine tuberculosis and its mode of transmission

knowledge examined in questionnaire	Responders out of 100 (%)
Had noticed respiratory problems in their cattle	30(30%)
Aware of bovine tuberculosis (TB)	22 (22%)
Know that cattle transmit bovine TB to humans	15 (15%)
Know that humans transmit TB to cattle or vice versa	0 (0%)
Know that milk is a source of infection	23(23%)
Know that meat is a source of infection	17(17%)
Drink raw milk	37(37%)
Eat raw meat	45(45%)
Use the same watering point with animals	48(48%)
Share the same house with animals	30(30%)

Table 8. Public health concerns of TB patients

TB patients status examined in questionnaire	Responders out of 50
Know that cough spray is a source of infection	68(68%)
Had any previous contact with human TB person	2(4%)
Had any type of contact with cattle or other animals	20(40%)
Drink raw milk	24(48%)
Eat raw meat	35(70%)

5. DISCUSSION

Tuberculosis remains a major global health problem causing high morbidity and mortality among millions of people each year (WHO, 2014). Tuberculosis caused by *M. bovis* is clinically indistinguishable from tuberculosis caused by *M. tuberculosis* and globally the proportion of human tuberculosis caused by *M. bovis* is estimated to 3.1% of all forms of which 2.1% of pulmonary and 9.4% of extra pulmonary forms (Cosivi *et al.*, 1998).

Ethiopia is one of the countries with highest number of livestock resource in Africa and animal tuberculosis is known to be endemic and wide spread in the country. However, in spite of high prevalence both human and animal tuberculosis in the country, the emphasis given on bovine tuberculosis to the Gambella region is very little and so far no research were carried out on BTB in Gambella Region. Infection of cattle with *M. bovis* constitutes a human health hazard as well as an animal welfare problem. Furthermore, the economic implications in terms of trade restrictions and productivity losses have direct and indirect implications for human health and the food supply (Zeweld, 2014).

In the present study an attempt was made to determine the prevalence of bovine tuberculosis in Gambella town municipal abattoir and identifying risk factors associated with bovine tuberculosis, to isolate and molecular characterization of *Mycobacterial* isolates from slaughtered cattle and from human TB patients and to investigate the potential risk factors for zoonotic transmission of mycobacterial infections from animal to human and vice versa.

Based on detailed post mortem inspection the prevalence of BTB in slaughtered cattle was found to be 13.2%, which moderately high and this result was comparable with other pervious research reports carried out on cattle originated from extensive and pastoral production system of Ethiopia; 11.50% by Abdurohman (2009) in Butajira, and 11% by Mamo *et al.* (2013) in Afar, but less than a result from 19.8% record from cattle slaughter

in rural Tanzania (Cleaveland *et al.*, 2007). The result of the present prevalence study was higher than findings by various other authors Biffa *et al.*, 2010a who reported 4.2% prevalence in cattle slaughtered at in Yabello municipal abattoir and 4.5% at Hosaana abattoir by Teklu *et al.*, (2004). In addition, the result were also higher than previously reported by other researcher in Northern and central parts of the country (Nemomsa *et al.* (2014) (9%); Zeru *et al.* (2013) (6.4%); Romha *et al.* (2013) (5.8%). This difference in prevalence of tuberculous lesions could be due to the difference in origin or types of production system and breed of animals that are slaughtered in the abattoirs.

In respective of small sample size due to wondering of the Felata breed from place to place, association of breed with prevalence of BTB showed a statistically high significant difference among different local breed of cattle, ($P = 0.000$) animals which might be related to the genetic difference of the breeds, Other previous studies also showed different breeds could result in difference in susceptibility to BTB infections (Ameni *et al.*, 2007).

