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PREVALENCE OF BOVINE TUBERCULOSIS IN SLAUGHTER CATTLE IN KENYA: A PATHOLOGICAL AND MICROBIOLOGICAL STUDY. 11

A thesis submitted to the University of Nairobi in partial fulfilment of the Masters degree in
Applied Microbiology (Bacteriology option).

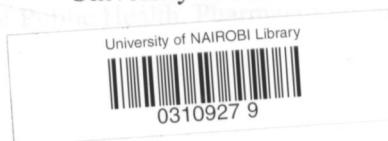
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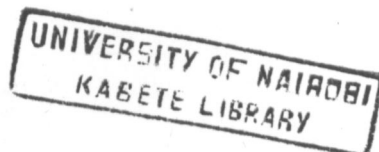
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DECLARATION

This thesis is my original work and has not been presented for a degree in any other

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LIST OF ABBREVIATIONS AND ACRONYMS

AFB.....	acid fast bacilli
ASAL	Arid and semi-arid lands
BSC.....	Bio-safety cabinet
DNA.....	Deoxyribonucleic acid
HIV/AIDS.....	Human immunodeficiency virus/acquired immunodeficiency syndrome
KMC.....	Kenya Meat Commission
L-J.....	Lowenstein-Jensen media
MAC.....	Mycobacterium avium complex
MoLD.....	Ministry of Livestock Development
MGIT.....	Mycobacterial growth indicator tubes
MOTT.....	Mycobacteria other than tuberculosis
MTBC.....	<i>Mycobacterium tuberculosis</i> complex
CTRL.....	Central Tuberculosis Reference Laboratory

OADC.....Oleic acid, Albumin, Dextrose, Catalase

OIE.....Office International des Epizooties

PANTA.....Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, Azlocilin

PCR.....Polymerase chain reaction

PZA.....Pyrazinamide

SITT.....Single intradermal tuberculin test

TB..... Tuberculosis

TST.....Tuberculin skin test

WHO.....World Health Organisation

ZN..... Ziehl-Neelsen

ABSTRACT

Bovine Tuberculosis, caused by *Mycobacterium bovis* primarily in cattle is one of the most important zoonotic diseases with a worldwide distribution. *M. bovis* is the principal agent of tuberculosis in a wide range of domestic and wild animals and has no geographical boundaries. In Africa, the disease is poorly controlled. Frequent droughts, malnutrition, high levels of poverty and immune-suppressing disease are common feature that favour the spread of bovine tuberculosis in Africa. Currently, the human HIV/AIDS pandemic has made *M. bovis* a serious public health threat and there is increasing evidence that its infections may be much more significant than generally considered. Bovine tuberculosis has been confirmed in domestic animals, wildlife and humans in the countries bordering Kenya. However, it remains a neglected zoonosis in Kenya despite the existence of uncontrolled cross-border movement of livestock and the country having one of the highest HIV-TB burdens in Africa. This study aimed at investigating the presence of *M. bovis* among slaughter cattle in Kenya. The objective of the study was to document and estimate the prevalence of bovine tuberculosis due to *M. bovis* using routine abattoir meat inspection procedures and microbiological analysis. The second objective was to review and collate secondary data on bovine tuberculosis in slaughter cattle in Kenya by examination of annual national meat inspection reports. The study on the prevalence of *M. bovis* was carried out from July to November 2009 at Kenya Meat Commission, (KMC), Athi River and Njiru abattoirs. The two abattoirs are located in the outskirts of Nairobi city and slaughter cattle, sheep and goats from different parts of the country. They supply part of the daily beef requirements to over 3.2 million

inhabitants of Nairobi. Lesioned tissue samples were randomly collected from bovine carcasses using routine abattoir inspection. Laboratory analysis was carried out at the Central Tuberculosis Reference Laboratory (CTRL) and the Department of Veterinary Pathology, Microbiology and Parasitology, University of Nairobi. Thirty six tissue samples were embedded in paraffin wax, sectioned, stained with hematoxylin-eosin and evaluated microscopically. Samples from 176 cattle were cultured for isolation of mycobacteria in Lowenstein-Jensen (L-J) and mycobacterial growth indicator tubes (MGIT). Tissue sections, direct sample smears and culture smears were stained by the standard Z-N staining technique for detection of acid fast bacilli (AFB). Mycobacterial isolates were subsequently analysed by molecular methods using GenoType® MTBC (Hain Lifescience, Nehren, Germany) DNA strip technology. All the slaughter cattle were zebu and came from 18/47 districts covering about 77.4% of the total land size of Kenya. A total of 929 cattle; 666 females and 263 males were examined for tuberculous lesions. Of the carcasses examined, 176 [18.95%, 95% CI: (16.43-21.48)] had lesions suggestive of tuberculosis which were detected in 18.32% and 20.53% females and males respectively. Statistically there was no significant difference between prevalence of bovine tuberculosis in males and females ($p>0.05$). Z-N staining of direct smears revealed 63/176 [35.80%; 95%: (28.64-42.96)] acid fast bacilli (AFB) positive cattle giving a prevalence of 6.78% (95% CI: 5.16-8.40). Sixty four acid fast smear positive from L-J cultures subjected to molecular analysis confirmed *M. bovis* in 19/64 (32.8%), *M. tuberculosis* in 2/64 (3.1%) and mycobacteria other than tuberculosis (MOTT) in 43/64 (67.2%). Prevalence of *M. bovis* was found to be 2.05% [95% CI: 1.13-2.96] by molecular analysis. The secondary data analysis revealed that detection of bovine tuberculosis by meat inspectors during routine abattoir meat inspection is low. This study documents for the first

