ABATTOIR BASED STUDY OF EPIDEMIOLOGY OF CAPRINE TUBERCULOSIS USING CONVENTIONAL AND MOLECULAR TOOLS

BY

BENTI DERESSA GELALCHA

A thesis submitted to School of Graduate Studies of Addis Ababa University and Freie Universität Berlin in partial fulfillment of the requirements for the degree of Masters in Transboundary Animal Disease Management

DECEMBER, 2011

BISHOFTU, ETHIOPIA

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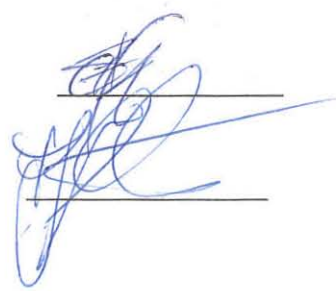

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LIST OF ABBREVIATIONS

AFB	Acid Fast Bacilli
AIDS	Acquired Immuno-Deficiency Syndrom
ALIPB	Aklilu Lemma Institute of Pathobiology
ATGC	Adenine Thiamine Guanine Cytosine
BCG	Bacillus Calmette-Guerin
bp	Base Pair
BTB	Bovine Tuberculosis
CFSPH	Center for Food Security and Public Health
CMI	Cell Mediated Immunity
DNA	Deoxyribonucleic Acid
DR	Direct Repeat Region
DTH	Delayed-type Hypersensitivity
EAI	East African-Indian
EDTA	Ethyl Diamine Tetra Acetate
HCl	Hydrochloric Acid
HIV	Human Immuno Deficiency Virus
IS	Insertion Sequence
L-J	Lowenstein-Jensen
MDR-TB	Multi Drug Resistant Tuberculosis
$MgCl_2$	Magnesium Chloride
MIRU-VNTR	*Mycobacterial* Interspersed Repetitive Units-Variable Numbers Tandem Repeats
ml	milliliter
MTBC	*Mycobacterium tuberculosis* Complex
NaH2PO4	Sodium Hypophosphate
NaOH	Sodium hydroxide
ng	Nanno gram
OIE	World Organization for Animal Health (Office international des epizooties)
PCR	Polymerase Chain Reaction
PZA	Pyrazine Amide

RD	Region of Differentiation
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal Ribonucleic Acid
rpm	Revolutions per Minute
SpolDB	Spoligotype Data Base
TB	Tuberculosis
TCH	Thiophen Carboxylic Hydrazide
VNTR	Variable Number Tandem Reapets
WHO	World Health Organization
µl	Microliter

ABSTRACT

Tuberculosis is an infectious disease occurring in several animal species including domestic and wild animals, as well as humans. A cross-sectional study was conducted from March to September 2011 on 1990 randomly selected male goats slaughtered at Luna Export Abattoir to estimate the prevalence of tuberculosis (TB), evaluate associated risk factors, and molecularly characterize the causative agents. Postmortem examination, mycobacterial culturing, Genus typing, region of difference (RD) based polymerase chain reaction (PCR) and spoligotyping were the techniques used in the study. The overall prevalence of caprine tuberculosis was 3.5 % (69/1990).There was marked variation in TB prevalence between the age categories and it was significantly higher in older goats (>1.5 year) (χ^2 =6.436; P=0.011).Variation of the disease between the different goat types and origins were not statistically significant (P>0.05). Mediastinal lymph node was the most severely (0.52±0.084) and frequently (42%) affected tissue of goats. Culture and acid fast bacillus positivity was confirmed in 12 % (8/69) of the goats with grossly visible tuberculous lesion. Up on further molecular characterization using multiplex PCR, five of the culture positive isolates showed signal for the Genus *Mycobacterium*, of which four were identified to be members of the *Mycobacterium tuberculosis* complex. Deletion typing targeting RD4 performed on the four isolates showed the evidence of the presence of RD4 in three isolates which appeared on the gel. Further characterization of the three isolates using RD9 deletion typing has showed that all of them were *M. tuberculosis.* Spoligotyping of the isolates confirmed that one of the isolate is SIT53where as the other two were new strains. The isolation of *M. tuberculosis* from goats in this study suggests evidence of transmission from humans. This also raises the concern of the risk of transmission from goat-to-human and the need to raise awareness among pastoralists consuming raw milk and meat derived from goats.

Keywords: *Caprine tuberculosis, Epidemiology, Molecular typing, Abattoir, Ethiopia*

1. INTRODUCTION

Tuberculosis (TB) is a chronic bacterial disease of animals and humans characterized by the progressive development of specific granulomatous tubercle lesions in affected tissues (Thoen *et al.*, 2006). The disease remains a major cause of human morbidity and mortality worldwide, especially in developing countries of Asia and Africa. *Mycobacterium tuberculosis* complex (MTBC) is the major cause of human and animal TB (Brosch *et al.*, 2002; Kaufmann and Shaible, 2003).

Animal TB is widely distributed in developing countries where there are no or minimal control measures (Ofukwu *et al.*, 2008).In Ethiopia, the endemic nature of tuberculosis in cattle has long been reported (Hailemariam, 1975) and is one of the countries where tuberculosis is wide spread in both human and cattle populations (Ameni, 1996; Kiros, 1998; Assegid *et al.*, 2000; Ameni *et al.*, 2003b; Asseged *et al.*, 2004; Ameni *et al.*, 2007; WHO, 2008). However, due to the prevailing production system and inadequate animal health infrastructures, the actual prevalence of the disease has been unknown (Abdo, 1993; Assegid *et al.*, 2004; Biffa *et al.*, 2009; Ameni *et al.*, 2010).

M. bovis, a major agent responsible for bovine tuberculosis (Radostits *et al.*, 2000; CFSPH, 2009) has an exceptionally wide host range. It has been reported to be an important pathogen in a number of other farmed animals (Collins *et al.*, 1993; O'Reilly and Daborn, 1995; Hermans, 1998). Cattle, goats and pigs are found to be most susceptible to *M. bovis*; while sheep and horses are showing a high degree of natural resistance (Radostits *et al.*, 2000; Thoen *et al.*, 2006).

Goats may become infected with *M. bovis* when sharing pasture with infected cattle (Ayele *et al.*, 2004) at watering points, market places and sharing the same shelter at night. Goats are relatively more susceptible; and if they are maintained with infected herd of cattle, the incidence may reach as high as 70% (Radostits *et al.*, 2000). These animals have historically been regarded as the most common accidental spillover host in areas with endemic bovine TB (Morris *et al.*, 1994).

Caprine tuberculosis caused largely by *M. bovis* and *M. caprae* (Aranaz *et al.*, 1999; Daniel *et al.*, 2009), poses an increasing risk to goat health and production worldwide (Crawshaw *et al.*, 2008; Cadamus *et al.*, 2009). It is reported that the disease is widespread in areas where goats co-graze with cattle herds that are not subject to rigorous TB test and slaughter regimes (O'Reilly and Daborn, 1995; Cousins, 2001; Ayele *et al.*, 2004; Nigussie, 2005).

In Ethiopia, mixed farming of cattle and goats is a common practice; livestock move freely from one region to another and from farm-to-farm; Thus, this practice poses a high risk of inter- and intraspecies transmission and spread of *M. bovis* infection in animals (Ameni *et al.*, 2010). This mixed farming of small and large ruminants is especially a risk to goats in countries like Ethiopia where bovine tuberculosis are endemic (Hailemariam, 1975) and existing reports (Ameni *et al.*, 2001, 2007a; Berg *et al.*, 2009) have shown a high prevalence of bovine TB ranging from 3.4% in small holder production system to 50% in intensive dairy production system.

Ejeta and Hiko (2010) reported a 4.2% abattoir-based prevalence of caprine tuberculosis; isolated two members of MTBC and showed the importance of the disease for the first time in Ethiopia. However, the study employed conventional methods which are suboptimal to establish strong epidemiological links of the disease among susceptible species.

Regardless of the substantial contribution of goat for meat and milk production which is mostly consumed raw particularly by pastoralists, study of TB in animals is biased to cattle in the country. Reports of status of TB in caprine species are lacking; the nature of caprine species and strains of MTBC which would give insight as to the pattern of transmission between susceptible animals remains unstudied. If a control program founded on sound scientific knowledge is to be initiated, a reliable baseline data on the prevalence of the disease across a range of eco-epidemiological settings, generation of molecular epidemiological data to identify transmission pattern of the disease as well as assessment of predisposing factors are the minimum prerequisites.

Therefore, the general objective of this study was to investigate abattoir based epidemiology of caprine tuberculosis.

Specific objectives:

- To estimate abattoir-based prevalence of caprine tuberculosis using detailed meat inspection
- To assess potential risk factors associated with the occurrence of caprine tuberculosis
- To characterize *Mycobacterium* isolates in slaughtered goats with lesions suggestive of TB using molecular tools

2. LITRATURE REVIEW

2.1. The Genus *Mycobacterium*

Mycobacteria belong to the Kingdom of Bacteria; Phylum of Actinobacteria; Order of Actinomycetales; Family of Mycobacteriaceae, and the Genus of *Mycobacterium*. The genus *Mycobacterium* includes: the *Mycobacterium tuberculosis* complex (MTBC), *Mycobacterium avium* complexes (MAC), other pathogenic *Mycobacteria*, and numerous species of saprophytic microorganisms present in soil and water (Dwight and Yuang, 1999).The phylogenetic analysis of the genome of pathogenic *Mycobacteria* has shown that most of them except *M.avium* belong to a single genetic species: the *Mycobacterium tuberculosis* complex (Haddad *et al.*, 2004).

2.1.1. Morphology, staining, and growth characteristics of *Mycobacterium*

Mycobacteria are thin rods of varying lengths, non-motile, non-capsular, non-spore-forming, aerobic and oxidative bacilli. Though *Mycobacteria* are cytochemically gram positive, they often resist staining with gram stain (Biberstein and Hirsh, 1999).Thus, they cannot be classified as either gram-positive or gram-negative bacteria (Brooks *et al.*, 2004). The cell wall is rich in lipids of high molecular weight that it is waxy at room temperature and successful penetration by the aqueous based staining solutions is prevented (Dwight and Yuan, 1999).Once stained, the rods cannot be decolorized with acid solutions; hence the name acid-fast bacteria.

Several staining techniques are available but Ziehel-Nelson stain is the one commonly used in most laboratories (Russell *et al.*, 2002).Thus, this thick waxy coat is responsible for acid fastness, hydrophobicity, and resistance to many disinfectants, common laboratory stains, antibiotics and injury. It probably also contribute to the slow growth rate of some species by restricting the uptake of nutrients (Rastogi *et al.*, 2001).

Because of the complexity of their cell wall, this group of organisms is fastidious; most *Mycobacteria* grow slowly, with generation times ranging from 12 to 24 hours. This is

extremely slow compared to other bacteria which have division times measured in minutes (Dwight and Yuan, 1999).

Based on different generation times, *Mycobacteria* can be divided into slow and rapid growers. Slow growers require more than 7 days forming visible colonies on solid medium, whereas rapid growers form colonies within 7 days. All the members of the MTBC are slow growers. Therefore, the inoculated media may have to be incubated at 37°C for 8 to 12 weeks (Quinn *et al.*, 1999).

2.2. Mycobacterium tuberculosis complex (MTBC) and their evolutionary development

Mycobacteria that cause tuberculosis in mammals form the MTBC. Members of the MTBC are highly related with remarkable nucleotide sequence homogeneity, despite varying pathogenicity, host preference, geographical range and epidemiology (O'Reilly and Daborn, 1995).

The MTBC comprises seven members which include *M. tuberculosis,* the primary causative agent of human TB; *M. bovis*, which has wide host range but primarily bovid pathogen includes the vaccine strain *M. bovis* BCG, which is responsible for bovine TB and *M. africanum*, the main causative agent of TB in West Africa (Kallenius *et al.*,1999); *M. microti,* which is a pathogen of voles and rarely infects humans (Rastogi *et al.*,2001); *M. canettii*, a rare MTBC strain that produces smooth and glossy colonies, with all known cases so far isolated from individuals having been to the horn of Africa (Van Embeden *et al.*,1997;Pfyffer,1998);*M. pinippedii*, also known as the seal bacillus (Cousins ,2001); and *M. caprae*, primarily isolated from goats (Aranaz *et al.*,1999).

It has been speculated that it is from *M. bovis* that *M. tuberculosis* evolved by specific adaptation of an animal pathogen to the human host (Niemann *et al.*, 2000). This hypothesis was proposed before the whole genome sequence of *M. tuberculosis* was available and before comparative genomics uncovered several variable regions in the members of the MTBC. Based on the presence or absence of an *M. tuberculosis* specific deletion (TbD1), *M .tuberculosis* can be divided in to ancestral and modern strain.

Brosch *et al.* (2002) proposed an evolutionary pathway for *mycobacteria* of the MTBC (Figure 1), based on the loss of chromosomal regions and on sequence polymorphism of selected genes. The analysis of these regions of difference (RD) suggests that *M. bovis* is not a progenitor of *M. tuberculosis* as previously thought. In fact, it appears that the *M. tuberculosis* lineage appeared long before the *M. bovis* lineage and that at the origin of the *M. bovis* lineage, members of the *M. tuberculosis* complex were already human pathogens. Recent data obtained by Samper *et al.* (2002) based on the observation of a high degree of nucleotide diversity in a small group of loci, has led the authors to postulate that the *M. tuberculosis* lineage emerged approximately 35,000 years ago, which is twice as high as previous estimate. On the other hand, *M. bovis* strains belong to a common lineage with *M. africanum* and *M. microti*. This lineage is characterized by the deletion of the RD9 region and by the subsequent loss of RD7, RD8, and RD11 (Haddad *et al.*, 2004). *M. canetti* and ancestral *M. tuberculosis* lack none of these deleted regions and seem to be direct descendants of ancestral tubercle bacilli. Consequently, the common ancestor of bacilli could well have been human pathogen already (Brosch *et al.* 2002). Deletion analysis in conjunction with molecular typing and analysis of specific mutations was shown to be a very powerful approach for the study of the evolution of the tubercle bacilli and for the identification of evolutionary markers (Haddad *et al.*, 2004; Smith *et al.*, 2006).

