ADDIS ABABA UNIVERSITY
COLLEGE OF HEALTH SCIENCE
SCHOOL OF VETERINARY MEDICINE

ISOLATION AND CHARACTERIZATION OF MYCOBACTERIAL SPECIES CAUSING TUBERCULOUS LESIONS IN SLAUGHTERED CAMELS IN EASTERN ETHIOPIA

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A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfilment of the requirements for the Degree of Master of Science in Tropical Veterinary Microbiology

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ABSTRACT

A cross sectional study was conducted on 293 apparently healthy camels slaughtered at Jigjiga, Harar, Aweday and Dire Dawa abattoirs between December 2010 and April 2011. The objective of the study was to isolate and characterize Mycobacterial species causing tuberculous lesions in slaughtered camels through postmortem examination, mycobacteriological culturing, and multiplex polymerase chain reaction (PCR). A prevalence of 12.3% (36/293) was recorded on the basis of postmortem examination and the difference was statistically significant in females (χ^2=7.474, P=0.006) and in poor body condition (χ^2=7.323, P=0.025) camels when compared to their counter parts. About 79.5% of tuberculous lesions were found in lungs and associated lymph nodes in thoracic cavity and 15.9% in the lymph nodes of the head indicating inhalation as principal route of infection.Of the 36 camels with grossly suspicious TB lesion organs and tissues cultured mycobacterial growth was observed in 66.7% (24/36). Genotyping of the 17 isolates using multiplex polymerase chain reaction identified 88.2% (15/17) of them as member of mycobacteria other than *Mycobacterium tuberculosis complex* and only one as member of *Mycobacterium tuberculosis complex*. The relatively higher isolation of nontuberculous mycobacteria from most of camel TB lesions in this study could indicate their pathogenic or opportunistic role in the pathology of camel TB signifying the need to further examine these species as cause of camel TB so as to effectively elucidate its pathology, and enhance the effort in the control of the disease.

Key words: Camels, Genotyping, *Mycobacterium tuberculosis complex*, Nontuberculous mycobacteria, Prevalence, Tuberculosis

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LIST OF ABBREVIATIONS

AFB	Acid Fast Bacilli
AIDS	Acquired Immune Deficiency Syndrome
ALIPB	Aklilu Lemma Institute Pathobiology
BCG	Bacille Calmette-Guérin
BCS`	Body Condition Score
bp	base Pairs
BTB	Bovine Tuberculosis
^{o}C	Degree Celsius
CACIA	Central Australian Camel Industry Association
CSA	Central Statistic Agency
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside Triphosphate
DR	Direct Repeat
DTH	Delayed-Type Hypersensitivity
DVM	Doctor of Veterinary Medicine
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
FVM	Faculty of Veterinary Medicine
GC	Gaunosine and Cytosine
H37Rv	*Mycobacterium tuberculosis* H37Rv
HCL	Hydrochloric Acid
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon Gamma
IS	Insertion Sequence
IUATLD	International Union Against Tuberculosis and Lung Disease
LAM	Lipoarabinomannan
LJ	Löwenstein Jensen
MAPIA	Multiantigen Print Immunoassay
MHC	Major Histocompatbility
MIRU	Mycobacterial Interspersed Repetitive Units
MTBC	*Mycobacterium tuberculosis* complex

N	Normality
NALC	*N*-Acetyl-L-Cysteine
NaOH	Sodium Hydroxide
NAP	p-nitro-a-acetylamino-ß-priophenone
NIHCE	National Institute for Health and Clinical Excellence
OADC	Oleic Acid-albumin-Dextrose-Catalase
OIE	Office International des Epizooties/ World Organization for Animal Health
PCR	Polymerase Chain Reaction
PGRS	Polymorphic GC-rich Repetitive Sequence
PIM	Phosphatidyl Inositol Mannoside
PPD	Purified Protein Derivative of Tuberculin
PPE	Pro Pro Glu protein family
RD	Region of Difference
RFLP	Restriction Fragment Length Polymorphism
SPSS	Statistical Package for the Social Sciences
16SrRNA	16Sequence Ribosomal Ribonucleic Acid
TB	Tuberculosis
TbD1	*M. tuberculosis* specific deletion 1
TAE	Tris-Acetate EDTA
UAE	United Arab Emirates
UK	Kingdom
UNDP	United Nations Development Programme
USAID	United States Agency International Development
VNTRT	Variable-Number Tandem Repeat Typing
UV	Ultra Violet
WHO	World Health Organization
ZN	Ziehl-Neelsen

ABSTRACT

A cross sectional study was conducted on 293 apparently healthy camels slaughtered at Jigjiga, Harar, Aweday and Dire Dawa abattoirs between December 2010 and April 2011. The objective of the study was to isolate and characterize Mycobacterial species causing tuberculous lesions in slaughtered camels through postmortem examination, mycobacteriological culturing, and multiplex polymerase chain reaction (PCR). A prevalence of 12.3% (36/293) was recorded on the basis of postmortem examination and the difference was statistically significant in females (χ^2=7.474, P=0.006) and in poor body condition (χ^2=7.323, P=0.025) camels when compared to their counter parts. About 79.5% of tuberculous lesions were found in lungs and associated lymph nodes in thoracic cavity; and 15.9% in the lymph nodes of the head indicating inhalation as principal route of infection.Of the 36 camels with grossly suspicious TB lesion organs and tissues cultured mycobacterial growth was observed in 66.7% (24/36). Genotyping of the 17 isolates using multiplex polymerase chain reaction identified 88.2% (15/17) of them as member of mycobacteria other than *Mycobacterium tuberculosis complex* and only one as member of *Mycobacterium tuberculosis complex*. The relatively higher isolation of nontuberculous mycobacteria from most of camel TB lesions in this study could indicate their pathogenic or opportunistic role in the pathology of camel TB signifying the need to further examine these species as cause of camel TB so as to effectively elucidate its pathology, and enhance the effort in the control of the disease.

Key words: Camels, Genotyping, *Mycobacterium tuberculosis complex*, Nontuberculous mycobacteria, Prevalence, Tuberculosis

1. INTRODUCTION

Pastoralism refers to the use of extensive grazing rangelands for livestock production. It is one of the key production systems in the dry land Africa (FAO, 2001). Pastoralism covers about 25% of the earth's terrestrial surface, and it is an important economic and cultural way of life for between 100 and 200 million people throughout the world surface (WISP, 2008). Pastoral production system accounts for the livelihood of 50-100 million people in developing countries while 60% of this population lives in more than 21 African countries confined to the most arid regions of the continent (Sheik-Mohamed and Velema, 1999; UNDP, 2007). In eastern Africa, Ethiopia has the largest pastoralist population (7-8 millions) representing around 20 ethnic groups (Markakis, 2004). The major ethnic groups in Ethiopia are Somalis, Afar, Kereyu and Borena pastoral communities occupying the Eastern and southern lowlands of the country (Nori, 2005). Ethiopia's camel population is estimated to be 0.807 million head (CSA, 2010). The eastern part of Ethiopia is considered the heartland of the camel population, because it is home to two-thirds of the nation's camels. As in most dry lands of Africa and Asia, in Ethiopia camels are the principal source of income and food for millions of pastoralists. In addition, camels play a central role in providing draught power and determining the wealth and social status of pastoralists (Andrew, 1993).

In pastoral communities of Somali, Afar and Borena, camels are kept almost entirely for milk production (Getahun and Belay, 2002). In these communities, camel milk is consumed raw, and this habit combined with close physical contact with their animals create a potential public health concern for transmission of zoonotic diseases such as TB from animals to the pastoralist (Gezahegne *et al.*, 2011).

Tuberculosis is a chronic disease caused by bacteria of the genus *Mycobacterium* that affects many animal species. It is characterised by development of tubercles in the organs of most species and forms tubercles in the organs of most species (Eberlein, 2007). The disease had already been diagnosed around the turn of the 19^{th} century in dromedaries in Egypt (Littlewood, 1888) and in India (Lingard, 1905; Leese, 1908). *M. tuberculosis, M. bovis* and atypical mycobacteria have been isolated from dromedaries (Elmossalami *et al.,* 1971; Osman, 1974 ;Wernery *et al.,* 2002). Kinne *et al* (2006) described a TB case in dromedary in the United Arab Emirates with typical lesions in the both lungs. The report described a

mycobacterial strain of the ante loped of the *Mycobacterium tuberculosis complex*, closest to *Mycobacterium bovis*.

Camel TB is generally sporadic and camels are not considered to be maintenance host (infection can persist by intra species transmission alone, and may also be transmitted to other species) and infection is via contact (Cousins and Florisson, 2005). Leese (1969) reported that it is frequently found in Egyptian camels, while Mason (1917) indicated that in one Cairo abattoir the incidence of tuberculous carcasses was 2.8%. Tubercular lesions were found to affect the liver and lungs, or the lungs alone, or were sometimes generalized throughout the body. Mason concluded that camel tuberculosis was caused by the same bacilli that cause the bovine type (*Mycobacterium bovis*). Apart from these it was also reported in Somalia (Wernery *et,al.,* 2002), Pakistan (Zubair *et al.*, 2004), and Australia (Manefield and Tinson, 1997).

Although the camel is susceptible to bovine tuberculosis, it would appear that the disease is not an important one. In spite of their susceptibility to infection, camels are very resistant to the effects of the disease, and a long period elapses before they become cachectic and emaciated (Mukasa-Mugerwa, 1981). However in the other hand since bovine TB is caused by *M.bovis* and is a zoonotic disease implies the greatest importance of the disease as direct cause of infection to human mainly through the consumption of raw milk.

The zoonotic risk arising from camel milk should be considered because camel milk is usually consumed in its raw state. In particular in the pastoralists of the Horn of Africa where no treatment of milk is allowed either it is consumed raw or when it just soured (Eberlein, 2007). Donchenko *et al.* (1975) isolated *M. bovis* strains from camel milk samples in Russia. Further more *M. bovis*, *M .avium*, *M.kansasii* (Strauss, G. 1995) and *M.tuberculosis* were isolated (Zubier *et al.*, 2004) in necropsy examination of camels.

Ethiopia is a country where the impact of TB is particularly important and it is amongst the three African countries with highest burden of human TB cases. Regrettably, in the pastoral production system, in the low land areas, despite the presence of huge livestock population, the actual prevalence of bovine TB is not yet known. In this production system, the study process can be complicated by the frequent movement of animals for water (watering point) and the live stock markets. To this effect, animals came together from different directions and

due to the enclosure of the animals overnight may expose to the increased transmission of the disease (Shitaye *et al.*, 2007).

Regarding TB in dromedaries of Ethiopia there are only very few studies conducted so far. In 1979, Richard for the first time report the existence of the disease in his work entitled "The Study of the pathology of the *dromedary* in Borana Awraja (Ethiopia)" (Wernery *et al.*, 2002). Further report was made in recent years indicating the existence of the disease and specifying some of its causative agents (Gezahegne *et al.*, 2009; Gezahegne *et al.*, 2011).

As preliminary work these studies called for the need for further investigation of the pathology of camel TB, and identification of its causative agents. The need for further investigation of the disease arise from its very crucial importance both to alleviate the large paucity of information on the pathology, and the causative agent of TB in camels of pastoral region of the country and also more importantly to encourage and enhance the effort in the control of the disease and reduce its risk of zoonosis to the pastoralist community of Ethiopia.

Therefore the general objective was:

- Estimating the prevalence of TB in camels and characterization of mycobacteria from TB suspicious lesions of camels.