time that bovine tuberculosis due to *M. bovis* is prevalent among slaughter cattle in Kenya. The study further revealed that bovine tuberculosis is wide spread in the arid and semi arid (ASAL) areas of Kenya where the bulk of beef animals and over 40% of milk consumed in Kenya come from. The presence of the human tuberculosis pathogen, *M. tuberculosis* in the lungs of two bovine shows the risk tuberculous patients pose to animals. The study also found that MOTT can produce in cattle localised lesions which macroscopically and histologically resemble lesions caused by *M. bovis* and these findings further suggest that *M. bovis* is not the only *Mycobacteria* responsible for bovine tuberculosis. The low rate of detection of lesioned carcasses by the meat inspectors during routine abattoir meat inspection poses great health risk to the consumers and could be a major obstacle in Kenya's international livestock trade. With the rising incidence of TB and the ever increasing opportunistic mycobacteria-HIV co-infection in Kenya, stringent veterinary public health particularly meat inspection procedures should be enhanced to control zoonotic tuberculosis and other mycobacterial transmission from food animals to humans. However, further research to determine the extent of bovine tuberculosis in animals and humans in Kenya is needed through joint veterinary and medical surveillance programmes.

CHAPTER ONE

1.0. INTRODUCTION

Bovine Tuberculosis, one of the most important zoonotic diseases known to humans (Biffa *et al.*, 2010a), is an infectious, chronic and progressive disease with a worldwide distribution (Shitaye *et al.*, 2007). In cattle the disease is primarily caused by two members of the *Mycobacterium tuberculosis* complex (MTBC), namely *Mycobacterium bovis* and *Mycobacterium caprae* (Pavlik *et al.*, 2002), the later being the major cause of tuberculosis in cattle and also the predominant agent of zoonotic tuberculosis in humans in central Europe (Prodinger *et al.*, 2002; Kubica *et al.*, 2003). However, *M. bovis* is endemic in Africa (Ayele *et al.*, 2004). Although cattle are considered as the primary host of *M. bovis* (OIE, 2009), it has one of the broadest host ranges of all known pathogens and has been shown to be the principal agent of tuberculosis in a wide range of domestic and wild animals (O'Reilly and Daborn, 1995) and has no geographical boundaries (Kazwala *et al.*, 2006). Transmission of *M. bovis* can occur between animals, from animals to humans and vice versa and rarely, between humans (HPA, 2009). In cattle and other domestic animals, *M. bovis* spreads within and between animal species mainly by aerosol and ingestion routes. Man acquires the disease from animal sources by consumption of unpasteurised contaminated milk, ingestion of raw and undercooked meat from infected cattle (Cosivi *et al.*, 1998; Radostits *et al.*, 2000; Thoen *et al.*, 2006), directly by aerogenous route (Anonymous, 1994), through direct contact with material contaminated with nose and mouth secretions from infected cattle (Beals, 2007) and post mortem examination of infected carcasses ((Kaneene and Pfeiffer, 2006).

The economic losses due to bovine tuberculosis worldwide are estimated to account for over \$ 3 billion annually (Steele, 1995) through production losses resulting from loss of production efficiency, losses associated with meat condemnation and restricted trade. Currently, the human HIV/AIDS pandemic has made *M. bovis* a serious public health threat (Daborn *et al.*, 1996; Thoen *et al.*, 2006) and there is increasing evidence that *M. bovis* infections may be much more significant than generally considered (Shitaye *et al.*, 2007). The zoonotic importance of bovine tuberculosis cannot be under-estimated since more than 94% of the world population lives in countries in which control of the disease in cattle is limited or absent (Cousins, 2001). The world health organization (WHO) in conjunction with FAO and OIE, recently classified bovine tuberculosis as a neglected zoonotic disease with special reference to developing countries (Michael *et al.*, 2009). According to Daborn *et al.* (1996), there is a substantial lack of knowledge of the distribution, epidemiological patterns, and zoonotic implication of *M. bovis* in Africa.