There is a statistically significant difference in the prevalence of the disease ($P = 0.000$) between BCS, the prevalence being the highest in poor body condition (32.6 %) as compared to medium (12.7%) fatty (good) animals (6.3 %) respectively which in agreement with study resulted by Nemomsa (2014). This could due to related to the weak protective immune response in poor body conditioned animals as compared to good one that may result extensive lesions and wasting of the body condition as well as its chronicity nature of the disease. The present result is consistent with previous reports which indicated that animals with good BCS have relatively good immunological response to the infectious agent than animals with medium BCS (Radostits *et al.*, 1994; Radostatit *et al* 2007).

In this study, gross tuberculosis lesions were found most frequently in the lymph nodes of thoracic cavity (50%), mesenteric lymph node (25.6%), followed by lymph nodes of the head (24.4%). This finding is significantly different from previous studies done in Ethiopia (Tamiru *et al.*, 2013) where 70 and 70.7% TB lesions were reported in lungs and

associated lymph nodes, respectively. However, the distribution of TB lesion in the current study significantly similar with reports from Mexico (Ndukum *et al.*, 2010) where 49.2% of lesions involved the thoracic lymph node. The result, therefore, indicate that the primary route of infection was through the respiratory route which can also spread to other parts of the body as described previously (Radostits *et al.*, 2007).

Corner (1994) has reported that up to 95% of cattle with visible TB lesions could be identified by examination of the lung and associated lymph nodes. This finding indicates that inhalation might be the principal route of TB infection in cattle. Therefore, during postmortem examination, focus should be given on lungs and associated lymph nodes. However, the presence of lesions in mesenteric lymph nodes also indicates that the additional infection could also occur through ingestion (Radostits *et al.*, 1994).

In this study, culture positivity in primary culture media was found low and confirmed in 23.49% (31/133) despite slightly lower than that reported by Ameni *et al.* (2007), 56% culture positivity. This low isolation rate of mycobacteria may have resulted from reduced sensitivity of culture arising from prolonged storage at field sites and the freeze-thaw cycles that occurred during transportation and contamination of tissue samples (OIE, 2009). Furthermore, the presence of caseous and/or calcified lesions and even lesions resembling tuberculous lesions may not always found to be of mycobacterial origin; they can be caused by any other intracellular organisms or parasites, or viable mycobacteria may not be present in calcified lesions (Corner, 1994).

In the present study, interestingly, *M. tuberculosis* strain SIT523 was isolated and characterized with spoligotyping from cattle cranial Mediastinal lymph node tissue and the result implies the occurrence of reverse zoonosis in the study area where human strains could be transmitted to cattle. The transmission to cattle could be through different routes including ingestion of feed contaminated with infected sputum and/or urine from *M. tuberculosis* infected farmers. Humans suffering from active TB are the most probable source of *M. tuberculosis* in animals, with infection spread via sputum, and rarely urine or faeces (Thoen and Steele, 1995) or respiratory route as in rural area of

Ethiopia, grazing cattle are commonly brought into the farmers' households at night where they may become infected via aerosol transmission from humans (Ameni *et al.*, 2013)... Previous studies in Ethiopia had confirmed transmission of *M. tuberculosis* from farmers to their cattle, goat and camel (Berg *et al.*, 2009; Ameni *et al.*, 2011; Gumi *et al.*, 2012; Mamo *et al.*, 2012) supporting the result of the present study. Moreover, in a study conducted in India from extra pulmonary tissue of tuberculous cattle, 15–28% of the animals were discovered to be infected with *M. tuberculosis* (Aranaz *et al.*, 1996). Another study from Nigeria also identified one tissue isolate as *M. tuberculosis* by spoligotyping and VNTR (Prasad *et al.*, 2005). Even though human to cattle transmission of *M. tuberculosis* has been reported, it is generally held that disease in cattle due to *M. tuberculosis* is less severe than that caused by *M. bovis* and the identification of *M. tuberculosis* in cattle by itself is intriguing (Tsegaye *et al.*, 2010). On to this, the identification of *M. tuberculosis* from cattle tissues requires further investigation.