The specific objectives were:

- To estimate the prevalence of TB in camels at Jigjiga, Harar, Aweday and Dire Dawa abattoirs.
- To isolate, identify and characterise mycobacterial species causing pathological lesions compatible with bovine tuberculosis in slaughtered camel.
- To detect and assess possible risk factors for infection and transmission of the causative agents in camels.

2. LITERATURE REVIEW

2.1. The genus *Mycobacterium*

Mycobacteria belong to the Kingdom of Bacteria; Phylum of *Actinobacteria*; Order of *Actinomycetales*; Family of *Mycobacteriaceae*, and the Genus of *Mycobacterium*. (Haddad *et al.*, 2004) The genus shares an unusually high genomic Deoxyribonucleic Acid Gaunosine and Cytosine (DNA G+C) content (62–70%) and production of mycolic acids with closely related genera, *Nocardia* and *Corynebacterium*, within *Actinomycetales* (Harris, 2006; Saviola and Bishai, 2006). The Genus *Mycobacterium* includes many pathogens known to cause serious diseases in mammals, including tuberculosis and leprosy. The phylogenetic analysis of the genome of pathogenic mycobacteria has shown that all (except *M. avium*) belong to a single genetic species: the *Mycobacterium tuberculosis* complex (Haddad *et al.*, 2004).

2.1.1. *Mycobacterium tuberculosis* complex (MTC)

The *Mycobacterium tuberculosis* complex (MTC) is the causative agent of tuberculosis (TB) in humans and animals (Niemann *et al.*, 2000). It comprises different species of mycobacteria that include *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium canettii*, *Mycobacterium bovis* and *Mycobacterium bovis* BCG (Aranaz, *et al.*, 1996; van Soolingen, *et al.*, 1997). Newly recognized additions to the MTC include *Mycobacterium caprae*, which affect goats and was originally classified as *M. tuberculosis subsp caprae* or *M. bovis subsp caprae* (Aranaz, *et al.*, 2003) and *Mycobacterium pinnipedii*, which affects pinnipeds (Cousins, *et al.*, 2003).

Mycobacterium tuberculosis

This is the predominant cause of human TB and arguably the most successful of human bacterial pathogens. In 1888, Robert Koch first described this bacillus and it is thus sometimes referred to as Koch's bacillus (Asiimwe, 2008). It has been speculated that it is from *M.bovis* that *M. tuberculosis* evolved by specific adaptation of an animal pathogen to the human host (Stead, 1995).These hypotheses were proposed before the whole genome

sequence of *M.tuberculosis* (Cole, 1998) was available and before comparative genomics uncovered several variable genomic regions in the members of the MTC (Brosch, 2002). Based on the presence or absence of such conserved regions, a degree of relatedness to the last common ancestor of the MTC was proposed that the lineages of *M.tuberculosis* and *M.bovis* separated before the *M.tuberculosis* specific deletion TbD1 occurred. From this analysis it is clear that *M.bovis* cannot have been the ancestor of *M.tuberculosis* but, rather, appears to be descended from *M.tuberculosis* or to have emerged independently (Cole, 2002).

Mycobacterium africanum

This species is heterogeneous and has characteristics that appear to lie between *M. bovis* and *M.tuberculosis* and was first isolated from a Senegalese patient suffering from pulmonary TB in 1968(Asiimwe, 2008).On the basis of geographic origin and biochemical properties, *M.africanum* species have been subdivided into two major subgroups : those from West Africa are subtype I and are closer to *M.bovis* , while those from East Africa are closer to *M.tuberculosis* and are subtype II (Niemann *et al.,* 2003). Recent surveys show difference in distribution of *M.africanum* in various regions of Africa(Sola *et al*, 2003).

Mycobacterium bovis and Mycobacterium bovis BCG

M.bovis is the causative agent of TB in cattle and other mammals and the disease in humans is clinically indistinguishable from that due to infection with *M.tuberculosis.* It has been observed that the risk factors for *M. bovis* infection in both animals and humans especially in many African settings where domestic animals are an integral part of human social life are close contact , food hygiene practices like consumption of unprocessed dairy and meat products and HIV/AIDS infection (Cosivi *et al*, 1998); while other studies have noted reactivation disease due to *M.bovis* even in areas where cattle TB is well controlled (Yates *et al.*, 1993). *M.bovis* BCG (Bacillus Calmette-Guerin) has its origin from a virulent *M.bovis* strain. Calmette and Guerin performed 230 in vitro passages of *M. bovis* until the organism lost its virulence.While this strain has been used worldwide as a live attenuated vaccine, it may cause disease but only after vaccination with BCG (Asiimwe, 2008).

Mycobacterium microti

M.microti, which causes TB mainly in small rodents such as voles, had earlier been considered non pathogenic for humans (Kremer *et al.*, 1998).Considering the DNA sequences of the 16s rRNA gene and the 16 S-to-23S internal transcribed spacer region, *M.microti* proved to be a member of the MTC. Primary isolation and differentiation by classical biochemical tests are complicated by very slow growth of this species but spoligotyping permits the simultaneous detection and typing of *M.microti* (van Sooligen *et al.*, 1998). This technique has been applied in two retrospective studies of *M.microti* infections in the Netherlands and England, enabling identification of cases in llamas, cats, and ferrets, as well as in four humans, only one of whom was described as imminocompetent. Niemann *et al.* (2000) reported two cases of *M. microti* infection causing pulmonary TB in Germany. The isolates were identified as *M. microti* of llama and vole types, according to spoligotype patterns, which displayed reactions with or only 2 of the 43 spacers (Asiimwe, 2008)

Mycobacterium caprae

When isolates from the MTC cultured from caprine pathological tissue samples in Spain were biochemically and genetically characterised, they segregated from other members of the complex. The results of this testing, together with the repeated association of this micro-organisms with goats , suggested that a new member of this taxonomic complex not matching any of the classical species had been identified. The name *M. tuberculosis subsp. capre subspnov.* was proposed for theses isolates (Aranaz *et al.,* 1996). Besides biochemical (sensitivity to pyrazinamide) and epidemiological features, strains of this unusual member of the MTC showed a special combination of *pncA*, *oxxyR* , *katG* and *gyrA* gene polymorphisms, while sequence analysis of the *gyr B* gene in these strains revealed special nucleotide substitution not found in other members of the MTC that could be used to differentiate caprine mycobacterial strains from *M. bovis* and other members of the MTC . It was proposed that *M. tuberculosis susp. capre* be elevated to species status , as *Mycobacterium capre comb. nov., spnov.* (Aranaz *et al.*, 2003).

Mycobacterium pinippedii

Tuberculosis was first diagnosed in seals (pinnnipeds) in Australia (Cousins *et al.*,1993) in 1993. When mycobacterium species isolated from the lungs of 2 of seals were studied to determine the similarity of the strains to each other, the secretary protein MPB70, present in *M. bovis*, was not detected in the wild seal isolates using sodium dodecylsuphate poly acrylamide gel electrophoresis and Westeren blotting techniques. Further analysis of protein and DNA fragment profiles indicated that seal TB isolates formed a unique cluster within the MTC. When a comparison of MTC isolates from seals in Australia, Argentina, Uruguay, Great Britain and New Zealand was undertaken to determine their relationships to each other and their taxonomic position within the complex (Cousins *et al.*,1993), biochemical testing clearly confirmed that the seal isolates belonged to the MTC. In most cases, the isolates grew preferentially on media that contained sodium pyruvate, and slight differences from typical *M. bovis* isolates were note. It was further shown that all isolates contained the sequences IS6110, IS1081, mpb70 and mtp40, yet failed to produce detectable MPB70 antigen. The *pncA* gene contained CAC (His) at codon 57 and the *oxyR* gene showed G at position 285, similar to *M. tuberculosis* , *M. microti* and *M. africanum*. The seal isolate spoligotypes formed a cluster that was clearly different from those of all other members of the MTC. The name *Mycobcterium pinnipedii sp. nov*. was proposed for this novel member of MTC(Cousins *et al.*,1993).

"Mycobateterium canettii"

The name *"M. canettii"* is in quotation since it does not appear the official List of Bacterial Names with Standing in Nomenclature (Asiimwe, 2008).In an attempt to characterize an unusual mycobacterial strain isolated from 2–year-old Somali patient with lymphadenitis, van Soolingen *et al.* (1997) applied various molecular methods to a bacillus that produces smooth and glossy colonies, which is highly exceptional for this species. In another study, Miltgen *et al.* (2002) identified an unusual strain of mycobacteria from two patients with pulmonary TB its smooth, glossy morphotype and, primarily, its genotypic characteristics. Spoligotyping and IS6110 RFLP revealed that it was *M. canettii*. All known cases of TB caused by *M. canettii* have been contracted in the Horn of Africa, as was also the case of a Swiss patient described by Pfyffer *et al.* (1998). The prevalence of *M. canettii* isolates may be underestimated in the routine microbiology laboratory due to their close similarity to *M. tuberculosis*, particularly as

the smooth colony type easily reverts to give a stable rough colony type in *M.canetti.* (Asiimwe, 2008).

2.2. Genomics and evolutionary relationships of MTBC

Comparative analysis with the genome sequences of *Mycobacterium tuberculosis* complex (MTBC) promises to expose the genetic basis for the phenotypic differences between the tubercle bacilli, offering unparalleled insight into the virulence factors of the MTBC (Gordonafl *et al.*, 2001). The use of deletion analysis in conjunction with molecular typing and analysis of specific mutations was shown representing a very powerful approach for the study of the genomics and evolution of the tubercle bacilli and for the identification of evolutionary markers (Gordonafl *et al.,* 2001; Brosch *et al.*, 2002).

Because of the unusually high degree of conservation in their housekeeping genes, it has been suggested that the members of the complex underwent an evolutionary bottleneck at the time of speciation, estimated of having occurred roughly 15,000 – 20,000 years ago (Brosch *et al.*, 2002). Differential hybridization arrays based on regions of difference challenged the previous *M. bovis* genome analysis that *M. tuberculosis* human adapted variety of *M. bovis* that was acquired from cattle. The irreversible DNA material uncovered by the *M. bovis* genome sequencing project and systematic analysis of polymorphisms in a large panel of strains indicate quite a different scenario from the previous. The analysis indicates that the common ancestor of the tubercle bacilli resembling to *M. tuberculosis* or *M. canettii* (Brosch *et al.*, 2002; Fritsche *et al.,* 2004).

The complex includes *M. canettii, M. tuberculosis, M. africanum, M. microti, M. bovis, M. caprae* and *M. pinnipedii* (Fig.2). The group characterized by more than 99.95% similarity at the nucleotide level and identical 16S rRNA sequences (Tauro *et al.*, 1996; Brosch *et al.*, 2002). However, a comparative genomic analysis shows that minor genome changes have a profound effect on the host tropisms, phenotypes and pathogenicity (Brosch *et al.*, 2002). Successive DNA deletions, starting by the loss of region RD9 from *M. tuberculosis* originate *M. africanum, M. microti*, and also after loss of RD4 *M. bovis*. Moreover, a further deletion of RD1 during *in vitro* adaptation of *M. bovis* gives avirulent *M. bovis* BCG strain (Fig.2) (Brosch *et al.*, 2002).