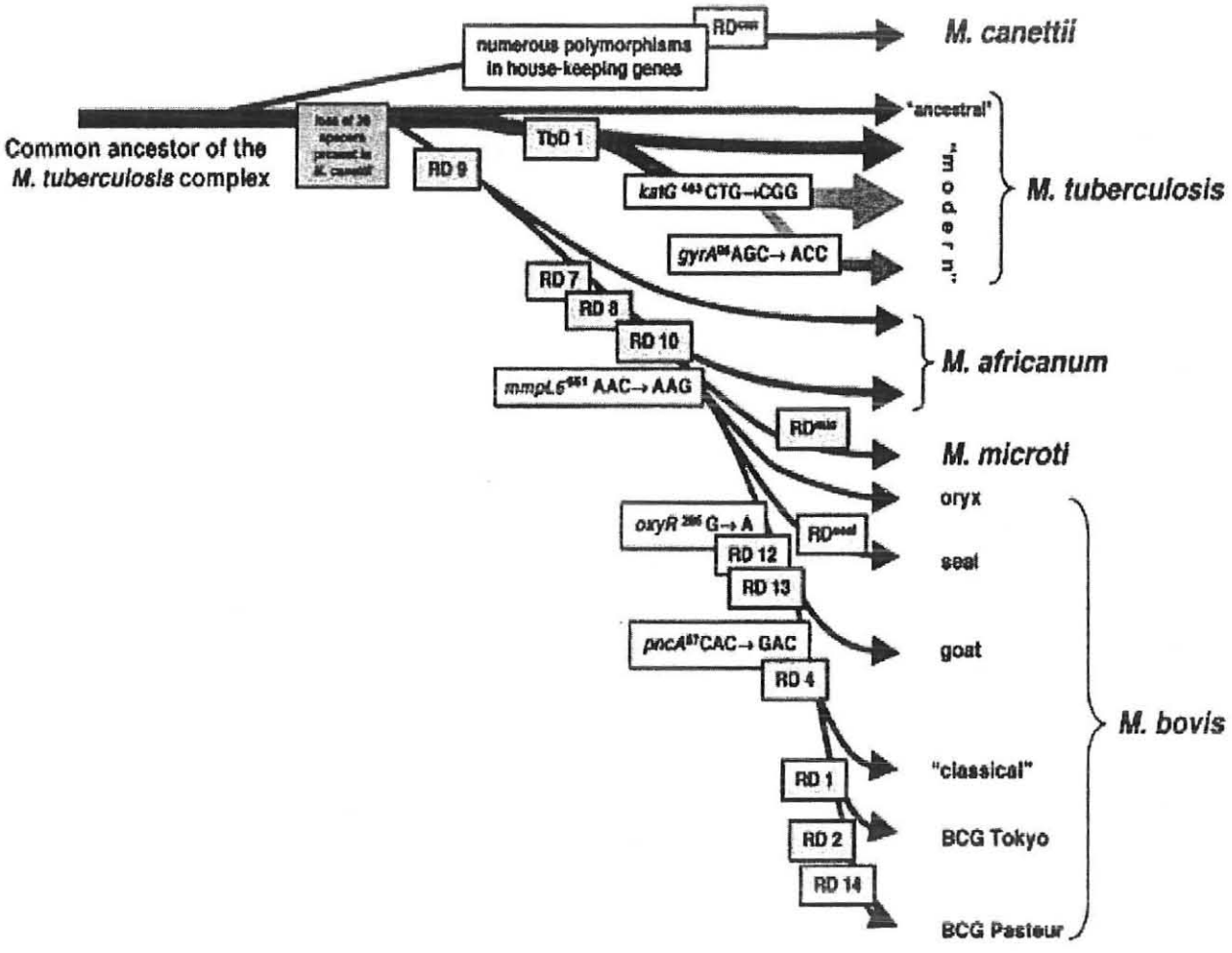


Figure 1: Scheme of the proposed evolutionary pathway of the tubercle bacilli.
Source: (Brosch *et al.*, 2002)

2.3. Identification of *Mycobacterium tuberculosis* complex (MTBC)

Complete identification of *Mycobacteria* isolates is important in order to collect information on their epidemiology and help in policy formulation and enabling appropriate public health measures to be put in place (Huard *et al.*, 2006). Various biological and molecular mycobacterial characteristics have been utilized to identify MTBC isolates.

2.3.1. Phenotypic identification of the MTBC

A series of classical tests based upon growth, colony morphology, and biochemical properties have been traditionally used to segregate members of the MTBC (Niemann *et al.*, 2000; Kremer *et al.*, 2005).

Biochemical analysis for the differentiation within the MTBC include nitrate reduction on modified Dubos broth, niacin accumulation test, growth in the presence of thiophen-2-carboxylic hydrazide (TCH), catalase activity at room temperature, and growth characteristics on Lebek and on bromocresol purple medium (Neimann *et al.*, 2000).

Typically, in laboratories that use biochemical tests, only classical *M. tuberculosis* is thought to be resistant to TCH; likewise monodrug resistance to pyrazinamide (PZA) has been listed only for *M. bovis* and *M. bovis* BCG, and a preference for microaerophilic conditions differentiates *M. africanum* and *M. bovis* from other members of the complex (Kremer *et al.*, 2005).Interestingly, while *M. bovis* was thought to be best identified by screening those isolates of the MTBC that have any PZA resistance. It has recently been shown that the sensitivity for detection of *M. bovis* is lowered to 82% when only PZA-monoresistant isolates are screened (De Jong *et al.*, 2005).

In general, these phenotypic methods of identification of MTBC isolates to the species level relied mostly on a series of biochemical tests. However, the biochemical reactions of isolates of the same species may vary from each other and from time to time, and in many cases no definitive identification is obtained (Soini and Musser, 2001).Most of these tests can be slow, cumbersome, imprecise, non-reproducible and time-consuming (Niemann *et al.*, 2000). Moreover, various laboratories use different method and may also use different criteria to interpret results of the same test. This lack of standardization creates ambiguity in interpretation of the results (Sola *et al.*, 2003) which implicates the necessity to complement or replace these classical tests and thus, laboratories are increasingly using molecular methods for *Mycobacterium* identification.

2.3.2. Genotypic identification of the MTBC

The development of DNA fingerprinting techniques for typing mycobacterium isolates during the last decade has led to an increasing number of studies of the molecular epidemiology of tuberculosis. These have shown to be useful in studying tuberculosis outbreaks, identifying sources and chains of infection, and distinguishing re-infection from endogenous reactivation (Van Soolingen *et al.*, 1990; Van Soolingen *et al.*, 1995) which were previously difficult to identify using biochemical procedures.

Genus typing of MTBC

This is a multiplex PCR-based technique which is able identify bacteria in the genus mycobacterium and also differentiates MTBC from *M. avium* complex, *M. intracellularae* and other mycobacterial species (Warren *et al.*, 2009). This assay provides a rapid and accurate method to identify presence of various members of the MTBC when it is used with growth-positive cultures (Richter *et al.*, 2003; Neonakis *et al.*, 2007). Overall, the Genus typing of MTBC assay provides a reliable direct method for identification of members of the MTBC; with an excellent specificity in many studies (Richter *et al.*,2003; Somoskovi *et al.*, 2008).The techniques, however, cannot differentiate between individual members of the MTBC, and a secondary typing method such as deletion analysis is needed (Somoskovi *et al.*, 2008).

Genomic deletion (Regions of Difference) analysis

The *Mycobacteria* grouped in the MTBC are characterized by 99.9% similarity at the nucleotide level and identical 16SrRNA sequences. However, comparative genomics have revealed several variable genomic regions in the members of the MTBC. Differential hybridization arrays identified 14 regions of difference (RD1-14), ranging in size from 2 to 12.7 kb, that were absent from bacillus Calmette-Guerin Pasteur relative to *M. tuberculosis* H37Rv (Gordon *et al.*, 1999). Brosch *et al.*, (2002) studied the distribution of 20 variable regions resulting from insertion-deletion events in the genomes of the tubercle using strains of *M. tuberculosis*, *M. africanum, M. canettii, M. microti, and M. bovis*. They showed that the majority of these polymorphisms did not occur independently in the different strains of the

MTBC but, rather resulted from ancient, irreversible genetic events in common progenitor strains.

Parsons *et al.* (2002) investigated whether a simple molecular amplification method for determination of the presence or absence of specific RDs of the genome of the MTBC could be used by clinical laboratories as a rapid and simple assay for the precise identification of individual members of the MTBC. Accordingly, these regions, primarily RD9 and TbD1 but also RD1, RD2, RD4, RD7, RD8, RD10, RD12, and RD13 represent powerful diagnostic tools for the rapid and unambiguous identification of members of the MTBC. Genetic approaches for differentiation could replace the often-confusing traditional division of the MTBC into rigidly defined subspecies (Huard *et al.*, 2006).

2.3.3. Strain identification of MTBC

It is well studied and generally accepted that different strains of *M. tuberculosis* have distinctive epidemiological and clinical characteristics such as virulence and clinical presentation, and that behavior in animal models appears to be strain-dependent (Asiimwe *et al.*, 2008)). Some *M. tuberculosis* strains are noted for their wide dissemination and acquisition of drug resistance (Glynn *et al.*, 2002) while others tend to predominate limited locales (Van Soolingen *et al.*, 1995; Chihota *et al.*, 2007). As the phylogenic level, distribution of specific *M. tuberculosis* clones or multi-drug resistant TB strains (MDR-TB) can be easily followed by molecular typing methods; country-wise ,regionally and even at a global level (Mathema *et al.*, 2006).The most widely used tools for epidemiological strain typing of *Mycobacterium* are spoligotyping (kamerbeek *et al.*,1997), IS6110 restriction fragment length polymorphism (IS6110-RFLP) (Van Embeden *et al.*, 1993) and variable number of tandem repeat DNA; in *Mycobacteria* termed *Mycobacteria* interspersed repetitive units (MIRU-VNTR) analysis (Supply *et al.*,2001).

Spoligotyping

Spacer oligonucleotide typing or 'spoligotyping' is based on the detection of DNA polymorphism in the direct repeat (DR) region in the genome of MTBC (Kamerbeek *et al.*, 1997). This MTBC-specific genomic region contains 36 bp DRs, interspersed by unique DNA

spacers of 35–41 bp in length. The numbers of DRs per strain and thereby the presence of particular spacer sequences differs significantly between strains. To visualize the DNA polymorphism in the DR region of MTBC, the spacer sequences are initially amplified by PCR, using primers based on the DR sequence. The reverse primer is biotin-labelled and hence, also the PCR product. In the first generation spoligotyping, 43 oligonucleotides are used, based on the DNA sequence of spacers in *M. tuberculosis* strain H37Rv and BCG vaccine strain P3. These oligonucleotides are covalently linked to a Biodyne C membrane in parallel lines. The denatured PCR products are allowed to hybridize perpendicular to the oligo lines in the reversed line blot hybridization (Kremer *et al.*, 1999). The hybridization is detected by adding streptavidine-peroxidase conjugate and a substrate, which results in a chemiluminescence reaction that is detected on film (Van Solingen *et al.*, 2003).

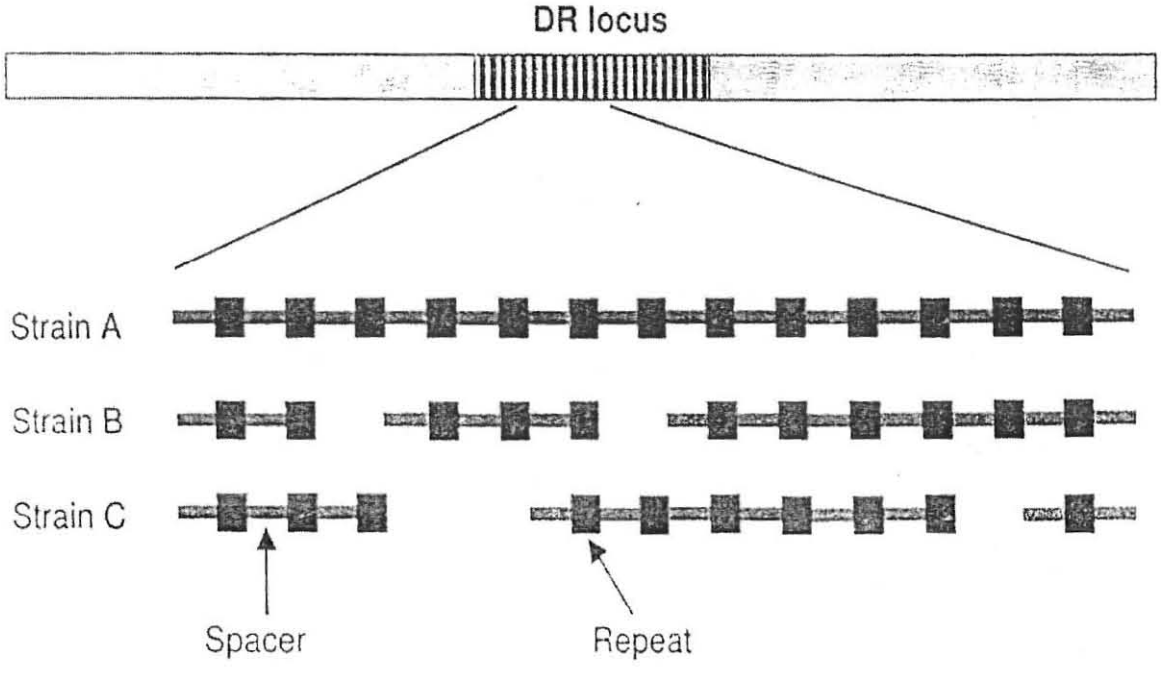


Figure 2: Schematic view of the DR locus and three examples of spoligotype patterns

Source: (Kamerbeek *et al.*, 1997)

Spoligotyping can be directly applied to clinical material thus allowing epidemiological typing of bacteria from stored clinical specimens, material from slides of Ziehl-Neelsen staining and even to non-viable specimens, paraffin-embedded samples (Van der Zanden *et*

al., 1998; Van Solingen *et al.*, 1999). Spoligotyping can simultaneously detect and type the bacteria: *M. bovis* (Kamerbeek *et al.*, 1997), *M. microti* (Kremer *et al.*, 1998), *M. canettii* (Van Soolingen *et al.*, 1997) and *M. caprae* (Aranaz *et al.*, 1999) can be readily recognized by distinct characteristic patterns. Spoligotyping, unlike IS*6110* genotyping, which requires approximately 2 μg of bacterial DNA, can be performed with considerably less DNA and in a fraction of time. It also allows genotyping of boiling-prepared or impure DNA (Chihota *et al.*, 2007). In addition, the degree of differentiation achieved by spoligotyping is higher than that of IS*6110*-RFLP for *M. bovis* (Kamerbeek *et al.*, 1997). One of the major drawbacks to spoligotyping is that it can only identify polymorphisms within the DR cluster, whereas RFLP typing can detect genetic differences arising from multiple loci (Kamerbeek *et al.*, 1997).

Generally, this genotyping technique is relatively easy to perform, cheap, rapid and reproducible (Kremer *et al.*, 1999; Glynn *et al.*, 2002). However, strains with less than five IS*6110* copies can often be further subdivided by this technique; its discriminatory power is far less for other strains than is IS*6110* RFLP typing. In any case, spoligotyping can be used as a screening method to differentiate between strains since differences in spoligo patterns are almost always indicative of differences on the basis of other genetic markers (Van Solingen *et al.*, 2003).

Restriction fragment length polymorphisms analysis (RFLP)

The first, most widely applied and standardized molecular typing method for MTBC isolates is IS*6110* restriction fragment length polymorphism (RFLP) typing. It is based on the detection of the repetitive sequence IS*6110* which can be found in most isolates of the MTBC (Small *et al.*, 1994; Van Soolingen *et al.*, 1999). MTBC strains differ in the number of IS*6110* copies present in their genome, ranging from 0 to 25, and also in the genomic positions where these elements are inserted (Van Soolingen *et al.*, 1991; Mendiola *et al.* 1992; Diaz *et al*, 1998).It has been the gold standard method for genotyping of *M. tuberculosis* (Van Embeden *et al.*, 1993) which has a relatively larger number of IS6110 copies.

