STUDY ON *MYCOBACTERIUM BOVIS* USING CONVENTIONAL AND MOLECULAR METHODS IN CATTLE SLAUGHTERED IN KOMBOLCHA ELFORA MEAT PROCESSING PLANT

BY

FEKADU DESTA

JUNE 20, 2008

DEBER ZIET, ETHIOPIA

ADDIS ABABA UNIVERSITY
FACULTY OF VETERINARY MEDICINE

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A thesis submitted to the school of Graduate studies of Addis Ababa University in partial fulfillment of the requirement for the Degree of MSc in Tropical Veterinary Epidemiology

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ACKNOWLEDGEMENTS

All the bacteriological work was done at Aklilu Lemma Institute of Pathobiology (ALIPB) and PCR and Spoligotyping at Armauer Hansen Research Institute (AHRI).

My special thanks and appreciation goes to Dr Yilkal Asfaw and Dr. Gobena Ameni, my advisors, for their intellectual advice, provision of materials and meticulous correction of this manuscript. In addition I would like to remember the financial support by Dr Gobena Ameni.

I would like to thank Head officials and members of Kombolcha ELFORA meat processing plant for their arrangement and collaboration during the study.

I would like also to thank Dr Getahun Ejeta for his help in correction of the manuscript and for his academic and valuable suggestions.

Special thank to Ato Hailu Negatu, Dr Rebuma, W/t Surane Gemeda and Ato Nega for their expert technical support.

My deepest gratitude goes to my sweet heart Hana Estemech for her continuous moral support.

Thanks to all friends working in Kombolcha ATVET College, above all to Moltotal Habte and Wubshet for their encouragement and moral support.

At last but not the least I thank my uncle Ato Getachew Desta and his wife W/ro Mulunesh Mamo and the rest of the family members for their love and concern to me, which I enjoyed all my life.

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ABBREVIATIONS

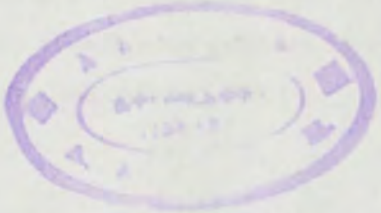
AFB	Acid Fast Bacilli
AIDS	Acquired Immune Deficiency Syndrome
BCG	Bacillus Calmette-Guèrin
bp	Base Pairs
BTB	Bovine Tuberculosis
CMI	Cell Mediated Immunity
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside Triphosphate
DR	Direct Repeat
FAO	Food and Agriculture Organization
FNA	Fine Needle Aspirate
HIV	Human Immunodeficiency Virus
ILRI	International Livestock Research Institute
IS	Insertion Sequence
LJ	Löwenstein Jensen
MDR	Multi Drug Resistance
MoA	Ministry of Agriculture
MOTT	Mycobacterium Other than Tuberculosis
MTC	*Mycobacterium tuberculosis* complex
OIE	Office International des Epizooties
°C	Degree centigrade
PGRS	Polymorphic GC-rich Repetitive Sequence
PCR	Polymerase Chain Reaction
RD	Region of Difference
RFLP	Restriction Fragment Length Polymorphism
SEM	Standard Error of Mean
SNPs	Single Nucleotide Polymorphisms
rpm	Revolutions Per Minute
TB	Tuberculousis
UV	Ultra Violet
WHO	World Health Organization
$\chi 2$	Chi-square
%	Percentage

ABSTRACT

A cross-sectional study was conducted on 1138 heads of cattle at Kombolcha ELFORA meat processing plant, South-Wello Administrative Zone, Amarha Regional State from November 2007 to May 2008. The objectives of the study were to estimate the prevalence of bovine tuberculosis in cattle slaughtered at Kombolcha ELFORA meat processing plant, and to isolate and characterize the strains of mycobacteria from tuberculousis suspicious lesions. Detailed post mortem examination, bacteriological culturing, regions of difference-bases polymerase chain reaction and spoligotyping were used. The prevalence of bovine tuberculosis was 5.0 % (57/1138) in cattle slaughtered at Kombolcha ELFORA meat processing plant on the basis of detailed post-mortem examination. There was no significance difference in prevalence between male and female ($P > 0.05$), as well as among the age groups ($P > 0.05$). Mycobacteria were able to be isolated from 28 of the 57 cattle with gross lesion. A total of 49 isolates were recovered from the different tissues of these 28 cattle, and 20 isolates showed signal for *M. tuberculosis* complex species of which 19 were *M. bovis* while one isolate was *M. tuberculosis*. Further characterization of the strains of *M. bovis* using spoligotyping revealed the presence of five different cluster of spoligotyping pattern, which include Ethiopian *M. bovis* train 1, SB1176, SB0134, SB0133 and new strain. The binary number representation of the new strain is 1100000101010110111111111111111111101111100000 where 1 indicates the presence of a spacer and 0 indicates a loss. The new spoligotyping was not reported previously from elsewhere to the *M. bovis* database (www.mbovis.org). The findings of this study indicated that tuberculous lesions were common in cattle slaughtered at Kombolcha ELFORA meat processing plant, and hence detailed post mortem meat inspection procedures are required to minimize the risk of its transmission to the public through meat consumption. In addition, appropriate cooking of meat is required before consumption.

Key words

Bovine tuberculousis; *Mycobacterium bovis*, Pathology scoring, Molecular typing



1. INTRODUCTION

Bovine tuberculosis (BTB) is a chronic bacterial disease of animals and human caused by *Mycobacterium bovis*, slow growing and acid-fast bacillus (AFB) (Quinn *et al.*, 1994). It is still continuing to be a significant local problem with a global perspective, despite intensive efforts over a number of decades (Pollock *et al.*, 2006). *Mycobacterium bovis* is a member of the *M. tuberculosis* complex, a group that also includes *M. tuberculosis*, *M. africanum*, *M. canetti* and *M. microti* and also the recently described species *M. caprae* and *M. pinnipedii* (Cousins *et al.*, 2003).

Tuberculosis (TB) in humans caused by *Mycobacterium bovis*, the agent of TB in cattle, has been reduced to low levels in industrialized countries as a result of milk pasteurization and animal tuberculosis control programs. However, in economically developing countries, where pasteurization is only sporadically practiced and animal tuberculosis control programs are frequently suboptimal or non-existent, bovine tuberculosis in humans remains a substantial public health problem (Cosivi *et al.*, 1998).

Developing nations of the world have nearly two-thirds of the world livestock population, but produce less than the developed world's meat and milk production due to poor management and high prevalence of livestock diseases such as mastitis, respiratory diseases, tuberculosis, etc (ILRI/FAO, 1995). Tuberculosis is one of the important diseases not only due to its effect on animal production and productivity but also due to its public health importance (O'Reilly and Daborn, 1995).

In sub-Saharan Africa, humans and animals share the same microenvironment and water holes, especially during drought and the dry season, thereby potentially promoting the transmission of *M. bovis* from animals to humans. According to Cosivi *et al.*, (1998), 60% of the African, 47% of the Asian, and 38% of the Latin American and Caribbean countries have reported the occurrence of BTB from sporadic to enzootic levels. Approximately 85% of the cattle and 82% of the human populations of Africa live in areas where BTB is either partly controlled or not controlled at all (Cosivi *et al.*, 1998). In such countries, where BTB is still common and pasteurization of milk is not practiced, an estimated 10 to 15% of human TB cases are caused by *M. bovis* (Ashford *et al.*, 2001).

In Ethiopia, BTB has been found to affect a higher proportion of exotic breeds than local zebus, which has been conferred through prevalence studies of BTB in different parts of Ethiopia (Ameni, 1996; Kiros, 1998; Ameni *et al.*, 2001). The economic impact of BTB has also been reported (Gezahegne, 1991). Thus, BTB is still a great concern in many developing countries and Ethiopia is one of those where BTB is considered as prevalent disease in cattle populations. Its zoonotic implication has also significantly indicated an increasing trend to be of public health hazards (Kiros, 1998; Regassa, 2005).

The World Health Organization (WHO) estimates that if the effectiveness of tuberculosis control does not improve substantially, the number of TB cases will pass the 200 million mark in early 2001 and by 2020 nearly 1 billion people will be newly infected, because of a combination of demographic factors, population movements, the expanding HIV epidemic and increasing drug resistance (Kochi, 1994). Unlike many other diseases affecting the developing world, TB can be controlled and treated. Better case finding and treatment would considerably reduce the risk of transmission (Rodrigues and Smith, 1990).

The advent of genetic engineering has provided alternative deoxyribonucleic acid (DNA) based strategies, which have the potential to overcome non-specific reactions. The development of gel electrophoresis for the size separation of DNA fragment, followed by the availability of restriction endonucleases which cleave DNA at defined sites lead to the development of restriction fragment analysis of bacterial DNA. This technique produces a pattern of fragments or finger prints, which uniquely characterizes the strain from which the DNA were isolated (Collins *et al.*, 1994). Polymerase chain reaction (PCR) is a biochemistry and molecular biology technique for enzymatically replicating DNA without using a living organism. It is a method that efficiently increases the number of DNA molecules in logarithmic and controlled fashion (Kamerbeek *et al.*, 1997).

The lack of quarantine and the smuggling of live animals across boundaries, which is very common among east Africa countries, promote the transmission of *M. bovis* from one country to another. It is therefore necessary to investigate the molecular epidemiology of *M. bovis* within and across countries so that the strains circulating in human beings, cattle, and wildlife can be identified. The most molecular typing method applied to *M. bovis* in developed countries is spoligotyping (Durr *et al.*, 2000), and restriction fragment length polymorphism

(LFLP) using the insertion sequence (IS) 6110 is the preferred typing method for *Mycobacterium tuberculousis* (Van Soolingen *et al.*, 1993).

Spoligotyping is a PCR-based method that exploits polymorphisms within the direct repeat (DR) region of the chromosome (Groenen *et al.*, 1993; Kamerbeek *et al.*, 1997), and subsequent differential hybridization of the amplified products with membrane bound oligonucleotides complementary to the variable spacer regions localized between the DRs, and have been used for the investigation of the molecular epidemiology of *M. bovis* and *M. tuberculousis* with a low copy-number of IS6110 (Brunello *et al.*, 2001). Strains that are similar or different can be distinguished by their spoligotype patterns, characterized by the number and identity of spacers (Van Soolingen *et al.*, 1995). The presences of the spacer sequences vary in different strains and are visualized by a spot on a fixed site of the hybridization membrane. A simultaneous detection and strain differentiation based on this method has been developed (Kamerbeek *et al.*, 1997). The method is simple, rapid and robust but lacks discrimination.

Eradication of BTB was a major component of the anti-tuberculosis strategy in all the nations that eliminated the disease (Barwinek and Taylor, 1996). Molecular epidemiology is an essential element in BTB campaigns. But, data on molecular epidemiology of mycobacteria in the country in general and Amhara Regional State in particular, are lacking. On the assumption that different strain clusters of *M. bovis* might exist in cattle slaughtered, this study is intended to investigate strain type of *M. bovis* causing diseases in the slaughtered cattle.

Therefore the objectives of this study are:

- To estimate the prevalence of BTB in cattle slaughtered at Kombolcha ELFORA meat processing plant.
- To assess the distribution and severity of tuberculosis lesion in slaughtered cattle.
- To characterize the species of mycobacteria and strain identification of *M. bovis* prevailing in the study site based on molecular technique, PCR and spoligotyping.

2. LITERATURE REVIEW

2.1. Mycobacteria

The genus *Mycobacterium* is classified under the order Actinomycetales and family Mycobacteriaceae (Quinn *et al.*, 1999). The genus, *Mycobacterium* include a number of species, some being pathogenic to man and animals, some are opportunistically pathogenic while others are essentially saprophytic living in water and soil (Thoen and Bloom, 1985). The classic species of *Mycobacterium* that cause disease in man and animals include. *M. bovis*, *M. tuberculosis*, *M. paratuberculousis*, *M. avium*, *M. leprae* and *M. lepraemurium*. Tuberculousis in mammals is caused by *M. tuberculousis* complex (*M. bovis*, *M. tuberculosis*, *M. microti* and *M. africanum*) and by *M. avium* in birds (Bhata and Ichpujani, 1994). *Mycobacterium* species other than the *M. tuberculosis* complex that cause TB like diseases in man and animals are commonly called 'atypical mycobacteria' (Quinn *et al.*, 1999). They have been classified into four groups by Runyon in 1959 as, photochromogenic, scotochromogenic, nonchromogenic and rapid growers based on growth rate and formation of pigments (Carter and Chengappa, 1991). Atypical mycobacteria are not pathogenic to man and animals except in certain situations such as direct inoculation into wounds or introduction into immunocompromised hosts due to immunosuppressive therapy or due to HIV (Thoen and Bloom, 1985); however, they are very important during diagnosis as they sensitize man/ animals to tuberculin test (Carter, 1986).

2.2. Animal Tuberculosis

Tuberculosis in farmed cattle, like that in many other animal species, is caused by *M. bovis* and can result in a chronic, granulomatous disease, mainly of the respiratory tract. Cattle can become infected in numerous ways, with animal age and behavior, existing environment and climate, and prevailing farm practices having significant influence. Inhaling *M. bovis* is the most probable and important route, as lesion distribution and pathology in field cases show predominant involvement of the upper and lower respiratory tract and associated lymph nodes (Pritchard, 1988; Corner, 1994; Neill *et al.*, 1994; Whipple *et al.*, 1996). Ingestion of *M. bovis* directly from swallowing infected milk or from contaminated pastures, water or fomites is considered secondary to respiratory spread (Menzies and Neill, 2000). Genital

transmission can occur also if the reproductive organs are infected, but this remains extremely rare (Neill *et al.*, 1994).

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.*, 1992). The droplet nucleus lodges within the respiratory tract, giving rise to a primary complex, usually considered to involve the lungs and associated lymph nodes. This initial host reaction, to challenge with tubercle bacilli, leads to the generation of detectable immune responses, subsequent pathology and clinical presentation. Bacilli are phagocytosed by macrophages, which subsequently interact with cells involved in innate and acquired immunity. The resulting immune responses in cattle are complex and it is evident that cell mediated immune (CMI) responses are usually dominant in a spectrum of immune responses, which occur after infection (Pollock and Neill, 2002). Antibody responses following challenge are rarely evident immediately and often would indicate a response relationship with bacterial load (Pollock and Neill, 2002).