In molecular characterization of isolates from human tuberculosis patients, *M. tuberculosis* was the predominant species causing TB in human and the genetic diversity of the isolate on the spoligopattern was 45.45%, which was higher than previous reports in other part of Ethiopia where 39% of spoligotype based genetic diversity were reported in Afar PTB patients (Mamo *et al.*, 2013). The difference might be related to difference on geographic and sociocultural difference among the studied population which might affect the transmission pattern of the organism. The most common spoligotype identified from TB patient was the SIT 289, in agreement with previous study (Ameni *et al.*, 2011) which also reported the same SIT289 strains in pulmonary TB patients of central Ethiopia. In the present study, the predominant lineage was unknown according to TB-insight database analysis. Similar, unknown lineage had been previously reported from patients from Northwestern Ethiopia (Belay *et al.*, 2014) and this indicates the need for further investigation.

In the present study, the questionnaire survey of the respondents showed that 22 % of them were aware of BTB with no knowledge about zoonosis of the disease. This disagrees with report from Tamiru *et al.*, (2013) 80.7% of them were aware of BTB with low level knowledge about zoonoses of the disease. Our result was

comparable with the study on assessment of the knowledge of cattle owners about BTB in Wuchale Jida district, Ethiopia showed that 38.3% (36 of 94) of the respondents knew that cattle can have tuberculosis, and 30.8% (29 of 94) recognized that BTB is zoonotic (Ameni *et al.*, 2003). Ameni *et al.*, (2007) have indicated that lack of understanding regarding the zoonotic of BTB, food consumption behavior and poor sanitary measures is the potential risk of BTB to public health. The proportion of BTB contributes to total tuberculosis cases in humans depends on the prevalence of the disease in cattle, consumer habits, socio-economic conditions, level of food hygiene (Ashford *et al.*, 2001) and medical prophylaxis measures in practice (Tigre *et al.*, 2011). According to the result of the present study, 45% consume unpasteurized or raw milk. Similarly, studies conducted in different parts of Ethiopia indicated the habits of raw milk consumption. The current result on habit of milk consumption was lower than 85.7% report from Jimma town, Ethiopia (Tigre *et al.*, 2011). Study conducted in Wuchale Jida district indicated 52.1% (49 of 94) households“ has habit of consuming raw milk (Ameni *et al.*, 2003), which is significant when compared with the current result. No one of the respondents in our study were found to be aware about the transmission of the disease from cattle to human and vice versa.

In our study, keeping cattle in close proximity to their house and calves in their house was a common practice of households. This indeed can facilitate transmission of the causative agent from animal to human or vice versa. According to Bogale (1999), conditions such as customs of consuming raw milk, keeping cattle in close proximity to the owner house and using cow dung for plastering wall or floor and as source of energy for cooking do exacerbate the chance of spread of tuberculosis as zoonosis in Ethiopia.

6. CONCLUSIONS AND RECOMMENDATIONS

The result of the present study has shown that bovine tuberculosis was prevalent in cattle slaughtered at Gambella municipal Abattoir with moderately high prevalence (13.2%). This study also revealed that a high proportion of tuberculous lesion in the thoracic cavity lymph nodes and which implies that respiratory route was the major means of transmission. Isolation and molecular characterization of one *M. tuberculosis* isolate (SIT523 strain) from animal tissue sample suggested the occurrence of transmission of the agent between the communities and animals that implies reverse zoonosis. The high genetic diversity (45.5%) of the human *M. tuberculosis* isolates (SIT289, SIT134, SIT1634, SIT142, and the new one) and presence of clustering of the isolates might indicate the recent transmission pattern and circulation of the agents in the study communities.

Lack of awareness regarding BTB and its routes of transmission in the study population was high and existence of habits of consumption of raw animal product and sharing of the same microenvironment with their livestock could be potential risk factors for zoonotic transmission of the disease.

On the basis of findings of the present study, the following recommendations are forwarded

- Further study should be conducted with larger sample size and geographic coverage to elucidate the role of *M. tuberculosis* complex in human and animal.
- With the finding of promising result on molecular characterization using few samples; a broader study to investigate the molecular epidemiology in human and animal tuberculosis is essential.