Generation of IS*6110* RFLP patterns require a well-grown MTBC isolate. DNA is extracted, purified and digested with the restriction enzyme *Pvu*II. This restriction enzyme cleaves the IS6110 element at a single site. The *Pvu*II restriction fragments are separated overnight on an

agarose gel and subsequently transferred to a DNA membrane.IS*6110*-containing restriction fragments are visualized by adding a peroxidase labeled probe with a DNA sequence complementary to the right-hand part of the IS*6110* sequence in a hybridization buffer onto the DNA membrane. The hybridizing restriction fragments are detected by a chemiluminescence reaction initiated by two substrates and the RFLP patterns are detected on a light sensitive film (Van Solingen *et al.*, 2003).

This tool has found use in identifying laboratory cross-contamination, differentiating recurrent TB caused by treatment failure or relapse from exogenous re-infection, population-based studies as well as in identifying settings for TB transmission. However, the technique is considered laborious, time-consuming, technically demanding and has an extra shortcoming of inability to discriminate strains with low copy number of the IS6110 element (kremer *et al.*, 2005).

Mycobacterial interspersed repetitive units variable numbers tandem repeats (MIRU-VNTR)

This is PCR-based method that analysis multiple loci containing variable numbers of tandem repeats (VNTR) of different families of interspersed genetic elements collectively called mycobacterial interspersed repetitive units (MIRU). Currently, the most used version of this method (MIRU-VNTR) is based on the analysis of 12 loci (Supply *et al.*, 2001).A standardized format has now been proposed and is comprised of 24 loci, 15 of which were defined as comprising a discriminatory subset based on higher variability within the different clonal complexes studies. This new panel has been evaluated (Allix *et al.*, 2008) and permits accurate and high-resolution analysis.

2.5. Epidemiology of caprine and bovine tuberculosis

2.5.1. Etiology

Mycobacterium bovis and *Mycobacterium caprae* are member of the *M. tuberculosis* complex (MTBC) that comprises the closely related species: *M. tuberculosis, M. bovis, M. bovis BCG, M. africanum, M. microti, and M. caprae, M. canetii, and M. pinnipedii.* These

species are the causative agents of tuberculosis (TB) in humans and animals (Carlton *et al.*, 2010).

2.5.2. Source of infection

The source of tubercle bacilli is mostly tuberculous individual. Humans perpetuates *M. tuberculosis*; cattle *M. bovis*; and chickens *M. aviam* (Dwight and Yuan, 1999).Organisms are excreted in the exhaled air, in sputum, feces (from both intestinal lesions and swallowed sputum from pulmonary lesions), milk, urine, vaginal and uterine discharges, and discharges from open peripheral lymph nodes. Animals with gross lesions that communicate with airways, skin, or intestinal lumen are obvious disseminators of infection to in-contact susceptible species. Cattle in the early stages of the disease, before any lesions are visible, may also excrete viable *Mycobacteria* in nasal and tracheal mucus (McIlroy *et al.*, 1986).

Shedding of *M. bovis* in milk, urine and faeces is now regarded as a relatively insignificant feature of bovine tuberculosis in developed countries (Morris *et al.*, 1994; Cassidy *et al.*, 1999). However, it is recognized that *M. bovis* can be isolated from nasal mucus (Cassidy *et al.*, 1999) and probably this contributes to spreading infection (Neill *et al.*, 2005).

2.5.3. Transmission and routes of infection

In cattle as well as in other animal hosts, the route of transmission of *M. bovis* can be deduced by the pattern of lesions observed in slaughtered animals. Animals with lesions restricted to the thoracic cavity are presumed to have been infected by the inhalation of aerosols, while those with lesions in mesenteric lymph nodes are thought to have acquired the infection by ingestion (Pollock and Neil, 2002).

In cattle, the majorities of lesions are found in the upper and lower respiratory tract and associated lymph nodes (Neill *et al.*, 1994) even in those at pasture suggesting respiratory routes the most probable routes of infection. Contrary to this finding, in the recent report by Ameni *et al.* (2007a), 94.5 % of the lesions were detected in mesenteric lymph nodes in animals that are kept on pasture. In fact, the development of tuberculosis lesions which invade the airways is thought to be required to facilitate active excretion and aerosol spread of

M. bovis (Menzies and Neill, 2000).Respiratory excretion and inhalation of *M. bovis* is considered to be the principal route through which cattle-to-cattle transmission occurs in housed cattle. Infection by inhaling *M. bovis* bacilli, or possibly a single bacillus, in an aerosol droplet is a concept that is generally accepted (Neill *et al.*, 1994). Droplets of contaminated water, eructation while ruminating contaminated pastures or inhaling contaminated dust particles can also be an alternative way of aerogenous infection (CFSPH, 2007).

Infection by ingestion of *M. bovis* directly from infected animals or from contaminated pastures is possible when feces contaminate the feed and communal drinking water and feed troughs but a large infective dose is required. The drinking of infected milk by young animals is a common method of transmission where the disease is endemic (Radostits *et al.*, 2000). This routes of infection is considered secondary to respiratory spread, as deduced from the minor presence of mesenteric lesions in cattle cases (Menzies and Neill, 2000; CFSPH, 2007).

Other uncommon routes of infection include: intrauterine infection at coitus, by the use of infected semen or of infected insemination or uterine pipettes, and intramammary infection by the use of contaminated teat siphon or by way of infected cups of milking machines (Radostits *et al.*, 2000). The importance of all these routes of transmission varies between species (CFSPH, 2007).

In some areas of the world certain wildlife species appear to be a significant maintenance host and reservoir for infection in cattle. For instance, Buffaloes (Syncerus caffer) in South Africa (Keet *et al.*,1996) and Uganda, Badgers (Meles meles) in United Kingdom and Ireland, Bison(*Bison bison*) in USA ,Brushtail possum (*Trichosurus vulpecula*) and Deer (*Cervus elaphus)* in New Zealand, Feral pig (*Suis scrofa*) in Spain and Italy act as maintenance hosts in these countries (De Lisle *et al.*,2002).

A main trait of *M. bovis* is its broad host range, *causes* disease in a wide range of domestic, wildlife animals as well as in humans. Thus, the epidemiology of *M. bovis* is very complex and includes transmission within and between domestic and wildlife animals, as well as from animals to humans or vice versa (Figure 3) (Aranaz *et al.*, 1999; Beit *et al.*, 2005).

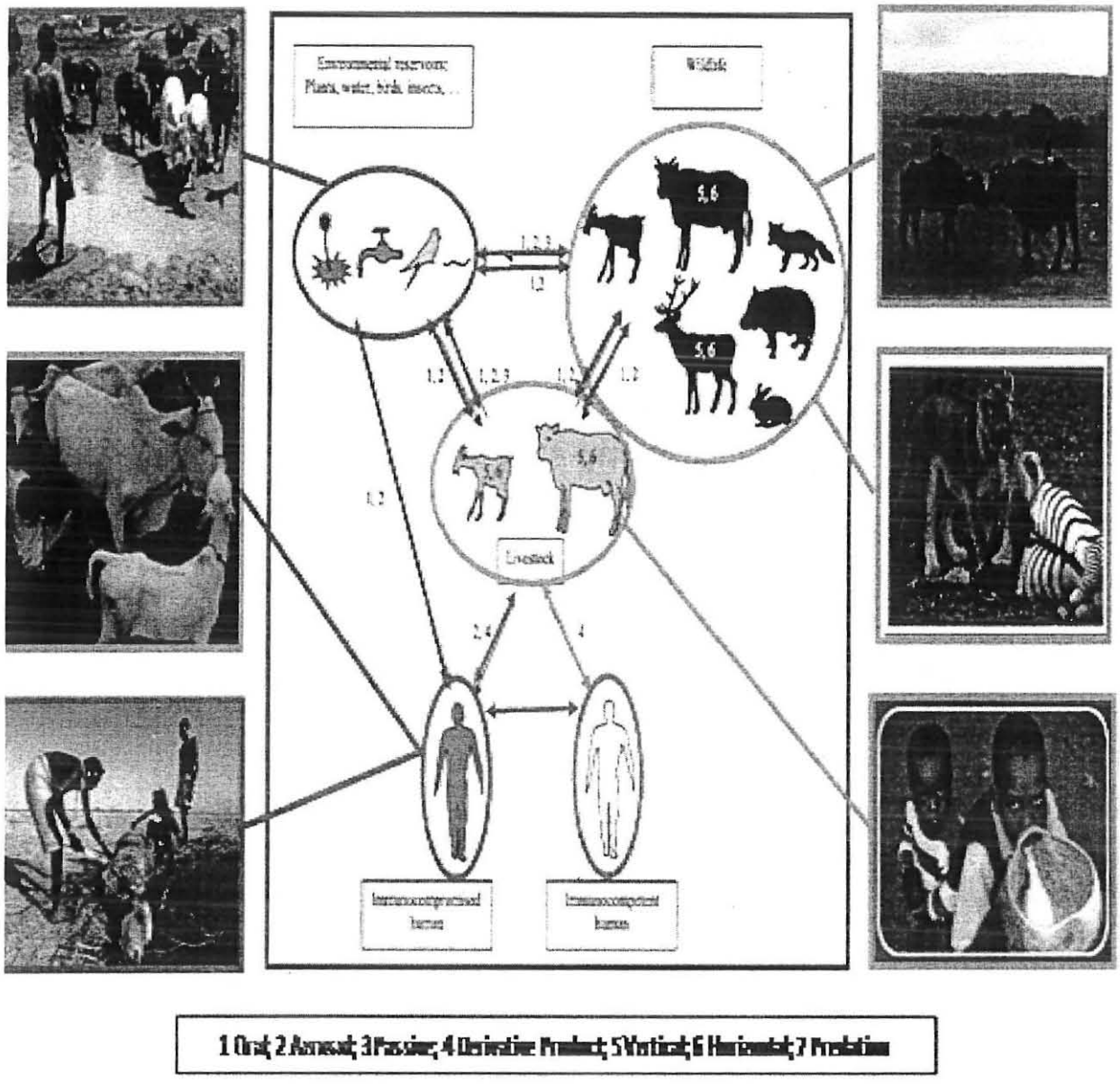


Figure 3: Illustration of possible transmission pathways of *M.bovis* between the environment, wildlife, livestock and humans.

Source: (Geoghegan and Getz, 2009).

2.5.4. Risk factors

Various host, agent, environmental and management risk factors have been identified to predispose the animal to *Mycobacterium* infections as detailed below.

Age

One of the main individual risk factors identified by numerous studies in both developed and developing countries is the age of animals. The likely hood of exposure increases with age; older animals are more likely to have been exposed than younger ones, as shown by several cross-sectional studies (O'Reilly and Daborn, 1995; Ameni *et al.*, 2007; Shitaye *et al.*, 2007; Ameni *et al.*, 2008; Biffa *et al.*, 2009). Similarly, studies in Canada and Northern Ireland indicated an increased incidence of bovine TB with increased age (Amanfu, 2006). An alternative explanation is that, reactivation occurs under stress or in old age since *Mycobacteria* in a latent state may become subject to less stringent control by host systems.

Genetic variation

Genetic variation in resistance to *M. bovis* infection in animal is manifested at the species and breed levels. Species difference in susceptibility to *Mycobacterium* in animals has long been documented. Cattle, goats and pigs are found to be most susceptible to *M. bovis*, while sheep and horses are showing a high level of natural resistance (Radostits *et al.*, 2000; Thoen *et al.*, 2006). Goats are quite susceptible and if they are maintained in association with infected herds of cattle the incidence may be as high as 70% (Radostits *et al.*, 2000).

There is paucity evidence whether there is variation in resistance to *M. bovis* between the different breed of goats but informations are available for cattle. Experimental *M. bovis* infection of calves of taurine cattle and zebus indicated marked resistance in zebu calves, while calves of Ankole (taurine cattle of African origin) and European breeds were susceptible (Caramichael, 1940) as cited in (Ameni *et al.*, 2007). Zebu (*Bos taurus indicus*) type cattle are thought to be much more resistant to tuberculosis than European cattle (Bos taurus taurus) and the effects on these cattle are much less severe (Radostits et *al.*, 2000). In Ethiopia, (Ameni *et al.*, 2007) have also demonstrated differences in susceptibility of cattle to bovine TB; indicating that *Bos t.indicus* cattle are more resistant than *Bos t.taurus* under identical husbandry condition with even more severe pathology in the latter.

Agent risk factors

The presence of lipid substance in the genus *Mycobacterium* increases its resistance to conditions that are detrimental to other non-sporing types of bacteria. The cell wall makes a substantial contribution to the hardiness of this genus (Bhamidi, 2009). The organism is moderately resistant to heat, desiccation, and many disinfectants. Thus, the survival of the organism in the environment is influenced by temperature, moisture, exposure to the desiccating effect of sunlight and ultraviolet light (Dwight and Yuan, 1999).

Although direct sunlight kills the bacillus after few minutes of exposure, much of the animal exudates and fecal smear in which the microorganism is present usually protects it from drying direct sun rays. The organism can survive for long periods in feces, darkness and moist soil but most studies show that survival on pasture is measured in weeks rather than months and that environmental contamination of pasture is not of major importance in the epidemiology of the disease in cattle (Morris, 1994; Radostits *et al.*, 2000).

Management and other risk factors

Poorly ventilated cattle housing provides an ideal environment for the spread of pathogens (Smith *et al.*, 2005) and predisposes the animal to the disease. High humidity provides an ideal environment for transmission of the organism, when compared with the environment in which cattle are kept at pasture (Andrews *et al.*, 1998).

High stocking intensity or herd-size is also one of the major bovine TB herd-level risk factors as higher prevalences of intradermal test positives were recorded in larger herds (Kazwala ,1997).This is because the more cattle there are on a farm, the greater the probability that one of them will acquire the infection. The closer the animals are in contact, the greater is the chance that the disease will be transmitted (Radostists *et al.*, 2000; Palmer *et al.*, 2002).

The disease incidence grows more in intensive dairy farms than beef ranches. Factors that play role in predisposing animals to infection include type of production (dairy cattle are at risk), suckling (calves become infected by ingesting contaminated milk), and presence of wild

reservoirs of *M. bovis* infection for grazing cattle (Quinn *et al.*, 1999).Cattle movement, purchase, grazing field boundaries or short swards and spreading cattle slurry on the fields are a potential risk for transmission of the disease between cattle (Thoen *et al.*, 2006). A smaller study in Italy showed that mixed dairy and beef enterprises were at greater risk of breakdown than either dairy or beef herds, which may have been due to increased likelihood of cattle movement, a major risk factor (Marangon *et al.*, 1998). Moreover, the severity of bovine TB was significantly greater in cattle kept indoors at a higher population density than in those kept on pasture (Ameni *et al.*, 2006).

Moreover ,studies conducted in central Ethiopia (Regessa *et al.*,2008) has showed a high prevalence of bovine tuberculosis in animals owned by farmers with active tuberculosis as compared to those having no tuberculosis suggesting that the former is one of the risk factor.

Nutrition

It is to be expected that nutritional deficiencies are associated to reduce resistance in cattle may enhance bovine tuberculosis infection (Phillips *et al.*, 2002). There is epidemiological evidence of an association between the provision of mineral licks and *M. bovis* infection. An Irish study found that the provision of mineral licks reduced the risk of a herd acquiring *M. bovis* infection in a study of breakdown herds, with an odds ratio of 2.7 (Griffin *et al.*, 1995). Circumstantial evidence also suggests that deficiencies in vitamins A and C, calcium and protein, as well as carbohydrate excess, are likely to increase the risk of cattle acquiring *M. bovis* infection (Thoen *et al.*, 2006).