Bovine tuberculosis has been reported in domestic animals in all the countries that border Kenya; in Tanzania (Durnez *et al.*, 2009), Somalia (Gracey *et al.*, 1999), Uganda (Oloya *et al.*, 2007), Sudan (Manal *et al.*, 2005) and Ethiopia (Shitaye *et al.*, 2006). It has also been reported in wild life in many African countries (O'Reilly and Daborn, 1995). Cases of human tuberculosis due to *M. bovis* have also been reported in Ethiopia (Kidane *et al.*, 2002), Tanzania (Mfinanga *et al.*, 2004), and Uganda (Oloya *et al.*, 2008) accounting for 18–30% of all *Mycobacterium tuberculosis* complex (MTBC) strains isolated from human patients, in rural settings (Kazwala *et al.*, 2001; Mfinanga *et al.*, 2004; Cleaveland *et al.*, 2007). The

correlation between the prevalence of *M. bovis* infection in humans and that in local cattle population highlights the potential threat of this disease for humans (Daborn *et al.*, 1996).

Piers and Wright (1946, cited by Sula *et al.*, 1960), reported that very little bovine tuberculosis was being reported by Veterinary Officers in Kenya, though post-mortem results from selected districts gave an incidence ranging between 0.005% and 4%. Terara *et al.* (1985), reported the occurrence of bovine tuberculosis in Wild olive Baboons (*Papio cynocephalus anubis*) in Masai game reserve and Sapolsky and Else, (1987) traced the source of the infection as ingestion of abattoir offal from infected slaughtered cattle in a village slaughterhouse near Mutito Adei, Kenya. In an intradermal tuberculin skin test (SITT) study conducted in dairy cattle in Dagoretti division, a 10% prevalence of bovine tuberculosis was reported (Kang'ethe *et al.*, 2007). However SITT does not discriminate against cattle sensitized to tuberculin test by *M. bovis* and other members of MTBC, *Mycobacterium avium* complex (MAC) and environmental *Mycobacteria* (OIE, 2009).

During the period 1930- 1945, extra-pulmonary tuberculosis accounted roughly for one-third of all tuberculosis infections treated in Kenyan hospitals (Sula *et al.*, 1960) and *M. bovis* was highly suspected because during the same period more than 30% of extra-pulmonary tuberculosis cases in children in Europe were caused by *M. bovis* infection (Cosivi *et al.*, 1995; Ayele *et al.*, 2004). However, a study to isolate and type *Mycobacteria* species from cervical lymph gland specimens from African (Kenyan) patients in 1958 did not isolate *M. bovis* (Sula *et al.*, 1960). A recent study conducted in Narok district in 2000, did not isolate any *M. bovis* in humans with extra pulmonary tuberculosis despite a 17% incidence of extra-

pulmonary tuberculosis in patients (Koech, 2001). Although Kenya is ranked 13th and 5th on the list of 22 high-burden tuberculosis countries in the world and Africa respectively (Anonymous, 2009a), the contribution of *M. bovis* to this burden remains unknown.

The bulk of beef cattle, sheep, goats and camels, about 50% of wildlife outside the national parks and approximately 20% of Kenya's population, are found in the arid and semi-arid lands (ASAL) which cover about 80% of the country. The cattle kept here are the highly adapted indigenous Zebu which also contributes about 40% of the total cattle milk production in Kenya (Anonymous, 2010). These ASAL areas stretch across the countries neighbouring Kenya and animals move extensively, even across the borders in search of water and pastures. According to a report by Aklilu *et al.* (2002), unofficial cross-border movement of livestock from Somali, Ethiopia, Sudan and Tanzania is an important source of livestock slaughtered in Nairobi and other parts of Kenya. Cross-border movement of livestock also occurs between Kenya and Uganda (Michael *et al.*, 2008). Trekking is the favourite mode of transport particularly for the livestock from pastoral areas to the livestock markets from where the animals are transported on trucks to Nairobi (Aklilu *et al.*, 2002). Others still trek to Nairobi especially those from pastoralists of Kajiado and Narok districts (personal communication with the pastoralist, July, 2009). Movement of infected cattle has been shown to pose a clear transmission risk of *M. bovis* (Gopal *et al.*, 2006) and cattle movements are a significant predictor of the distribution of bovine tuberculosis (Gilbert *et al.*, 2005).