The majority of tuberculous cattle were previously reported to have lung tissue lesions (Francis, 1958). However, subsequent studies have shown lung lesions in only a small proportion of tuberculous cattle (Neill *et al.*, 2001). Such lesions usually occur singly, and are extremely small in size (diameter <1 cm) and hence are often difficult to find. Tuberculous lesions are found most frequently in the bronchial and/or mediastinal lymph nodes; lymph nodes of the head region are the second most frequent site and in many instances lesions in the retropharyngeal and submaxillary lymph nodes exist in the absence of detectable lung lesions (Neill *et al.*, 2001). Interestingly, more recent investigations have revealed involvement of the tonsillar tissues (Palmer *et al.*, 1999). The uncommonness of these reports probably arises from the limited number of head examinations carried out on tuberculous cattle at abattoir inspection. It is not common to find tuberculous lesions in the mesenteric lymph nodes only. When present they probably result from ingestion of a heavy bacterial load, such as from drinking infected milk or arise from dissemination from primary lesions at other sites (Francis, 1958; Morris *et al.*, 1994). Generalized tuberculousis in cattle is now seen infrequently in countries with active control programmes. It is characterized by lesions in organs such as liver, kidneys and udder or in the meninges and serous cavities arising from primary lesions possibly in the lung or alimentary tract.

2.3. Molecular genetics of *Mycobacterium tuberculosis* complex

Members of the MTC are highly related mycobacteria exhibiting remarkable nucleotide sequence level homogeneity despite varying in pathogenicity, geographic range, certain physiological features (such as colony morphology as well as profiles of resistance and susceptibility to inhibitors), epidemiology and host preference (Eisenach *et al.*, 1986; Frothingham *et al.*,1994).

Various biological and molecular mycobacterial characteristics have been utilized to identify MTC isolates but have limited applicability as MTC taxonomical tools. Although certain *Mycobacterium* species-specific gene sequence differences work well to differentiate mycobacteria other than MTC (MOTT) from each other and from the MTC, to date none can discriminate the individual MTC subspecies due to genetic invariance in the target loci (Brunello *et al.*, 2001; Frothingham *et al.*, 1994). In contrast, a series of classical test based upon growth, phenotypic, and biochemical properties have been traditionally used to segregate members of the MTC (Haas *et al.*, 1997a; Niemann *et al.*, 2000). However, together these tests can be slow, cumbersome, imprecise, nonreproducible, and time-consuming, and they may not give an unambiguous result in every case and may not be performed by every laboratory. To complement the classical tests for determination of MTC species, well-defined MTC lineage and subspecies restricted single-nucleotide polymorphisms (SNPs) have been used to specify certain MTC groupings through sequence analysis and/or digestion of PCR products followed by restriction fragment length polymorphism (PCR-RFLP) analysis (Frothingham *et al.*, 1999; Goh *et al.*, 2001; Haas *et al.*, 1997b; Niemann *et al.*, 2000). However, these loci on their own are unable to differentiate all of the MTC subspecies. Likewise, molecular genetic MTC typing assays (e.g. variable number of tandem repeat analysis, mixed linker PCR, and IS6110 RFLP) that have been designed to reveal interstrain relationships (Frothingham *et al.*, 1999; Haas *et al.*, 1997a; kremer *et al.*, 1999) cannot be used as efficient taxonomic tools to unambiguously classify individual MTC strains. Spacer oligonucleotide typing (spoligotyping) is the only DNA-based methodology for which most MTC members are believed to have signature features (Aranaz *et al.*, 1996; Gutierrez *et al.*, 1997; Kamerbeek *et al.*, 1997; kremer *et al.*, 1999). However, spoligotype are not necessarily exclusive to none MTC member nor are they restricted, as strains can waver from the expected minimal consensus spoligotype pattern for MTC subspecies.

2.4. Molecular epidemiology of *Mycobacterium bovis*

Different studies on *M. bovis* are carried out to improve the traceability of infections and identification of the origin of the outbreak. One thousand two hundred sixty six *M. bovis* isolates were genotyped in France and observed an apparently high level of heterogeneity of 161 different clusters and a low frequency of the two main spoligotype clusters (Haddad *et al.*, 2001). In contrast, similar molecular studies in island countries like Great Britain or Australia showed a low level of heterogeneity and a high frequency of the main spoligotype clusters (Clifton-Hadley *et al.*, 1998; Cousins *et al.*, 1998). Again very few such studies have been carried out in developing countries like Africa. Some studies in Cameroon and Tanzania have shown similar results to those made in Britain or Australia with a high homogeneity and thereby indicate a high recent transmission rate (Kazwala *et al.*, 1997; Njanpop-Lafourcade *et al.*, 2001).

There are also studies which look at transmission pathways of *M. bovis* between different animal species, from animal to human and from human to human. Result of spoligotype clusters which include 9 strains isolated from wild boar and 11 strains isolated from cattle, thus confirming the possibility of transmission between the two animal species (Serraino *et al.*, 1999). Clusters containing *M. bovis* were also isolated from humans and cattle using the combination of the RFLP methods IS6110 and PGRS (Van Soolingen *et al.*, 1995). One of the first results indicating but not proving *M. bovis* zoonotic transmission between cattle and humans in Africa is shown in a study from Tanzania, where the same *M. bovis* spoligotype was isolated from man and cattle (Kazwala *et al.*, 2005). Moreover, molecular epidemiological studies showed the transmission of *M. bovis* multi drug resistance (MDR) tuberculousis between HIV-1-positive patients (Guerrero *et al.*, 1997). It is suggested that transmission of *M. bovis* took place within hospitals and that advanced HIV-1 immunosuppression was associated with the development of MDR tuberculousis. As with *M. tuberculosis*, molecular epidemiology can also develop a better understanding of the sources and modes of *M. bovis* transmission thereby enabling more effective control measures to be implemented in bovine eradication programs.

2.5. Status of bovine tuberculousis in Ethiopia

Detection of BTB in Ethiopia is carried out most commonly on the basis of tuberculin skin testing, abattoir meat inspection and rarely on bacteriological techniques. However, the current status on the actual prevalence rate of BTB at a national level is yet unknown. In Ethiopia, screening of cattle by the tuberculin skin test was sporadic until 1984. But, while the higher prevalence rate of the disease has been observed after the two year survey in government and some "parastatal" dairy farms, then it was decided to embark on a routine BTB survey, on these dairy farms, in particular using single and comparative intradermal skin tests (Alemu, 1992). Since then tuberculin skin test and abattoir meat inspection surveillances have been undertaken in different parts of the country at various times.

2.5.1. Bovine tuberculousis in animal populations

Most of the surveys carried out in Ethiopia have been based on tuberculin skin testing and abattoir inspection reports of animals in a particular locality. Bovine tuberculousis is one of the endemic infectious diseases that have long been recorded in Ethiopia (FAO, 1967; Hailemariam, 1975) and the infection has been detected in cattle and rarely in other species of domestic animals (FAO, 1972).

The disease is considered as one of the major livestock diseases that results in high morbidity and mortality (MoA, 1984). Furthermore, in recent years, Ethiopia has reported the occurrence of BTB in the years 1992–1996 and 2001 (FAO-OIE-WHO Animal health year books, 1992–1997; OIE 1998-2001, cited by Ayele *et al.*, 2004; Zinsstag *et al.*, 2006). However, still there is lack of knowledge about the actual prevalence and distribution of the disease at a national level.

Despite this, the economic impacts and zoonotic importance of BTB infection are either not well studied or documented. A study undertaken in previous years, 1984–1986, has shown the prevalence rate of 16.7% of BTB based on tuberculin skin tests on the government state farms and other "parastatal" dairy farms (Alemu, 1992). Among the recently undertaken studies, the prevalence rate of BTB ranges from 3.4% in a small holder production system to 50% in intensive dairy productions in the country (Kiros, 1998; Ameni and Roger, 1998; Ameni *et*

al., 2003a; Ameni *et al*., 2003b; Asseged *et al*., 2001; Regassa, 2001; Regassa, 2005) (Tables 1, Tables 2 and Tables 4).

In Ethiopia, exotic breeds were found to be more susceptible than cross and local breeds to *M. bovis* with manifestation of high incidence and prevalence rates (Kiros, 1998; Regassa 2005; Ameni *et al*., 2006).

In addition, a herd prevalence rate of 42.6% to 48.6% was found to be higher than the prevalence rate of individual animals (7.9% to 18.7%), that may indicate that the herd size can favor the transmission of BTB in intensive dairy farms in particular (Ameni *et al*., 2003b; Shitaye *et al*., 2006.)

Table 1 Prevalence of bovine tuberculosis detected by tuberculin skin test in a traditionally managed extensive production system in six farming areas in different districts in Ethiopia

Area of study	No of cattle			Reference:
	Tested	Positive	%	
Assella*	281	25	8.9	Teshome, 1986
Debre-Birhan	76	11	14.5	Tadele, 1998
Kombolcha	53	12	22.6	Tadele, 1998
Dessie	34	4	11.8	Tadele, 1998
West Wellega	353	12	3.4	Regassa, 2001
North Shewa	1041	169	16.2	Regassa, 2005
Total	1838	233	12.9	

*A comparative intradermal tuberculin skin test was used; positive results are given for bovine tuberculin

Table 2 Prevalence of BTB in small holder dairy farms based on tuberculin skin test in seven farming areas in different districts in Ethiopia

Areas of study	No of cattle			Reference
	Tested	Positive	%	
Holleta*	389	25	6.4	Teshome, 1986
Selale*	1528	78	5.1	Teshome, 1986
Wolaita-Sodo*	416	59	14.2	Regassa, 1999
Fiche**	735	31	4.2	Gemta, 2000
Wuchale-Jida*	763	60	7.9	Ameni *et al.*, 2003a
Assella*	514	18	3.5	Redi, 2003
Addis Ababa	473	61	12.9	Alemu, 1992
Total	4818	332	6.9	

*Comparative skin tests with bovine and avian tuberculin

**A single intradermal skin test with bovine tuberculin

Table 3 Prevalence rates of BTB detected by abattoir meat inspection in cattle in different city abattoirs in Ethiopia

City abattoirs	**No. of cattle**			Reference
	Examined	Positive	%	
Addis Ababa	81944	123	0.15	Hailemariam, 1975
Addis Ababa	1350	20	1.48	Asseged *et al.*, 2004
Addis Ababa	984	34	3.46	Shitaye *et al.*, 2006
Debre-Zeit	3934	7	0.18	Hailemariam, 1975
Dire-Dawa	7453	4	0.05	Hailemariam, 1975
Gonder	12525	3	0.02	Hailemariam, 1975
Hossana	751	34	4.53	Teklu *et al.*, 2004
Kombolcha	57965	265	0.46	MoA, 1973
Makele	39875	730	1.83	Hailemariam, 1975
Nazareth	1125	58	5.16	Ameni and Wudie, 2003
Wolaita-Sodo	402	32	7.96	Regassa, 1999
Wondo-Genet	38303	207	0.54	Hailemariam, 1975
Total	246611	1517	0.62	

Table 4 Prevalence of BTB detected by tuberculin skin tests in intensive dairy farms in 13 farming areas in different districts

Areas of study	No of cattle			Reference
	Tested	Positive	%	
Addis Ababa	843	80	9.5	Teshome, 1986
Addis Ababa	2098	392	18.7	Shitaye *et al.*, 2006
Ambo	133	37	27.8	Ameni *et al.*, 2003b
Asella	281	23	8.2	Alemu, 1992
Debre-Birhan*	51	3	5.9	Tadele, 1998
Debre-Zeit	739	308	41.7	Teshome, 1986
Debre-Zeit	788	234	29.7	Kiros, 1998
Debre-Zeit	281	185	65.8	Ameni *et al.*, 2003b
Dessie*	127	6	4.7	Tadele, 1998
Dessie*	121	89	73.6	Ameni *et al.*, 2003b
Holleta	70	17	24.3	Ameni *et al.*, 2003b
Kombolcha*	179	96	48.7	Tadele, 1998
Mojo	493	338	68.6	Teshome, 1986
Mullo	525	162	30.9	Teshome, 1986
Repi	481	310	64.4	Anonymous, 1999
Sebeta	37	4	10.8	Ameni *et al.*, 2003b
Sellale	44	3	6.8	Ameni *et al.*, 2003b
State dairy farms**	6940	1217	17.5	Alemu, 1992
Ziway*	205	56	27.3	Ameni *et al.*, 2003b
Total	14454	3560	24.6	

*test conducted only by a single intradermal skin test with bovine tuberculin.

**tuberculin skin test conducted in the years 1987 to 1991 on state dairy farms around Addis Ababa

2.5.2. Bovine tuberculousis in human populations

The role of BTB causing tuberculousis in humans has not been studied adequately. However, very few studies have indicated the isolation of the causal agent of BTB from humans. With respect to this Kiros, (1998) demonstrated that out of 85 sputum samples taken from 28 dairy farm workers and 57 tuberculous patients, 48 samples were positive for acid fast bacilli, of

which 14 (29.2%) were niacin negative indicating *M. bovis* and 34 (70.2%) *M. tuberculousis* 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. tuberculousis*, respectively.

Furthermore, a higher prevalence of BTB was found in cattle owned by tuberculous patients than in cattle owned by nontuberculous owners, which suggests the significant role of *M. bovis* in the incidences of tuberculousis in humans (Regassa, 2005). In addition, Kidane *et al*., (2002) indicated that *M. bovis* along with other MTC species were found to be responsible for tuberculous lymphadenitis in humans.

The occurrence of *M. bovis* in humans against the background of the high HIV/AIDS incidence in Eastern and Southern Africa implies that the risk of spillover of zoonotic BTB to rural communities is rapidly increasing (Zinsstag *et al*., 2006). Thus, the correlation between the prevalence of *M. bovis* infection in humans and that of local cattle populations highlights the potential threat of this disease for humans (Daborn *et al*., 1996; Regassa, 2005), most notably in developing countries like Ethiopia, where drinking raw milk is a common practice in rural areas in particular.