The risk of infection with *M. bovis* increases with immunosuppression; although it has not been scientifically demonstrated in cattle and other livestock species to date .The evidence is extrapolated from studies of *M. tuberculosis* in mice (Menzies and Neill, 2000). Genetic mechanisms of non-specific immunity could eliminate a low-dose *M. bovis* challenge: bronchial mucus, efficiency of the mucociliary escalator, active non-specific macrophages in the lungs (and their lysosomal enzymes) as well as their destructive efficiency are some examples (Phillips *et al.*, 2002; Morris, 2007).

Immunosuppressive disease

The effect of concurrent immunosuppressive disease on *M. bovis* infection in cattle and other livestock species does not appear to have been investigated. However, the major influence of HIV infection in humans on the risk of subsequent infection with *M. tuberculosis* or other *Mycobacteria* is well documented (Glynn *et al.*, 2002; Mukadi *et al.*, 2001). A severe outbreak of *M. bovis* infection in housed calves with concurrent bovine viral diarrhoea (BVD) infection has been reported (Monies and Head, 1999).Concurrent infection with feline immunodeficiency virus (FIV) and *M. bovis* in farm cats has also been reported (Monies *et al.*, 2000).

2.5.5. Distribution and prevalence bovine and caprine tuberculosis

Bovine tuberculosis (BTB) is distributed throughout the world and has been reported in every continent except Antarctica; in general where there are cattle there is, or has been, bovine tuberculosis. The exceptions are those countries that successfully used test and a slaughter programme to identify and cull infected cattle (Amanfu, 2006).

Animal TB is widely distributed in developing countries, where there are no or minimal control measures (Shitaye *et al.*, 2007). In developing countries, such as in 46% of African, 44% of Asian and 35% of the South American and the Caribbean countries, sporadic occurrences and (particularly in Africa 11%) enzootic occurrences of bovine TB have been reported (Cosivi *et al.*, 1998; Oloya *et al.*, 2007).

Prevalence data on BTB infection in Africa is scarce. However, there is sufficient evidence to indicate that it is widely distributed in almost all African countries and is found to be of high prevalence in some animal populations (Ayele *et al.*, 2004; Zinsstag *et al.*, 2006a). In Ethiopia, more recent reports suggest that the prevalence of bovine TB may range from 3.4 % in smallholder production systems to 50% in intensive dairy production systems (Kiros, 1998; Asseged *et al.*, 2000; Ameni *et al.*, 2003b; Regassa, 2005; Ameni *et al.*, 2007; Berg *et al.*, 2009).

Bovine tuberculosis also affects other farmed animals; among which goats are most commonly affected species. Tuberculosis in goats has been diagnosed in France, Italy, Great Britain, Portugal, Germany, USA, Africa, India, Australia and New Zealand (Cousins *et al.*, 1993; O'Reilly and Daborn, 1995; Malone *et al.*, 2003). In goats, tuberculosis causes a gradual decrease in dairy production, poor body condition, chronic intermittent cough, lymphadenitis and finally death (Crawshaw *et al.*, 2008).

Caprine (goat) tuberculosis is a zoonotic disease with a high prevalence in certain countries (O'Reilly and Daborn, 1995), whose principal etiological agents are *M. caprae* and *M. bovis* (Aranaz *et al.*, 1999; Crawshaw *et al.*, 2008).

Caprine tuberculosis is reported to be common in Mediterranean countries (Aranaz *et al.*, 1999; Cotter *et al.*, 1996) and often it is a very severe disease (Cotter *et al.*, 1996). Study conducted on goat in Spain (Montserrat *et al.*, 1997) isolated 23 *M. bovis* strains. The spoligotypes revealed that caprine strains form a separate and well differentiated group that they referred as the caprine genotype.

In Nigeria, cadamus *et al.* (2009) found a 4.47% lesion suggestive of TB out of the total 1,387 goats screened in abattoir; and isolated four strains of *M. bovis* and one strain of *M. tuberculosis.* Spoligotyping identified the *M. tuberculosis* isolate as belonging to the East African Indian (EAI) families in the SpolDB4 database. All *M. bovis* isolates were M. *bovis bovis*, not *M. bovis caprae*, according to their deletion typing profile (Warren *et al.*, 2009) which suggests transmission from cattle rather than transmission from the goat reservoir.

In Algeria, which is known to be TB-infected country, a 6.03% (n=995) of goat tuberculosis was reported in a study conducted at two slaughter houses in Wilaya of Bejaia (Naima *et al.*, 2011).

In Ethiopia, (Nigussie, 2005; Hiko and Ejeta, 2010) reported for the first time a 4.3 and 4.2 % prevalence of caprine tuberculosis respectively in export abattoirs and showed the importance of the disease in goats. Similarly, Tefess *et al.* (2011) also conducted cross sectional study of caprine tuberculosis on goats at Adami Tulu Agricultural Research Centre and the

surrounding smallholder farmers and reported a prevalence of 3.1 and 9.6% at the center and farmers' house hold respectively.

Generally , reports of caprine TB are rising in several countries in recent years; even in those following test and slaughter policy (Crawshaw *et al.*, 2008; Cadamus *et al.*,2009;Daniel *et al.*,2009; Michel *et al.*, 2010; Quintas *et al.*, 2010; Sharpe *et al.*, 2010) posing a growing threat to goat farming and public health.

2.5.6. Molecular epidemiology of bovine tuberculosis in Ethiopia

Molecular epidemiology is a powerful approach for monitoring infectious diseases. Molecular epidemiological data help clarify the sources of infection and the major routes of the transmission and spread of bovine tuberculosis (TB) and the risk factors involved (Neill *et al.*, 2005).They are also necessary for eco-systemic analyses of transmission chains within and between wildlife, livestock and humans (Haas *et al.*, 1993).

In Ethiopia, a number of molecular epidemiological studies have been conducted on bovine tuberculosis though it is non-existent in caprine species. Accordingly, several unique and previously reported strains of *Mycobacterium* have been reported from different parts of the country as shown in Table 1.

Table 1: Spoligotypes of *Mycobacterium*, host and the abattoir/areas where isolates were collected.

Spoligotype	abattoir/areas	Host	Reference
SB1476*	Gimbi,Bitajira,Gonder,Jinka	cattle	Berg *et al.*,2009
SBO134	Addis, Gonder	cattle	Berg *et al.*,2009;Biffa *et al*,2010;Tsegaye *et al.*,2010
SBO133	Addis Gimbi,Jinka,Wello,Akaki	Cattle and Camel	Ameni *et al,2007b,2010;*Berg *et al.*,2009;Biffa *et al*,2010;Tsegaye *et al.*,2010 ;Mamo *et al.*,2011
SBO1176*	Addis, Gonder Wello, Butajira, kombolcha	cattle	Ameni *et al,2007b,2010;* Berg *et al.*,2009; Biffa *et al*,2010;Tsegaye *et al.*,2010
SIT59	Wello	cattle	Berg *et al.*,2009
SB1477*	Jinka, Butajira, Addis	cattle	Berg *et al.*,2009
SB1489*	Addis		Berg *et al.*,2009
SIT1742	Butajira	cattle	Berg *et al.*,2009
SIT 1	Gimbi	cattle	Berg *et al.*,2009
SIT149	Gimbi	cattle	Berg *et al.*,2009; Tsegaye *et al.*,2010
SB1488*	Jinka	cattle	

Continued from table 1

spoligotype	abattoir/areas	Host	Reference
SIT1688	Jinka,Melge-wendo	cattle	Berg *et al.*,2009
SB1517*	Addis,Yebello	cattle	Biffa *et al*,2010
SB1518*	Addis	cattle	Biffa *et al*,2010
SB1265	Hawassa	cattle	Biffa *et al*,2010
SB1468	Adama	cattle	Biffa *et al*,2010
SIT262	kombolcha	cattle	Ameni *et al.*,2010
SIT451	kombolcha	cattle	Ameni *et al.*,2010
SB1490*	kombolcha	cattle	Ameni *et al.*,2010
SB1491*	kombolcha	cattle	Ameni *et al.*,2010
SB1492*	kombolcha	cattle	Ameni *et al.*,2010
SBO912	Addis, Adama	cattle	Ameni *et al.*,2010; Biffa *et al.*,2010
SIT 121	Addis	cattle	Tsegaye *et al.*,2010
SB1519*	Addis	cattle	Biffa *et al*,2010
SB1520*	Addis	cattle	Biffa *et al*,2010
SB1521*	Adama	cattle	Biffa *et al*,2010
SB1522*	Adama	cattle	Biffa *et al*,2010
SB1953*	Akaki	camel	Mamo *et al.*,2011

SIT-denotes that the isolate is *M. tuberculosis*

SB-denotes that the isolate is *M. bovis*

Star (*)-indicates that the isolate is unique and reported from Ethiopia for the first time.

2.6. Pathogenesis and pathology of tuberculosis

Mycobacteria have evolved as pathogens to survive in macrophages and to overcome multifactorial hostile innate and acquired host immune mechanisms, so that they can survive and eventually multiply and transmit to other animals (Carlton *et al.*, 2010).

The pathogenesis of bovine tuberculosis is not as well understood as that for human tuberculosis (Neill *et al.*, 2005) as most studies have been performed with *M. tuberculosis.* However, the presence of homologous genes in other *Mycobacterium* and the close relationship between these microorganisms suggest the use of similar virulence determinants and mechanisms of pathogenicity (Carlton *et al.*, 2010).

After infection, the bacteria may localize in tissues related to the route of infection and associated lymph nodes. 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 a*l., 2000).

The *Mycobacteria* are then taken by the alveolar macrophages from the primary focus to the circulation and establishes in regional lymph nodes. Cellular responses attempting to control the disease result in the accumulation of large number of phagocytes and lead to the formation of a macroscopic lesion 'tubercle' (Pallock *et al.*, 2006; Thoen, 2010).

The cell mediated immunity (CMI) emerges 10-14 days after infection and triggers the release of cytokines from T-lymphocytes that activate the bacteriostatic effect of macrophages and accelerate the recruitment of additional blood-borne mononuclear cells into the site resulting in delayed type of hypersensitivity reaction (DTH) (Theon., 2010).

As the process progresses, monocytes mature into epitheloid cells and multi-nucleated giant cells of the Langhan's type (Pallock *et al.*,2006; Carlton *et al.*, 2010) that form the center of the young tubercle which will be surrounded by lymphocytes, plasma cells, monocytes and an outer boundary of fibrous connective tissue. Caseous necrosis results from the delayed type hypersensitivity reaction. The gross appearance of the tubercle is usually firm yellow to white

and on section a yellowish caseous necrotic material or calcified tissue is observed (Neill *et al.*, 1994; Quinn *et al.*, 1999).

2.7. Zoonotic importance of bovine and caprine tuberculosis

Bovine tuberculosis, apart from being the most important disease of intensification with a serious effect on animal production, also has a significant public health importance (O'Reilly and Daborn, 1995).

The global prevalence of human TB caused by *M. bovis* has been estimated to be 3.1% of all human TB cases. This comprises of 2.1% and 9.4% of pulmonary and extra-pulmonary TB cases respectively (Cosivi *et al.,* 1998; Ayele *et al.,* 2004). This figure is considered low probably due to the difficulty in discriminating between species and strains of the MTBC using conventional methods; as *M. bovis* infection is largely clinically, radiologically and pathologically indistinguishable from *M. tuberculosis* infection (Amanfu, 2006).

It has been estimated (Cosivi *et al.,* 1998) that approximately 85 % of cattle and 82% of human populations in Africa live in areas where bovine TB is either partly controlled or not controlled at all. In these countries, pasteurization of milk is rarely practiced, the habit of eating raw meat is deeply established, and there is frequent close contact between humans and animals (Ofukwu *et al.,* 2008).In such countries an estimated 10 to 15 % of human TB cases have been attributed to *M. bovis* (Ashford *et al.,* 2001).

In Ethiopia, very few studies have indicated the isolation of the causal agent of bovine tuberculosis (BTB) from humans. In line with this, Kiros (1998) demonstrated that out of 85 sputum samples taken from 28 dairy farm workers and 57 tuberculous patients; where 48 samples were positive for acid fast bacilli, of which 14(29.2%) were niacin negative indicating *M. bovis* and 34 (70.2%) *M. tuberculosis* isolates.

Similarly, Regassa (2005) demonstrated that, out of 87 sputum and 21 fine needle aspiration (FNA) human samples, 42 mycobacteria species were identified by culture, of which, 7 (16.3%) and 31 (73.8%) were found as *M. bovis* and *M. tuberculosis,* respectively. In

addition, Kidane *et al.* (2002) indicated that *M. bovis* along with other MTBC species was found to be a cause for tuberculous lymphadenitis in humans. Ethiopian Ministry of Health (MoH, 2005) showed a significant rise in the incidence of extrapulmonary TB in the country, although the extent attributed to *M. bovis* is not known.

3. MATERIALS AND METHODS

3. 1. Study site

This study was conducted in Luna Modern Export Abattoir which is located in Modjo town. The town is the center of Lume district in eastern Showa administrative zones of Oromia regional state and it is located at 73 km south east of Addis Ababa. The abattoir is privately owned, export type that slaughters mainly goats but also few sheep for export purpose. Only male goats and sheep are slaughtered in the abattoir.

According to the manager of the abattoir, 200-600 sheep are slaughtered every Thursday and Sunday, and 500 to 1700 goats are slaughtered every day based on the demand from the importers. Sheep and goats are slaughtered separately one after the other and the same personnel are involved in slaughtering both species of animals. The slaughtering process involves bleeding "Halal" method followed by hanging and washing the skin with tap water.

The abattoir has one main slaughter hall with one over head rail. The rail is higher and serves for both small ruminant and cattle slaughter operations. In addition, the abattoir has one emergency slaughter hall, one detaining room and four chilling rooms. Furthermore, head, skin and gastrointestinal content collection rooms are available separately.

3. 2. Study population

All study goats were local breeds; and originated from different areas of the country mainly from Arsi, Borana, Jimma, Somali and South Wello representing different agro- ecological zones. All animals were managed under extensive production system either as mixed crop–livestock production system or as a pastoral system of production. Animals were purchased at different local markets and transported to the abattoir using double –decked trucks. At the abattoir, animals were fed, watered, and rested before slaughter for 24 to 72 hrs. Antemortem examinations of animals and meat inspections were routinely conducted by state

veterinarians. Only apparently healthy male goats that passed the antemortem examination were included in the study.

In the abattoir, there was no pre-slaughter tuberculin skin testing scheme. Thus, there was no information about the health of the animals with respect to tuberculosis except a fore-mentioned antemortem inspection for general examination of animal health.