Considering the presence of bovine tuberculosis in all the neighbouring countries, uncontrolled cross-border movement of livestock, long distance trekking of livestock to the markets, animals (domestic/wild life) and humans sharing the same eco-system and the high

HIV-TB burden, the public health and socioeconomic importance of this neglected zoonosis in Kenya cannot be overlooked.

1.1 JUSTIFICATION

Animal and human health is closely related and food animals, especially cattle serve as a reservoir of diseases of public health importance. Since *M. bovis* is endemic in Africa and has one of the broadest host ranges of all known pathogens affecting domestic and wild animals and humans, it is an important socioeconomic and public health disease. The safety of food of animal origin with regard to infection by *M. bovis* is worth giving consideration, taking into cognizance the current tuberculosis crisis ravaging the world. Consumption of unpasteurized milk and raw or undercooked meat is commonly practiced by some communities in Kenya. In addition, other risk factors such as high levels of poverty, poor nutrition, human and livestock/wildlife sharing territory, uncontrolled livestock movement and high HIV-TB burden increases the probability of bovine tuberculosis presence in cattle and humans in Kenya. As such, fundamental questions regarding the presence, prevalence, distribution, socioeconomic and public health importance of *M. bovis* in Kenya remains unanswered. The information that this study will generate will be useful in developing strategies for controlling bovine tuberculosis in Kenya. The study will therefore contribute towards improving human health and economic growth leading to poverty alleviation among the rural population.

1.2. OBJECTIVE OF THE STUDY

1.2.1. Overall objective

The overall objective of the study is to document the presence of bovine tuberculosis and estimate its prevalence among slaughter cattle in Kenya as a basis for the future national programme of prevention, control and eradication.

1.2.2. Specific objectives

1. Estimate the prevalence of bovine tuberculosis by:
 - i. Examination of bovine carcasses using routine abattoir inspection for tuberculous lesions in two selected abattoirs in the out skirts of Nairobi.
 - ii. Culture and isolation of *M. bovis* from the samples.
 - iii. Molecular speciation of isolated members of MBTC.
2. Review and collate secondary data on tuberculosis in slaughter cattle in Kenya by examination of annual national meat inspection reports.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1 AETIOLOGY

Tuberculosis in cattle and other domestic animals is caused by two members of the *Mycobacterium tuberculosis* complex (MTBC): *Mycobacterium bovis* and *Mycobacterium caprae* (Pavlik *et al.*, 2002; Prodinger *et al.*, 2002; Erler *et al.*, 2004; Shitaye *et al.*, 2007), the latter being the major cause of tuberculosis in cattle and also the predominant agent of zoonotic tuberculosis in humans in central Europe (Prodinger *et al.*, 2002; Kubica *et al.*, 2003). Occasional occurrence of tuberculosis due to *Mycobacterium tuberculosis* with concurrent tuberculous lesions has been reported in cattle and other animals (Pavlik, 2006). The genus *Mycobacterium* is the only genus in the family *Mycobacteraceae* (Wayne and Kubica, 1986). Many species within the genus *Mycobacterium* are prominent pathogens, members of MTBC being the most important. MTBC comprises of closely related group of mycobacteria that characteristically exhibit 99.9% similarity at the nucleotide level, with identical 16S rRNA sequences but differ widely in terms of their host tropisms, phenotypes and pathogenicity (Boddinghaus *et al.*, 1990). They include *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. microti*, *M. bovis BCG* and *M. canetti* (van Soolingen *et al.*, 1997) and other members of the MTBC previously considered as being *M. bovis* which have now been accepted as new species (Thoen *et al.*, 2006; OIE, 2009). These include *Mycobacterium caprae* (Aranaz *et al.*, 2003) which in some countries is considered a primary pathogen of goats and *Mycobacterium pinnipedii* (Cousins *et al.*, 2003), a pathogen of fur seals and sea lions. These two new species are also known to be zoonotic (OIE, 2009).

Mycobacterium bovis are obligate intracellular, non-spore forming, non-motile, slightly curved or straight rods measuring 0.2-0.6µm by 1.0-10µm, which may branch (Biet *et al.*, 2005; Thoen and Barletta, 2006). Tubercle bacilli are strict aerobes that grow on complex organic media such as Lowenstein-Jensen (L-J) which contains among other ingredients, whole egg (Hirsh *et al.*, 2004). In contrast to most members of MTBC, *M. bovis* is able to grow in reduced oxygen atmosphere (Murray *et al.*, 2003). The organism grows poorly on Lowenstein-Jensen media (L-J), but the growth is stimulated if glycerol is replaced with 0.4% sodium pyruvate (OIE, 2009). Colonies on egg-based media are small, rounded, moist, nonphotochromogenic and break easily (Quinn *et al.*, 1994; Murray *et al.*, 2003). Growth of *M. bovis* generally occurs within 3–6 weeks of incubation depending on the media used (Anonymous, 2009b; OIE, 2009).