2.6. Genotyping

2.6.1. Polymerase chain reaction

Currently, much attention is being focused on the use of PCR. The principle of the PCR technique is based on the amplification of a given DNA sequence to a large number of copies that can be identified by separation on gel electrophoresis and, subsequently, either with or without probing with a labeled oligonucleotide specific for the amplified DNA fragment. During the last decade, several such unique sequences have been reported for the *M. tuberculousis* complex. The usefulness of PCR for detection of tubercle bacilli in clinical specimens has been confirmed in several recent studies, with sensitivities and specificities ranging from 60% to 100%. Contamination is a problem that all PCR laboratories must face, since the product of the reaction contains millions of suitable templates that can be carried back to the next assay on finger tips, pipettes and clothing or in aerosols. With mycobacteria there is the additional difficulty of extracting DNA from within the cells and this is one of the

important limiting factors in determining the sensitivity of PCR assays for *M. tuberculousis* in clinical material (Zumarraga *et al.*, 1999). There have been many reports in recent years and several groups have published encouraging results involving considerable number of samples, but routine mycobacteriological laboratories are not yet convinced of its practical utility (Zumarraga *et al.*, 1999).

2.6.2. Spoligotyping

This method is based on the evaluation of the presence or absence of 43 spacer DNA sequences between the 36 bp direct repeats (DRs) in the genomic DR region of MTC strains (Kamerbreek *et al.*, 1997) (Fig. 1). Spoligotyping is based on amplification of the DR region and subsequent differential hybridization of the amplified products with membrane bound oligonucleotides complementary to the variable spacer regions localized between the DRs. Strains that are similar or different can be distinguished by their spoligotype patterns, characterized by the number and identity of spacers (Van Soolingen *et al.*, 1995) (Table5). The presence of the spacer sequences varies in different strains and are visualized by a spot on a fixed site of the hybridization membrane. The differentiating power of spoligotyping is less than IS6110 typing when high copy number strains are being analyzed, but it is superior for the evaluation of low copy number strains. It distinguishes *M. tuberculousis* and *M. bovis* and can be used with culture negative specimens (Kamerbreek *et al.*, 1997). A simultaneous detection and strain differentiation based on this method has been developed (Kamerbeek *et al.*, 1997). The method is simple, rapid and robust but lacks discrimination.

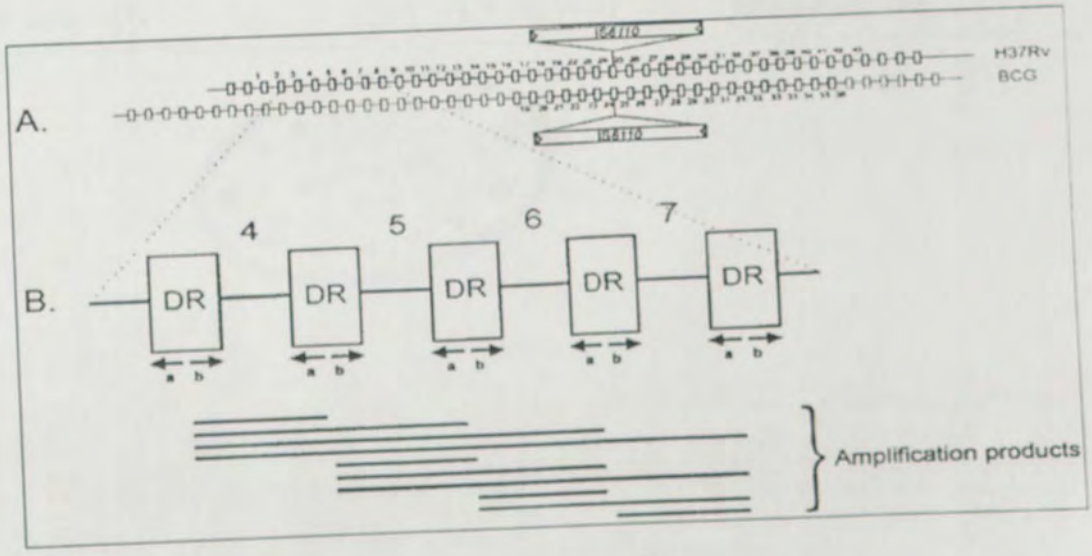


Fig 1 (A) Structure of the DR locus in the mycobacterial genome. *M. tuberculousis* H37Rv and *M. bovis* BCG contain 48 and 41 DRs, respectively (depicted as rectangles), which are interspersed with unique spacers varying in length from 35 to 41 bp. The (numbered) spacers used correspond to 37 spacers from *M. tuberculosis* H37Rv and 6 from *M. bovis* BCG.
(B) Principle of in vitro amplification of the DR region by PCR. Shown is the combination of fragments that would be produced by in vitro amplification of a DR target containing only five continuous DRs (Kamerbeek *et al.*, 1997).

Table 5 Diagnostic spoligo spacer missing for *M. tuberculosis* family members, *M. africanum* and host dapted *M. bovis* strains

***M. tb* family members**	**Spacer lacking**	**Reference:**
M. tuberculosis (Beijing)	1-34	Ferdinand *et al.*, 2004
M. tuberculosis (Haarlem)	31, 33-36	Ferdinand *et al.*, 2004
M. tuberculosis (Latin America)	21-24, 33-36	Ferdinand *et al.*, 2004
M. tuberculosis (East African India)	29-32, 34	Ferdinand *et al.*, 2004
M. tuberculosis (Central Asia)	4-7, 23-34	Ferdinand *et al.*, 2004
M. tuberculosis (Cameroon)	23-25, 33-36	Ferdinand *et al.*, 2004
M. africanum (Type I)	9, 39	Smith *et al.*, 2005
M. bovis (antelope)	9, 16, 39	Smith *et al.*, 2005
M. bovis (seal/vole)	3, 9, 16, 39-43	Smith *et al.*, 2005
M. bovis (caprine)	3, 9, 16, 39-43	Smith *et al.*, 2005
M. bovis (cattle)	3, 9, 16, 39-43	Smith *et al.*, 2005
M. bovis BCG	3, 9, 16, 39-43	Smith *et al.*, 2005

3. MATERIAL AND METHODS

3.1. Study area and population

A cross-sectional study on BTB was conducted from November 2007 to May 2008 in Kombolcha town of Debub Wello Zone of Amhara Regional State. Kombolcha is a city and *Woreda* in north-central Ethiopia. Located immediately southeast of Dessie, with an altitude of less than 1,500 meters above sea level (m.a.s.l) and a latitude and longitude of 11°4′37″N, 39°44′42″E, respectively.

Based on figures from the Central Statistical Agency in 2005, Kombolcha has an estimated total population of 68.766 of which 36,102 are males and 32,664 females. The *Woreda* has an estimated area of 8.66 square kilometers, which gives Kombolcha a density of 7,940.60 people per square kilometer.

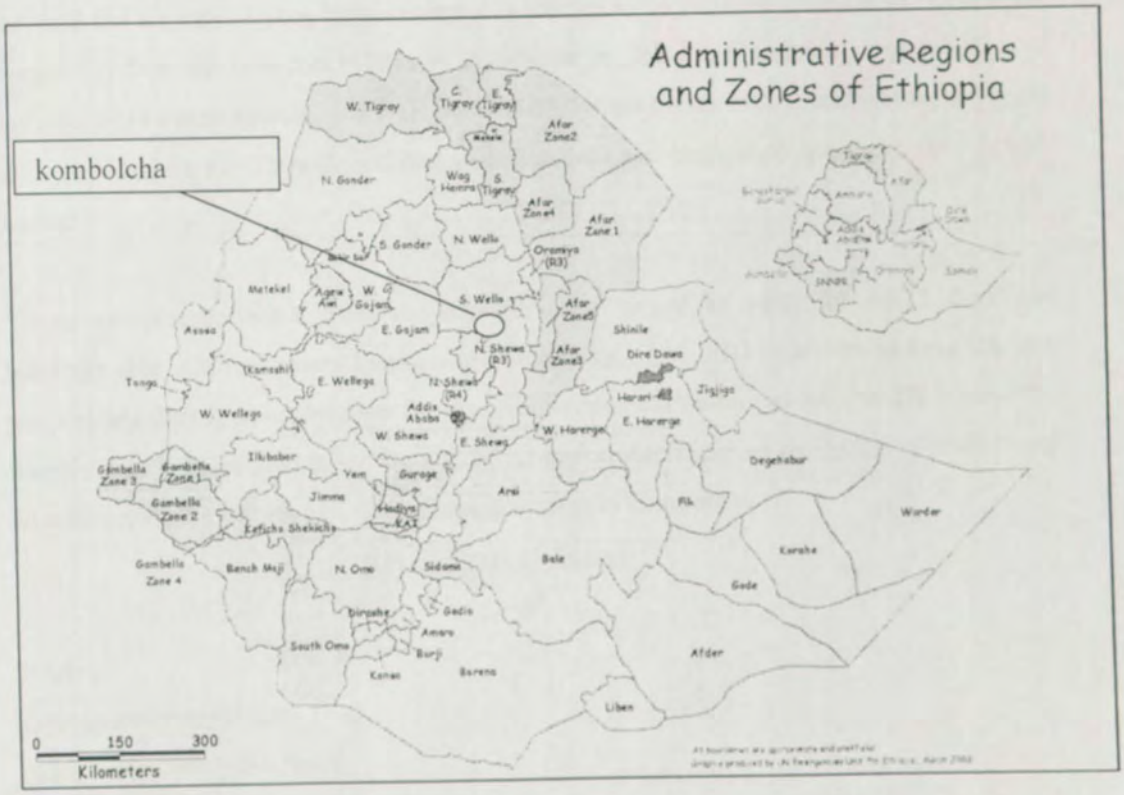


Fig 2 Administrative regions and zones of Ethiopia

Although South-Wello, North-Wello, North-Shewa, South-Gonder, Eeat-Gojam, Oromia (Bati) Zone and Afar Region were the major sources of cattle, the exact area of origin of the animals could not be traced since they were not individually identified. As a result, none of the cattle examined had a history of tuberculousis and they were, therefore, not tested for tuberculousis before slaughter. However, all the cattle brought to the abattoir were found to be apparently normal at ante-mortem examination. The animals are routinely bled by cutting the jugular vein after casting; and a senior meat inspector performed both the ante-mortem and routine post-mortem inspections.

3.2. Study design and Sampling

The study design for this abattoir based survey was cross sectional study, which was conducted to determine the prevalence of BTB in the study area. The study was designed to meet the overall objective of the project in identifying and characterization of strains of *M. bovis* in the country. Tissue lesion samples suspected to be positive for BTB were collected aseptically from the lung lobes (left apical, left cardiac, left diaphragmatic, right apical, right cardiac, right diaphragmatic and right accessory), lymph nodes of the head (retropharyngeal and mandibular), lymph nodes of lungs (mediastinal and bronchial) and mesenteric lymph nodes.

Tissue samples collected from the abattoir were stayed at +4°C for 10-15 days and transported to Aklilu Lemma Institute of Pathobiology (ALIPB) in icebox to keep the low temperature during transportation for mycobacteriological culture at ALIPB TB laboratory. Sample size is calculated based on a (22.6%) prevalence reported by Tadele, (1998) based on tuberculin skin test on extensive farming system in kombolcha.

$$n = \frac{Z^2 . Pexp. (1-Pexp.)}{d^2}$$

Where:-

n = required sample size

Pexp. = expected prevalence

d = desired absolute precision

Z = constant from normal distribution at a given confidence interval.

3.3 Body condition scoring.

The body condition of each of the study animal was scored using the guidelines established by Nicholson and Butterworth, (1986). Accordingly, on the basis of observation of anatomical parts such as vertebral column, ribs, spines, tip of tail, etc., the study animals were classified as lean (score, 1 to 3), medium (4 to 6), or fat (7 to 9).

3.4. Detailed post-mortem examination

The lymph nodes included in the examination are mandibular, retropharyngeal, left and right bronchial, mediastinal, mesenteric, inguinal and other tissue specimens such as the lungs, liver, and kidneys were subjected to a detailed post-mortem examination in the abattoir under a bright light source. The tissues were cut into slices of 2 cm using separate surgical blades. The slices were then examined for the presence of abscesses and tubercles (Gracy, 1986; Mambule, 1984).

During observation of tuberculous lesions in various parts of the carcass; lung, intestine, liver, and corresponding lymph nodes, the whole carcass was condemned. In the case of localized lesions, partial condemnation of organs is undertaken. During this period, 1138 animals were slaughtered and inspected. This figure comprises 437 males and 701 females culled because of old age or other reproductive problems. The age structures of most of the animals were similar, as most were culled for the same reason, because of old age. Moreover, in majority the cases the body conditions were similar, as they were required for canning.

3.5 Pathology scoring.

A total of 57 animals were pathology scored. The lungs and lymph nodes were removed for the investigation of lesions. The seven lobes of the two lungs, including the left apical, left cardiac, left diaphragmatic, right apical, right cardiac, right diaphragmatic and right accessory lobes, were inspected externally and palpated. Then, each lobe was sectioned into about 2-cm-thick slices to facilitate the detection of lesions. Similarly, lymph nodes, namely, the mandibular, retropharyngeal, mediastinal, left and right bronchial, hepatic and mesenteric

lymph nodes, were sliced into thin sections (about 2 mm thick) and inspected for the presence of visible lesions. When gross lesions suggestive of BTB were found in any of the tissues, the animal was classified as having lesions. The severity of the gross lesions was scored by the semi quantitative procedure developed by Vordermeier *et al.*, (2002), with minor modifications to facilitate performance under field conditions (Ameni *et al.*, 2006). Briefly, lesions in the lobes of the lungs were scored separately as follows: 0 = no visible lesions; 1 = no gross lesions but lesions apparent on slicing of the lobe; 2 = fewer than five gross lesions; 3 = more than five gross lesions; 4 = gross coalescing lesions. The scores for the individual lobes were added up to calculate the lung score. Similarly, the severity of gross lesions in individual lymph nodes was scored as follows: 0 = no gross lesions; 1 = small lesion at one focus; 2 = small lesions at more than one focus; 3 = extensive necrosis. Individual lymph node scores were added up to calculate the lymph node score. Finally, both lymph node and lung pathology scores were added up to determine the total pathology score per animal.