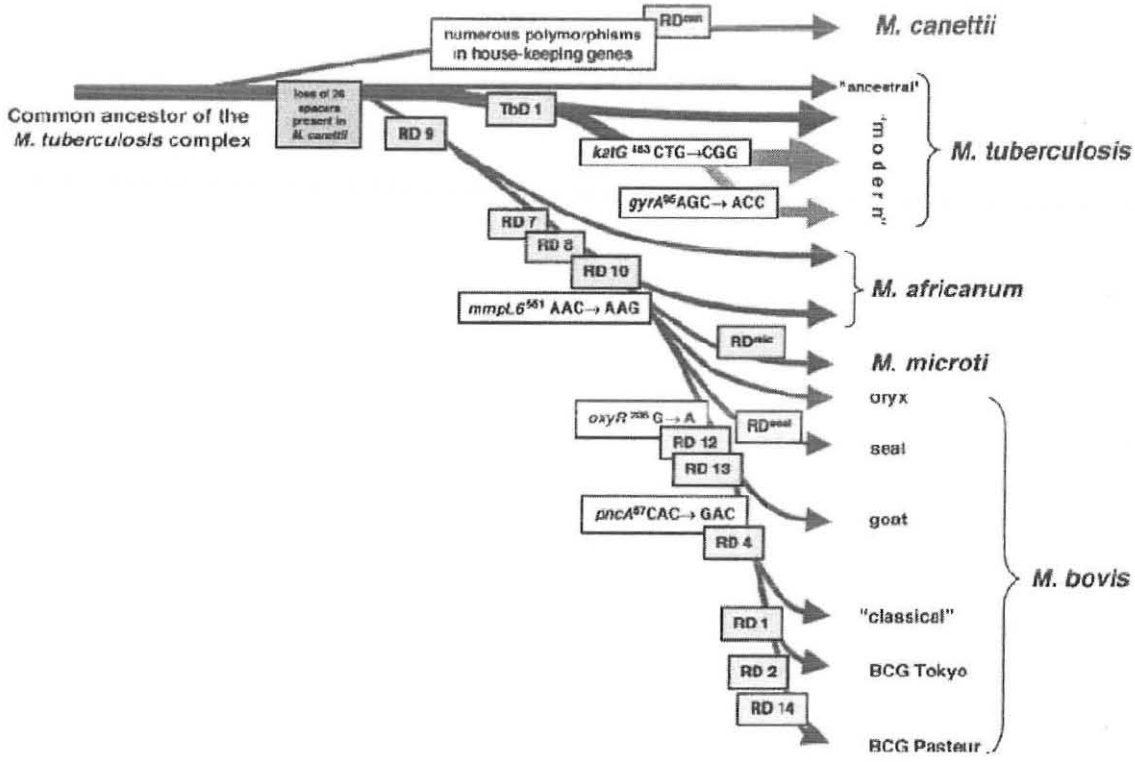

Figure 1: Proposed evolution of mycobacterial pathogens from a common ancestral *M. cannetti* strain and phylogenetically informative mutations in the lineages leading to *M. bovis.* The scheme bases on the presence or absence of conserved deleted regions and on sequence of polymorphisms in five selected genes.

Source: Brosch *et al.* (2002).

2.3. Virulence Factors of MTBC

Virulence factors of mycobacteria are the special properties that enable them to infect, survive, multiply and cause disease in their host (Collins, 2001; Thoen *et al.*, 2006).Most studies of mycobacterial virulence determinants have been performed on *M. tuberculosis* rather than on other members. However, the presence of homologous genes in other members of the complex and the close relationship between these microorganisms indicate the use of similar virulence determinants and mechanisms of pathogenicity. Complex lipids, extracted from both virulent and attenuated strains of mycobacteria, have been extensively evaluated by *in vivo* or *in vitro* systems to obtain information on their significance in disease (Chua and Deretic, 2004).

Therefore, the cell wall core of mycobacteria is composed of three covalently attached molecules: 10 peptidoglycan, arabinogalactan, and mycolic acid. Glycolipid complexes present in the cell wall of virulent and attenuated strains of mycobacteria are extensively examined to obtain information on their significance in granuloma formation. An important property of virulent tubercle bacilli is their ability to form cord factors when grown in liquid culture medium (Chua and Deretic, 2004; Thoen and Barletta, 2004).

On the other side, the effects of mutations in cell wall components could have indirect effects that extend well beyond the physical integrity of the wall itself (Barry, 2001). Moreover, differential hybridization arrays within MTBC identified 14 regions of difference (RD1–14) that were absent from *M. bovis* BCG Pasteur relative to *M. tuberculosis* H37Rv (Fig.2). In addition, loss of RD1 has been demonstrated as the mechanism of virulence attenuation in *M. bovis* BCG during *in vitro* adaptation (Behr *et al.*, 1999; Gordon *et al.*, 1999; Brosch *et al.*, 2002).

2.4. Growth requirements and cultural characteristics

Samples before being tested for mycobacterial growth must be decontaminated to remove rapidly growing micro flora. Decontamination procedures take advantage of the thick lipid envelope of mycobacteria and its natural resistance to chemical agents. *N*-Acetyl-L-cysteine (NALC) (0.5-2.0%) liquefies sputum samples. The compound has not only minor inhibitory effects on mycobacteria but also does allow other antibacterial agents to access and neutralize contaminating organisms. Sodium hydroxide (NAOH 4%) is another decontaminating used to inactivate contaminating bacteria with only modest inhibitory effects against mycobacteria (Isenberg, 1992; Metchock *et al.*, 1999; Pfyffer, 2003)

Once decontaminated, specimens may be used to inoculate either a solid or liquid growth medium. The solid growth media are available as either egg- or agar-based. Both types of solid media contain malachite green dye, which inhibits the growth of many other bacteria. The commonly used Löwenstein-Jensen (LJ) medium is egg-based, whereas Middlebrook 7H10 and 7H11 are agar-based media, growth can also be assayed in a liquid medium such as Middlebrook 7H9. For the culture of tissue, sputum, milk specimens and others clinical samples the LJ medium is the first choice. Because it is easy to prepare, least expensive while supporting good growth of tubercle bacilli, can be stored in the refrigerator for several weeks,

contamination during preparation is limited for it can be inspissated after being placed in bottles. In addition, the malachite green added to the media suppresses the growth of non mycobacterial organisms while providing green background during readings of colonies (Woods *et al.*, 1997). Furthermore, the International Union Against Tuberculosis and Lung Disease (IUATLD) is recommended the modified LJ medium containing glycerol favours the growth of *M. tuberculosis* while LJ medium without glycerol but containing pyruvate enhances the growth of *M. bovis.* In addition, both should be used in countries or regions where patients may be infected with either organism (Woods *et al.*, 1997). Furthermore, the growth of *M. bovis* in liquid medium is diffused, but on a solid medium is dry, sparse, delicate and non luxuriant (Quinn *et al.*, 1999) while the growth of *M. tuberculosis* in solid medium is dry, rough colonies with sometimes nodular or wrinkled surfaces (Pfyffer, 2003).

However, solid growth media has some limitations. Because they are both labour and time consuming i.e. it may take five to eight weeks before cultures become positive, especially if specimens contain few bacilli or if decontamination procedures have been overly harsh. To shorten this, in most developed countries, the Bactec (Becton-Dickinson) system is used. With regard to this, samples are decontaminated as necessary and added to vials containing Middlebrook 7H12 or 7H9 media, an antibiotic mixture and 14C-labelled palmitic acid (Pfyffer *et al.*, 2002). If mycobacterial growth occurs, 14C palmitic acid is utilized and $14CO_2$ is produced. In this method growth of mycobacteria may be detected within 5-7 days, but positive results require further testing to distinguish between tubercle bacilli and other mycobacteria (Pfyffer *et al.*, 2002).

2.5. Morphology and staining characteristics

Mycobacteria are non-motile, non-spore forming, pleomorphic bacilli or coccobacilli. In tissue samples they appear as rods, which may be straight, curved, or in the forms of clubs. They occur singly, in pairs or as small bundles (Quinn *et al.*, 1999). The distinguishing features of pathogenic mycobacteria are the formation of characteristic cords on acid fast staining (Thoen and Barletta, 2004). The mycobacterial cell wall is triple layered comprising a basal peptidoglycan layer and an intermediate arabinogalactan mycolate complex. The outer layer is lipid rich, comprising surface rope-like structure of peptidoglycolipid (Thoen and Barletta, 2004).

Mycobacterium species may be invisible by Gram staining. Therefore, special acid-fast staining procedures are necessary to promote the uptake of phenol dyes and higher temperature in the classical Ziehl Neelsen staining allow penetration of the carbol fuchsin stain more easily. When counterstained with methylene blue, the red to pink stained acid fast bacilli (AFB) are highlighted against a blue background which facilitates their microscopic recognition (Metchock *et al.*, 1999).

2.6. Diagnostic tests for mycobacterial infections

To date, the diagnosis of tuberculosis has not improved significantly particularly in developing countries and still relies heavily on staining and culture of sputum and tissues or other clinical specimens which were developed more than 100 years ago. Even though, culturing is golden standard diagnostic technique, but not presumptive between species. In response to this demand, new rapid diagnostic methods: molecular diagnostic techniques are urgently sought (Wei-Juin, 2002; Heekyung *et al.*, 2004). In the coming sections different diagnostic techniques are reviewed.

2.6.1. Ante mortem examination

Bovine tuberculosis is tentatively diagnosed by its clinical symptoms (Seifert, 1996). Clinical signs such as chronic debilitation, moist cough, low-grade fever and enlargement of local lymph nodes may suggest infection with tuberculosis (OIE, 2004). Nevertheless, other techniques are required to confirm the diagnosis.

2.6.2. Smear microscopy

Smear microscopy is still among the most rapid and inexpensive ways to diagnose tuberculosis and a rapid means to identify the most contagious patients with high specificity and also with high positive predictive value for tuberculosis (>90%) for respiratory specimens (Kaufmann and Hahn, 2003). However, the overall sensitivity of the smear ranges from 22 to 80% from clinical specimens, and in addition to this, species diagnosis is impossible (Metchock *et al.*, 1999; Kaufmann and Hahn, 2003).

2.6.3. Immunodiagnosis

The diagnosis of camelid tuberculosis in living animals faces many difficulties. None of the tests available can diagnose tuberculosis with certainty (Wernery *et al.*, 2002).

Tuberculin test

The tuberculin skin tests are the international standard for ante-mortem diagnosis of BTB in cattle herds and individual animals. They are based on eliciting cell-mediated immunity dependent delayed-type hypersensitivity (DTH) response, to the intradermal injection of tuberculin, a crude protein extract from supernatants of mycobacterial cultures (Pollock *et al.*, 2006). The tuberculin skin test, involves the injection of 0.1 ml *M. bovis* purified protein derivatives (PPD) intradermally and observation of the injection site at 72 hours in cattle, and any inflammatory response is classified as a reactor cattle with suspicious reactions to the caudal fold test are subsequently subjected to a comparative cervical test to determine their relative responsiveness to biologically balance of *M. bovis* and *M. avium* subspecies *avium* PPD. Cattle infected with *M. bovis* will develop more induration (measured as an increase in skin thickness at the site of injection) in response to the *M. bovis* PPD than to the *M. avium* PPD. The increase in skin thickness at each injection site is plotted on a scattergram. The results are classified as negative, doubtful, or reactor (positive) based on inflammatory response of skin swelling (Pollock *et al.*, 2006).

Intradermal tuberculin testing, which is the classical diagnostic test, often gives nonspecific reactions in camelids. Several papers report non-specific skin reactions in camels. The literature contains reports of dromedary camels with positive responses to intradermal tuberculin testing that range from 1.9% reactors in India to 37% in Kenya (Wernery *et al.*, 2002).

A program to control tuberculosis in camelids based only on intradermal tuberculin tests will face severe deficiencies. One of the main reasons for false-positive or false-negative reactions is not the structural compounds of the camelids skin but the presence of atypical mycobacterial antigens that are common in camelids. The common antigens shared with *Nocardia* and *Corynebacteriae* further negatively affected the specificity of these tests (Wernery *et al.*, 2002).