Although cytochemically Gram-positive, the *Mycobacteria* do not take up the dyes of the Gram-stain because the cell walls are rich in lipids, mycolic acid forming the bulk of these lipids (Quinn *et al.*, 1994; Murray *et al.*, 2003). Their most noted staining property is their acid fastness and once a dye has been taken up by the cells they are not easily decolourized, even by 3% hydrochloric acid in ethanol (Hirsh *et al.*, 2004) or 25% sulphuric acid. The rods stain irregularly and often have a beaded appearance (Quinn *et al.*, 1994). When numerous and actively multiplying, the bacilli are strongly acid fast and show an evident and distinctive tendency to form hydrophobic bundles (Palomino *et al.*, 2007). *M. bovis* can survive for several months in the environment, particularly in cold, dark and moist conditions, in buildings, on transport vehicles, on pasture and in slurry (O'Reilly and Daborn, 1995; Ayele *et al.*, 2004; Anonymous, 2009b). The organism has been reported to survive in cow faeces

for more than 5 months in winter, 4 months in autumn, 2 months in summer, and in soil for up to 2 years (Wray, 1975). Manure fertilisation of arable land is common practice in developing countries; survival of *M. bovis* in soil and slurry therefore implies pasture and vegetable contamination, representing a potential source of infection to animals and humans, respectively (Ayele *et al.*, 2004). The survival of *Mycobacterium bovis* in the environment is affected by exposure to direct sunlight (Menzies and Neill, 2000).

Mycobacteria are easily killed by heat, 60⁰C for at least 15-20 minutes (Ananthanarayan and Paniker, 2006) and by UV light but not freezing or desiccation and are more resistant to acids, alkalis and some chemical disinfectants than are most other non-spore forming bacteria (Murray *et al.*, 2003). Malachite green, quaternary ammonium compounds, hexachlorophene and chlorohexidine are bacteriostatic at best. Formaldehyde vapour, chlorine compounds, 70% ethanol, hydrogen peroxide and 2% alkaline glutaraldehyde are effective in killing *M. bovis*. Chemicals which are inactivated in the presence of organic matter (e.g. alcohols) cannot be relied on to disinfect mucous or protein-containing materials (Murray *et al.*, 2003). With iodophors, the bactericidal effect depends on the contents of available iodine as well as on the presence of organic matter (Best *et al.*, 1990).

2.2 DISEASE EPIDEMIOLOGY

2.2.1 Host range

Mycobacterium bovis has one of the broadest host ranges of all known pathogens and has been shown to be the principal agent of tuberculosis in a wide range of domestic and wild animals (O'Reilly and Daborn, 1995). Cattle are considered to be the primary hosts of *M. bovis* (OIE, 2009). The mammalian host range of *M. bovis* includes North American bison (*Bison bison*), buffaloes (*Syncerus caffer*), elk (*Cervus elaphus*), domestic and wild pigs (*Sus scrofa*), goats (*Capra hircus*), camels (*Camelus bacterianus*), dogs (*Canis familiaris*), cats (*Felis catus*), sheep (*Ovis aries*), possums (*Trichosurus vulpecula*), badgers (*Meles meles*), mink (*Lutreola vison*), ferrets (*Putorius furo*), non-human primates and humans (O'Reilly and Daborn, 1995; Thoen *et al.*, 1995; Keet *et al.*, 1996; Radostits *et al.*, 2000; OIE, 2009). This list is non-exhaustive but shows the wide range of *M. bovis* susceptible mammalian hosts.

2.2.2 Geographical distribution

Although bovine tuberculosis was once found worldwide, in many developed countries it has been controlled through policies of test and slaughter while in developing countries, it is widespread (Ayele *et al.*, 2004; Anonymous, 2009b). However, despite the rigorous control and eradication programmes in developed countries, bovine tuberculosis has persisted while in developing countries, it has been on the increase (Cosivi *et al.*, 1998; Gilbert *et al.*, 2005). In Africa, the disease is present in almost all countries, affecting both domestic and wild animals (Anonymous, 1994; Ayele *et al.*, 2004). *M. bovis* infection has been reported in domestic ruminants in Senegal, Burkina Faso, Mauritania, Ghana, Nigeria (Benkirane, 1998,