3.6. Isolation of mycobacteria

Samples from 57 postmortem positive cattle were further processed for isolation of mycobacteria in accordance with the Office International des Epizooties (Anonymous, 2000) protocols. Briefly, tissue specimens for culture were collected in sterile universal bottles in 5 ml of 0.9% saline solution and then transported to the laboratory by maintaining a cold chain. In the laboratory, the specimens were sectioned using sterile blades and then homogenized with a mortar and pestle. The homogenate was decontaminated by adding an equal volume of 4% NaOH by centrifugation at 3,000 rpm for 15 min. The supernatant was discarded, and the sediment was neutralized by 1% (0.1 N) HCl acid using phenol red as an indicator. Neutralization was achieved when the color of the solution changed from purple to yellow (Anonymous, 2004). Next, 0.1 ml of suspension from each sample was spread onto a slant of Löwenstein Jensen (LJ) medium. Duplicate slants were used, one enriched with sodium pyruvate and the other enriched with glycerol. Cultures were incubated aerobically at 37°C for about 5 to 8 weeks with weekly observation for growth of colonies.

3.7. Molecular characterization of mycobacteria

3.7.1. Polymerase chain reaction

Initial identification of mycobacteria species was based on the rate of growth, pigment production and colony morphology. Species belonging to the *Mycobacterium tuberculousis* complex show a slow growth rate. Growth of *M. bovis* is enhanced by pyruvate for further characterization of the species using molecular techniques.

Extraction of mycobacterial genomic DNA was conducted as previously described (Cousins *et al.*, 1991), with minor modifications. One colony from each isolate was suspended in 100ml of sterile distilled water and heat killed to 80°C for 20 minute. The heat treated suspensions were kept preserved in deep freeze (-20°C) until tested.

DNA amplifications were done in 25 µl reaction volumes consisting: Two µl of genomic DNA used as a template, 12.5µl hot taq ($MgCL_2$, dNTP; Taq polymerase and PCR buffer) for each sample, 5µl internal primer per sample, 1µl forward and reverse primer per each sample and 3.5 µl per sample of distilled water. The reaction mixture will then be heated in thermal cycle as follows: 95^0C for 15 minutes for enzyme activation; 94^0C for 1 minute for denaturation; 60^0C for 2 minute for annealing; 72^0C for 3 minute for extension; and finally 72^0C for 10 minutes for post extension, involving 35 cycles all in all. The amplified DNA were visualized after gel electrophoresis at 100V for 40 minute in 2% agarose gel stained with ethidium bromide and was viewed in UV-transilluminator (Zumarraga *et al.*, 1999).

3.7.2. Spoligotyping

All samples with positive amplification for mycobacterial DNA were subjected to further analysis by spoligotyping (Kamerbeek *et al.*, 1997). Briefly, the extracted DNA was amplified with primers specific for the whole DR region, while a biotin labeled reverse primer was used to obtain biotin labeled PCR products. Subsequently, the PCR products were perpendicularly hybridized on a membrane to lines of immobilized spacer-oligos that represent spacers of known sequence. Finally, the hybridization signals were visualized by autoradiography.

Strains that are similar or different can be distinguished by their spoligotype patterns, characterized by the number and identity of spacers (Van Soolingen *et al.*, 1995). The presences of the spacer sequences varies in different strains and are visualized by a spot on a fixed site of the hybridization membrane.

3.8. Data collection and analysis

During the study, individual animal ante-mortem examination findings will be recorded. Age categorization will be made using dental eruption and wear as described by Amstuz, (1998). Body condition scoring will be made using the method developed for Zebu cattle (Nicholson and Butterworth, 1986). The type and stage of TB lesion, the frequency of affection of anatomical sites will be also recorded for individual tuberculous suspected cattle.

The prevalence of tuberculosis was calculated as the number of cattle with tuberculosis lesion divided by total number of cattle slaughtered during the study. The chi-square test was applied for statistical evaluation of results (Matouskova *et al.*, 1992). A P value <0.05 were considered statistically significant.

4. RESULT

4.1. Abattoir prevalence of bovine tuberculousis.

The prevalence of BTB was 5.0 % (57/1138) on the basis of detailed post-mortem examination. There was no significance difference in prevalence between male and female ($P > 0.05$), as well as among the age groups ($P > 0.05$) (Table 6).

Table 6 Association of *Mycobacterium bovis* infection with various host risk factors

Variable	No of animal examined	Number	%	P-value
Sex				
Male	437	18	4.1%	P=0.329
Female	701	39	5.6%	
Age				
<2 year	74	2	2.7%	P=0.634
2-5 year	492	26	5.3%	
>5 year	572	29	5.1%	
Body conditior				
Lean	398	17	4. 3%	P=0.217
Medium	623	37	5. 9%	
Fat	117	3	2.6%	
Origin				
High land	731	32	4.4%	P=0.191
Low land	407	25	6.1%	

4.2. Pathology scoring

The distribution of lesions and the severity of the disease were established in the 57 post mortem positive cattle. Lung lesions were detected in sixteen, and forty five animals had at least one lesion in their lymph nodes. The lesions were more frequent and severe in the diaphragmatic lobes of both lungs than the apical lobes (Table 7 and Table 8). The mediastinal, bronchial and retropharyngeal lymph nodes were the most frequently and severely affected thoracic lymph nodes. Lesions were also found in the mesenteric lymph

nodes of 19 cattle. However, 2 of these cattle also had lesions in the lymph nodes of the thoracic cavity (Table 7, Table 8).

The severity of pathology of BTB is shown in (Table 9), the mediastinal lymph nodes constituted the most severely affected lymph node (mean pathology score ± standard error of the mean (SEM), 0.38 ± 0.95), followed by left bronchial (0.25 ± 0.76) and right bronchial (0.23 ± 0.73) lymph nodes.

Table 7 Severity and distribution of lesions of BTB pathology score in the lungs of cattle (n= 57)

Lung lobe	**Number of animal with TB lesions(%)**	**Frequency of pathological score**					
		0	1	2	3	4	Total score
Left apical	9(15.8%)	48	2		3	4	27
Left cardiac	11(19.3%)	46	0	2	5	4	33
Left diaphragmatic	15(26.3%)	42	2	1	7	5	45
Right apical	10(17.4%)	47	2	1	4	3	28
Right cardiac	11(19.3%)	46	3	2	2	4	29
Right diaphragmatic	15(26.3%)	42	1	5	5	4	42
Right acessory	11(19.3%)	46	1	3	4	3	31

Table 8 Severity and distribution of lesions of BTB pathology score in the lymph nodes of cattle (n= 57)

Site	**Number of animal with TB lesions(%)**	**Frequency of pathological score**				
		0	1	2	3	Total score
Mandibular	4(7.0%)	53			4	12
Retropharyngeal	16(28.1%)	41	2	3	11	41
Mediastinal	23(40.4%)	34	2	6	15	59
Left bronchial	18(31.6%)	39	4	5	9	41
Right bronchial	15(26.3%)	42	2	6	7	35
Hepatic	2(3.5)	55	1		1	4
Mesenteric	19(33.3)	38	3	6	10	45

Table 9 Mean pathology scores of the lymph nodes and lung of cattle (n= 57)

Lung		Lymph nodes	
Site	Mean ±SEM	Site	Mean ±SEM
Left apical	0.47 ±0.16	Mandibular	0.21±0.10
Left cardiac	0.61 ±0.17	Retropharyngeal	0.72±0.16
Left diaphragmatic	0.79±0.19	Mediastinal	1.04±0.18
Right apical	0.49±0.16	Right bronchial	0.67±0.15
Right cardiac	0.51±0.16	Left bronchial	0.67±0.15
Right diaphragmatic	0.74±0.18	Hepatic	0.07±0.06
Right accessory	0.54±0.16	Mesenteric	0.79±0.16

4.3. Bacteriology

Mycobacteria were able to be isolated from forty nine percent (28/57) of the animals with visible TB lesion of at least one lymph node or other tissue sample. The highest proportion of culture positivity (21.1%) was observed in mediastinal lymph nodes, while the lowest percentage was observed in the hepatic (3.5%) and mandibular (1.8%) lymph nodes (Fig. 3).

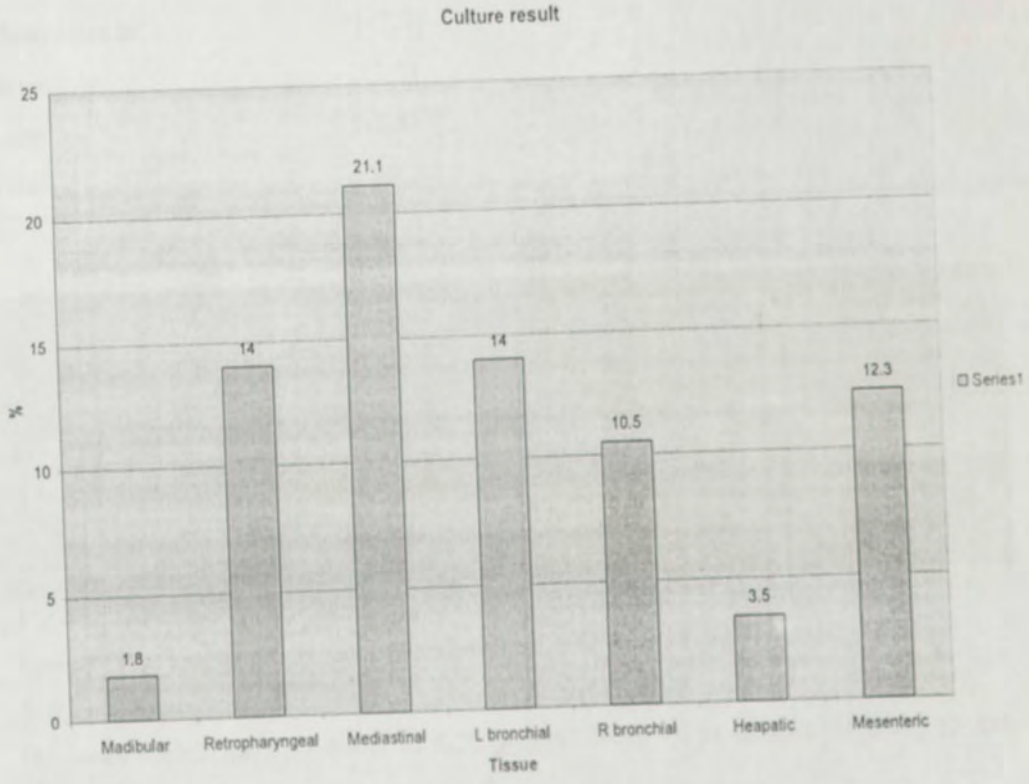


Fig 4 Rate of culture positivity of 57 animals with gross lesion

4.4. Regions of difference-bases polymerase chain reaction

This study employed region of difference-bases PCR typing for identification of species of mycobacteria isolated from animal tissue samples and found the technique to be of value in identification of MTC isolates to species level. A total of 49 isolates were recovered from the different tissues of 28 culture positive cattle, and 20 isolates showed signal for *M. tuberculousis* complex species of which 19 were *M. bovis* while one isolate was *M. tuberculousis* (Table 10) (Fig. 4).

Table 10 Culture and PCR result from tissue samples

Examined samples	Culture Positive	PCR	
	(%)	*M .bovis*	*M. tuberculousis*
Mandibular ln	1(1.8)	0	0
Retropharyngeal ln	8(14)	3	1
Mediastinal ln	12(21.1)	8	0
Left bronchial ln	8(14)	5	0
Right bronchial ln	6(10.5)	1	0
Mesenteric ln	7(12.3)	0	0
Hepatic ln	2(3.5)	2	0
Lung	5(8.8)	0	0
Total	49	19	1

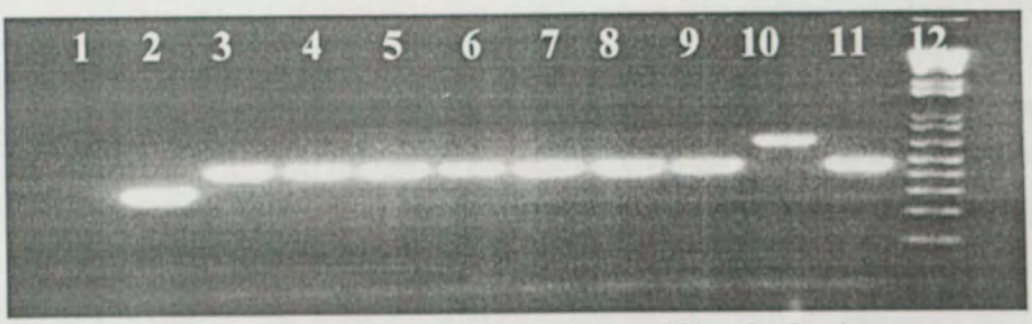
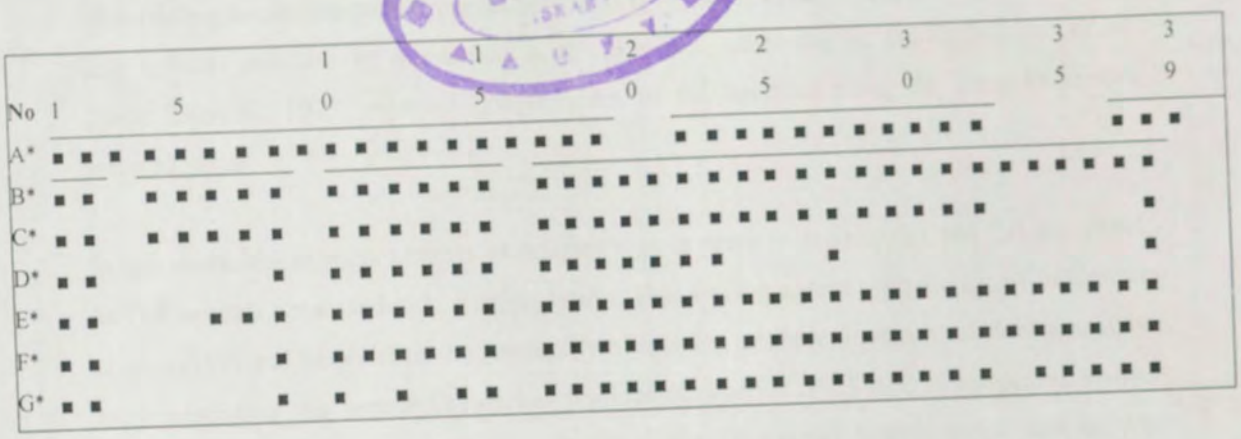


Fig 4 Picture showing some of the region of difference-base PCR typing Result.