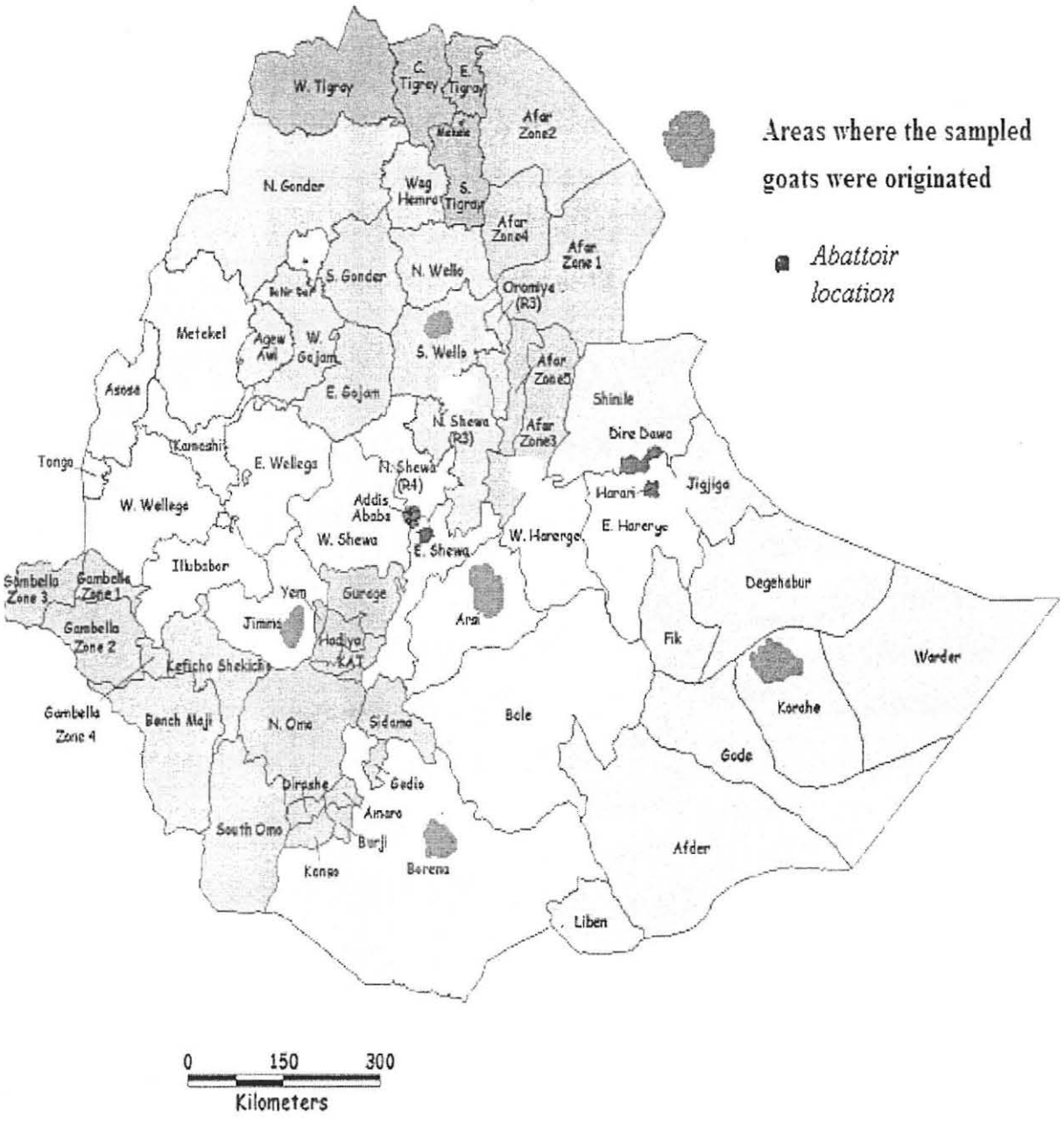


Figure 4: Map of Ethiopia showing areas where the slaughtered goats were originated

Source: (UN, 2000)

3. 3. Sample size determination

The total sample size was calculated based on the predetermination of the following parameters: 95% level of confidence, 2% desired level of precision and 4.2% expected prevalence of caprine tuberculosis (Hiko and Ejeta, 2010) was considered to obtain the sample size according to (Thrusfield, 2005).

$$n = \frac{(1.96)^{2} * P_{exp} * (1 - P_{exp})}{d^{2}}$$

Where:

n = required sample size

P_{exp} = expected prevalence

d = desired absolute precision

Using the above formula, the minimum sample size required was about 386. But to increase the precision, a total of 1990 goats were sampled. The number of animals sampled from specific geographical origin was determined based on the expected prevalence previously reported by Hiko and Ejeta (2010) for a given area.

3. 4. Study design

A cross-sectional study was the design used in the study. It involved stratification of the study population according to geographic origin and goat-type to estimate the prevalence of caprine tuberculosis and assess the potential risk factors of the disease. The goat type was defined according to their origin and the physical description given by ILRI (1996). In the abattoir, animals from the same origin were kept in a separate premises or holding room and slaughtered as one batch. Study animals were also categorized into two age groups conventionally; ≤ 1.5 years as young, and >1.5 years as adult by means of dentition as described for African indigenous goats (Steele, 1996).

3. 5. Sampling methodology

The selection of the goat population included in the study was based on systematic random sampling (from those slaughtered each day from specific locality). The animals were selected by employing systematic random sampling when they were moving in line to the slaughter hall. The selected animals were identified using permanent marker, kept separately and released for slaughter one after the other. The sampling interval was obtained by dividing the total number of animals from specific geographical origin slaughtered within that day by the estimated daily sample size. The first animal was selected randomly from the first batch, then, every n^{th} animal from the same origin was included in the sample according to (Thrusfield, 2005). Accordingly, on average twelve animals were sampled during every study day. Thus, the total number of animal slaughtered in the particular day from particular origin was divided by twelve and every twelfth animal was selected after random selection of the first animal until the daily sample size was met.

3. 6. Detailed postmortem examination and Pathology scoring

Postmortem inspection was performed following the procedure described by (Corner, 1994). Detailed postmortem examination involving visual inspection, palpation and incision of the lungs, lymph nodes and other organs such as liver, kidneys was conducted. All the seven lobes of the two lungs, and lymph nodes of the head (retropharyngeal, mandibular), thoracic cavity (mediastinal and bronchial), mesenteric, and hepatic lymph nodes were examined thoroughly. TB cases were defined based on gross lesion characteristics supplemented by the knowledge of pathogenesis and routes of lesion dissemination in the body (Hancox, 2000; Pollock *et al.*, 2005).

The suspected organs/tissues were cut into approximately 2-cm-thick slices to facilitate the detection of lesions using separate sterile scalpel blades. The cut surfaces and internal portions were examined visually under bright light sources for the presence of lesions compatible with TB (Gracy, 1986; Asseged *et al.*, 2004). When gross lesions compatible with TB were detected in any of the tissue, the carcass was classified as having lesions (positive). Tissues with lesions suggestive of TB were collected for bacteriological culture into sterile

universal bottles with 5ml of 0.9% saline solution. The collected tissues were labeled and sealed separately; and kept in icebox with solid packs to keep the cold chain. The sample was transported to Aklilu Lemma Institute of Pathobiology (ALIPB) possibly in the same day and stored at +2 to +8 °C until mycobacteriological culturing was carried out.

Pathology scoring was conducted on those tissues with abscesses and tubercle lesions to determine the severity of the lesions based on semi-quantitative procedure developed by Vordermeier *et al.* (2002);but with minor modifications to facilitate performance under field conditions (Ameni *et al.*, 2006) as cited by Mamo *et al.* (2011). Briefly, lesions in the lobes of the lungs were scored separately as follows: 0=no visible lesions; 1=no gross lesions but lesions apparent on slicing of the lobe; 2=fewer than five gross lesions; 3=more than five gross lesions; 4=gross coalescing lesions. The scores for the individual lobes were added up to calculate the lung score. Similarly, the severity of gross lesions in individual lymph nodes was scored as follows: 0=no gross lesions; 1=small lesion at one focus; 2=small lesions at more than one focus; 3=extensive necrosis. Individual lymph node scores were added up in order to calculate the lymph node scores. Then, both lymph node and lung pathology scores were added up to determine the total pathology score per animal.

3. 7. Laboratory examinations

3. 7. 1. Culturing and identification of *Mycobacteria*

Specimen processing and culturing for isolation of *Mycobacteria* was carried out at TB laboratory of Aklilu Lemma Institute of Pathobiology (ALIPB) in accordance with World Organization for Animal Health protocols (OIE, 2010).. In the laboratory, individual tissues specimens were sectioned using sterile blades in sterile petri dishes to obtain fine pieces and then homogenized with a mortar and pestle. The homogenate was decontaminated using 2 ml of 4% NaOH for 15 minutes, centrifuged at 3,000 rpm for another 15 minutes. The supernatant was discarded and the sediment was neutralized by 1% (0.1N) HCl using phenol red as indicator. Neutralisation was considered to be achieved when the color of the solution was changed from purple to yellow.

Thereafter, 0.1ml of the suspension was inoculated onto a duplicate set of Löwenstein-Jensen slants; one supplemented with 0.4% sodium pyruvate (L-J pyruvate) and the other with glycerol (standard L-J). Cultures were incubated aerobically at 37°C for at least eight weeks with weekly observation for discernible growth. Initial identification of Mycobacterial species was based on the rate of growth, colony morphology and confirmed by detection of acid fast bacilli (AFB) by Ziehl–Neelsen staining (Mcilroy *et al.,* 1986). Positive cultures were heat killed in water bath at 80°C for 45 minutes. The heat killed isolates was stored at -20°C for further molecular typing.

3. 7. 2. Mycobacterial Genus typing

Mycobacterial genus typing is a multiplex polymerase chain reaction (PCR) which differentiate *M. tuberculosis* complex from *M. avium* complex, *M. intracellularae* and other mycobacterial species. It was conducted as described previously by Wilton and Cousins, (1992). Heat killed AF positive samples was used as source of DNA template.

DNA amplifications was done in thermocycler with 20 µl reaction volumes consisting: 5 µl of genomic DNA as a template, 8 µl HotstarTaqMasterMix (MgCL2, dNTP, Taq polymerase and PCR buffer) (Qiagen, United Kingdom) for each sample, 0.3 µl of each of the six primer per sample and 5.2 µl of Qiagen water per sample. The primer name and sequence used for amplification in genus typing is given in table 2.

Table 2: Primers used and PCR product size in genus typing of *Mycobacterium* isolates

Primer name	Primer sequence	Required amount	Product size
MYCGEN-F	AGAGTTTGATCCTGGCTCAG	35ng/ µl	1030 bp
MYCGEN-R	TGCACACAGGCCACAAGGGA	35ng/ µl	
MYCAV-R	ACCAGAAGACATGCGTCTTG	35ng/ µl	180 bp
MYCINT-F	CCTTTAGGCGCATGATGTCTTTA	75ng/ µl	850 bp
TB1-F	GAACAATCCGGAGTTGACAA	20ng/ µl	372 bp
TB1-R	AGCACGCTGTCAATCATGTA	20ng/ µl	

M. tuberculosis strains (H37Rv) and *M. avium* were used as positive control while Qiagen water was used as negative control. The reaction mixture was then be heated in Programme Thermal Controller (Applied biosystem; PTC- 100^{TM}) cycle using the following amplification programs: 95°C for 10 minutes for enzyme activation; 95°C for 1 minute for denaturation; 61°C for 0.5 minute for annealing; 72°C for 2 minutes for extension; involving 35 cycles all in all and final extension at 72°C for 10 minutes.

A 1.5% agarose gel was prepared and the products were electrophoresed in 10xTAE running buffer. Ethidium bromide at ratio of 1:10, 100bp DNA ladder, and orange 6x loading dye were used in gel electrophoresis.

All members of the genus *Mycobacterium* are expected to produce a band of 1030 bp, *M. avium* or subspecies such as *M. avium* subspecies *paratuberculosis* produces a band of 180 bp, *M. intracellularae* a band of 850 bp while members of *M. tuberculosis* complex produce a band with 372 bp (Mamo *et al.*, 2011).

3. 7. 3. Region of Difference (RD) deletion typing

This deletion typing protocol differentiate species of the *M .tuberculosis* complex from *M. avium*, *M. intracellulare* and other *Mycobacterium* species (Gordon *et al.,* 1999). The method was applied to heat-killed bacterial suspensions. For deletion typing, the procedure described by Cadamus *et al.* (*2009*) was followed.

The RD4 deletion typing was carried out on isolates that showed band for *M. tuberculosis* complex by multiplex PCR. Each sample was tested in a separate PCR tube. Primers directed against the RD4 were used to generate a deletion profile that would allow species identification of the isolate. RD4 is 12.7 kb genetic segment that is deleted from *M. bovis* BCG strain, but present in *M. microti*, *M. africanum*, and *M. tuberculosis* (Wilton and Cousins, 1992).

RD9 deletion typing was performed on isolates that showed presence of Region of Difference four (RD4) during RD4 deletion typing. RD9 is 2030 bp gene segment of *M. tuberculosis* and PCR analysis using flanking primers revealed that RD9 is absent in *M. bovis*, *M. microti*, and

M. africanum (Gordon *et al.*, 1999). Primers directed against RD4 and RD9 as well as the size of PCR product size expected in the presence or absence of the respective region of difference is given in table 3.

Table 3: Primers used for deletion typing of MTBC isolates and sizes of PCR products

Locus	Primer name	Primer sequence	present	deleted
RD4	RD4_intF	ACACGCTGGCGAAGTATAGC	335 bp	446 bp
	RD4_flankF	CTCGTCGAAGGCCACTAAAG		
	RD4_flankR	AAGGCGAACAGATTCAGCAT		
RD9	RD9_IntR	CTGGACCTCGATGACCACTC	339 bp	472 bp
	RD9_FlankF	GTGTAGGTCAGCCCCATCC		
	RD9_FlankR	GCCCAACAGCTCGACATC		

The HotStarTaq Master Mix systems from Qiagen were used for PCR with primers shown in table 4. The reaction mixture consists of: 10 µl of HotStarTaq master mix, 0.3 µl x 3 of each primer (flank_R, F and Int) of the respective deletion typing, 2 µl DNA template and 7.1 µl Qiagen water to a final volume of 20 µl. *M. tuberculosis* H37Rv and *M.bovis* 2122/97 were used as positive control while Qiagen water was used as negative control. The mixture was heated in Programme Thermal Controller (Applied biosystem; PTC- 100^{TM}) using an initial hot start of 95°C for 15 minutes followed by 35 cycles of 95°C for 1 minute; 55°C for 1 minute; and 72°C for 1 minute; a final extension step of 72°C for 10 minutes to complete the cycle. PCR products were electrophoresed in 1.5% agarose gel in 10x TAE running buffer. Ethidium bromide at ratio of 1:10,100bp DNA ladder and orange 6x loading dye were used in electrophoresis.

Finally, bands were visualized using alpha innotech, version 1.2.0.1 (Alpha Innotech Corporation) in a multi–image^{TM} light cabinet. The presence of RD4 (*M. tuberculosis, M. africanum*) gives a product size of 335 bp while its absence (*M. bovis)* gives a product size of 446 bp. The presence of RD9 (i.e. *M. tuberculosis*) gives a product size of 339 bp and its absence (*M. africanum* or *M. bovis*) gives a product size of 472 bp using the primers given in table 3 (Cadamus *et al.*, *2009*).