Serological tests

It is obvious that camels are susceptible to tuberculosis but this disease is very difficult to diagnose on clinical grounds. A definitive diagnosis of tuberculosis requires the culturing and specification of the organism. Considerable efforts have been undertaken in the development of serological tests for the diagnosis of tuberculosis, but they still remain inadequate for the clinical application of this disease (Wernery *et al.*, 2002).

The ante mortem tests, such as lymphocyte transformation and ELISA tests have also not been very reliable in undomesticated mammals because of false-negative and false-positive reactions. This is also true for tuberculosis testing in camelids. However, it is recommended that several tests be used to aid in the diagnosis of tuberculosis in camelids (Fowler, 1998).

2.6.4. Post-mortem inspection

During necropsy, the lymph nodes, especially those associated with the head, thorax and abdomen are closely examined with good light source. Careful examination of as few as 6 pairs of lymph nodes, the lungs and the mesenteric lymph nodes can result in 95% of inspected animals with macroscopic lesions being identified. However, this method is not useful to determine the significance of animals that give a positive reaction in diagnostic tests but do not have visible lesions. Non visible lesion reactors may be due to early infection, poor necropsy technique or infection with mycobacteria other than *M. tuberculosis* complex. Above all, it is not always possible to differentiate TB lesions by gross examination from those of other infections (Croner, 1994; Vordermeier *et al.*, 2002).

2.6.5. Histopathological techniques

For histopathology, fixed samples (in 10 percent formalin) from lesions are required (Quinn *et al.*, 1999) and histologic sections are stained with hematoxylin-eosin (Biberstein and Hirsh, 1999). Microscopic appearance of a typical BTB lesion consists of a peripheral zone of mononuclear cells, fibroblasts and giant cells with central caseous necrosis (Quinn *et al.*, 1999). The presumptive diagnosis of mycobacteriosis can be made if the tissue has characteristic histological lesions such as caseous necrosis, mineralization, epitheloid cells,

multinucleated giant cells and macrophages. The presence of acid-fast organisms in histological sections may not be detected (OIE, 2004).

2.6.6. Isolation and identification of *Mycobacterium*

A definitive diagnosis of tuberculosis requires the culturing and specification of the organism. The presence of *Mycobacterium* species in clinical samples and post-mortem specimens may be demonstrated by examination of stained smears and confirmed by cultivation of the organism on Lowenstein-Jensen medium (OIE, 2004). Cultures are incubated for 8 weeks at 37 °C. When growth is visible, smears are prepared and stained by the Ziehl-Neelsen staining technique.

Growth of *M. bovis* generally occurs within 3-6 week incubation. *M. bovis* will grow on Lowenstein-Jensen medium with pyruvate, but *M. tuberculosis* grow well when glycerol is added to the medium. Characteristic growth patterns and colonial morphology cannot provide a presumptive diagnosis of species (OIE, 2004).

2.6.7. Molecular diagnostic techniques

Molecular diagnostic techniques allow investigators to determine whether the species or strain causing disease in one case is identical to that causing disease in another case, and hence whether transmission could have occurred. In addition, the level of genetic relatedness of *Mycobacterium* isolates can be determined (Durr *et al.*, 2000; Kaufman and Hahn, 2003; van Soolingen *et al.*, 2003).

Polymerase chain reaction (PCR)

DNA amplification by PCR provides a rapid and sensitive method for the detection of *M. tuberculosis* complex. The development of any species-specific PCR assay requires that the target gene or DNA fragment be present in all the isolates from the particular species of interest and be absent from all other unrelated species (Jahans and Worth, 2006). The PCR technique is much faster than culture, provides detection of *M. bovis* when rapidly growing *Mycobacterium species* are present in the sample and may be able to detect the presence of *M. bovis* in samples even when organisms have become nonviable. It could be used as a rapid

screening technique which would be complementary to culture of tissue specimens for the routine diagnosis of bovine tuberculosis (Jahans and Worth, 2006). Generally, a sample found to be positive by either culture or PCR is regarded as a true positive. However, a negative result by either method means that *M. bovis* was not detected in the sample of tissue that was tested. Other samples of tissue from the same animal could harbour *M. bovis.* Therefore, a negative test result from PCR does not provide conclusive proof that an animal is not infected with *M. bovis,* especially if the animal originated from an infected or suspect herd. PCR requires sophisticated laboratory equipment, facilities, and advanced technical skills (Jolly *et al.,* 2007). The most common PCR based molecular typing methods for *Mycobacterium* species are multiplex PCR (mycobacterial genus typing), 16SrRNA, RD4, RD9, RD10 deletion typing (Durr *et al.*, 2000; Saunders and McFadden, 2003; van Soolingen *et al.,* 2003; Ayele *et al.*, 2004; Harris, 2006).

A conventional PCR generally only uses one set of primers (Jahans and Worth, 2006). Several genes and insertion sequences have been targeted in attempts to develop PCR assays to differentiate between mycobacteria in general and between members of the *M. tuberculosis* complex in particular. PCR amplification of genomes such as 16S rRNA, mtp40 gene, mpb70 gene, pncA gene, the oxyR gene and the regions of difference (RD) (Haddad *et al.,* 2004) are used to differentiate the genus *Mycobacterium* or, *M. tuberculosis* from *M. bovis* isolates.

Spoligotyping (spacer oligonucleotide typing)

The genomic advances in molecular typing have improved our understanding of the dissemination of MTBC and helped improve our ability to distinguish among strains. The genomics of the MTBC carry a single region on the chromosome called the direct repeat (DR) locus and this single locus is composed of a series of 43 identical DR sequences. Each DR is bp long, interspersed by spacer sequences varying in length from 35 to 41bp and DNA polymorphism in this locus allow for strain typing (Kamerbeek *et al.,* 1997; Costello *et al.*, 1999). Spoligotyping is now considered as standard alternative molecular techniques for strain typing (Supply *et al.*, 2000; Allix *et al.*, 2004; Sophie *et al.*, 2006). The technique is PCR-based that evaluate the polymorphism of the direct repeat copy number at several loci and have been used to identify different strains of the complex (Skuce and Neill, 2001; Gibson *et al.*, 2004; Sophie *et al.*, 2006).

Moreover, the DR region is the basis of a novel mycobacterial strain-typing technique called "reverse line blot hybridization," or spoligotyping. In this, polymerase chain reaction (PCR) products are amplified using RFLP primers specific for the DR sequences. Thus, products of multiple sizes are generated, representing all the spacer sequences present in an isolate's genome (Fig.2). Using a specialized dot-blot apparatus, this product mixture is then hybridized against a membrane to which the 43 individual spacer sequences have been covalently linked, generating a specific pattern (spoligotype) of positive and negative hybridization signals (Fig.3) (Kamerbeek *et al.*, 1997). Each spacer is then assigned the value of 0 (negative hybridization signal) or 1 (positive signal). The 43 spacers are then divided into a set of 14 triplets with one extra, and each set is then converted to a 15-digit octal code (Fig.2) (Kamerbeek *et al.*, 1997).

Differences between spoligotype patterns are mainly the result of deletions of spacer sequence regions. Interestingly, the absence of spacers 3, 9, 16, and 39–43 is a characteristic of all *M. bovis* strains (Costello, 1999) and is thought to be a result of the deletion of this region by homologous recombination instead of genetic divergence from the *M. tuberculosis* spacer sequence (Caimi., 2001).

Spoligotyping has the advantage of being significantly less technically demanding than other fingerprinting, with a much shorter turnaround time. In addition, the degree of differentiation achieved by spoligotyping is higher than that of IS*6110* RFLP for *M. bovis* (Kamerbeek *et al.*, 1997). However, the major drawback to spoligotyping is that it can only identify polymorphisms within the DR cluster. Despite this limitation, spoligotyping is capable of providing satisfactory sensitivity for routine molecular epidemiologic analyses at both regional and global levels (Caimi, 2001).

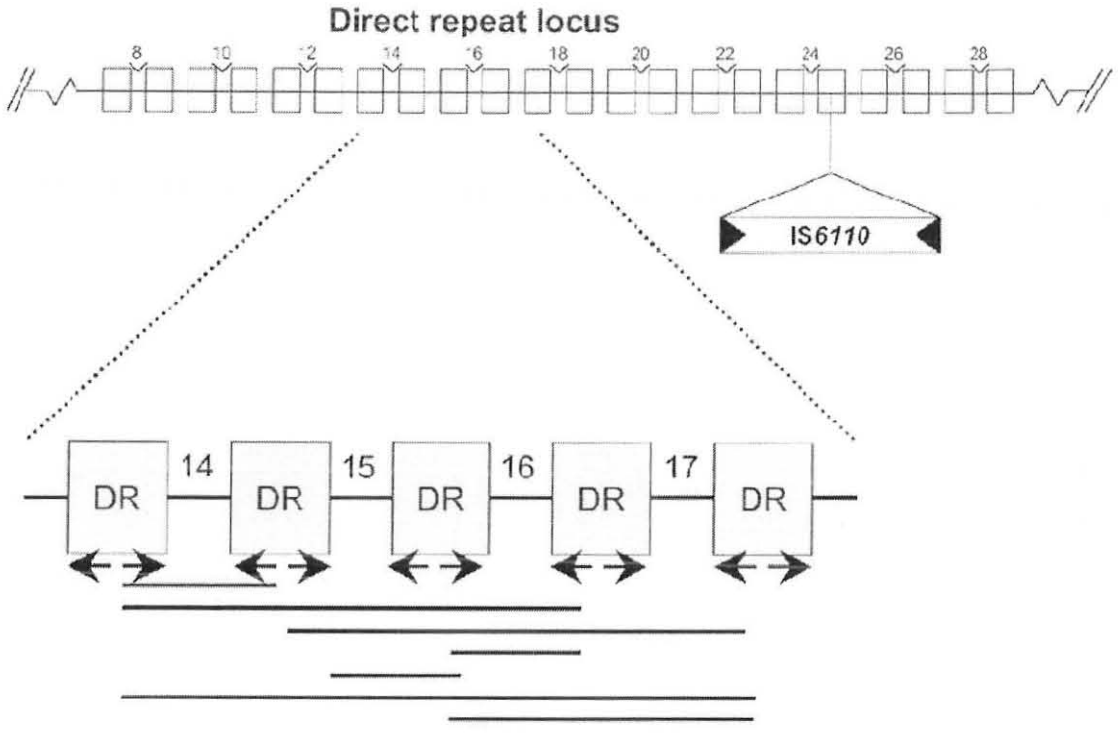

Figure 2: Schematic diagram of a segment of the direct-repeat locus in the mycobacterial genome. The direct-repeat sequences are depicted as rectangles, interspersed by variable length spacer regions, labeled 8–28. The in vitro amplification of the DR region by PCR is represented by the expanded diagram, which illustrates a series of five direct repeats containing spacer regions 14–17. Spoligotyping primers directed against terminal sequences of the DR region are shown as outward facing arrows, whereas the variable-length polymerase chain reaction products generated from these primers are indicated as solid lines below the diagram.