- Public health awareness campaigns should be launched and needed to raise community awareness about the risk of BTB transmission through consumption of raw/under cooked meat; and the zoonotic implication of BTB/, route of reverse zoonosis are of extreme importance for effective implementation of TB control measures.

- Establishment of collaboration between physician and veterinarians to trace back positive patient to get profile of their cattle in the slaughterhouse across the region so as to estimate the regional prevalence of BTB as well as identification and characterization of the *M. tuberculosis* complex, and evaluation of their pathogenicity in bovine is essential.

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8. APPENDICES

Appendix 1. Sample registering sheet

No.	Source of Animal		Sex	Age	Breed	Bo. con.	Lesion samples taken from							
	Low land	High land					Liver	Intestine	Lung	Kidney	Lymph nodes			
										Bronchia	mediastin	Retp.	Mes	Mand

Appendix 2. Questionnaire to interview farmers and animal owners

Date _____

Name _____

Age _____

Sex _____

Region _____

Tribe _____

Zone _____

Wereda _____

Village _____

1. Educational status:
 - a. Illiterate
 - b. Basic writing & reading
 - c. Primary (Grade 1 to 6)
 - d. Junior secondary (grade 7 to 8)
 - e. Secondary (Grade 9 to 12)
 - f. Diploma
 - g. Degree and above
2. Which species of domestic animals do you own?
 - a. Cattle
 - b. Sheep
 - c. Goats
 - d. Horse/mules/donkeys
 - e. Poultry
3. Which breed of cattle do you own?
 - a. Local
 - b. Cross
 - c. Exotic
4. Purpose of keeping cattle for.
 - a. Draft
 - b. Milk for home use
 - c. Milk for sell
 - d. Others
5. How many cattle do you have?
 - a. Less than 10
 - b. 10-20
 - c. More than 20
6. Have you noticed respiratory problems in your cattle?
 - a. Yes
 - b. No
7. Do you know bovine tuberculosis?
 - a. Yes
 - b. No
8. How do you manage cattle?
 - a. Free grazing
 - b. Stall feeding
9. Do you practice mouth chew tobacco or local medications to your cattle?
 - a. yes
 - b. No
10. Do you mix your cattle with other cattle?
 - a. Yes
 - b. No
11. If yes where?
 - a. Watering point
 - b. Grazing field
 - c. Market

12. Is there any contact of your cattle with wild animals? a. yes b. No
 If yes which type, in order of priority? 1..... 2
 3.....
13. Do you use the same watering point with animals? a. Yes b. No
14. Do you share the same house with your animals? a. Yes b. No
15. Do you know that tuberculosis can be transmitted from cattle to man and vice versa? a. Yes b. No
16. If yes, would you please indicate some ways?
17. Habit of drinking milk a. Raw b. Boiled c. Mixed
18. Do you boil milk? a. Yes b. No
19. Reason for boiling? a. Fear of milk born disease b. Culture
20. Habit of eating meat? a. Cooked b. Raw c. Mixed
21. Habit of eating blood? a. Cooked b. Raw c. Mixed
22. If you sell milk/milk products, who buys it?
 a. Local people b. Milk collection unit c. Others (specify).....
23. Do you have tuberculosis patient in your family or farm worker?
 a. Yes b. No
24. If yes how many? -----
25. If you have TB patient in your family or farm worker, indicate the type of TB?
 a. Pulmonary b. Extra pulmonary c. Do not known
26. How long have you been sick? a. Less than a year b. More than a year
 c. Others
27. Have you ever taken any treatment? a. yes b. No
28. if yes, a. Traditional b. Drugs

Thank you!