3. 7. 4. Spoligotyping

Spoligotyping were performed at ALIPB; following the procedure described by (Kamerbeek *et al.,* 1997) and according to the spoligotype kit supplier's instructions (Ocimum Biosolutions Company, Iisselstein, The Netherlands).The direct repeat (DR) region were amplified by PCR using oligonucleotide primers derived from the DR sequence(DRa:5'-GGT TTT GGG TCT GAC GAC-3' and DRb:5'-CCG AGA GGG GAC,GGA AAC-3'). A total volume of 25 µl of the following reaction mixture was used for the PCR: 12.5 µl of HotStarTaq master mix (Qiagen). This solution provides a final concentration of: 1.5 µM MgCl2 and 200mM of each deoxynucleotides triphosphates, 2 µl of each primer (20 p mol each), 5 µl suspension of heat-killed cells (approximately 10 to 50ng), and 3.5 µl distilled water. The mixture were heated for 15 minutes at 96°C and then subjected to 30 cycles of 1 minute at 96°C; 1 minute at 55°C, 30 seconds at 72°C and a final extension at 72°C for 10 minutes.

The amplified product were hybridized to a set of 43 immobilized oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus. After hybridization, the membrane was washed twice for 10 minutes in 2x SSPE (1x SSPE is 0.18 M NaCl, 10 µM NaH2PO4, and 1 µM EDTA (pH 7.7)-0.5% sodium dodecyl sulfate at 60°C and then incubated in 1:4000 diluted streptavidin peroxidase (Boehringer) for 45 to 60 minutes at 42°C. The membrane were washed twice for 10 minutes in 2x SSPE-0.5% sodium dodecyl sulfates at 42°C and rinsed with 2x SSPE for 5 minutes at room temperature. Hybridizing DNA (presence or absence of the unique spacers) were detected by the enhanced chemiluminescence method (Amersham) and by exposure to X-ray film (Hyperfilm ECL, Amersham) which detects light signals and thereby produces a pattern which allows for typing of isolates as specified by the manufacturer.

3.8. Data collection, management and analysis

For each individual animal examined, information relevant to epidemiological investigation like age, goat type and geographical origin were recorded on a data sheet. Presence or

absences of tuberculous lesions, lesioned organs or tissues with their pathology score were all recorded on database established in Microsoft® Excel for Windows 2007.

Descriptive statistics were used to estimate prevalence of carcass with tuberculous lesion across the individual factors, frequency of anatomical location and severity of the lesion. Uni- and multivariate logistic regression were used to investigate possible associations between the prevalence and the explanatory variables (age, goat type and origin of the animals). P value <0.05 and 95% confidence level were used for statistical significance. All statistical analysis was carried out using SPSS version 18.0 (SPSS INC. Chicago, IL).

4. RESULTS

4.1. Prevalence of tuberculosis in goats and associated risk factors

These results were based on post-mortem examinations of the sampled goats. Based on post-mortem examinations the prevalence of TB in sampled goats was 3.5% (2.7-4.3% at 95% confidence interval) on the basis of lesion suggestive of TB. It was 2.5% in young and 4.6% in the older age group. The highest (4.9%) and lowest (2.1%) prevalence of caprine TB were recorded in goats originated from Borena and Somali areas respectively.

Table 4: Individual variables and prevalence of TB in slaughtered goats

Variables	Carcass examined	No. TB positive	Prevalence (%) at 95%CI
Age			
≤1.5 year	1048	26	2.5 [1.55-3.45]
>1.5 year	942	43	4.6 [3.26- 5.94]
Origin			
Borena	472	23	4.9 [2.95- 6.85]
Arsi	360	13	3.6 [1.68- 5.52]
Jimma	386	15	3.9 [1.97- 5.83]
South Wello	386	10	2.6 [1.01- 4.19]
Somali	386	8	2.1[0.67 3.53]
Goat type			
Somali	858	31	3.6[2.35-4.85]
Arsi-Bale	360	13	3.6 [1.68- 5.52]
Central lowlands	386	15	3.9 [1.97- 5.83]
Afar	386	10	2.6[1.01- 4.19]
Total	1990	69	3.5[2.69-4.31]

Among the goat types, the Afar and Central Lowlands were the least (2.6%) and most (3.9%) affected goat types respectively (Table 4). The result of analysis of the risk factors affecting prevalence of TB in goats is presented in Tables 5 and 6. Accordingly, there was marked variation in TB prevalence between the age categories. It was significantly higher in older animals (>1.5 year) ($P<0.05$) while variation of the disease between the different goat types and origins were not statistically significant ($p>0.05$).

Table 5: Univariate association of the proposed potential risk factors with caprine TB

Variables	Carcass examined	No (% positive)	χ^2 -value	P-value
Age			6.436	0.011*
≤1.5 year	1048	26(2.5)		
>1.5 year	942	43(4.6)		
Origin			6.140	0.189*
Borena	472	23 (4.9)		
Arsi	360	13 (3.6)		
Jimma	386	15 (3.9)		
South wello	386	10 (2.6)		
Somali	386	8(2.1)		
Goat type			1.165	0.76(NS)
Somali	858	31(4.5)		
Arsi-Bale	360	13 (3.6)		
C. Lowlands	386	15 (3.9)		
Afar	386	10 (2.6)		

C-central

*-the value is significant

NS- not significant

Those variables with P-value less than 0.2 in univariate analysis were taken to multivariate logistic regression.

Table 6: Multivariate analysis of risk factor with presence of TB lesion in goats

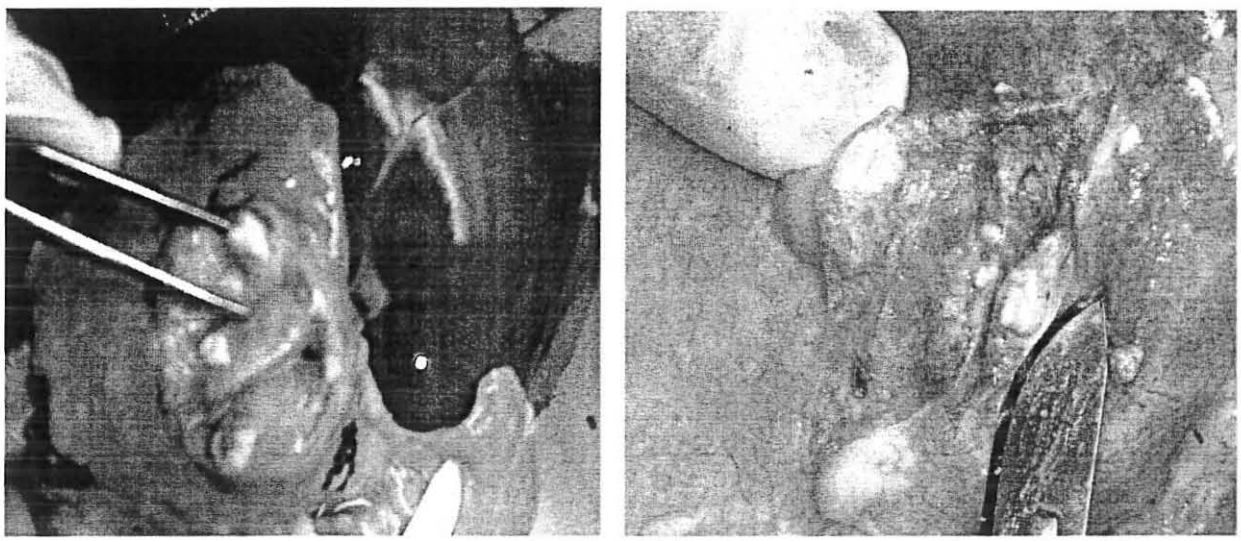
Variable	No. of carcass examined	OR	P-value	95% CI
Age				
≤1.5 year	1048	1	1	
>1.5 year	942	1.88	0.013*	1.142 - 3.096
Origin				
Somali	386	1	1	
Borena	472	2.14	0.034*	1.068 - 5.473
Arsi	360	1.83	0.187 (NS)	0.746 - 4.462
Jimma	386	1.78	0.196 (NS)	0.744 - 4.258
South wello	386	1.24	0.65 (NS)	0.485 - 3.187

*-the value is significant

NS- not significant

4.2. Distribution and magnitude of pathology

Characteristic TB lesions (Figure 5) were observed in different tissues; the distribution and severity of lesions were established in the 69 carcass with lesions compatible with the disease.



A B

Figure 5: TB lesions on bronchial lymph node and goat lung

Purulent bronchial lymphadenitis with few areas of necrotic pneumonia (A) Randomly distributed, irregularly shaped, soft white foci of TB lesion on the external and cut surfaces of goat lung (B).

From total TB lesions encountered from all organs and lymph nodes examined, approximately 97% were observed in the lungs and lymph nodes of the thoracic cavity. The lesions were observed more frequently in the mediastinal (42%) and bronchial (33%) lymph nodes than in the other lymph nodes. Likewise, the mediastinal lymph node was the most severely affected (0.52±0.084) organ followed by bronchial lymph node (0.45±0.083). Among the lung lobes, right and left apical lobes were the most severely and frequently lesioned as compared to other lobes. The frequency and mean severity of tuberculous lesion of goat is shown in Table 7.

Table 7: Distribution and mean severity of TB lesion in goats' tissues

Tissue	Total	Number (%positive)	Mean± SEM
Lymph nodes			
Mediastinal	69	29(42%)	0.52±0.084
Bronchial	69	23(33%)	0.45±0.083
Retropharyngeal	69	2(2.8%)	0.04±0.032
Mesenteric	69	1(1.4%)	0.03±0.029
Hepatic	69	1(1.4%)	0.04±0.043
Lung lobes			
Right apical	69	15(21.7%)	0.45±0.011
Right cardiac	69	12(17.4%)	0.2±O.057
Right diaphragmatic	69	5(7.2%)	0.12±0.053
Right accessory	69	4(5.8%)	0.09±0.04
Left apical	69	13(18.8%)	0.33±0.089
Left cardiac	69	10(14.5%)	0.28±0.085
Left diaphragmatic	69	8(11.6%)	0.14±0.052

SEM-standard error of the mean

4.3. Culture and Acid fast test result

Seventy-eight samples from suspected TB lesions were processed and cultured onto glycerol and pyruvate based Lowenstein Jensen media. Bacterial growth was observed in 14 %(11/78) of the sowed slants of which eight of them were confirmed to be acid fast bacilli (AFB).

4.4. Molecular characteristics of the isolates

4.4.1. Identification of the Genus *Mycobacterium*

All the 8 acid-fast bacilli (AFB) isolates were subjected to PCR amplification of *Mycobacterium* Genus specific16s rRNA fragment to know whether the isolates belong to the Genus *Mycobacterium* or not. This Genus typing revealed that five of the eight isolates showed the band for the Genus *Mycobacterium*. Furthermore, four of the isolates generated a PCR product of 372 indicating they belong to MTBC group where as one was atypical *Mycobacterium* (Figure 6).

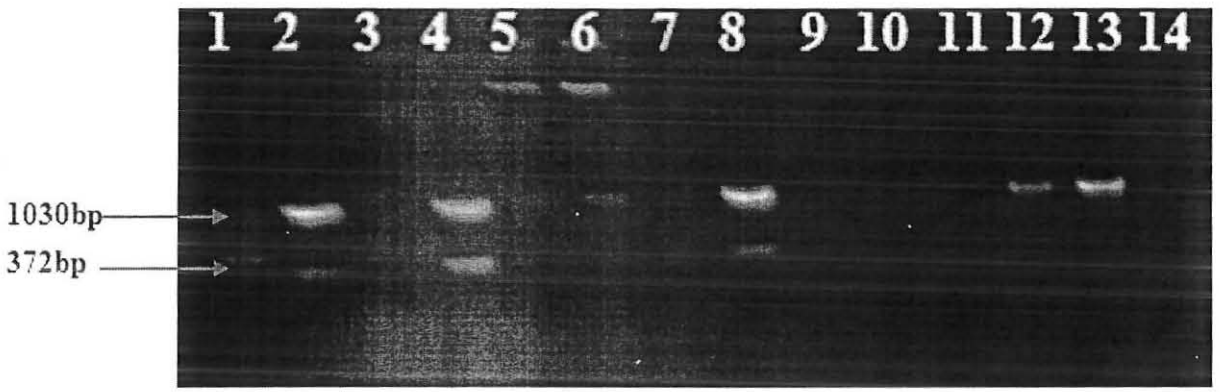


Figure 6: Gel Electrophoretic separation of PCR products by multiplex PCR genus typing of mycobacterial isolate from tuberculous tissue of goats:

Lanes: 1 = 100bp DNA Ladder, 2 = *M. tuberculosis* (positive control), 3 = Qiagen H2O (negative control), 4=*M. bovis*, 5 = *M. avium* complex (positive control) and 6 =*M. intracelularae* complex (positive control). Lanes 7-14 were samples from individual goats with tuberculous lesions. Lane 8, 10,11,12,13 were positive for the Genus *Mycobacterium* Lane 8 and 10-12 showed bands for *M. tuberculosis* complex (MTBC) and lane 13 was positive only for the Genus *Mycobacterium* while lane 7, 9 and 14 were negative.

4.4.2. Speciation of *Mycobacterium tuberculosis* complex

RD4 deletion typing result

To investigate if the isolates in the group of MTBC were *M. bovis*, deletion typing was performed to confirm the specific deletion of the RD4 region in *M. bovis* strains. Three of the samples generated a PCR product of 335 bp (Table 3) suggesting that RD4 is intact and the isolates were indeed not *M. bovis;* while one of the samples was not seen on the gel (Figure 7).

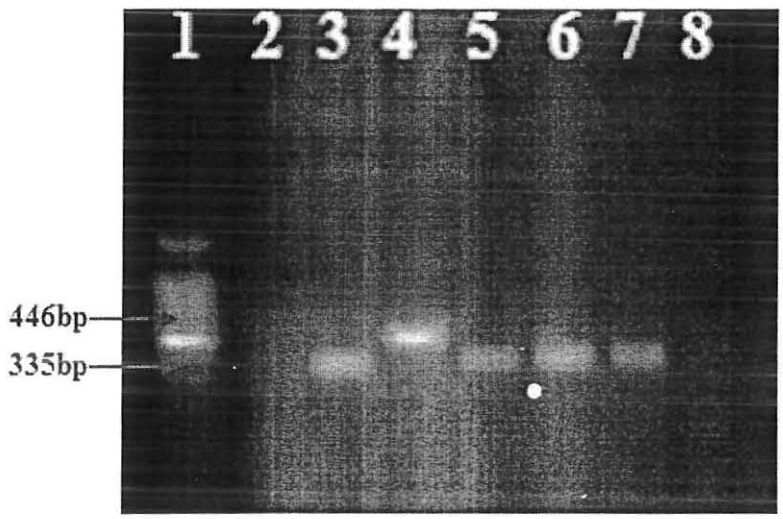


Figure 7: Electrophoretic separation of PCR products of RD4 deletion typing:

Lanes: 1=100bp DNA Ladder, 2=Qiagen H2O (negative control), 3=*M. tuberculosis* (positive control), 4=*M. bovis* (positive control). Lanes 5- 8 were isolates which showed positive signal for MTBC in genus typing. Lane 8 was negative while Lane 5, 6 and 7 showed a positive signal for the presence of RD4.

RD9 deletion typing result

The three isolates which showed presence of RD4 suggesting that they could be either *M. tuberculosis* or *M. africanum* were further characterized using RD9. In RD9 deletion typing all the three isolates generated a PCR product of 339 bp confirming that they are *M. tuberculosis* (Figure 8).