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

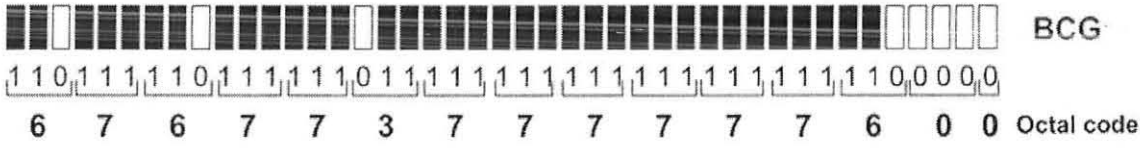

Figure 3: Diagram demonstrating the hybridization pattern (spoligotype) and octal code derivation for *M. bovis* BCG. Positive hybridization signals are represented by solid black rectangles, and negative signals are indicated by empty rectangles. The numbers directly below rectangles are the assigned value of 0 or 1, for negative and positive hybridization signals, respectively. The octal code is derived from these values by first allocating the

individual signals into 14 sets of triplets plus one additional singlet. Within each triplet, the first position is arbitrarily assigned the value of 4, the second position is assigned the value of 2, and the third position is assigned the value of 1. These values are then summed for each positive signal within the triplet to arrive at a single digit. The 15 individual digits then form the unique octal code for an isolate.

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

In general, the finding of genetic differences between two strains of a *Mycobacterium* species implies that they presumably originate from different sources, which is a fundamental question to be answered during a disease outbreak. Confidence in this assumption can be increased by using more than one typing system to detect genetic differences, as various genetic markers target changes or mutations within different parts of the mycobacterial genome (van Soolingen *et al.*, 1995; Kaufmann and Hahn, 2003; Thoen and Barletta, 2004).

2.7. Molecular epidemiology of *Mycobacterium tuberculosis complex*

The development of DNA fingerprinting techniques for typing *Mycobacterium tuberculosis* complex isolates during the last decade has led to an increasing number of studies of the molecular epidemiology of tuberculosis. The techniques have led to improvements in the identification and recognition of subspecies of the *Mycobacterium tuberculosis* complex such as *Mycobacterium tuberculosis* H37Rv, *M. bovis* BCG and *M. microti* (Ferdinand *et al.*, 2004; Smith *et al.*, 2005; Sophie *et al.*, 2006) which were previously difficult to identify using biochemical procedures. Two new species have been identified within the complex, namely *M. canettii* which is the most divergent subspecies within the complex, and *M. tuberculosis* subspecies. *caprae* subspecies. *nov.* which has been isolated mainly from goats (Aranaz *et al.*, 1999; Ferdinand *et al.*, 2004; Smith *et al.*, 2005)

Strain typing in different geographic areas has revealed that the population structure of *M. tuberculosis* complex isolates differs significantly between settings with a low and a high incidence of tuberculosis. This has led to extensive research on selective advantages of predominant genotypes, such as the 'Beijing' genotype of *M. tuberculosis*, which is highly prevalent in Asia. Selection of predominant *M. tuberculosis* genotypes may have implication for research on the development of new tuberculosis vaccines (Ferdinand *et al.*, 2004; Smith *et al.*, 2005).

The recent introduction of whole genome analysis has provided possibilities to associate the presence or absence of particular genes present in complex with pathogenicity and transmissibility (Ferdinand *et al.,* 2004). This genetic marker, with a low turnover, will also provide new insight in the evolutionary development of the *M. tuberculosis* complex and offers possibilities for a renewed identification of taxons within the complex (Smith *et al.*, 2005; Sophie *et al.*, 2006).

2.8. Distribution

Based on estimated number of incident cases in human, India, China, and Indonesia are the top among the world's 22 high-burden tuberculosis (TB) countries. Ethiopia ranks seventh in this report. The country had an estimated 314,267 TB cases in 2007, with an estimated incidence rate of 378 cases per 100,000 populations (WHO, 2009).

However, the distribution and incidence of zoonotic tuberculosis probably remains underestimated, as a distinction between the *Mycobacterium* species, i.e. *M. bovis* and *M. tuberculosis*, is not systematically performed, especially in developing countries (Anonymous, 2006; Sarah *et al.*, 2007). Industrialized countries have eradicated or drastically reduced the prevalence of *M. bovis* infection in both animals and humans through animal tuberculosis control and elimination programmes, improvements in milk hygiene, pasteurization and awareness creation on the zoonotic and economic significance of *M. bovis* infection. In developing countries, however, animal TB is widely distributed because control measures are not applied or are applied sporadically and milk pasteurization is rarely practiced (Cosivi, 1998; Amanfu, 2006).

Data on the occurrence of animal tuberculosis in developing countries like Africa are generally scarce. It is difficult to measure the precise morbidity of the disease in some countries and only qualitative assessment often not supported by laboratory or field investigations, are submitted to OIE (Amanfu, 2006; Sarah *et al.*, 2007).

In Ethiopia bovine tuberculosis is endemic, although the epidemiology and zoonotic importance of the disease is not well known due to lack of nation-wide investigation. However, the disease has frequently been reported in Ethiopia from small scale studies, though the magnitude of the impact of BTB on animal health is largely unknown. Since the

disease is known to affect a large host range (cattle, goat, sheep, camels, humans, and wildlife) it makes its assessment difficult (Ameni *et al.*, 2006; Ameni *et al.*, 2007; Yimenu and Demissie, 2008; Gezahegne *et at.*, 2009).

2. 9. Risk factors to mycobacterial infections

2.9.1. Host factors

One of the main host risk factors identified by numerous studies in both developed and developing countries is the age of animals. The duration of exposure increases with age; older animals are more likely to have been exposed than younger ones (Kazwala *et al.*, 2001; Cleaveland *et al.*, 2007; Inangolet *et al.*, 2008; Munyeme *et al.*, 2008). Since camel can live around 40 years (Kinne *et al.*, 2006) they are more affected than other domestic animals. Sex is the other risk factor which has been mentioned in studies carried out in cattle. Opinions diverge regarding its influence on the susceptibility to a *M. bovis* infection (Kazwala *et al.*, 2001). A cross-sectional study on imported cattle in Uganda revealed significantly more females positive to the skin test than males (Inangolet *et al.*, 2008). Sex-linked factors are probably related to management practices or behavioural habits. Regarding pastoralists in east Africa, they have preference to female animals. Therefore, based on the above analogous studies, it is most probably the risk factor of mycobacterial infection in camel. The other host factor is poor BCS an increased risk of a positive skin test result. Skin test reactors might have a poor BCS as a consequence of an advanced stage of BTB, as suggested by cross sectional study indigenous cattle carried out in Tanzania; animals suffering from a clinically advanced BTB often present a low BCS as a result of the long-lasting pathological process (Kazwala *et al.*, 2001).

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

Vertical transmission of *M. bovis* was described as host risk factor from an infected dam to her calf through congenital infection in utero (Ozyigit *et al.*, 2007). Ingestion of contaminated colostrum/ milk is another way of BTB transmission from cow to calf. The risk is more important in developing countries where control measures are not very effective (Phillips *et al.*, 2003). An animal is infected through autoinfection. For example, the oral route might further emit contaminated aerosols during the rumination process. The animal might inhale these contaminated aerosols and a subsequent respiratory infection can occur (Phillips *et al.*, 2003) and the same is true from respiratory to oral route.

2.9.2. Agent factors

All *Mycobacterium* species share a characteristic cell wall, thicker than in many other bacteria, which is hydrophobic, waxy, and rich in mycolic acids/mycolates. The cell wall consists of the hydrophobic mycolate and a peptidoglycan layers held together by a polysaccharide, arabinogalacta. The cell wall makes a substantial contribution to the hardiness of this genus (Bhamidi, 2009). Optimum growth temperatures vary widely according to the species ranging from 25 °C to over 50 °C. For example, *M. bovis* is highly susceptible to sunlight, but it can survive for several months in the environment, in moist soil and in darkness that remains viable for long periods (>6 months) in frozen tissues (Jahans and Worth, 2006).

Some species become difficult to culture (i.e. they are fastidious), sometimes they take over two years to develop in culture. Some species also have extremely long reproductive cycles. For example, *M. leprae* may take more than 20 days to proceed through one division cycle (for comparison, some *E. coli* strains take only 20 minutes), and also *M. tuberculosis* and *M. bovis* take 16-24 hours to proceed one division cycle. These making laboratory culture a slow process (Kaufmann and Hahn, 2003). In addition, the availability of genetic manipulation techniques still lags far behind that of other bacterial species (Parish and Brown, 2009).

Mycobacteria can colonize their hosts without the hosts showing any adverse signs. For example, billions of people around the world are infected with *M. tuberculosis* without never knowing it for they do not develop symptoms. Also, mycobacterial infections are notoriously difficult to treat. The organisms are hardy due to their cell wall which is neither truly Gram negative nor positive and unique to the family. They are naturally resistant to a number of

antibiotics that work by destroying cell walls such as penicillin. Also, because of this cell wall, they can survive long exposure to acids, alkalis, detergents, oxidative bursts, lysis by complement and antibiotics which eventually leads to antibiotic resistance. The other distinguishing features of pathogenic mycobacteria are the formation of characteristics cords on acid fast staining (Thoen and Barletta, 2004).

2.9.3. Environmental and management factors

Housing and feeding conditions are other important risk factors for animal tuberculosis since transmission of BTB between animals is mainly aerogenic, close contact between animals becoming a major risk factor (Menzies and Neill, 2000; Ameni *et al.*, 2006). The severity of BTB was significantly greater in cattle kept indoors at a higher population density than in cattle kept on pasture. Therefore, close contact facilitates the transmission of infective aerosols between animals. In this regard, mixing animals from different herds is common in Africa (Ameni *et al.*, 2006). Moreover, pastoralists in east Africa move from place to place with their livestock including camels and also mixing is common with other herds. This close proximity may favour the transmission of the infection between infected and susceptible animals and/or herds. The disease incidence grows more in intensive dairy farms than beef ranches. Factors that play role in predisposing animals to infection include type of production (dairy cattle are at risk), suckling (calves become infected by ingesting contaminated milk), and presence of wild reservoirs of *M. bovis* infection for grazing cattle (Quinn *et al.*, 1999).

Cattle movement, purchase, grazing field boundaries or short swards and spreading cattle slurry on the fields are a potential risk for transmission of the disease between cattle (Thoen *et al.*, 2006).

Circumstantial evidence suggests that an adequate intake of mineral, vitamin and protein could reduce the susceptibility of cattle to BTB infection (Thoen *et al.*, 2006). Although weather patterns implicate in the susceptibility of herds, it is concluded that some reduction in the susceptibility of cattle to *M. bovis* infection can be achieved by modifications of the management practices to minimize the weather risk factors (Thoen *et al.*, 2006).The arrival of an infected animal in a BTB free herd serves as one of the major risk factors for introducing of the disease (Gopal *et al.*, 2006).

Movement of animals was recently shown to be a critical risk factor in a study conducted on analyzing cattle movements. For instance, molecular typing demonstrated animal movements as greatly responsible for most outbreaks reported (Gopal *et al.*, 2006). This specific factor retains a major impact on animal movements from an endemic zone to a BTB-free zone (Oloya *et al.*, 2007; Munyeme *et al.*, 2008).

2.9.4. Co- infection with HIV/ADIS

One of the greatest challenges in the fight against the global TB epidemic is the HIV epidemic. The vast majority of people carrying this dual infection live in developing countries (Cosivi *et al.,* 1998; WHO, 2009). The occurrence of both infections in one person makes TB infection very likely to progress 5% to 15%, depending on their level of immunosuppression to active disease. Before 15 years HIV seroprevalence rates greater than 60% found in TB patients in various African countries (Raviglione *et al.,* 1995).

The epidemic of HIV infection in developing countries, particularly countries in which *M. bovis* infection is present in animals and the conditions favor zoonotic transmission, could make zoonotic TB a serious public health threat to persons at risk (Grange *et al.*, 1994; Cosivi *et al.,* 1995). For instance, a study based on genotyping of isolates from Co-infection with TB and HIV/AIDS in Ethiopia has shown of higher zoonotic tuberculosis infection in HIV positive patients 9% (Kidane *et al.*, 2002).