Lanes 1: Non-template 2: *M. tuberculousis* H37Rv 3: *M. bovis*, 4: 9,23MDR/P (*M. bovis)* 5: 9,30BL/P (*M. bovis)* 6: 9,30MDR/P (*M. bovis)* 7: 9,64MDR/P (*M. bovis)* 8:10,29MDR/P (*M. bovis)* 9: 13,27BR/P (*M. bovis)* 10: 6,20HP/G (*M. bovis)* 11: 18,46BL/G (*M. bovis)* 12: 1kb DNA marker ladder

4.5. Spoligotyping patterns

Further characterization of the strains of *M. bovis* using spoligotyping indicated the isolates had five different cluster of spoligotyping pattern, which includes Ethiopian *M. bovis* strain 1(EMbs1), SB1176, SB0134, SB0133 and new strain. The binary number representation of the new strain is 11000001010101101111111111111111101111100000, where 1 indicates the presence of a spacer and 0 indicates a loss. Spacers 3, 9, 16 and 39 to 43, which were absent from *M. bovis* strains, were absent from all the isolates. In addition did not have spaces 4 -7, 11, 13, and 33. Thus the slaughtered cattle appeared to be infected with different strain of *M. bovis* (Fig.5).



*A= H37RV, B= BCG, C=ETHbov1, D= SB1176, E= SB0134, F= SB0133 G= New (9, 64 MDR/G)

Fig.5 Spoligotyping patterns of the 20 isolates obtained from 28 culture positive cattle examined at Kombolcha ELFORA meat processing plant. The isolates were found to be grouped in to five clusters of strains *M. bovis*, namely: ETHbov1 SB1176, SB0134, SB133, and New strain. The new strain is unique and no strain with a similar spoligotype pattern has been reported to the *M .bovis* spoligotype data base so far and did not have spacers 3-7, 9, 11, 13, 16, 33.

5. DISCUSSION

In this study, detailed laboratory examination revealed 5% tuberculous animals. This was higher than previous findings where lower prevalence has been reported (Nsengawa and Otaru, 1987; Radostits *et al.*, 2000; Asseged *et al.*, 2004). On the contrary, the finding was similar with a report of Ameni and Wudie, (2003), where the prevalence rate was 5.2%.

In the present study, gross tuberculousis lesions were found most frequently in lymph nodes of the thoracic cavity; (40.4%) mediastinal, (31.6%) left bronchial and (26.3%6) right bronchial, than in lymph nodes of the head; (28.1%) retropharyngeal and mandibular (7%). This finding is consistent with previous reports (Pritchard, 1998; Whipple *et al.*, 1996), and may indicate infection by the aerosol route. However, other studies (Miliano-Suazo *et al.*, 2000; Solomon, 1975) reported lymph nodes of the head as being the most frequently infected tissue.

In this study, the average number of infected tissues per infected carcass was 2.0 and about half of animals possessed only a single lesion. This finding agrees with the report of Corner *et al.*, (1990) and emphasizes the possibility of missing a tuberculous carcass during routine meat inspection. As opposed to earlier findings (Corner *et al.*, 1990; Woldesenbet *et al.*, 2002), tuberculous lesions were not found in the sublingual lymph nodes and in the mammary gland. This may be the result of a lower level of generalised tuberculousis in this environment compared to other environments.

The absence of significant differences in infection rates among age groups and between sexes was in consistent with previous reports (Asseged *et al.*, 2004; Woldesenbet *et al.*, 2002). This indicates the presence of other factors that may play a significant role in the spread of tuberculousis in this environment. Possible risk factors include management (as related to confinement), herd size and location, relation to proximity to already infected farms (Asseged *et al.*, 2000). Other factors, such as nutrition, also influence the susceptibility of cattle to BTB. In a case control study Griffin *et al.*, (1993) found an association between recurrent herd outbreaks of BTB and the presence of rough grazing, which suggested that nutritional deficiencies lead to reduced resistance to BTB. Doherty *et al.*, (1995) demonstrated significantly lower numbers of lymphocyte subpopulations in nutritionally deficient cattle. Susceptibility to *M. bovis* infection may also be enhanced in cattle

persistently infected with immunosuppressive viruses, such as bovine viral diarrhea virus or bovine immunodeficiency virus (Menzies *et al.*, 2000). However, a higher proportion of females were infected, possibly due to their longer productive life and other stressful factors (such as pregnancy, parturition, lactation, etc.) associated with female animals (Miliano-Suazo *et al*., 2000; Radostits *et al*., 2000).

The frequency and severity of the lesions were higher in the thoracic lymph nodes than the mesenteric lymph nodes. This finding agrees with results of previous studies, where greater than 90% occurrence of TB lesions in the respiratory system was reported in developed countries (Collins, 1996; Corner, 1994; Francis, 1972; Lepper *et al.*, 1973; McIlroy *et al.*, 1986; Neill *et al.*, 1994; Whipple *et al.*, 1996). In another study conducted on 2,886 cattle, 57% had lesions in the thoracic cavity, while only 3% had lesions solely in the mesenteric lymph nodes (Neill *et al.*, 1994). Therefore, respiratory excretion and inhalation of *M. bovis* are considered the main route by which animal-to-animal transmission occurs (Francis, 1972; Smyth *et al.*, 2001). However, in a very recent study Ameni *et al.*, (2007) demonstrated that shedding of *M. bovis* in the feces and ingestion of the bacilli with contaminated pasturage and/or water may be the main route of transmission in cattle kept on pasture, as the mesenteric lymph node was found to be the main lesion containing site. In contrary to this, in developed countries, shedding of *M. bovis* in urine and feces is considered to be an insignificant feature of disease transmission in cattle (Hardie *et al.*, 1992; Morris *et al.*, 1994).

In the present study, the chance of growing mycobacteria was less than 50% which could be due to either losses of the agent during freezing or delayed transportation from the site of collection. WHO, (1998) indicated losses of 5-10% due to contamination resulting from prolonged preservation and a loss of up to 60% due to decontamination procedure. Therefore, the use of proper time in culturing and application of standard laboratory technique could increase the chance of recovery of acid fast bacilli.

Identification of mycobacteria to species level is crucial since it provides a great deal of useful information on epidemiology and facilitates successful treatment of patients. The molecular techniques offer a rapid, reliable, specific and cost effective means of identification of mycobacterial species. In this study 20 isolates showed signal for *M. tuberculosis* complex species of which 19 were *M. bovis* while one isolate was *M.*

tuberculousis. Further characterization of the strains of *M. bovis* using spoligotyping indicated that the spoligotype patterns of all the isolates were not identical, suggesting that the animals in the area were infected with more than one strain of *M. bovis* some with the different spoligotype pattern from previously reported patterns either form Africa (Cadmus *et al.*, 1996; Njanpop-Lafourcade *et al.*, 2001), or from other parts of the world (Aranaz *et al.*, 1996; Goyal *et al.*, 1997; de La Salmoniere *et al.*, 1997). The predominance of a single *M. bovis* strain in a local area has been noted before; indeed, in the UK there is a distinct geographic localization of *M. bovis* molecular types (Smith *et al.*, 2003). To investigate the origin of strains, additional studies will be needed to survey the molecular types of *M. bovis* with a wider range including wildlife reservoirs.

6. CONCLUSION AND RECOMMENDATIONS

In Ethiopia, the endemic nature of infection due to *M. bovis* has long been reported. Moreover, recent studies showed that *M. bovis* was found to be the cause of cervical lymphadenitis in humans indicating the significance of BTB between human and cattle. The prevalence of BTB recorded in this study area was relatively higher than the previous report in the slaughter houses. The most frequent site to find tuberculous lesion are lymph nodes of the thoracic region and lung indicating the importance of respiratory routes as the main routes of transmission. Molecular study revealed the existence of different clusters of strains of *M. bovis* which gives clue for further investigation. The findings of this study indicated that tuberculous lesions were common in cattle slaughtered at Kombolcha ELFORA meat processing plant, and hence detailed post mortem meat inspection procedures are required to minimize the risk of its transmission to the public through meat consumption. In addition, appropriate cooking of meat is required before consumption.

Therefore based on the above conclusion, the following points are recommended:

- Abattoir inspection services should focus on lymph nodes of the thoracic region and head. On top of that detailed post mortem meat inspection procedures are required to minimize the risk of its transmission to the public through meat consumption.
- Further molecular typing of strains of *M. bovis* from the different regions of Ethiopia, which could be useful for mapping its molecular epidemiology in the country.
- Public education and creation of awareness in the society not to consume raw meat and other animal products should be encouraged.
- Integrating research and disease surveillance programs in-between the medical and veterinary institutions, as it helps and simplifies the designing of feasible control program against BTB infection and to minimize zoonotic threat of tuberculosis.

7. REFERECE

Alemayehu, M. (2003): Country Pasture/Forage Resource Profiles. 1st Edition, Suttie, J. M. and Reynolds, S. G., Ethiopia, Pp 1–36.

Alemu, T. (1992): Bovine tuberculosis in Ethiopia. MSc. Thesis, University of Edinburgh of Tropical animal health and production, United Kingdom.

Ameni, G., Aseffa, A., Engers, H., Young, D., Gordon, G., Hewinson, G. and Vordermeier, M. (2007): High prevalence and increased severity of pathology of BTB in Holsteins compared to Zebu breeds under field cattle husbandry in central Ethiopia. *Clin. Vacc. Immunol.*, **14**:1356-1361.

Ameni, G., Aseffa, A., Engers, H., Young, D., Hewinson, G. and Vordermeier, M. (2006): Cattle husbandry in Ethiopia is a predominant factor for affecting the pathology of BTB and gamma interferon responses to mycobacterial antigens. *Clin. Vacc. Immunol.*, **13**: 1030–1036.

Ameni, G. and Wudie, A. (2003): Preliminary study on BTB in Nazareth municipality abattoir of central Ethiopia. *Bull. Anim. Hlth. Prod. Afr.*, **51**: 125–132.

Ameni, G., Amenu, K. and Tibbo, M. (2003a): Bovine tuberculosis: Prevalence and risk factors assessment in cattle and cattle owners in Wuchale-Jida district, Central Ethiopia. *Int. J. Appl. Res. Vet. Med.*, **1**:17–25.

Ameni, G., Bonner, P. and Tibbo, M. (2003b): Across-sectional study of BTB in selected dairy farms in Ethiopia. *Int. J. Appl. Res. Vet. Med.*, **1**: 85–97.

Ameni, G., Regassa, A., Kassa, T. and Medhin, G. (2001): Survey on BTB and its public implication to cattle raising families in Wolaita-sodo, Southern Ethiopia. *Eth. J. Anim. Prod.*, **32**: 53.

Ameni, G. and Roger, F. (1998): Study on the epidemiology of BTB in dairy farms Debre-Zeit and Ziway, Ethiopia. In: Proceeding of the Twelve Conferences of Ethiopian Veterinary Association, Addis Ababa, Ethiopia, Pp 13–19.

Ameni, G. (1996): Bovine tuberculosis: Evaluation of diagnostic tests, prevalence and zoonotic importance. DVM thesis, Faculty of Veterinary Medicine, Addis Ababa University, Debre Zeit, Ethiopia.

Amstutz, H. E. (1998): Dental development. 8th Edition, Merck Veterinary Manual, Philadelphia, Pp 131-132.

Anonymous (2004): Manual of standards for diagnostic tests and vaccines for terrestrial animals. OIE, Paris, France.

Anonymous (2000): Manual of standards for diagnostics and vaccines. OIE, Paris, France.

Anonymous (1999): Dairy development enterprise. Annual report, Addis Ababa, Ethiopia.

Aranaza, A., Liebana, E., Mateos, A., Dominguez, L., Vidal, D., Domingo, M., Gonzolez, O., Rodriguez-Ferri, E. F., Bunschoten, A. E., Van Embden, J. D. and Cousins, D. (1996): Spacer oligonucleotide typing of *M. bovis* strains from cattle and other animals: A tool for studying epidemiology of tuberculousis. *J. Clin. Microbiol.*, **34**: 2734-2740.

Ashford, D. A., Whitnely, E., Raghunathan, P. and Cosivi, O. (2001): Epidemiology of selected mycobacteria that infect humans and other animals. *Rev. Sci. Tech. Off. Int. Epizoot.*, **20**:325-337.

Asseged, B., Woldesenbet, Z., Yimer, E. and Lemma, E. (2004): Evaluation of abattoir inspection for the diagnosis of *M. bovis* infection in cattle in Addis Ababa abattoir. *Trop. Anim. Hlth. Prod.*,**36** : 537-546.

Asseged, B., Lubke-Beker, A., Lemma, E., Kiros, T. and Britton, S. (2001): Bovine tuberculosis: A cross sectional and epidemiological study in and around Addis Ababa. *Bull. Anim. Hlth. Prod. Afr.*, **48**: 71–80.

Asseged, B., Lübke-Becker, A., Lemma, E., Taddele, K. and Britton, S. (2000): Bovine tuberculosis: A cross-sectional and epidemiological study in and around Addis Ababa. *Bull. Anim. Hlth. Prod. Afr.*, **48**: 71-80.

Ayele, W. Y., Neill, S. D., Zinsstag, J., Weiss, M. G. and Pavlik, I. (2004): Bovine tuberculosis: An old disease but a new threat to Africa. *Int. J. Tuberc. L. Dis.*, **8**: 924–937.