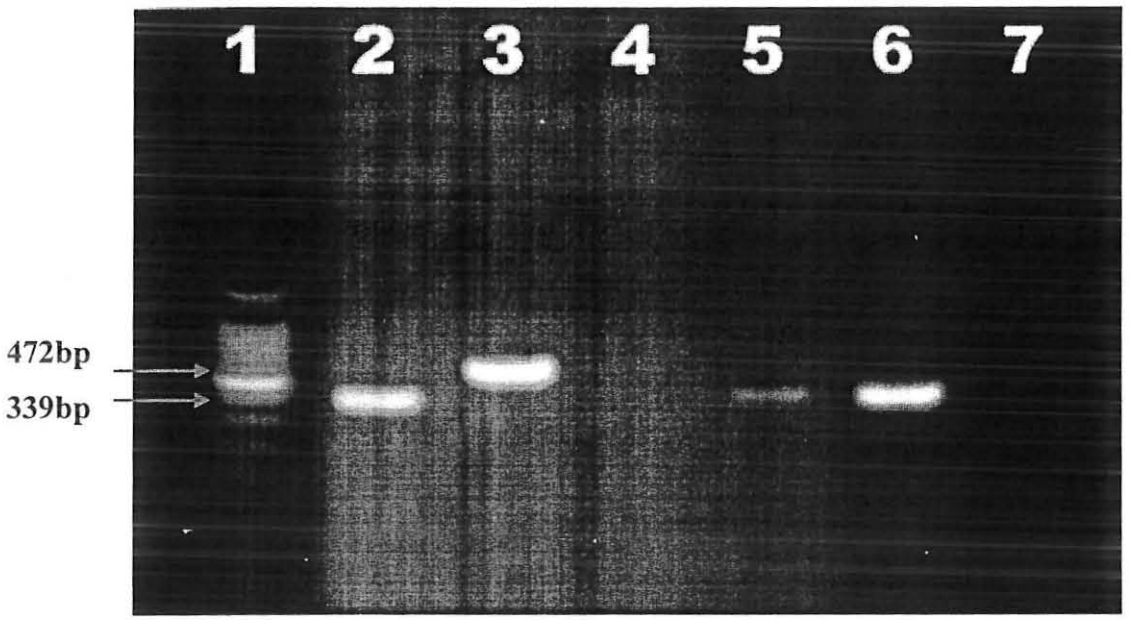


Figure 8: Electrophoretic separation of PCR products of RD9 deletion typing:

Lanes: 1=100bp DNA Ladder, 2= *M. tuberculosis* (positive control), 3= *M. bovis* (positive control), 4= Qiagen H2O (negative control), Lanes 5- 7 were isolates which generated a PCR product of 335 bp in RD4 deletion typing.

4.4.3. Spoligotyping of *Mycobacterium tuberculosis* isolates

Up on Spoligotyping typing, all the three isolates showed distinct patterns indicating all are different strains or spoligotypes (Figure 9).

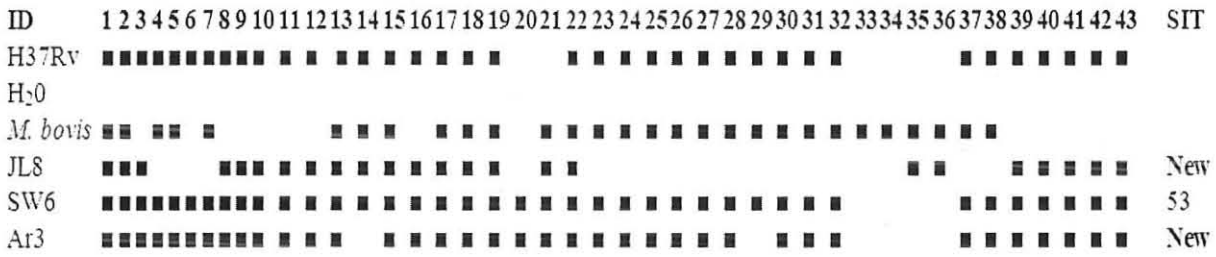


Figure 9: Spoligotype pattern of the controls (H37Rv, H20, and M.bovis) and the three samples (JL8, SW6 and Ar3).

Both positive control strains yielded standard patterns of spacer arrangements; absence of spacers 20-21 and 33-36 and presence of spacers 39-43 for *M. tuberculosis* H37Rv and absence of spacers 3, 9, 16 and 39-43 for *M. bovis* BCG. Feeding of the spacer patterns of the isolates in to the spoligotype international typing (SITVIT) data base generated an SIT53 for one of the isolates (SW6). However, the pattern of the other two isolates(Ar3 and JL8) were unique and not so far recognized by the database indicating that they are new strains and hence not given SIT identification.

5. DISCUSSION

In the past TB has been considered rare and a minor problem in goats; it was even thought that this species is naturally immune to TB. However, several studies showed that goats possess no particular resistance and are susceptible to the disease (Bruner and Gillespie, 1973; O'Reilly and Daborn, 1995; Liébana *et al.*, 1996). Recently, reports of caprine TB are increasing in several countries; even in those practicing a long standing test and slaughter policy (Crawshaw *et al.*, 2008; Cadamus *et al.*, 2009; Daniel *et al.*, 2009; Michel *et al.*, 2010; Quintas *et al.*, 2010; Sharpe *et al.*, 2010).

In this study, the occurrence and frequency of occurrence of TB was investigated in male goats originating from different areas and slaughtered at Luna Export Abattoir on the basis of post mortem examination. Further confirmatory tests were conducted on suspicious lesions using bacteriological and molecular biological techniques.

Compared to the previous very few studies carried out on goat TB in Ethiopia, this study was more comprehensive in that the number of goats included in this study was relatively higher; the origin and types of animals was more representatives of the reference population and advanced molecular techniques were used.

The overall prevalence of 3.5% reported in this study is similar with the prevalence reported by previous works in the country. For instance, Hiko and Ejeta (2010) reported a prevalence of 4.2% (n=1,536) in a study conducted in 2005 at Modjo Modem Export Abattoir. Preliminary study conducted on TB in small ruminants slaughtered at Helmex Export Abattoir also recorded similar prevalence of 4.3% (n=1152) on the basis of gross lesion (Nigussie, 2005). Likewise, a prevalence of 3.1% (n=193) was reported on goats kept at Adami Tulu research center using single intradermal tuberculin test (TST) (Tefess *et al.*, 2011). However, the same author also reported a higher prevalence of 9.6% (n=437) in goat kept at the house hold level in small holder farmers than the prevalence reported in this study. The deviation could be due to the larger number of animal tested being older in age as compared to our study where the majorities were young. Another explanation could be due to the high sensitivity of TST compared to postmortem examination.

Studies conducted in other countries have also reported similar results with the present study. Cadamus *et al.* (2009) reported an abattoir prevalence of 4.5% (n=1,387) in goats from Nigeria. In Pakistan, a prevalence of 2.4% was reported in goats at farms using TST (Javed *et al.*, 2010). In contrary to this, Edgiton (1989) recorded a higher prevalence of 7 % (n=532) than the present study in a flock of goat using TST in South Canterbury, Australia. Moreover, the prevalence recoded in this study is lower than that recorded in feral goat (31%) in New Zealand using TST (Sanson, 1988). In Portugal, Quintas *et al.* (2010) also reported a higher prevalence of TB (65%) in a herd of goats subjected to compulsory slaughter due to herd breaks down. More recently, Shanahan *et al.* (2011) reported a much higher prevalence of 66.3% in a dairy goat herd tested with TST on a farm in Ireland. This variation could be explained by the probable difference in the level of challenge, management, breed and the intrinsic difference between the two detection methods. In addition, Liébana *et al.* (1996) demonstrated that differences in the prevalence of caprine TB found in different countries may be caused by variation in subpopulations of *Mycobacterium.*

In general, reliable prevalence data of caprine TB in goats in both developed and developing countries are unrecorded (Daniel *et al.*, 2009). However, caprine TB is probably widespread in areas where goats co-graze with cattle herds that are not subject to rigorous TB test and slaughter regimes (O'Reilly and Daborn, 1995; Cousins, 2001; Ayele *et al.*, 2004; Nigussie, 2005; Hiko and Ejeta, 2010).

Age, origin and Goat types were the factors of variation which were taken in to account in the present study. Age of animals is one of the main individual risk factors affecting TB as identified by numerous studies in both developed and developing countries (O'Reilly and Daborn, 1995; Ameni *et al.*, 2007; Shitaye *et al.*, 2007; Biffa *et al.*, 2009). Likewise, the predominance of the disease in relatively older goats (χ^2=6.436, OR=1.88, $p<0.05$) reported in this study is in agreement with the works of Javed *et al.* (2010) who reported in goats of more than 6 years of age, the reactor animals to TST were 5, 8 and 11 times higher than goats of 4 -6, 3-4 and <3 years of age respectively. Similarly, several cross sectional studies conducted on bovine TB reported a higher prevalence of infection in older animals (O'Reilly and Daborn, 1995; Ameni *et al.*, 2007; Shitaye *et al.*, 2007; Biffa *et al.*, 2009). This could be explained by the fact that the likely hood of exposure increases with age; older animals are more likely to have been exposed than younger ones; added to the chronic nature of the

disease which takes a long time to develop a grossly visible lesion that are detected at postmortem examination. However, this study fails to agree with few previous works in Ethiopia who reported lack of association of age with goat tuberculosis (Nigussie, 2005; Hiko and Ejeta, 2010; Tefess *et al.*, 2011).This could be attributed to the smaller sample size used in these studies compared to our study, classification of age in to more than two categories which decrease the statistical power to significantly explain the role of the factor and lack of precise measurement of the age of the animals.

The prevalence of goat TB in all origins of the study animal was almost similar except for those originated from Borena and Somali with the highest and lowest record respectively. Localities specific prevalence of goat TB reported in this study were consistent with previous work of Hiko and Ejeta (2010) who reported a prevalence of 3.9, 5.2, 3.4 and 4.4% from Bale, Borena, Fentale and Jinka respectively. A Significantly higher prevalence ($p<0.05$, OR =2.14) was recorded for goats originating from Borena areas as compared to those from Somali region; although the goats types were the same, Somali goats, in both areas. In these two areas, the production system is predominantly pastoralism but in Somali, the pastoralists are keeping exclusively goats and camels as the area is arid and not suitable for cattle which could have posed a risk of *Mycobacterial* infection in goats. Contrary to this, in Borena, pastoralists are keeping large number of cattle and some goats which usually co-graze and share the same premises particularly in agro-pastoralists of the area. Consequently, there is potential for exposure to *Mycobacterium* in goat sharing the same environment with the cattle, the maintenance host of *M. bovis* and humans. However, only atypical *Mycobacterium* was isolated from goats coming from this area. Similar to our study, a relatively higher prevalence of 5.2% (n=384) of TB infection was reported by Hiko and Ejeta (2010) in goats whose origin was from Borena. Javed *et al.* (2010) have also found that caprine tuberculosis was significantly higher in goats kept with large ruminants. It is also stated that the incidence of goat tuberculosis may reach as high as 70% if they are maintained with infected herd of cattle (Radostits *et al.*, 2000).

According to this study, there was no significant variation in the prevalence of caprine TB in all the four goat types. The result lacks agreement with report from Pakistan which demonstrated a significant breed variation ($p<0.05$) of goats in reacting to TST (Javed *et al.*, 2010). This finding is also inconsistent with the reports of variation in breed susceptibility of

cattle to bovine TB in Ethiopia (Ameni, 2006; Ameni *et al.*, 2007); however, it is difficult to compare as the entire goat included in our study were local which are not well characterized to be considered as separate breed. The result of our study may necessarily not reflect that the occurrence of TB is not affected by goat type because most of the goat types in this study were from a different agro ecological zone which may have a different management system having the potential to confound the effect of this variable. Thus, we encourage further studies to be carried out to clarify genetic variation in TB resistance/susceptibility in different ecotypes/ genotypes of Ethiopian goats.

Macroscopic tuberculous lesions in goats are similar to those described in cattle as reported by Liébana *et al.* (1996).The present study showed that typical tuberculous lesion in goats was almost exclusively observed in the thoracic cavity. Only four TB lesions were detected in extra thoracic cavity. Similar study documented 97% of the gross TB lesions in the lungs and associated lymph nodes of the study goats (Hiko and Ejeta, 2010). Neima *et al.* (2011) also reported a 100% of TB lesion to be confined to the thoracic cavity in Algerian goats. Similarly, other authors had also reported the majority of the tuberculous lesions in the lungs and associated lymph nodes of Ethiopian cattle (Asseged *et al.*, 2004; Ameni *et al.*, 2010; Tsegaye *et al.*, 2010). Neill *et al.* (2005) suggested that the route of transmission of *Mycobacterium* can be deduced by the pattern of lesions observed in slaughtered animals. Thus, this could indicate that goats acquire the infection mainly through the respiratory route.

Both the frequency and severity of the lesions were highest in mediastinal lymph nodes followed by bronchial lymph nodes than lung lobes similar to the work on cattle by Ameni *et al.* (2010) who reported a highest severity and frequency of lesion on the mediastinal lymph nodes. However, the distribution and the magnitude of the maximum and minimum mean severity of pathology in lesioned tissue reported in our study is lower than the earlier reports for cattle by Ameni *et al.* (2010) and for camel (Mamo *et al.*, 2011).This variation could be explained by the fact that most of the goats in our study were young and thus, the agent could not find time to progress and cause extensive damage to the tissue. Another explanation could be a very good body condition of all the goats (export oriented) which might render them immunologically competent to limit the dissemination and restrict the pathogen to the lymph nodes.

In this study, mycobacteriological investigation of lesioned tissues showed that about 12 % (8/69) of the animals with gross TB lesions had colonies with confirmed acid fast bacilli. The recovery rate of *Mycobacterium* isolates in this study is lower than previous studies on: goats Hiko and Ejeta (2010), cattle Ameni *et al.* (2007, 2010) and camel (Mamo *et al.*, 2011). The lower success rate of growth of *Mycobacterium* in our study could be due to the few number tissues collected per animal as most of the positive animal presented lesion in only one of the tissues. This implies that the chance of culturing multiple or different tissue per diseased animal to increases the sensitivity of culture or *Mycobacterium* growth was low. Another justification could be due to the verity that in most of the samples, the extent of dissemination (severity) of lesion was very low (table 7); may be host immune responses were actively operating in the granulomatous inflammation and thus the pathogen may be fewer in number and most of them might lose viability during transportation, cold storage and tissue processing (decontamination).

In our study, four of the five isolates which were confirmed to be in the genus *Mycobacterium* belongs to MTBC group. Deletion typing for speciation of the isolates confirmed that all the three isolates which appeared on the final electrophoretic gel were *M. tuberculosis.* Further characterization of the isolates using spoligotyping indicates that one of the isolates were SIT53 strain where as the other were new strains.

The isolation of three *M. tuberculosis*, the primary agent of human tuberculosis, from goat in this study was unexpected. This is because in many countries, caprine TB in both domestic and feral goats occurs as a result of contact with cattle and wildlife reservoirs infected with *M. bovis* (Morris *et al.*, 1994; O'Reilly and Daborn, 1995; Cousins, 2001; Ayele *et al.*, 2004).Even recently caprine outbreaks of TB in the UK, Italy and Portugal have been shown to be caused by *M. bovis* (Crawshaw *et al.*, 2008; Daniel *et al.*, 2009; Quintas *et al.*, 2010).