Recently, there has been increased interest among public health officials in drug-resistant strains of *M. tuberculosis*, *M. bovis*, and *M. avium*. Because several such strains have been isolated from HIV-infected and non-immunocompromised humans. (Dupon and Ragnaud, 1992; Dankner *et al.*, 1993; Charles *et al.*, 2006)

HIV infected patients contract less of the pulmonary TB, and thus harder to detect by sputum microscopy, one of the cornerstones for TB diagnosis in resource limited settings, where HIV-TB co infections are actually highest (Asiimwe, 2008)

2.10. Transmission

There are different modes of spread of tuberculosis between camelid herds. One is the introduction of an infected animal into a non-infected herd (Bush *et al.*, 1990). Gatt Rutter and Mack in (1963) reported that in Egypt, tuberculosis did not occur in nomadic camels but in those belonging to farmers who kept them in close contact with cattle. The mode of transmission of tuberculosis is unknown in camelids, but it is presumed similar to that in cattle. In cattle it is mainly horizontal. It is believed that camelids suffering from pulmonary tuberculosis infect healthy animals via aerosols. The alimentary, congenital, venereal and cutaneous routes that may occur in cattle have not been described in camelids(Wernery *et al.*, 2002).

2.11. Public health significance of *M. bovis*

Shortly after *M. tuberculosis* and *M. bovis* were discovered to be unique organisms, controversy arose regarding the latter's ability to pose a significant health threat to humans. Robert Koch, for example, did not believe that bovine strains of tuberculosis represented a sufficient danger to humans such that their eradication was necessary (Dankner *et al.,* 1993). Subsequent work by a British Royal Commission and others showed, however, that humans could become infected by drinking contaminated milk, with the resulting disease commonly manifesting as lymphadenitis in children, or via inhalation of droplet nuclei produced by sick cattle, which could result in pulmonary disease (Dankner *et al.,* 1993). In response, *M. bovis* eradication programs were begun in cattle herds, along with widespread pasteurization of dairy products. These measures produced a sharp decline in the incidence of human *M. bovis.* By the 1990s less than 1% of human tuberculosis cases in industrialized countries were caused by this organism (O'Reilly and Daborn, 1995).

Nowadays zoonotic tuberculosis has been largely controlled in most developed countries.Because of pasteurization of cows' milk and systematic culling of cattle reacting to compulsory tuberculin tests. The risk of contracting *M. bovis* infection from animals appears to be extremely low in industrialized countries (Charles *et al.,* 2006).

On the other hand, in most developing countries, human TB is widely known to be caused by *M. tuberculosis*; however, an unknown proportion of cases are due to *M. bovis*. In Africa,

BTB is widespread throughout the content, but very little is known about risk factors for *M. bovis* infection in either human or cattle populations. A study in rural part of Tanzania already confirmed *M. bovis* in 7 of 65 (10.8%) human cervical adenitis cases, of which only one came from a household owning infected cattle with *M. bovis* (Sarah *et al.*, 2007). This infection in humans is under-reported as a result of the diagnostic limitations of many laboratories in distinguishing *M. bovis* from *M. tuberculosis*. For example, none of the national reports submitted to the OIE and WHO by African member states mention the importance of *M. bovis* in human TB cases. Moreover, consumption of unpasteurised milk and poorly heat-treated meat and close contact with infected animals represent the main sources of infection for humans (Ayele *et al.*, 2004).

In Ethiopia bovine tuberculosis is endemic, although the zoonotic importance of the disease is not well known like other developing countries (Ameni *et al.*, 2006; Ameni *et al.*, 2007; Yimenu and Demissie, 2008). Few study in different parts of the countries confirmed *M. bovis* infection in human. For example, a study conducted on cattle owned by TB patients had higher prevalence (24.3%) than cattle owned by non tuberculous owners (8.6%). And the study also noted that 16% of 42 human isolates were identified as *M. bovis* (Regassa *et al.*, 2008)

3. MATERIALS AND METHODS

3.1. Description of the study sites and population

3.1.1. Study sites

The study was conducted on camels slaughtered at Jigjiga, Harar , Aweday, and Dire Dawa municipality abattoirs brought from parts of Eastern Ethiopia pastoral areas. These regions are located 500–600 km east of Addis Ababa. The areas differ in both topography and climate. Harar is approximately 2000 m above sea level and the surrounding farming areas are semi-arid. Jigjiga is located at a lower elevation (1600 m), and Dire Dawa is in the Rift Valley at 1100 m. Dire Dawa and Jigjiga districts are hotter and drier and are generally more arid than those of the Aweday and Harar.

Meat inspection in this abattoir is routine procedure in cattle and small ruminants but camel carcass inspection was not properly performed by meat inspectors. The average number of camels slaughtered per week was 2 at Aweday and Harar, 4 at Dire Dawa and 7 in Jigjiga abattoirs.

3.2. Study population

The study was carried out on apparently healthy slaughtered camels. The camels slaughtered at the abattoir were both male and female originated from Eastern pastoral parts of the country.

3.3. Study design

A cross sectional study was carried out to determine the prevalence of camel TB in slaughtered camels in four municipal abattoirs found in Eastern Ethiopia Jijgiga, Harar, Aweday and Dire Dawa on the basis of PM examination and, mycobacterial culture and molecular characterization of the isolates to assess the circulating organisms. Census type sampling of slaughtered camels was used to collect all the tissue samples in between December 2010 and April 2011. Tissue samples from different lymph nodes, lung tissue parts

and other organs were aseptically collected exactly corresponding to other factors of the same camel.

3.4. Sampling procedures

Census type sampling of slaughtered camels was used to collect all the tissue samples. At first the work was established in Dire Dawa municipality abattoir where on average 4 camels was slaughtered per week but due to the low number of camel Aweday, Harar and Jigjiga abattoirs were included where an average number of camel slaughtered per week were 2,2, and 7 respectively .

3.5. Study methodology

3.5.1. Body condition scoring and age estimation

BCS estimation was done based on hump which reflects internal fat reserve and superficial body conformation such as ribs cage and coxal tuber according to (CACIA, 2001). On the basis of this the BCS of camels examined during the study period were scored as 1-5 (AnnexVI).Moreover age of the camel was determined based on teeth eruption. Even if some minor variation existed, the most convenient teeth for age estimation purposes were the incisors (front teeth), canines and the first of the premolars according to (CACIA, 2001) (Annex VII)

3.5.2. Post mortem inspection

For this study through postmortem examination was conducted following the procedure described by Croner (1994) on organs and tissue lymph nodes which are expected to have tubercle lesions. These tissues were dissected from the surrounding tissue and carefully visualized for any change in colour or morphology, palpated for detection of any alteration in its consistency and incised for examining the cut surface for the presence or absence of abscess and tubercles, these all were done under a bright light source.The lymph nodes included in postmortem examination were mandibular, retropharyngeal, left and right bronchial, mediastinal, mesenteric, hepatic and the lungs and other organs such as liver, gastrointestinal. The lobes of the two lungs, including the left apical, left cardiac, left

diaphragmatic, right apical, right cardiac, right diaphragmatic and right accessory lobes, were inspected and palpated externally. When gross lesions suggestive of tuberculous detected in any of the tissues, the tissue was classified as having lesions.

3.5.3. Isolation of mycobacteria

Mycobacterial isolation from tissue lesions

For mycobacteriological isolation tuberculous lesions from slaughtered camels were aseptically collected adapted from cattle in accordance with OIE (2004) 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, mandibular and parotid), lymph nodes of lungs (mediastinal and bronchial), mesenteric lymph nodes, hepatic lymph node and other organs. Tissue samples were put in sterile universal bottles with about 5 ml of 0.9% saline solution and kept in a deep freeze ($-20C^0$) in accordance with the proximity to the abattoir were collected (at Dire Dawa Veterinary Diagnostic and Investigation Laboratory, Harar and Jigjiga Veterinary Clinic) until being transported to Aklilu Lemma Institute of Patho Biology (ALIPB). Tissue samples were transported in icebox to keep the cold chain. Samples were kept at +2-8°C for mycobacteriological culturing at TB laboratory.

Then, the preserved samples were further processed for isolation of mycobacteria in accordance with the Office International des Epizooties (OIE, 2004) protocols. Thus, in the laboratory samples were processed under a biological safety cabinet the specimens were sectioned using sterile blades, scissors and homogenized with a mortar and pestle. The homogenate were decontaminated by adding an equal volume of 4% NaOH on the sample in order to remove other environmental bacteria. Then immediately centrifugation at 3,000 rpm for 15 minutes to concentrate the mycobactria. 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 (OIE, 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 8-12 weeks with weekly observation on growth of colonies.

3.5.4. Microscopic examination

After initial identification of mycobacterial species were made based on the rate of growth, pigment production and colony morphology. Ziehl-Neelsen staining was performed to confirm the presence of acid–fast bacilli (AFB) on positive cultures (Quinn *et al.*, 1994).

Direct smears were made from the positive cultures for direct microscopic examination and stained using the Ziehl-Neelsen staining technique. Smears were heat fixed, flooded with concentrated Carbol fuchsin, heated gently to steam for 10 minutes. Then the stain was poured off and the smears were washed with tap water and decolorize with 3% HCl in 95% alcohol for 1 minute, with slides being washed under tap water between each step. The smears were then counter stained with methylene blue for 3 minute , air dried and examined under the 100x oil immersion objective of a light microscope for the presence of Acid Fast Bacilli (AFB) (Quinn *et al.,* 1994).

AFB confirmed positive cultures were preserved with freezing media while at the same time heat killed in water bath at 80°C for 45 minutes. The freeze and heat killed isolates were put at (-20 °C) for further mycobacteriology and genotyping analysis.

3.5.5. Molecular characterization of mycobacterial isolates

Multiplex polymerase chain reaction (PCR) (Mycobacterial genus typing)

The multiplex PCR differentiate *M. tuberculosis* complex from *M.avium* complex, *M.intracellularae* and other mycobacterial species. Mycobacterial genus typing was conducted as described by Wilton and Cousins (1992). Heat killed AFB positive samples were used as source of DNA template. DNA amplifications was done in thermocycler with 20 µl reaction volumes consisting: 5 µl of genomic DNA as a template, 8 µl HotstarTaqMasterMix ($MgCl_2$, dNTP, Taq polymerase and PCR buffer) for each sample, 0.3 µl internal primer per sample, 0.3 µl forward and reverse primer per each sample and 5.2 µl per sample of Qiagen water. The primers used for amplification were MYCGEN-F, 5`AGA GTT TGA TCC TGG CTC AG 3` (35ng/µl); MYCGEN-R, 5`TGC ACA CAG GCC ACA AGG GA 3` (35ng/µl); MYCAV-R, 5` ACC AGA AGA CAT GCG TCT TG 3`(35ng/µl); MYCINT-F, 5`CCT TTA GGC GCA TGA TGT CTT TA 3`(75ng/µl); TB1-F, 5` GAA CAA

TCC GGA GTT GAC AA 3` (20ng/µl); TB1-R, 5` AGC ACG CTG TCA ATC ATG TA 3` (20ng/µl). *M. bovis* strain 2122/97 and *M. avium* were used as positive control while Qiegen water was used as negative control.

The reaction mixture was then heated in Programme Thermal Controller (Applied biosystem; PTC- 100TM) cycle using the following amplification program: 95^0C for 10 minutes for enzyme activation; 95°C for 1 minute for denaturation; 61^0C for 0.5 minute for annealing; 72^0C for 2 minutes for extension, involving 35 cycles all in all; and final extension at 72^0C for 10 minutes.