Barwinek, F. and Taylor, N. M. (1996): Assessment of socio-economic importance BTB in Turkey and possible strategies for control or eradication. Ankara: Turkish-German Health Information Project, General Directorate of Protection and Control, Pp 3–45.

Bhatia, R. and Ichpujanti, R. (1994): Mycobacterium In: essential of medical microbiology 1st edition, New-Delhi, Pp 285-292.

Brunello, F., Ligozzi, M., Cristelli, E., Bonora, S., Tortoli, E. and Fontana, R. (2001): Identification of 54 mycobacterial species by PCR-restriction frgment length polymorphism analysis of the *hsp*65 gene. *J. Clin. Microbiol.*, **39**: 2799-2806.

Cadmus, S. I. B., Atsanda, N. N., Oni, S. O. and Akang, E. E. U. (2006): Bovine tuberculousis in one cattle herd in Ibadan in Nigeria. *Czch. Vet. Med.*, **49**: 2602-2606.

Carter, G. R. (1986): The *Mycobacterium* In: essential of veterinary bacteriology and mycology. 3rd edition, Philadelphia, lea and febiger, Pp 185-193.

Carter, G. R. and Chengappa, M. A. (1991): The mycobacteria In: essential of veterinary bacteriology and mycology, 4th edition, Philadelphia, Lea and febiger, Pp 202-216.

Clifton-Hadley, R. S., Inwald, J., Hughes, S., Palmer, N., Sayers, A. R., Sweeney, K., Van Embden, J. D. A. and Hewinson, R. G. (1998): Recent advances in DNA fingerprinting using spoligotyping: Epidemiological applications in BTB. *J. Br. Catt. Vet. Asso.*, **6**:79-82.

Collins, J. D. (1996): Factors relevant to *M. bovis* eradication. *Irsh. Vet. J.*, **49**:241-243.

Collins, F. M. (1994): The immune response to mycobacterial infection: Development of new vaccine. *Vet. Microbiol.*,**40** : 95–110.

Collins, D. M. and Lisle, G. W. (1984): DNA restriction endonuclease analysis of *M. tuberculosis* and *M. bovis* BCG. *J. Gen. Microbiol.*, **130**: 1019–1021.

Corner, L. A. (1994): Postmortem diagnosis of *M. bovis* infection in cattle. *Vet. Microbio.*, **40**: 53-63.

Corner, L. A., Melville, L., McCubbin, K., Small, K. J., Mc-Cormick, B. S., Wood, P. R. and Rothel J.S. (1990): Efficiency of inspection procedures for the detection of tuberculous lesions in cattle. *Aust. Vet. J.*, **67**: 389–392.

Cosivi, O., Grange, J. M., Daborn, C. J., Raviglione, M. C., Fujikura, T. and Cousins, D. (1998): Zoonotic tuberculosis due to *M. bovis* in developing countries. *Emerg. Infect. Dis.*, **4**: 59–70.

Cousins, D. V., Bernardelli, A., Bastida, R., Cataldi, A., Quse, V., Dow, S., Redrobe, S., Duignan, P., Murray, A., Dupont, C., Ahmed, N., Collins, D. M., Ray-Butle, W., Dawson, D., Rodr´- guez, D., Loureiro, J., Romano, M. I., Alito, A., Zuma´rraga, M. and Bernardelli, A. (2003): Tuberculosis in seals caused by a novel member of the *M. tuberculosis* complex: *Mycobacterium pinnipedii*. *Nov. Int. J. Sys. Bacteriol.*, **53**: 1305–1314.

Cousins, D. V., Wilton, S. D. and Francis, B. R. (1991): Use of DNA amplification for the rapid identification of *M. bovis*. *Vet. Microbiol.*, **27:** 187–195.

Cousins, D., Williams, S., Liebana, E., Aranaz, A., Bunschoten, A., Van Embden, J. and Ellis, T. (1998): Evaluation of four DNA typing techniques in epidemiological investigations of BTB. *J. Clin. Microbiol.*, **36**:168-178.

Daborn C. J., Grange J. M. and Kazwala R. R. (1996): *Bovine tuberculosis* cycle: An African prospective. *J. Appl. Bacteriol.*, **81**: 275–325.

De La Salmoniere, Y. G., Torrea, G., Bunschoten, A., Van Embden, J. and Gicquel, B. (1997): Evaluation of spoligotyping in a study of the transmission of *M. tuberculousis*. *J. Clin. Micorobiol.*, **35**: 2210-2214.

De Kantor, I. N., Nader, A., Bernardelli, A., Giron, D. O. and Man, E. (1987): Tuberculosis infection in cattle not detected by slaughterhouse inspection. *J. Vet. Med.*, **34**: 202-205.

Doherty, M. L., Monaghan, M. L., Bassett, H. F., Quinn, P. J. and Davis. W. C. (1995): Effect of dietary restriction on cell-mediated immune responses in cattle infected with *M. bovis*. *Vet. Immunol. Immunopathol.*, **49**:307-320.

Durr, P. A., Hewinson, R. G. and Clifton-hadley, R. S. (2000): Molecular epidemiology of BTB In: *Mycobacterium bovis* genotyping. *Rev. Sci. Et. Tech. Off. Int. Epizoot.*, **19**: 675-688.

Eisenach, K. D., Cave, M. D., Bates, J. H. and Crawford, J. T. (1990): Polymerase chain reaction amplification of a repetitive DNA sequence specific for *M. tuberculosis*. *J. Inf. Dis.*, **161**: 977–981.

Eisenach, K. D., Crawford, J. T. and Bates, J. H. (1986): Genetic relatedness among strains of the *M. tuberculousis* complex analysis of restriction fragment heterogeneity using cloned DNA probes. *Am. Rev. Res. Dis.*, **133**: 1065-1068.

FAO (1972): Report to the Government of Ethiopia: Food and Agriculture Organization veterinary service and disease control. *Anim. Hlth. Ser.*, Rome, Italy.

FAO (1967): Report to the Government of Ethiopia: Food and Agriculture Organization, Veterinary Service and Disease Control. *Anim. Hlth Ser.*, Rome, Italy.

Ferdinand, S., Valetudie, G., Sola, C. and Rastogi, N. (2004): Data mining of *M. tuberculosis* complex genotyping results using mycobacterial interspersed repetitive units validates the clonal structure of spoligotyping-defined families. *Res. Microbiol.*, **155**:647-654.

Francis, J. (1972): Route of infection in tuberculosis. *Aust. Vet. J.*, **48**:578.

Francis, J. (1958): Tuberculosis in animals and man. Cassell and Co., London, United Kingdom.

Frothingham, R., Hills, H. G. and Wilson, K. H. (1994): Extensive DNA sequence conservation throughout the *M. tuberculousis* complex. *J. Clin. Micorbiol.*, **32**: 1639-1643.

Frothingham, R., Strickland, P. L. Bretzel, G., Ramaswamy, S., Musser, J. M. and Williams, D. L. (1999): Phenotypic and genotypic characterization of *M. africanum* isolates from West Africa. *J. Clin. Microbiol.*, **37**:1921-1926.

Gemta, M. B. (2000): A cross-sectional study on BTB in small holder dairy farms and implication in man in Fitche town, North Shewa. DVM Thesis, Faculty of Veterinary Medicine, Addis Ababa University, Debre-Zeit, Ethiopia.

Gezahegne, L. (1991): Economical aspect of condemned organs and parts due to cystycercosis, hydatidosis, fasciolosis and tuberculousis. Analysis report MoA, Addis Ababa, Ethiopia.

Goh, K. S. Legrand, E., Sola, C. and Rastogi, N. (2001): Rapid differentiation of '*M. canettii*' from other *M. tuberculousis* complex organisms by PCR –restriction analysis of the *hsp*65 gene. *J. Clin. Microbiol.*, **39**: 3705-3708.

Goyal, M., Saunders, N. A., Van Embden, J. D. A., Young, D. B. and Shaw, R. J. (1997): Differentiation of *M. tuberculousis* isolates by spoligotyping and IS6110 restriction fragment length polymorphism. *J. Clin. Microbiol.*, **35**: 647-651.

Gracy, J. F. (1986): Meat hygiene, 8th Edition, Bailliere Tindall, Eastbourne, Pp 350-360.

Groenen, P. M., Van Bunschoten, A. E., Van Soolingen, D. and Van Embden, J. D. (1993): Nature of DNA polymorphism in the direct repeat cluster of *M. tuberculosis*, application for strain differentiation by a novel method. *Molec. Microbiol.*, **10**: 1057–1065.

Griffin, J. M., Hahsey, T., Lynch, K., Salman, M. D., McCarthy, J. and Hurley, T. (1993): The association of cattle husbandry practices, environmental factors and farmers characteristics with the occurrence of chronic BTB in dairy cattle in Republic of Ireland. *Prev. Vet. Med.*, **17**:145-160.

Guerrero, A., Cobo, J., Fortun, J., Navas, E., Quereda, C., Asensio, A., Canon, J., Blazquez, J. and GomezMampaso. E. (1997): Nosocomial transmission of *M. bovis* resistant to 11 drugs in people with advanced HIV-1 infection. *Prev. Vet. Med.*, **350**:1738-1742.

Gutierrez, M., Samper, S., Jimenez, M. S., van Embden, J. D., Marin, J. F. and Martin, C. (1997): Identification by spoligotyping of a caprine genotype in *M. bovis* strains causing human tuberculousis. *J. Clin. Microbiol.*, **35**:3328-3330.

Haas, G. W., Bretzel, G., Amthor, B., Schilke, K., Krommes, G., Rusch-Gerdes, Sticht-Groh, V. and Bremer, H. J. (1997a): Comparison of DAN finger print patterns of isolates of *M. africanum* from East and West Africa. *J. Clin. Microbiol.*, **35**: 663-666.

Haas, G. W., Schilke, Brand, J., Amthor, B., Weyer, K., Fourie, G., Bretzel, B., Sticht-Groh, V. and Bremer, H. J. (1997b): Molecular analysis of *kat*G gene mutations in strains of *M. tuberculousis* complex from Africa. *Antimicrob. Agnt. Chemother.*, **41**: 1601-1603.

Haddad, N., Ostyn, A., Karoui, C., Masselot, M., Thorel, M. F., Hughes, S. L., Inwald, J., Hewinson, R. G. and Durand. B. (2001): Spoligotype diversity of *M. bovis* strains isolated in France from 1979 to 2000. *J. Clin. Microbiol.*, **39**:3623-3632.

Hailemariam, S. (1975): A brief analysis of activities of meat inspection and quarantine division. Department of Veterinary Service, MoA, Addis Ababa, Ethiopia.

Hardie, R. M. and Watson, J. M. (1992): *Mycobacterium bovis* in England and Wales. Past, present and future. *Epidemiol. Infect.*, **109**:23-33.

Hermans, P. W., Van Soolingen, D., Bik, E. M., De Haas, P. E., Dale, J. W. and Van Embden, J. D. (1991): Insertion element (IS)987 from *M. bovis* BCG isolated in a hot-spot integration region for insertion elements in *M. tuberculosis* complex strains. *Inf. Immuno.*, **59**: 2695–2705.

Hermans, P. W., Van Soolingen, D., Dale, J. W., Schuitma, A. R., McAdam, R., Catty, D. and Van Embden, J. D. (1990): Insertion element (IS)986 from *M. tuberculosis*. A useful tool for diagnosis and epidemiology of tuberculosis. *J. Clin. Microbiol.*, **28**: 2051–2058.

ILRI/ FAO, (1995): Livestock development strategies for low income countries. Proceedings of the joint ILRI/FAO round table on livestock development strategies for low income countries, Addis Ababa, Ethiopia.

Kazwala, R. R., Kusiluka, L. J., Sinclair, K., Sharp, J. M. and Daborn. C. J. (2005): The molecular epidemiology of *M. bovis* infections in Tanzania. *Vet. Microbiol.*, **1**: 1243-1245.

Kazwala, K. D., Sinclaire, J., Challans, D. M., Kambarage, J. M., Sharp, J., Van Embden, J. D. A, Daborn, C. J. and Nyange, J. (1997): Zoonotic importance of *M. tuberculosis* complex organisms in Tanzania: a molecular biology approach. Editions, Rabat, Morocco., Pp 199-204.

Kremer, K., Van Soolingen, D., Frothingham, R., Haas, W. H., Hermans, P. W. M., Martin, C., Palittapongarnpim, P., Plikaytis, B. B., Riley, L. W., Yakrus, M. A., Musser, J. M. and Van Embden, J. D. A. (1999): Comparison of methods based on different molecular epidemiological markers for typing of *M. tuberculosis* complex strains. Inter-laboratory study of discriminatory power and reproducibility. *J. Clin. Microbiol.*, **37**: 2607–2618.

Kamerbeek, J., Schouls, L., Kolk, A., Van Agterveld, M., Van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M. and Van Embden, J. (1997):

Simultaneous detection and strain differentiation of *M. tuberculosis* for diagnosis and epidemiology. *J. Clin. Microbiol.*, **35**: 907–914.

Kiros, T. (1998): Epidemiology and Zoonotic importance of BTB in selected sites of Eastern Shoa, Ethiopia. MSc. Thesis, Faculty of Veterinary Medicine, Addis Ababa University and Freie University of Berlin.

Kidane, D., Olobo, J. O., Habte, A., Negesse, Y., Aseffs, A., Abate, G., Yassin, M. A., Betreda, K. and Harboe, M. (2002): Identification of the causative organism of tuberculosis lymphadenitis in Ethiopia by PCR. *J. Clin. Microbiol.*, **40**: 4230–4234.

Kochi, A. (1994): Tuberculosis: Distribution, risk factors, mortality. *Immunobiol.*, **191**: 325–336.

Lepper, A. W. D. and Pearson, C. W. (1973): Tuberculosis of beef cattle. *Aust. Vet. J.*, **49**: 266-267.

Mambule, M. (1984): Bovine tuberculosis in Uganda. *Bull. anim. Hlth. Prod. Afr.*, **32**: 159-166.