Name of interviewer _____

Signature _____

Date _____

Appendix 3. Questionnaire to interview hospital coming patients and abattoir workers

Date _____ Name _____
Age _____ Sex _____ Region _____ Zone _____
Wereda _____ Village _____ Tribe _____

1. Educational status:
 - a. Illiterate
 - b. Basic writing & reading
 - c. Primary (Grade 1 to 6)
 - d. Junior secondary (grade 7 to 8)
 - e. Secondary (Grade 9 to 12)
 - f. Diploma
 - g. Degree and above
2. Species of animals commonly slaughtered in the abattoir
 - a. Cattle
 - b. Sheep
 - c. Goat
3. Which breed of cattle commonly slaughtered
 - b. Local
 - b. Cross
 - c. Exotic
4. Source of cattle to be slaughtered?
5. Number of cattle slaughtered in a day.....
6. Did the technician perform ante mortem examination?
 - a. Yes
 - b. No
7. Do you know bovine tuberculosis?
 - a. Yes
 - b. No
8. What type of lesion you has been encountered?
9. In which organ of the animal body the lesion commonly found?
 - a. Liver
 - b. Lung
 - c. Intestine
 - d. Lymph nodes
 - e. Others(specify).....
10. In which group of the cattle are mostly you found the lesion?
 - a. <2years
 - b. 2-4 years
 - c. 4-7 years
 - d. > 7 years
11. What do you recommend if you get TB lesion on the meat of slaughtered animal?
12. In which body condition group of animal do you found the TB lesion?
 - a. Emaciated
 - b. Tin
 - c. Medium
 - d. Fat
13. Do you know that tuberculosis can be transmitted from cattle to man and vice versa?

Appendix 4. LJ media preparation to culture the tissue and sputum samples

❖ LJ Pyruvate (LJP):

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

❖ LJ Glycerol (LJG):

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

❖ Egg Fluid

- ✓ Break 1000ml of eggs into a sterile beaker (2000ml).
- ✓ Add the fluid eggs to the autoclaved mineral solution.
- ✓ Using a Homogenizer, mix until homogeneous.
- ✓ Add 20ml of 2% Malachite Green.
- ✓ Stir for at least 10 minutes before dispensing.
- ✓ Filter the mixture through sterile muslin into a sterile round glass flask (2000ml) containing a sterile magnetic bar.

- ✓ Add: 3.0ml of Polymyxin B (100, 000 iu/ml), 0.75ml of Carbenicillin (0.2g/ml), 15ml of Fungizone (5mg/ml) and 3.0ml 1% Trimethoprim.
- ✓ Mix well and slowly on a magnetic mixer for at least one hour under UV light in a biosafety cabinet (without running the cabinet).
- ✓ Decant into two separate sterile Duran glass flasks and close each flask with sterile lids surmounted by a sterile dispenser.

Aliquoting the medium

- ✓ Dispense 8ml into each sterile test tube or 10 ml into each universal tube. Avoid air bubbles.
- ✓ Clean the dispenser top with paper tissue impregnate with 70% ethylic alcohol after every set of 10 tubes.
- ✓ Transfer the tubes containing the medium into a sterile crate.
- ✓ After dispensing the entire medium, range the tubes on a special rack and lean them to give a slope when placing in an oven.
- ✓ Tighten screw caps, slant them and coagulate by inspissation at 85 ° C for 50 min.
- ✓ Let cool.
- ✓ Give the medium batch a lot number and label the racks with this number.
- ✓ Label the universal lids or culture tube with colour codes.

Appendix 5. AFB-Smear preparation/ Ziehl-Neelsen staining of smears

- ✓ Label new, clean, unscratched microscope slide at one end with the relevant sample name/number. Avoid touching the surface of the slide.
- ✓ Thoroughly mix the specimen with a pipette (1ml) and place about one drop (or 2 – 3 loop full) on the slide.
- ✓ Using a loop spread the smear over a surface of about 1.5cm x 1cm.
- ✓ Allow the smear to air dry completely in the BSC I. Do not use heat for drying!
- ✓ Heat-fix the slide either by passing it through a flame three to four times with the smear side up. Alternatively, allow the slide to fix on an electric

slide warmer at 65 -75°C for 2-3 hours or overnight. Do not overheat or expose smears to UV light (6). Let the slide cool before staining.