Agaros gel electrophoresis

After amplification of mycobacterial DNA the PCR products were processed in agaros gel electrophoresis. 0.8 % agrose gel was prepared by dissolving agaros powder in 1x TAE buffer in a flask and melting in microwave oven then adding 1: 10 Ethidium bromide (10000 x dilutions) to melted agaros gel and pouring the melted agaros gel over gel casting tray assembled on a plane surface and combs were inserted into the agaros and allow to solidify for 30-45 minutes. The solidified agaros gel was immersed in an electrophoresis chamber filled with1xTAE running buffer. Then the combs were removed carefully. Then 10 µl (1:4 loading dye to PCR product) sample was loaded into the agaros wells (lanes). 5µl of 100bp DNA marker was loaded on the first lane (well). Then electrophoresis tark to the power supply was connected and separation of PCR products was achieved by running at 120V and 250mA for 40 minutes. The gel was removed from the electrophoresis apparatus with gloved hand and; it was examined on UV illuminator in the dark and visualized using in Multi–imageTM light cabinet using Alpha innotech version 1.2.0.1(Alpha Innotech Corporation). DNA molecules were identified as orange bands on the gel and were photographed using AlhpaImagerHP camera connected to a computer. The image was opened using AlhpaImagerHP software which was applied to manipulate the image and transferred to Microsoft power point for appropriate edition and documentation. Thus black and white were produced as presented in the results sections. All members of the genus Mycobacterium produce a band of 1030bp, *M. avium* or subspecies such as *M. avium subspecies paratuberculosis* produces a band of 180bp while members of *M. tuberculosis* complex produce a band with 372bp.

3.6. Data management and analysis

Data were classified, filtered, coded using Ms Excel 5, and transferred to STATA software V. 11. There after analyzed according to the different variables. Pearson chi-square was used to evaluate the statistical significance of variables. Bivariate and multiviarate regression analysis were performed using STATA 11. A probability value $P < 0.05$, and the 95% confidence interval for odds ratios (OR) does not include 1 was considered statistically significant.

4. RESULTS

4.1. Prevalence of tuberculosis and analysis of different risk factors

The prevalence of camel TB was 12.3 (36/293) on the basis of detail post mortem examination. Mycobacteria were isolated in 66.7% (24/36) of the camels with suspicious TB lesions.

The association of risk factors sex, age, and body condition with prevalence rate are-indicated in Table1.The difference in prevalence rates of TB was statistically significant in females (χ^2=7.474,P= 0.006) and in poor body condition (χ^2=,7.323,P= 0.025) camels when compared to their counter parts. However, no statistically significant difference was found for the different age categories.

Table.1: Association and Logistic regression analysis of host risk factors with post mortem TB lesion.

Characteristics		№ (%) Examined	№ (%) PM Positive	χ^2	P-Value	Crude OR (95%CI)	Adjusted OR (95%CI)
Sex	Female	141(48.1)	25(17.7)	7.474	0.006*	1	1
	Male	152(51.9)	11(7.2)			0.36* (0.17-0.77)	0.43 (0.19-0.97)
Age	≤ 5 years	19(6.5)	1(5.3)	1.125	0.656	1	1
	6-10years	111(37.9)	13(11.7)			2.4 (0.29-19.40)	1.33 (0.15-12.13)
	>10 years	163(55.6)	22(13.5)			2.80 (0.37-22.10)	1.29 (0.142-11.69)
BCS	Poor	126(43)	23(18.2)	7.323	0.025*	1	1
	Medium	152(51.8)	12(7.9)			0.38* (0.18-0.81)	0.52 (0.23-1.17)
	Good	15(5.1)	1(6.7)			0.32 (0.04-2.6)	1.06 (0.11-10.12)

BCS = Body Condition Scoring; χ2=Chi square; CI=Confidence Interval; OR=Odds Ratio; PM=Postmortem;*statistically significant

4.2. Distribution/location of TB lesion

Out of 36 animals with suspicious TB lesion, 24(66.67%) harbour the lesion on their lungs, 8 (22.22%) on lymph nodes and 4(11.11%) on both lungs and lymph nodes. Macroscopically the most common lesions encountered in affected organs and/or lymph nodes were encapsulated caseouse masses and circumscribed grayish white calcified masses of various sizes. Thick grayish white exudate, pus, was also found with some area of consolidation in retropharyngeal, sub-mandibular, prescapular lymph nodes and lungs.

Table.2: Distribution and tropism of tuberculous lesions in post mortem positive camels with lesions in at least one tissue or organ with their respective culture positivity.

Anatomical Site	Tissue/organ	№(%) of Positive Specimens	№(%) of Culture Positive
Head	Retropharyngeal ln.	4(9.1)	3(75)
	Sub mandibular ln.	3(6.8)	1(33.3)
	Parotid ln.	0	0
Total		**7(15.9)**	**4**
Thorax	Tracheobronchial ln.	3(6.8)	3(100)
	Lung	28(63.6)	14(50)
	Mediastinal ln.	4(9.1)	3(75)
Total		**35(79.5)**	**20**
Abdomen	Mesenteric ln.	1(2.3)	0
Total		**1(2.3)**	**0**
Carcass	Prescapular ln	1	0
Total		**1(2.3)**	**0**

In relation to distribution of tubercles lesion with anatomical sites the highest was found in lung and its associated lymph nodes in thoracic cavity (79.5%) followed by retropharengal and submandibular lymph nodes in the head region(15. 9%). In contrast the least lesion was found in abdomen (mesenteric lymph node) and carcass (prescapular lymph node) with 2.3% each.The average number of lesions per infected animal was 1.2 and about 81.8% of postmortem examination positive camels possessed only a single lesion.

4.3. Mycobacterial isolation

Of the 36 camels with grossly suspicious TB lesion in their organs and tissues cultured mycobacterial growth was observed in 66.7% (24/36).The rate of mycobacterial isolation was highest in the tracheobronchial lymph node (100%) (Table 2) followed by retropharyngeal and the mediastinal lymph nodes with similar percent (75%) where as lung and mandibular lesions were less often mycobacterial culture positive with positivity of 50% and 33.3%, respectively. The least of all mycobacterial culture positivity were found from mesenteric and prescapular lymph nodes lesions.

4.4. Molecular characterization of the isolates

Multiplex PCR.

Out of the 24 culture isolates *Mycobacterium* genus typing was carried out on the 17 culture isolates. From which 15 were identified as member of mycobacteria other than *Mycobacterium tuberculosis complex* and only one as member of *Mycobacterium tuberculosis complex.* Not any of the isolates were *M. avium complex* (Figure 4).

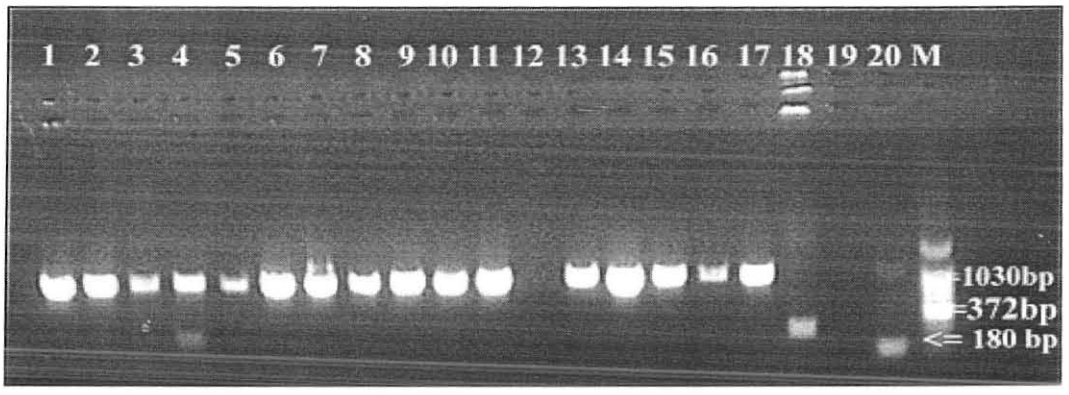

Figure: 4. Multiplex PCR results of mycobacteria isolated from camels with grossly suspicious TB lesion in their tissues and organs. Lanes 1-17 were isolates from individual camels with tubercles lesions. Lane 18 = *Mycobacterium tuberculosis complex* (positive control), Lane 19 = H_2O (negative control), Lane 20 = *Mycobacterium avium complex* (positive control), Lane M = 100bp DNA Ladder. Lane 1,2,3,5,6,7,8, 9, 10, 11, 13-17 were positive for genus *Mycobacterium* and Lane 4 was positive for *Mycobacterium tuberculosis* complex.Lane 12 was negative for all positive controls.

5. Discussion

The present study report a 12.3% (36/293) prevalence of camel TB based on detailed post-mortem examination and a prevalence of 8.19% (24/293) based on culture result. This finding is lower than the previous reports in Kenya based on intradermal tuberculin testing in camels (Wernery *et al.*, 2002) and while it is similar to the previously reported prevalence 12.5% by (Hussen, 2006) on cattle based on abattoir survey.But higher than the report of previous study in the Afar Region of Ethiopia by (Hussen, 2009), based on Comparative tuberculin test and in Dire Dawa and Akaki abattoirs by Gezahegne *et al.* (2009) and Gezahegne *et al.* (2011) based on detailed post-mortem examination. It is also higher than the prevalence reported in Egypt (Refai, 1992). Analogously the prevalence in this study is also higher than previous reports on abattoir survey for bovine TB in cattle (Teklu, *et al.*, 2004; Regassa, 1999).

However since the actual prevalence of bovine TB in pastoral production system is not yet known (Shitaye *et al.*, 2007) these reports on abattoir survey for bovine TB on cattle mainly originated from extensive husbandry system (more in high lands) might not give a great significance as indicative of prevalence of bovine tuberculosis in camel rearing pastoral community (in the low land areas).

Almost all of the camels slaughtered in the abattoir originate from the pastoral areas where the pastoralists depend on the milk of their camels for subsistence and where no treatment of milk is allowed either it is consumed row or when just soured. As *M.bovis* is inactivated by pasteurization, mainly raw camel milk plays a role in transmission of tuberculosis into human, even if *M.bovis* is not capable of growing in milk (Eberlein, 2007). Therefore the prevalence rate of 12.3% found in this study will show the greatest health implication of the disease on the pastoral community which was also further strengthen by the isolation of bacteria belonging to *Mycobacterium tuberculosis* complex group in this study.

Tuberculous lesions were found most frequently in the lungs and associated lymph nodes (81.8%) followed by lymph nodes of the head (15.9%) which is in consistence with Gezahegne *et al.* (2009) and Ashenafi *et al.* (2008) in camel and (Whipple *et al.*, 1996, Croner, 1994) where 60% and 56% TB lesions were reported, .in the lunges and associated lymphnode. Croner (1994) reported that up to 95% of cattle with visible TB lesion could be

identified by examination of lungs and associated lymphnodes; this finding suggests inhalation as the principal route of TB infection in cattle which is also true in camel due to the higher proportion of macroscopic lesions obtained in lunges and associated lymph nodes in this study. Presence of lesions in the mesenteric lymph nodes indicates the infection through ingestion (Radostitis *et al.*, 1994). However it is inconsistent with the report by Gezahegne *et al* (2011) which report higher distribution, and frequency, of lesions in the mesenteric lymph nodes than the thoracic.

The average number of infected tissues per infected camel was 1.2 while 81.8% of the camels had only a single lesion. This finding complies with previous report on camel (Ashenafi *et al.*, 2008, Gezahegne *et al.*, 2009).