Bonsu *et al.*, 2000), Mali (Muller *et al.*, 2008) and many other African countries. Closer to Kenya, it has been reported in domestic ruminants in Somalia (Gracey *et al.*, 1999), Sudan (Sulieman and Hamid, 2002) and Ethiopia (Shitaye *et al.*, 2006). Among the East African countries, *M. bovis* has been reported in cattle in Tanzania (Kazwala, 1998; Durnez *et al.*, 2009), Burundi (Rigouts *et al.*, 1996) and Uganda (Oloya *et al.*, 2007). In Kenya, *M. bovis* has been reported in wild baboons (Terara *et al.*, 1985; Sapolsky and Else, 1987). In humans, tuberculosis caused by *M. bovis* has been confirmed in several African countries (Nafeh *et al.*, 1992). Approximately 85% of cattle and 82% of the human population in Africa live in areas where bovine tuberculosis is prevalent and it is either partly controlled or uncontrolled (Cosivi *et al.*, 1998). According to Desta (2000), in Africa, the herd prevalence of bovine tuberculosis increased from 31% in 1996/97 to 34% in 1999 and the population prevalence also increased during the same period from 11.2% to 11.7%. This relatively high prevalence is more worrying when placed in the context of populations with high levels of human immunodeficiency virus (HIV) and high levels of poverty as is the case in Sub-Saharan countries.

2.3 PUBLIC HEALTH IMPORTANCE

The first bacteriologically confirmed case of bovine tuberculosis in man was reported in 1904 (Griffiths, 1914) and further investigations over the next three decades showed that 1-3% of all cases of respiratory tuberculosis resulted from *M. bovis* infections (Cutbill and Lynn, 1944). According to Cosivi *et al.* (1998), the global prevalence of human tuberculosis due to *M. bovis* was estimated at 3.1% of all human tuberculosis cases of which 2.15% and 9.45%

are pulmonary and extra-pulmonary respectively. Human tuberculosis due to *M. bovis* in developing countries is analogous to conditions in the 1930s and 1940s in Europe, where more than 30% of cervical lymphadenitis cases in children were caused by *M. bovis* infection (Cosivi *et al.*, 1995, cited by Ayele *et al.*, 2004). Recent studies estimate the prevalence of human tuberculosis due to *M. bovis* in developed countries at 0.5-7.2% and 10-15% in developing countries (De la Rúa-Domenech, 2006). It is estimated that in countries where pasteurization of milk is rare and bovine tuberculosis in cattle is common, 10-15% of human cases of tuberculosis are caused by *M. bovis* (Ashford *et al.*, 2001). In Burkina Faso, Vekemans *et al.* (1999), reported isolation of *Mycobacteria* in 26% of 60 retailed milk samples collected from markets while the Ministry of Health reported 1,334 human cases of zoonotic tuberculosis during the same period (Coulibaly and Yameogo, 2000). In Malawi, a survey of human sputum cultures from human tuberculosis patients revealed that 4.2% of the culture positive specimens were *M. bovis* (Ministry of Health, 1985 cited by Wedlock *et al.*, 2002). In Egypt, a study reported that 9 out of 20 randomly selected patients with tuberculosis peritonitis (extra-pulmonary) were infected by *M. bovis* (Nafeh *et al.*, 1992). There is increasing evidence that *M. bovis* infections may be much more significant than generally considered (Shitaye *et al.*, 2007).

The epidemiology of bovine tuberculosis has been affected in recent decades by the upsurge of human immunodeficiency virus (HIV) infection since many HIV-infected individuals are co-infected with tuberculosis and the incidence of the disease may rise in the coming years (Zumla *et al.*, 1999; Ayele *et al.*, 2004). This implies that the risk of spill over of zoonotic tuberculosis to rural communities is rapidly increasing particularly in eastern and southern Africa where high incidences of HIV/AIDS are being reported (Amanfu, 2006; Zinsstag *et al.*,

2006). Accurate identification of *M. bovis* and *M. tuberculosis* has not only epidemiological implication, but is also relevant for the management of patients, given that bovine strains are inherently resistant to pyrazinamide (PZA), a first line tuberculostatic drug (Grange and Yates, 1994). However the proportion of *M. bovis* within the TB and TB-HIV complex in Kenya and other African countries is unknown.

2.4. TRANSMISSION

Infectious animals may shed *M. bovis* in a number of ways: in faeces, milk, discharging lesions, saliva, vaginal secretions or semen and urine (Neill *et al.*, 1991; Anonymous, 2009b).