Reports of *M. tuberculosis* infection in goats are very rare both in Ethiopia and other countries. The single documented study in Ethiopia was by Hiko and Ejeta (2010) who reported two isolates of *M. tuberculosis* from goats' tissue in one of the export abattoir. In Nigeria, Cadamus *et al.* (2009) had also reported one strain of *M. tuberculosis* out of the five *Mycobacterium* species isolated from goats. The isolation of *M. tuberculosis* from goats in

this study suggests that goats might have acquired the infection from infected human owners as previously been reported (O'Reilly and Daborn, 1995).

Although there is paucity of information in goats; the fact that owners can be the source of infection has been established for cattle. In Slovenia, Ocepek *et al.* (2005) had confirmed that isolates from the cattle and farm worker who suffered from pulmonary tuberculosis were the same strains. Even in Ethiopia, Regessa *et al.* (2008) showed that cattle owned by farmers with active tuberculosis were four times more likely to have TB than cattle owned by those with no active tuberculosis. Accordingly, the authors' isolated two *M. tuberculosis* from milk of skin test positive cattle owned by TB patient and suggested possible transmission of the pathogen between the owners and their cattle. Similarly, Tsegaye *et al.* (2010) also isolated *M. tuberculosis* from a cow whose owner had died with the history of tuberculosis.

Thoen *et al.*(1981) described that animal attendants with active TB in the respiratory tract, urinary tract or gastrointestinal tract represent an active source of *M. tuberculosis* for animals; spreading the bacillus via the sputum, urine or faces. One of the *M. tuberculosis* isolates was SIT53 strain, one of the extremely drug resistant and frequent strains of *M. tuberculosis* in Ethiopia (Bruchfeld *et al.*, 2002; Agonafir *et al.*, 2010; Legesse, 2011). In Ethiopia, the prevalence of *M. tuberculosis* in humans is 643 cases per 100,000; the 7th highest TB burden in the world (WHO, 2008).The rural population in the country live in close contact with their animals, sharing the same shelter at night-time which poses high risk of transmission of *M. tuberculosis* from human-to-in-contact animals. Thus, transmission caused by close co-habitation of goats and humans with advanced TB may occur.

Although it was not expected, the isolation of *M. tuberculosis* from goat in this study should not be astonishing. This is in view of the high TB burden in human population and endemicity of the disease, added to the close contact between animal and human and absence of tuberculosis surveillance and control strategies in Ethiopia. The bigger concern is potential zoonotic threat posed by the disease particularly to the pastoralist communities consuming raw milk and meat derived from infected goats.

The *M. tuberculosis* isolates in this study were obtained from goats originating from Jimma, South Wello and Arsi zone. All are predominantly mixed crop-livestock farming area; in the

latter (Arsi), people drink raw goat milk usually by adding to coffee due to the belief that goat milk has curative properties. This suggests that transmission of *Mycobacterium* from goat to human is highly likely and caprine TB has the potential to become an emerging public health threat. This is supported by a report from Spain which clearly demonstrated that goats are also source of infection for humans (Gutiee´rrez *et al.*, 1997). The authors isolated three *M. bovis* spoligotypes which were of a typical caprine genotype from a person who had a history of close contact with goat herds.

Added to the zoonotic concern, caprine TB has a paramount veterinary importance as it poses a growing threat to goat health and production. With an emerging importance of goat meat export markets in Ethiopia, caprine tuberculosis has a potential to cause sanitary problems leading to economic losses associated with limitations of trade and removal of infected animals. Moreover, goats can also act as reservoirs of infection, facilitating transmission to susceptible in-contact animals as reported by Kubica *et al.* (2003). This is particularly a major risk in Ethiopia where a large number of livestock are freely mixed and managed together; a practice aiding inter-species transmission and spread of *Mycobacterium*.

6. CONCLUSION AND RECOMMENDATIONS

This study documented a lower and similar prevalence of caprine tuberculosis across a range of eco-epidemiological settings. A significantly higher prevalence was recorded in relatively older goats while no significant variation was observed among the other factors of variations, origin and goat types. However, this study should be interpreted in the context of its limitations in that all animals were male with a good body condition where most of them were very young. These could compromise representativeness of the sample and hence inference of the result to the target population. In addition, we also lacked information on the precise geographical origins of the animals and thus, there would be no chance of tracing back the herd or house hold from which positive animals sourced. To this end, we encourage a more intensified study involving screening of the goat using tuberculin skin test at the house hold level which helps know the true picture of the disease, protect the public health and prevent spread of the disease in the live stock.

The occurrence of most lesions in the thoracic cavity suggests that transmission of tuberculosis in goats is mainly through the respiratory route. Hence, during meat inspection, emphasis should be given to the lungs and its associated lymph nodes to detect or rule out tuberculous carcasses. The success rate of isolation of *Mycobacteria* from the suspicious lesion was low but interestingly two of the isolates obtained were confirmed to be new strains of *M. tuberculosis*. It is tempting to hypothesize that the isolation of *M. tuberculosis* from goats in this study is evidence of transmission from humans as SIT53 strain is one of the common *Mycobacteria* isolated from humans in Ethiopia. However, future studies are needed to find out if isolation of *M. tuberculosis* from goats is a 'spill over' effect from humans or if these strains (particularly the new strains) of *M. tuberculosis* are goats adapted genotypes. The latter also raises the concern of the risk for goat-to-goat and goat-to-human transmission and its impact on public health. Thus, we strongly recommend awareness creation to be made among pastoralists and other consumers of raw goat milk and meat as to the potential risk arising from these products. The finding of new strains of *M.tuberculosis* from goat clearly indicates the future threat the country is facing to control the disease if no measure is immediately taken to conquer the disease.

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8. APPENDICES

Appendix I: Antemortem and postmortem data collection format

Name of trader ----------

Specific area where most goats were bought---------- Date----------

ID	Origin	Goat type	Age	MD	BL	RPH	MS	HPL	SML	Lung lobes						

MD- mediastinal lymph node, BL-bronchial lymph node, RPH- Retropharyngeal lymph node, HPL- hepatic lymph node, MSL- mesenteric lymph node, SML-Submandibular lymph node

Appendix II: Preparation of Löwenstein-Jensen egg-based medium

1. Preparation of mineral salt solution

Approximate Formula* Per 600 mL

1.1 Ingredients

- Potassium dihydrogen phosphate anhydrous (KH2PO4) 2.4 g
- Magnesium sulphate (Mg SO4. 7H2O) 0.24 g
- Magnesium citrate (Mg3(C3H5O7(2)) 0.6 g
- Asparagines 3.6 g

1. Weigh and add the above listed minerals salt ingredients in to a flask that labeled with P (Pyruvate) and G (Glycerol),

2. Add 300ml distilled water to each flask,

3. Add 12ml of Glycerol (*M. tuberculosis*) to the flask labeled with G,

4. Add 10g of Pyruvate (*M. bovis*) to the flask labeled with P,
5. Dissolve the mineral salt ingredients to the distilled water by heating,
6. Autoclave the minerals salt solution at 121oc for 30 minutes to sterilize,
7. Cool at room temperature before use,
8. Keep the solution indefinitely and may be kept in suitable amounts in refrigerators.

2. Preparation of 2% malachite green solution

2.1 Ingredient

- Malachite green dye 2.0g
- Sterile distilled water 100 ml
- Using aseptic techniques dissolve the dye in sterile distilled water by placing the solution in the incubator for 1-2 hours.

3. Preparation of homogenized whole eggs

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

- Fresh hens' eggs, not more than seven days old, are cleaned by scrubbing thoroughly with a hand, brush in warm water and plain alkaline soap.
- Let the eggs soak for 30 minutes in the soap solution.
- Rinse eggs thoroughly in running water and soak them in 70% ethanol for 15 minutes.
- Before handling the clean dry eggs scrub the hands and wash them.
- Crack the eggs with a sterile knife into a sterile flask till 1000m obtained.
- Then beat with sterile egg whisk or by using magnetic stirrer until it fully homogenized

4. Preparation of the complete medium (Mixing of the different solution)

- Measure and add 500ml of homogenized eggs in to sterile flask that labeled with P and G,
- Add 300ml mineral salt solution to each flask(remember to add P labeled salt solution to P flask and labeled and G labeled mineral salt to G labeled),
- Add 10ml malachite green solution,
- Steer well the complete medium

- Distribute the complete egg medium in 6-8 ml volumes in sterile 14 or 28ml McCartney bottles or in 20 ml volumes in 20 x150 mm screw- capped test tubes and the tops tightly closed,
- Inspissate the medium within 15 minutes of distribution to prevent sedimentation of the heavier ingredients.

5. Coagulation of medium (Inspissation)

- Before loading heat the inspissations to 80oc to hasten the buildup of the temperature,
- Place the bottles in a slanted position in the inspissator and coagulate the medium at 80- 85o^{c} for 45 minutes.

6. Sterility checking

- After inspissations, the whole batch of media or a representative samples of culture bottles should be incubated at 35-37o^{c} for 24 hours as a sterility check

7. Storage

- Date the media for storage,
- Store or keep the dated media in the refrigerator for several weeks with caps tightly closed to prevent the medium from drying out.

Source: WHO (1998)

Appendix III: Culturing of tissue samples

1. Homogenization of tissue samples

- Remove the surrounding flash with sterilized blade/scissor
- Cut the tissue into pieces by using sterilized scissor
- Add these pieces of tissue into mortar that contain small amount of sterilized sand
- Then grind the tissue with pistel
- Add small amount of sterilized saline
- Filter the grinding tissue using sterilized sieve
- Take(harvest) the filter liquid, and add into centrifuge tube

2. Decontamination

- Add equal volume 4% NAOH to centrifuge tube
- Mix well for about 10 minutes

- Centrifuge the mixed liquid at 3000 rpm at 4_o^c for 15 minutes
- Decant the supernatant
- Mix the sediment

3. Neutralization

- Add drop of phenol red indicator to well mixed sediment and mix well until it becomes purple
- Add 1% HCL to the sediment and mix well until it becomes yellow

4. Inoculation

- Add 1-3 drop of neutralized sediments of the tissue into LJ media (one labeled with P and other with G)

5. Incubation

- Incubate at $37o^c$ with 10% $C0_2$ air at slant position for a week,
- Then put at upright position for 8 weeks at the some temperature and air condition until complete growth is observed.
- Monitor every week for growth

Appendix IV: Ziehl-Neelsen stain reagents preparation

1. Carbol-Fuchsin Solution:

Basic fuchsin	2.5 gm
Distilled water	250.0 ml
100% alcohol	25.0 ml
Phenol crystals, melted	12.5 ml

- Mix well, filter into brown bottle. Label bottle with date and initials, solution is stable for 1 year.

Caution: Carcinogen, toxin.

2. 1% Acid Alcohol:

Hydrochloric acid	10.0 ml
70% Alcohol	990.0 ml

- Mix well, label with date and initials, stable for 1 year

Caution: Flammable, corrosive

3. Methylene Blue

3.1. Stock Solution:

- Methylene blue 0.7 gm
- Distilled water 50.0 ml
- Mix well, filter into bottle. Label with date and initials, stable for 1 year.

3.2. Working Solution:

- Methylene Blue Stock 5.0 ml
- Distilled water 45.0 ml
- Pour into coplin jar, stable for 2 months.

Caution: Avoid contact and inhalation

Appendix V: Ziehl-Neelson staining procedure for AFB

- Mix well the sample/sputum/ isolated colonies and make the smear,
- Fix the smear with heat,
- Flood the slide with the carbon-fushsin stain (primary stain),
- Gently heat the carbon-fuchin stain until it steam and maintain steaming for 5 minutes,
- Wash the slide thoroughly with tape water,
- Decolorize the slide with acid alcohol (3% HCL) for 5 minutes,
- Wash thoroughly the slide with tape water,
- Flood the slide with the methyl blue/ malachite green (counter stain) for 1 minute,
- Wash thoroughly the slide with tape water,
- Wipe the back of the slide with cotton or gauze and dry it,
- Examine the slide with 40x magnification power to check for quality of smear and Staining,
- Add one drop of oil immersion and examine the slide with 100x magnification power

Source: WHO (1998)

9. CURICULUM VITAE

BENTI DERESSA GELALCHA

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A. OTHER PERSONAL DETAILS

Date of birth	12/05/1984
Nationality	Ethiopian
Sex	Male
Profession	Veterinarian
Position	Lecturer \
Marital status	Single
Language spoken	Afan Oromo, Amharic, English, Germany (little)

B.PROFESSIONAL OBJECTIVE

My career objective is to use veterinary knowledge to bring about a meaningful contribution in enhancing the livelihood of the society.

C.ACADEMIC BACK GROUND

1. Freie University of Berlin and Addis Ababa University

Degree MSc degree in transboundary animal disease management Jan 2010 –Dec 2011

2. Addis Ababa University 2006/2007-2008

Degree Doctor of Veterinary Medicine (DVM)

Cumulative grade point average (cGPA): **3.82/4.00** (Very Great Distinction)

Prize **Gold medal** and books

3. Mekelle University 2002/2003-2006

Academic Transcript of eight semesters

4. Dejazmach Geresu Duki Comprehensive Secondary School 1999-2002

Certificate Ethiopian Secondary School leaving certificate

cGPA 4.00/4.00 (Very Great Distinction)

Prize 500 ETB from the President of Oromia national state

5. Dilella Elementary and Junior School 1990-1998

Certificate Ethiopian primary and junior school leaving certificate

Score **91/100** (Great distinction)

D. WORK EXPERIENCE

Jimma University September 2008 to December 2009

Main responsibilities and position held:

- Teaching and conducting research
- Clinical studies course team leader
- Coordinator of Jimma University veterinary clinic center
- Secretary of Academic Commission of School of Veterinary Medicine

E. LIST OF PUBLICATION

1. Evaluation and characterization of reproductive performance and activities of jacks in and around Debre-zeit, central Ethiopia
2. Preliminary survey on Helminthes parasites of donkeys in Hawassa town, southern Ethiopia
3. Preliminary study on public health implication of Bovine tuberculosis in Jimma town, South west Ethiopia

H. HOBBIES

Watching movies, reading novels and doing physical exercise.

10. SIGNED DECLARATION SHEET

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

Name **Benti Deressa Gelalcha**

Signature _____

Date of submission _____

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

Dr. Gobena Ameni (DVM, PhD, Associate professor) _____

Prof. Franz J Conraths _____

ABATTOIR BASED STUDY OF EPIDEMIOLOGY OF CAPRINE TUBERCULOSIS USING CONVENTIONAL AND MOLECULAR TOOLS

By

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1. Dr. Gobena Ameni (AAU, Ethiopia)	
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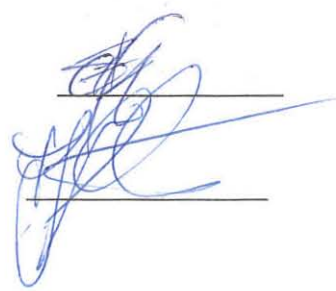

DECEMBER, 2011

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