The difference in prevalence rate based on sex was statistically significant (χ^2 = 7.474; P =0.006) which is in consistent with (Gezahegne *et al.*, 2011) in camel. The higher proportion of prevalence of the disease in female cattle is indicated as being possibly due to their longer productive life and other stressful factors (such as pregnancy, parturition, lactation, etc.) associated with female cattle (Miliano-Suuzo *et al.*, 2000; Teklu *et al.*, 2004 and Radostitis *et al.*, 1994) which also may apply true in female camel. The increased proportion of prevalence in female camel can be also due to the fact that almost all female camel slaughtered at the slaughter house were old (>10 years). As explained by Barwinek and Taylor (1996), this could be due to the fact that as the age increase the probability of acquiring tuberculous infection also increases, this might be the case in camels too.

In contrast to sex based study, the difference in prevalence rate and age was not statistically significant which is in consistence with previous reports (Gezahegne *et al.*, 2009; Gezahegne *et al.*, 2011). Animals older than ten years had the highest prevalence (13.5%) compared to other age groups. This is in consistent with Abdurrahman and Bornstein (1992); indicated TB as chiefly disease of old camels. This may be the fact that, in spite of their susceptibility to infection, camels are very resistant to the effects of the disease and long period elapses before they become chahectic and emaciated (Manson, 1917) and also to develop tuberculous lesion. Other researchers have also reported in cattle particularly that older animals are affected by TB (Kazwala *et al.*, 2001; Cleaveland *et al.*, 2007; Inangol *et al.*, 2008; Munyeme *et al.*, 2008) which could be due to the fact that older animals have weaker immune system.The reason mentioned above in case of older female by Barwinek and Taylor (1996) also hold true.

The statistically significance difference found between body condition score (poor body condition) (χ^2 = 7.323; P =0.025) and prevalence rate of camel TB, may arise from the resistance of camels to develop tuberculous lesion before they become chahectic and emaciated as indicated by Manson (1917).

Mycobacterium genus typing of the 17 isolates using multiplex polymerase chain reaction identified 88.2% (15/17) of them as member of mycobacteria other than *Mycobacterium tuberculosis complex* and only one as member of *Mycobacterium tuberculosis complex*. The isolation of nontuberculous mycobacteria from most of camel TB lesions having tuberculous nodules with granulomatous and caseous lesions in lymph nodes and lung of camel like those caused by *M. bovis* and *M. tuberculosis* in this study complies with previous report on camels by Gezahegne *et al.* (2011). Elmossalami *et al.* (1971) in Egypt and Strauss (1995) in Germany also reported the isolation of NTM from tuberculous like lesions in camel causing a similar lesions like those caused by classical forms of mycobacteria (*M. bovis* and *M. tuberculosis*). Moreover the isolation of NTM in Ethiopia, from cattle with tuberculous lesions in different regions of the country by Berg *et al.* (2009) and from wild animals and man by Tschopp *et al.* (2010) could further indicate their widespread geographical distribution and pathogenic or opportunistic role in the pathology of TB in a broad range of host.

6. CONCLUSIONS AND RECOMMENDATIONS

The present study recorded 12.3% prevalence of TB in camels on the basis of suspicious lesions. However, the proportion of isolation of *M. tuberculosis complex* from the suspicious lesion was low. Majority of the isolates from most of the suspicious lesions were Mycobacteria other than the *M. tuberculosis complex* which could suggest the pathogenic or opportunistic role of NTM in camel tuberculosis. Moreover, the common habit of consuming raw camel milk and meat in pastoral community may signify the possible potential role of risk factors for acquiring zoonotic TB.

On the basis of this conclusive remark, the following recommendations were forwarded:

- Further identification and characterization of the nontuberculos mycobacteria members that were most frequently isolated from suspicious TB lesions and investigation of their pathogenic or oportonistic role in the pathology of camel TB,

- Identification of the the nontuberculos mycobacteria members and one camel isolates of *M. tuberculosis* complex that were isolated from suspicious TB lesions at species and strain level using RD4 deletion typing and spoligotyping,

- Public education and creation of awareness in the society on safe use of camel milk and meat,

- Further investigation of the disease is also a basic requirement so as to effectively elucidate its epidemiological significance for public health and control of the disease in the region.

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8. ANNEXES

Annex I: Preparation of Löwenstein-Jensen egg-based medium (SOP)

1. Preparation of mineral salt solution

Approximate Formula* Per 600 mL

1.1. Ingredients

- Potassium dihydrogen phosphate anhydrous (KH2PO4) = 2.4 g
- Magnesium sulphate (Mg SO_4. $7H_2O$) = 0.24 g
- Magnesium citrate ($Mg_3(C_3H_5O_7)_2$) = 0.6 g
- Asparagines =3.6 g

1. Weigh and add the above listed minerals salt ingredients in to a flask that labeled with P (Pyruvate) and G (Glycerol),
2. Add 300ml distilled water to each flask,
3. Add 12ml of Glycerol (*M. tuberculosis*) to the flask labeled with G,
4. Add 10g of Pyruvate (*M. bovis*) to the flask labeled with P,
5. Dissolve the mineral salt ingredients to the distilled water by heating,
6. Autoclave the minerals salt solution at 121oc for 30 minutes to sterilize,
7. Cool at room temperature before use,
8. Keep the solution indefinitely and may be kept in suitable amounts in refrigerators.

2. Preparation of 2% malachite green solution

2.1. Ingredients

- Malachite green dye 2.0g
- Sterile distilled water 100 ml

Using aseptic techniques dissolve the dye in sterile distilled water by placing the solution in the incubator for 1-2 hours.

3. Preparation of homogenized whole eggs

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

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

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

- Measure and add 500ml of homogenized eggs in to sterile flask that labeled with P andG,
- Add 300ml mineral salt solution to each flask(remember to add P labeled salt solution to P flask and labeled and G labeled mineral salt to G labeled),
- Add 10ml malachite green solution,
- Steer well the complete medium
- Distribute the complete egg medium in 6-8 ml volumes in sterile 14 or 28ml McCartney bottles or in 20 ml volumes in 20 x150 mm screw- capped test tubes and the tops tightly closed,
- Inspissate the medium within 15 minutes of distribution to prevent sedimentation of the heavier ingredients.

5. Coagulation of medium (Inspissation)

- Before loading heat the inspissations to 80oc to hasten the build up of the temperature,
- Place the bottles in a slanted position in the inspissator and coagulate the medium at 80-85oc for 45 minutes.

6. Sterility checking

- After inspissation , the whole batch of media or a representative samples of culture bottles should be incubated at 35-37oc for 24 hours as a sterility check

7. Storage

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

Annex II: Culturing of tissue sample

1. Homogenization of tissue samples

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

2. Decontamination

- Add equal volume 4% NAOH to centrifuge tube
- Mix well for about 10 minutes
- Centrifuge the mixed liquid at 3000 rpm at 4oc for 15 minutes
- Decant the supernatant
- Mix the sediment

3. Neutralization

- Add drop of phenol red indicator to well mixed sediment and mix well until it becomes purple

- Add 1% HCL to the sediment and mix well until it becomes yellow

4. Inoculation

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

5. Incubation

- Incubate at 37oc with 10% C02 air at slant position for a week,
- Then put at upright position for 8 weeks at the some temperature and air condition until complete growth is observed.
- Monitor every week for growth

Annex III: Ziehl-neelsen stain reagents preparation

1. Carbol-Fuchsin Solution:

- Basic fuchsin 2.5 gm
- Distilled water 250.0 ml
- 100% alcohol 25.0 ml
- Phenol crystals, melted 12.5 ml
- Mix well, filter into brown bottle. Label bottle with date and initials, solution is stable for 1 year.

Caution: Carcinogen, toxin.

2. 1% Acid Alcohol:

- Hydrochloric acid 10.0 ml
- 70% Alcohol 990.0 ml
- Mix well, label with date and initials, stable for 1 year.

Caution: Flammable, corrosive.

3. Methylene Blue

3.1. Stock Solution:

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

3.2. Working Solution:

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

Caution: Avoid contact and inhalation

Annex IV: Ziehl-Neelson staining procedure for AFB

- Mix well the sample/sputum/ isolated colonies and make the smear,
- Fix the smear with heat,
- Flood the slide with the carbon-fishsin stain (primary stain),
- Gently heat the carbon-fuchin stain until it steam and maintain steaming for 5 minutes,
- Wash the slide thoroughly with tape water,
- Decolorelize the slide with acid alcohol (3% HCL) for 5 minutes,
- Wash thoroughly the slide with tape water,
- Flood the slide with the methyl blue/ malachite green (counter stain) for 1 minute, 72
- Wash thoroughly the slide with tape water,
- Wipe the back of the slide with cotton or gauze and and dry it,
- Examine the slide with 40x magnification power to check for quality of smear and staining,
- Add one drop of oil immersion and examine the slide with 100x magnification power.

Annex V: Preparation of freezing media

- For 450 ml of distilled water , add 2.35gm of 7H9 powder,
- Then add 1 ml of glycerol and mix,
- Autoclave at 121oc for 10 minutes,
- Let the solution to comes to 45oc,
- Then add 50 ml of Middle Brook ADC Enrichment (OADC)

2. Preparation of freezing medium

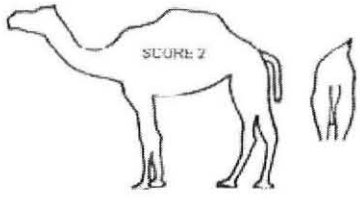
- Take 50ml of 7H9 media
- Add 25 ml of glycerol,
- Add 25 ml of distilled water,
- Mix well and thoroughly,
- Fliter into sterile container using 0.22 μm filter,
- Solution remains in use only for 3 weeks.

3. Freezing colony

- Take 1 ml of freezing media to 1.8 ml nanc tube
- Transfer 2-3 colonies from the LJ media using sterile loop to the nanc tube and also take a little LJ media with colonies and mix thoroughly.
- Label the nanc tube (similar with the label on the LJ media)
- Keep the freezed colonies contaning nanc tube at -20 o C or -80 oC

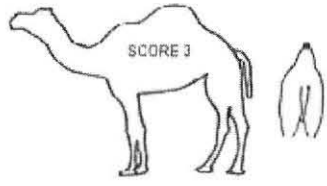
Annex VI: Description of body condition scoring in camel Hump score range is 1 - 5 based on the amount of fat in the hump

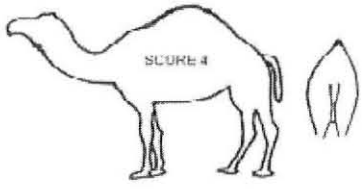
SCORE 1 Little or no fat in the hump sac, hump hairy and may be leaning to one side.



SCORE 2 Hump with moderate development rising 5% higher than chest depth, but may also be leaning to one side.

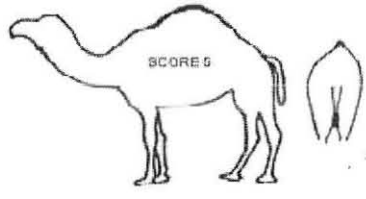
SCORE 3 Hump with good development and rising to 10% higher than chest depth. Hump is still sculptured inwards on both sides and still fits over the chest and abdominal area.





SCORE 4 Hump fully developed and rising to 15% higher than chest depth. Hump rounded outwards on both sides and runs from the shoulder to the rump.

SCORE 5 Hump over-extended and rising more than 15% higher than chest or the hump is so full that it is rounded on the sides like a semi circle.



Source: CACIA, 2001

9. SIGNED DECLARATION SHEET

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

Name: KALEAB ZEROM GEZAHAGN Signature _____ Date of submission _____

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

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