Maguire, H., Dale, J. W., McHugh, T. D., Butcher, P. D., Gillespie, S. H., Costetsos, A., Al-Ghusein, H., Holland, R., Dickens, A., Marston, L., Wilson, P., Pitman, R., Strachan, D., Drobniewski, F. A. and Banerjee, D. K. (2002): Molecular epidemiology of tuberculosis in London 1995 to 1997 demonstrating low rate of active transmission, **57**: 617–622.

McAdam, R. A., Hermans, P. W., Van Soolingen, D., Zainuddin, Z. F., Catty, D., Van Embden, J. D. and Dale, J. W. (1990): Characterization of a *M. tuberculosis* sequence belonging to the IS3 family. *Molec. Microbiol.*, **4**: 1607–1613.

McHugh, T. D. and Gillespie, S. H. (1998): Nonrandom association of IS6110 and *M. tuberculosis*: Implications for molecular epidemiological studies. *J. Clin. Microbiol.*, **36:** 1410–1413.

McHugh, T. D., Newport, L. E. and Gillespie, S. H. (1997): IS6110 homologs are present in multiple copies in mycobacteria other than tuberculosis. *J. Clin. Microbiol.*, **35**:1769–1771.

McIlroy, S. G., Neill, S. D. and McCracken, R. M. (1986): Pulmonary lesions and *M. bovis* excretion from the respiratory tract of tuberculin reacting cattle. *Vet. Rec.*, **118:** 718-721.

Menzies, F. D. and Neill, S. D. (2000): Cattle-to-cattle transmission of BTB. *Vet. J.*, **160**: 92-106.

Miliano-Suazo, F., Salmar, M. D., Ramirez, C., Payeur, J. B., Rhyan, J. C. and Santillan, M. (2000): Identification of tuberculousis in cattle slaughtered in Mexico. *Am. J. Vet. Res.*, **61:** 86-89.

MoA (1984): Livestock Sector Review. MoA, Addis Ababa, Ethiopia.

MoA (1973): Meat inspection and quarantine service. Department of Veterinary Service, MoA, Addis Ababa, Ethiopia.

Morris, R. S., Pfeifter, D. U. and Jackson, R. (1994): The epidemiology of *Mycobacterium* infection. *Vet. Microbial.*, **40**: 153-177.

Matouskova, O., Chalupa, J., Cigler, M. and Hruska, K. (1992): SPSS– Manual. 1st Edition, *Vet. Res. Inst.*, Brno, Pp 168.

Neill, S. D., Bryson, D. G. and Pollock, J. M. (2001): Pathogenesis of tuberculosis in cattle. Tuberculousis, **81**: 79–86.

Neill, S. D., Pollock, J. M., Bryson, D. B. and Hanna, J. (1994): Pathogenesis of *M. bovis* in cattle. *Vet. Microbiol.*, **40**: 41-52.

Neill, S. D., Hanna, J., Mackie, D. P. and Bryson, T. G. D. (1992): Isolation of *M. bovis* from the respiratory tract of skin test negative cattle. *Vet. Rec.*, **131:** 45-47.

Njanpop-Lafourcade, B. M., Inwald, J., Ostyn, A., Durand, B., Hughes, S., Thorel, M. F., Hewinson, G. and Haddad. N. (2001): Molecular typing of *M. bovis* isolates from Cameroon. *J. Clin. Microbiol.*, **39**: 222-227.

Nicholson, M. J. and Butterworth, M. H. (1986): A guide to condition scoring in zebu cattle. International Livestock Centre for Africa, Addis Ababa, Ethiopia, Pp 1-29

Niemann, S., Harmsen, D., Rusch-Gerdes, S. and Richter, E. (2000): Differentiation of clinical *M. tuberculousis* complex isolates by *gyr*B DNA sequence polymorphism analysis. *J. Clin. Microbiol.*, **38**: 2331-2334.

Nsengawa, G. R. and Otaru, M. M. (1987): Prevalence of TB in the dairy farms in southern Tanzania. *Bull. Anim. Hlth. Prod. Afr.*,**35** : 251–252.

O'Reilly, L. M. and Daborn, C. J. (1995): The epidemiology of *M. bovis* infections in animals and man: a review. *Tuberc. L. Dis.*, **76**: 1–46.

Palmer, M. V., Whipple, D. L., Rhyan, J. C., Bolin, C. A. and Saari, D. A. (1999): Granuloma development in cattle after intra tonsillar inoculation with *M. bovis*. *Am. J. Vet. Res.*, **60**: 310–315.

Patel, S., Wale, S. and Saunders, N. (1996): Hemi-nested inverse PCR for IS6110 fingerprinting of *M. tuberculosis* strains. *J. Clin. Microbiol.*, **34**: 1686–1690.

Pollock, J. M. and Neill S. D. (2002): *Mycobacterium bovis* infection and tuberculosis in cattle. *Vet. J.*, **163**:115-127.

Pollock, J. M., Rodgers, J. D., Welsh, M. D. and McNair, J. (2006): Pathogenesis of BTB: The role of experimental models of infection. *Vet. Microbiol.*, **112**: 141-15.

Pritchard, D. G. (1988): A century of BTB 1888-1988: conquest and controversy. *J. Comp. Pathol.*, 99: 356-399.

Quinn, P. J., Carter, M. E. and Markey, B. K. (1999): An imprint of Harcourt Publishers. *Clin. Vet. Microbiol.*, Pp 156

Quinn, P. J., Carter, M. E., Markey, B. and Carter, C. P. (1994): *Mycobacterium* species. In: Mosby-Year Book, London, Europe. Pp 156-169.

Radostits, O. M., Gay, C. C., Blood, D. C. and Hinchcliff, K. W. (2000): Diseases caused by *Mycobacterium* species. In: Veterinary Medicine: a textbook of the diseases of cattle, sheep, pigs, goats and horses, 9th Edition, Harcourt Publishers Ltd, London, Pp 909-919.

Redi, N. (2003): Prevalence of BTB and zoonotic implication in Asela Town, South East Ethiopia. DVM Thesis, Faculty of Veterinary Medicine, Addis Ababa University, Debre-Zeit, Ethiopia.

Regassa, A. (2005): Study on *M. bovis* in animals and human in and around Fiche, North Shewa zone, Ethiopia. MSc. Thesis, Faculty of Veterinary Medicine, Addis Ababa University, Debre-Zeit, Ethiopia.

Regassa, F. (2001): Herd prevalence of CBPP and BTB and Dictyocaulosis in Bodji Wereda in West Wellega. DVM Thesis, Faculty of Veterinary Medicine, Addis Ababa University, Debre-Zeit, Ethiopia.

Regassa, A. (1999): Preliminary study on BTB in Wolaita-Sodo, South Ethiopia. DVM Thesis, Faculty of Veterinary Medicine, Addis Ababa University, Debre-Zeit, Ethiopia.

Rodrigues, L. C. and Smith, P. G. (1990): Tuberculosis in developing countries and methods for its control. Transactions of the Royal Society of Tropical Medicine and Hygiene, **84**: 739–744.

Serraino, A., Marchetti, G., Sanguinetti, V., Rossi, M. C., Zanoni, R. G., Catozzi, L. Bandera, A., Dini, W., Mignone, W., Franzetti, F. and Gori, A. (1999): Monitoring of transmission of tuberculousis between wild boars and cattle. Genotypical analysis of strains by molecular epidemiology techniques. *J. Clin. Microbiol.*, **37**: 2766-2771.

Shitaye, J. E., Getahun, B., Alemayehu, T., Skoric, M., Treml, F., Fictum, P., Vrbas, V. and Pavlik, I. (2006): A prevalence study of BTB by using abattoir meat inspection and

tuberculin skin testing data, histopathological and IS*6110* PCR examination of tissues with tuberculous lesions in cattle in Ethiopia. *Vet. Med.*, **51**: 512–522.

Smith, N. H., Kremer, K., Inwald, J., Dale, J., Driscoll, J. R., Gordon, S. V., van Soolingen, D., Glyn, H. R. and Maynard, S. J. (2005): Ecotypes of the *M. tuberculosis* complex. *J. Theor. Biol.*, **2**:562-567.

Smith, N. H., Dale, J., Inwald, J., Palmer, S., Gordon, S. V., Hewinson, R. G., and Smith, J. M. (2003): The population structure of *M. bovis* in Great Britain: Clonal expansion. Proceedings of the National Academy of Sciences of the United State of America, **100**: 15271-15275.

Smyth, A. J., Welsh, M. D. Girvin, R. M. and Pollock, J. M. (2001): In vitro pensiveness of $\gamma\delta$ T cells from *M. bovis* infected cattle to mycobacterial antigens: Predominant involvement of $WC1^-$ cells. *Infect. Immun.*, **69**:89-96.

Solomon, H. (1975): A brief analysis of the activities of the Meat Inspection and Quarantine Division. MoA, Addis Ababa, Pp 57.

Tadele, A. D. (1998): Evaluation of diagnostic tests, prevalence and zoonotic importance of BTB in Ethiopia. DVM Thesis, Faculty of Veterinary Medicine, Addis Ababa University, Debre-Zeit, Ethiopia.

Teklu, A., Aseeged, B., Yimer, E., Gebeyehu, M. and Woldesenbet, Z. (2004): Tuberculous lesions not detected by routine abattoir inspection: The experience of the Hossana municipal abattoir, Southern Ethiopia. Review of Science and Technology, OIE, **23**: 957–964.

Teshome, M. (1986): Bovine tuberculosis in state dairy farms in and around Addis Ababa. Study Report, MoA, Ethiopia.

Thierry, D., Brisson-Noel, A., Vincent-Levy-Frebault, V., Nguyen, S., Guesdon, J. L. and Gicquel, B. (1990): Characterization of a *M. tuberculosis* insertion sequence, IS6110, and its application in diagnosis. *J. Clin. Microbiol.*, **28**: 2668–2673.

Thoen, C. O. and Bloom, B. R. (1995): Pathogenesis of *M. bovis* In: Thoen, O. O. and Steel, J. H. Edition. *M. bovis* infection in animal and humans, Iwa state University, Ames, Pp 3-14.

Van Embden, J. D. A., Cave, M. D., Crawford, J. T., Dale, J. W., Eisenach, K. D., Gicquel, B., Hermans, P. and Martin, C. (1993): Strain identification of *M. tuberculosis* by DNA fingerprinting: Recommendations for a standardized methodology. *J. Clin. Microbiol.*, **31**: 406–409.

Van Soolingen, D., Qian, L., De Haas, P. E., Douglas, J. T., Traore, H., Portaels, F., Qing, H. Z., Enkhsaikan, D., Nymadawa, P. and Van Embden, J. D. (1995): Predominance of a single genotype of *M. tuberculosis* in countries of East Asia. *J. Clin. Microbiol.*, **33**: 3234–3238.

Van Soolingen, D., De Haas, P. E., Hermans, P. W., Groenen, P. M. and Van Embden, J. D. (1993): Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *M. tuberculosis*. *J. Clin. Microbiol.*, **31**: 1987–1995.

Vordermeier, H. M., Chambers, M. A., Cockle, P. J., Whelan, A. O., Simmons, J. and Hewinson, R. G. (2002): Correlation of ESAT-6-specific gamma interferon with pathology in cattle following *M. bovis* BCG vaccination against experimental bovine tuberculosis. *Infect. Immun.*, **70**: 3026-3032.

Whipple, D. L., Bolin, C. A. and Miller, J. M. (1996): Distribution of lesions in cattle infected with *M. bovis*. *J. vet. diagn. Invest.*, **8**: 351-354.

Woldesenbet, Z. (2002): Evaluation of abattoir inspection for the diagnosis of *M. bovis* infection in cattle at Addis Ababa abattoir. DVM thesis, Faculty of Veterinary Medicine, Addis Ababa, Pp 27.

WHO (1998): Laboratory services in tuberculosis control: Global Tuberculousis programme, WHO, Geneva. Switzerland.

Zainuddin, Z. F. and Dale, J. W. (1989): Polymorphic repetitive DNA sequences in *M. tuberculousis* detected with a gene probe from a *M. fortuitum* plasmid. *J. Gen. Microbiol.*, **135**: 2347–2355.

Zinsstag, J., Kazwala, R. R., Cadmus, I. and Ayanwale, L. (2006): *Mycobacterium bovis* in Africa. In: Thoen C. O., Steele J. H. Gilsdorf M. F. 2nd Edition, *M. bovis* Infection in Animals and Humans. Blackwell Publishing Professional, Ames, Iowa, USA, Pp 199–210.

Zumarraga, M., Bigi, F., Alito, A., Romano, M. I. and Cataldi, A. (1999): A 12.7-kb fragment of the *M. tuberculosis* genome is not present in *M. bovis*. *Microbiol.*, **145**: 893–897.