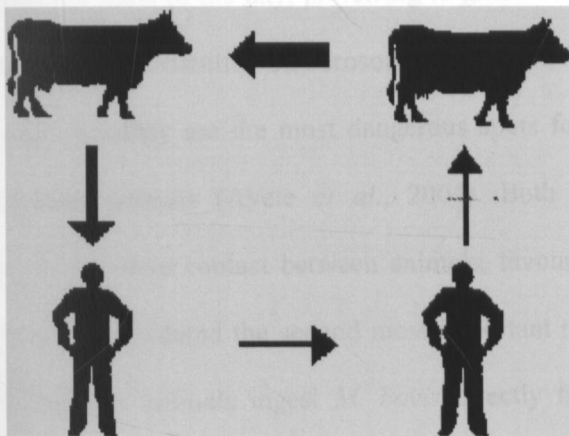


Figure 1: Cycle of *Mycobacterium bovis* transmission between cattle and humans. The thickness of the arrows suggests probability (Grange and Collins, 1987).

2.4.1 Animal to animal transmission

The distribution of tuberculous lesions in cattle, as shown in meat inspection reports, leaves no doubt that thoracic disease is much more common than the disease in the abdominal organs (Steele and Ranney, 1958). According to a review by Ayele *et al.* (2004), inhalation of *M. bovis* is the most probable and principal route of bovine infection and is facilitated by close and prolonged contact between infected and healthy animals. In Africa where extensive livestock farming is practiced especially by the pastoralists, grazing animals usually gather at night for protection against predators, at water points such as ponds, wells and streams, vaccination centres, artificial insemination stations, market/auction yards and in dipping tanks thus favouring aerosol transmission (Ayele *et al.*, 2004; Shitaye *et al.*, 2007). Due to the high ambient temperature in the tropics, animals tend to concentrate under trees or other shaded areas for parts of the day, preferring to graze in the morning and late in the afternoon further increasing probability of aerosol transmission of bovine tuberculosis. Salt supplementing points possibly are the most dangerous spots for nose-to-nose and mouth-to-mouth contact between animals (Ayele *et al.*, 2004). Both intensive and extensive livestock farming promotes close contact between animals, favouring the spread of *M. bovis*. Ingestion of *M. bovis* is considered the second most important route of infection (Menzie and Neill, 2000). Susceptible animals ingest *M. bovis* directly from infected animals or from contaminated pasture, water or utensils (Menzie and Neill, 2000; Kaneene and Thoen, 2004). This route is particularly important in calves nursing from infected cows (Anonymous, 2009b). Vertical transmission of *M. bovis* has been shown to congenitally occur via the umbilical vessels, as a sequel to uterine infection of the dam (Pritchard, 1988; O'Reilly and Daborn, 1995) and that approximately 1% of calves born from tuberculous cows are likely to be congenitally infected

(Francis, 1947). However, Cutaneous, genital and congenital infections are rare (Anonymous, 2009b).

2.4.2 Animal to human transmission

Bovine tuberculosis is becoming increasingly important due to the susceptibility of humans to the disease caused by *M. bovis* (Kleeberg, 1984). The primary source of infection to humans is consumption of unpasteurised milk and inhalation due to close association between humans and animals (Daborn and Grange, 1993). According to Francis (1947, cited by O'Reilly and Daborn, 1995), bacteriological testing of milk samples indicates that *M. bovis* is excreted in the milk of about 1-2% of infected cattle. The bacilli are however shed in large numbers directly from infected mammary tissue into the milk and a single infected cow can therefore contaminate the bulk milk produced by 100 other cows and still cause infection in susceptible humans (Coetzer and Tustin, 2005). Milk-borne infection is the main cause of non-pulmonary tuberculosis in areas where bovine tuberculosis is common and uncontrolled (Grange and Yates, 1994; Daborn *et al.*, 1996; Cosivi *et al.*, 1998). Raw or undercooked meat and meat products from infected cattle can also be a source of infection in man (Kazwala *et al.*, 1998; Pavlik *et al.*, 2002; Etter *et al.*, 2006). Trans-cutaneous transmission is an important route when performing post mortem examination of infected carcasses (Kaneene and Pfeiffer, 2006). In countries with a relatively high prevalence of bovine tuberculosis, abattoir workers, veterinarians and animal handlers are the groups most exposed to infection due to direct contact with *M. bovis* infected cattle (Ayele *et al.*, 2004; Yuni and Tooru, 2007). Inhalation of dust particles or bacteria-containing aerosols shed by infected cattle is an

important route especially in rural dwellers (Daborn *et al.*, 1996). A higher prevalence of tuberculosis in cattle owned by tuberculous patients than in cattle owned by non-tuberculous owners has been reported suggesting the significant role of *M. bovis* infection in the incidences of human tuberculosis (Regassa, 2005; Fetene *et al.*, 2011). In developing countries, the conditions for *M. bovis* transmission to humans not only exist unchanged, but the human population has a greater vulnerability due to poverty, HIV and reduced access to health care (Ayele *et al.*, 2004).