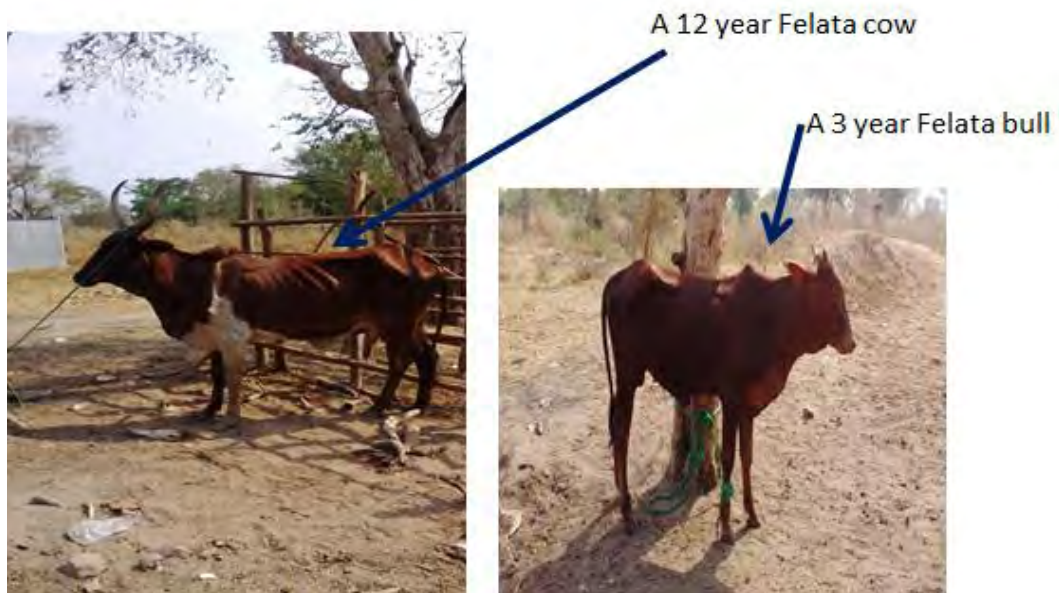
- ✓ Put the glass slides with the fixed smears on a staining rack (don't stain more than 12 smears at a time).
- ✓ Flood the entire slide with basic fuchsin solution.
- ✓ Heat the slides slowly until slight steam rises. Do not boil!
- ✓ Maintain steaming for 3-5 minutes (do not let slides dry, add basic fuchsin onto the slides if you note too much of evaporation). Do not let boil!
- ✓ After 5 minutes of basic fuchsin reaction, gently wash off all free stain completely from each slide with distilled water (using forceps, slope the slide one by one to remove the water completely).
- ✓ Destain the smear by covering the glass slides with acid-alcohol solution and incubate for a maximum of 3 minutes.
- ✓ Wash the slides with distilled water (using forceps, slope the slide one by one to remove the water completely).
- ✓ Counter stain the smear by covering the glass slides with methylene blue solution and incubate for 2 minutes.
- ✓ Wash the slide thoroughly with distilled water (using forceps, slope the slide one by one to remove the water completely).
- ✓ Leave the smear to dry before examination under the microscope.

Identification of AFB is done by examining the stained smears under a microscope using the (100x) oil immersion objective. Mycobacteria and other acid-alcohol resistant bacteria appear red-coloured, whereas the background and other nonacid-alcohol resistant material will appear blue-coloured. Clustering in small groups is a particular characteristic of *M. paratuberculosis*. *M. bovis* will appear as short thick acid-fast rods with cord formation being a characteristic property of strains of the *M. tuberculosis* complex. *M. avium* can be pleomorphic and may appear as coccoid or long thin AFB.

Appendix 6. Ethical clearance



Appendix 7. Different breeds of animals during the study period



Appendix 8. Description of body condition scores (BCS) (1 [thin] to 9 [obese])

Thin (poor)

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

Borderline (Optimum) = medium

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

Fat (good)

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