8. ANNEXE

Annex1. Description of body condition scoring

1. Condition Score 1(L-) marked emaciation- the animal could be condemned ante-mortem
2. Condition Score 2 (L) transverse processes project prominently, spine appear sharply
3. Condition score 3 (L+) individual dorsal spines are pointed to the touch, hips, tail-head and ribs are prominent
4. Condition score 4 (M-) ribs hips and pins are clearly visible, muscle mass between hooks and pins are slightly concave.
5. Condition score 5 (M) ribs usually visible, little fat cover, dorsal spines are barely visible
6. Condition score (Mt) the animal is smooth, dorsal spines can not be seen, but are easily felt.
7. Condition score7 (F-) animal is smooth and well covered but fat deposit are not marked
8. Condition score8 (F) fat cover in critical areas can easily be seen and felt; transverse process can be seen or felt
9. Condition score9 (F+) heavy deposits of fat is clearly visible on tail-head, brisket, dorsal spines ribs and hooks

   Source: (Micholson and Butter worth, 986)

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

Annex 2. Age determination

Age (years)	Characteristic Change
1 ½ - 2	I1 erupts
2 - 2 ½	I2 erupts
3	I3 erupts
3 ½ - 4	I4 erupts
5	All incisors and canine are in wear
6	I_1 is level and the neck has emerged from gum
7	I_2 is level and the neck is visible
8	I_3 is level and the neck is visible,
9	I4 may be level
10	I4 is level and the neck is visible
11	The dental star is squire in 1_1 and in all teeth by 12 years
12	The teeth that are not fallen out are reduce to small round pegs
	* Canine of ruminant is usually combined as fourth incisor

Source: De Lahunta and Habel, (1986)

Annex 3. DNA extraction from Biopsy Sample

1. Homogenize 1-5g tissue sample in 10 ml of PBS. The homogenate can be stored at 20^0C
2. Transfer 1000 ml of the homogenate in to 1.5 ml eppendorf and digest in with 5ml protenase K(10mg/ml) by incubating for 1.5-2 hrs at 60^0C water bath with occasional missing.
3. Add 900ml of PBS Containing 0.005% tween 80 and centrifuge at 12,000 x 9 for 10min
4. Carefully remove the supernant and re suspend the pellet in 100 ml TE buffer (0.1 x 5)
5. Add 10ml SDS (10%) and 2ml proteinase K (10mg/ml), vortex and incubate in 60^0C water bath for 1hr
6. Add 111 of 5M NaCl in vortex
7. Add 13ml of CTAB/NaCl solution, Vortex until it becomes milky white and incubate in 60^0C water bath for 1hr
8. Add an equal volume of a 24:1 mix of chloroform isoamly alcohol vortex and centrifuge for 5min at 12,000 x 9
9. Transfer the upper case carefully to new eppendorf tube
10. Add 0.6 x volume isopropanol (Child) and incubate 30 min t -70^0C
11. Centrifuge for 15min at 12,000 x 9 and remove the supernatant in 2 steps
12. Remove the supernatant until approximately 5000 ml is left and centrifuge it for 5 min at 12,000 x 9 and remove the remaining liquid without touching the pellet.
13. Add 1 ml of ice cold 70% ethanol and centrifuge for 5 min at 12,000 x 9
14. Carefully remove the supernatant in 2 step as in step 11 above
15. Let the pellet dry (5min) and dissolve in 20ml of 1 x TE buffer and store at -20^0C

Annex 5. Polymerase chain reaction procedures

1. PCR- Mix

Tag polymerase	1-2
Primrers	0.1 µL
Deoxy nucleotide triphosphate/dNTPs/	100 µm
PCR-Buffer	As applied with the enzyme
Template DNA	$10^5 - 10^6$ target molecules

2. PCR amplication Cycle

	Temperature	Time
Cycle1 /Denaturation	94^0C	30 sec
Cycle 2 /Annealing	60^0C	30sec
Cycle2/extension	72%	60sec

3. Gel-separation

A 2% agarose gel stained with ehtidium bromide is used.

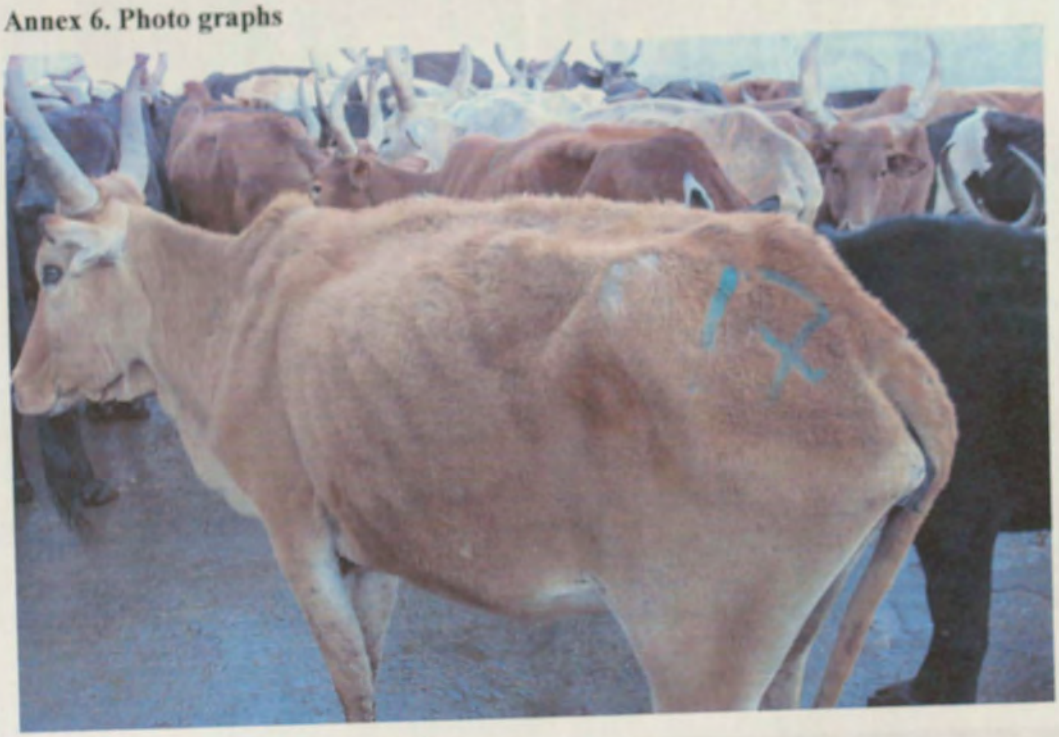
10µl of the product is loaded with 2µl loading buffer

2µl of a 100 bp DNA Molecular weight marker is loaded with 2µ l loading buffer in a single outside well

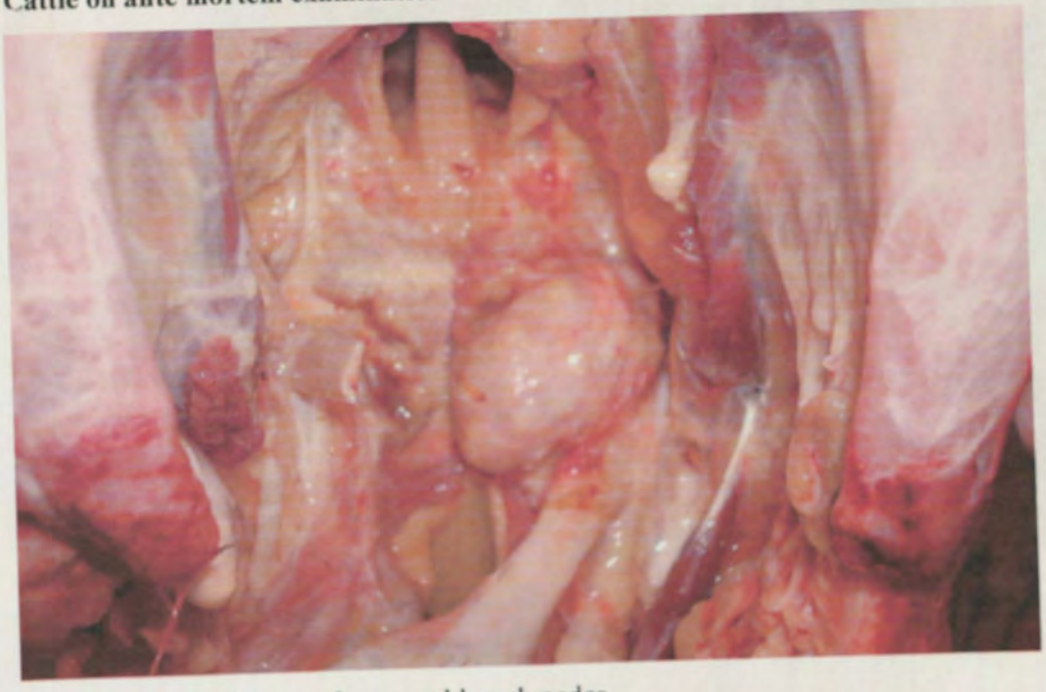
Gel electrophoresis is performed at 100 to 120 volt for 30 min

Analysis is done by using an automated photo documentation system.

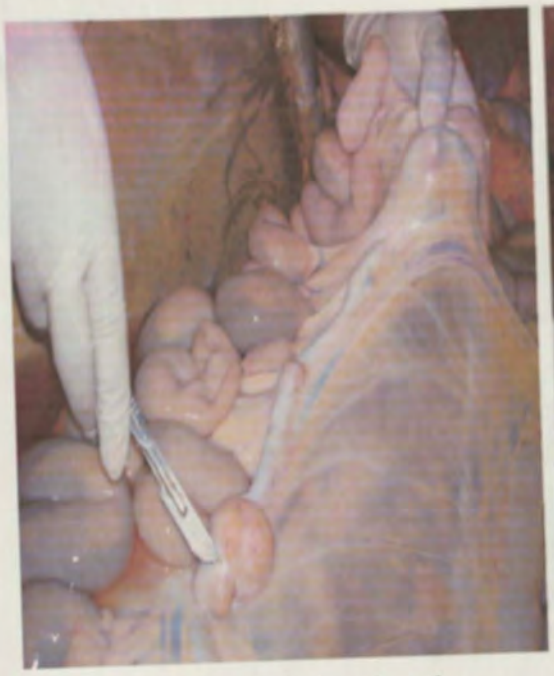
Annex 6. Photo graphs

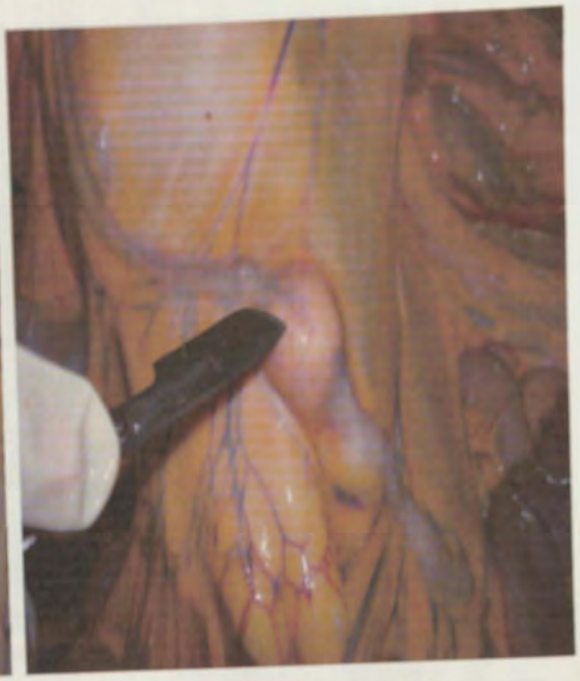


Cattle on ante mortem examination

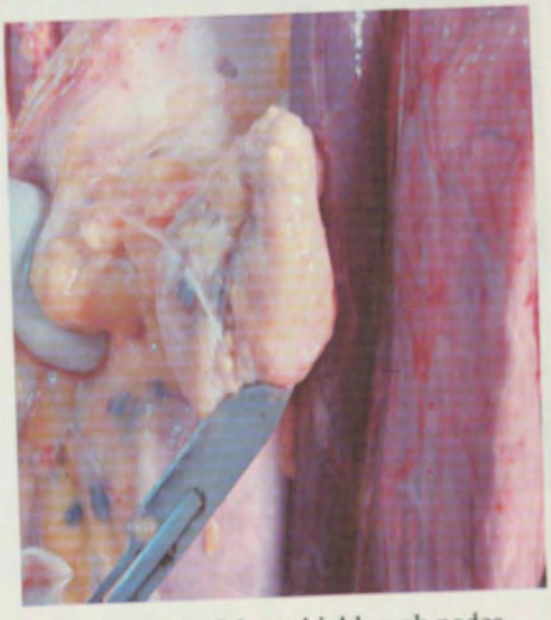


TB lesion on medial retropharyngeal lymph nodes

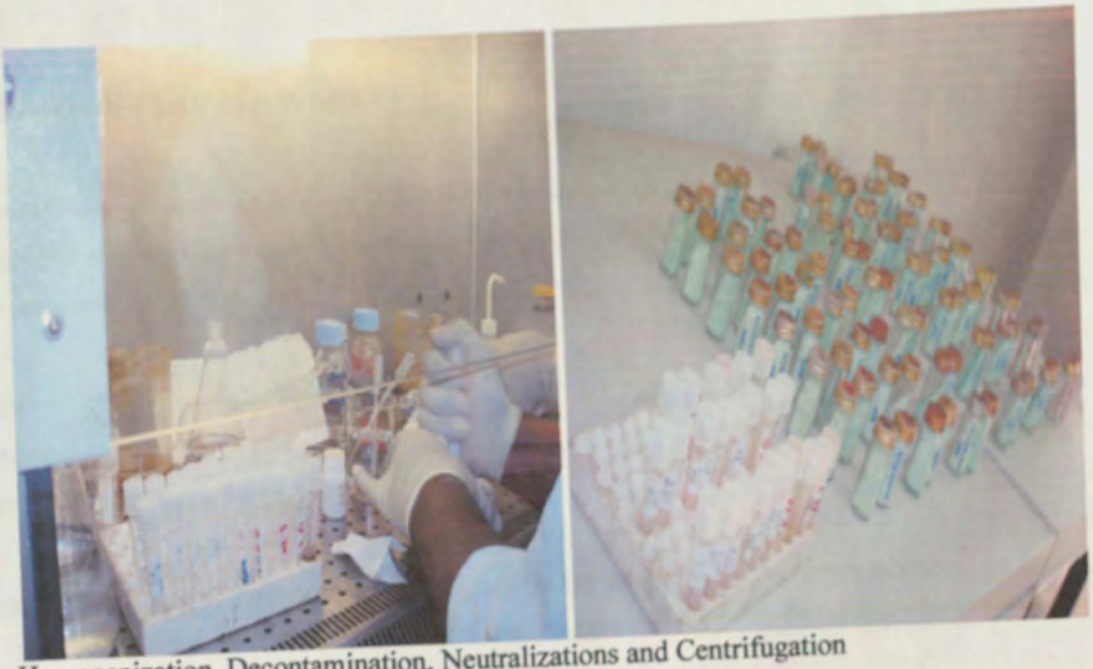




TB lesion on mesenteric lymph nodes



TB lesion on Left bronchial lymph nodes



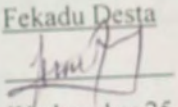
Homogenization, Decontamination, Neutralizations and Centrifugation

Inoculation and Incubaion

9. SIGHED DECLARATION SHEET

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

Name Fekadu Desta

Signature 

Date of submission Wednesday 25, 2008

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

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2. Dr Gobena Ameni (DVM, PhD cande, Assitant professor) _____