2.3 PATHOGENESIS

2.3.1 Animal exposure to cattle

2.4.3 Human to animal transmission

Human to animal transmission of bovine tuberculosis is well documented and patients with smear-positive pulmonary tuberculosis pose danger to animals (Sjogren and Hillerdal, 1978). Contaminated pastures with *M. bovis* by humans with genitourinary tuberculosis represent a source of infection for animals (Grange and Yates, 1994).

2.4.4 Human to human transmission

Although human-to-human transmission of *M. bovis* is considered rare and the rate of transmission seems insignificant compared to animal-to-human infection (O'Reilly and Daborn, 1995), several recent lines of evidence indicate that it may be more important than originally believed (LoBue, 2006). Reports of cases of patients with pulmonary *M. bovis* have suggested that human-to-human transmission does occur, even in non-immunosuppressed persons (Evans *et al.*, 2007; Sunder *et al.*, 2009). Human-to-human transmission of multidrug

resistant strains of *M. bovis* from a HIV-TB co-infected patient to an immunocompetent person has been reported in Spain (Long *et al.*, 1999). Transmission among HIV-infected and alcoholics is important (Anonymous, 2009b). This may be important especially in Africa where *M. bovis* infection is enzootic, pasteurisation of milk is not routinely practised and HIV infection in humans is prevalent (Cosivi *et al.*, 1998).

2.5 PATHOGENESIS

Aerosol exposure of cattle to *M. bovis* is considered the most frequent route of infection; gross lesions usually involve the lungs and thoracic lymph nodes (Cosivi *et al.*, 1998). Cattle exposed by ingestion of food or water contaminated with *M. bovis* often develop primary foci in lymph nodes associated with the intestinal tract (Pollock and Neill, 2002). Aerosol exposure leads to involvement of the lungs and associated lymph nodes. The muco-ciliary clearance by mucous and epithelial cilia in the upper respiratory passages provides a defence against infection by inhalation of mycobacteria (Philips *et al.*, 2002). The estimated size of terminal ending bronchiole is about 20µm as compared to 1-4 µm for an acid-fast bacillus (Thoen and Barletta, 2006) and therefore the acid fast bacilli are able to reach the alveoli. In the lungs, the bacteria are phagocytized by alveolar macrophages and this is the first event in the host-pathogen interaction (Raja, 2004). The pathogenic mycobacteria modulate the normal progression of phagosome into an acidic hydrolytically active compartment and avoid the development of a localized, productive immune response that could activate the host cell (Clemens and Horwitz, 1996; Russell, 2003). This guarantees an environment that protects it from the effector mechanisms of the host and allows it to replicate inside the macrophages

(McDonough, 1993). This sharply contrasts with the accepted view that macrophages afford an infective initial barrier to bacterial infections (Russell, 2001). Recent studies indicate that pathogenic mycobacteria are able to modulate macrophage apoptosis to an extent dependent on the intracellular bacterial burden and this benefits its intracellular growth and dissemination to adjacent cells (Rodrigues *et al.*, 2009). When entering into apoptosis, infected macrophages release apoptotic bodies containing mycobacterial antigens, which are engulfed by uninfected dendriatic cells, processed and subsequently presented via major histocompatibility complex class I, making the activation of CD8⁺ T cells possible through a mechanism of cross-priming (Winau, 2004). The cellular hypersensitivity that develops, contributes to cell death and tissue destruction resulting in caseous necrosis. In some instances, liquefaction and cavity formation occur as a result of enzymatic action on proteins and lipids. Rapture of these cavities into the bronchi allows aerosol spread of the tubercle bacilli (Thoen and Barletta, 2006). Phagocytosis induce a localized proinflammatory response that leads to recruitment of mononuclear cells which constitute the building block for the granuloma, the tubercle that defines the disease, from neighbouring blood vessels (Thoen and Barletta, 2006). The cellular responses attempting to control the infection results in the accumulation of large number of phagocytes and finally the formation of macroscopic lesion, the tubercle (Ayele *et al.*, 2004; Thoen and Barletta, 2006).The granuloma consist of infected macrophages surrounded by giant cells, uninfected macrophages and lymphocytes (Russell, 2001).

2.6 PATHOLOGY

Bovine tuberculosis in animals and humans is characterized by formation of granulomatous lesions in tissues and organs, more significantly in the lungs, lymph nodes, intestines and kidney among others. Tuberculous lesions are most commonly observed in the lymph nodes of the thorax, followed by the head and abdomen, particularly the bronchial, mediastinal, retropharyngeal lymph nodes and they may be the only tissue affected (Liebana *et al.*, 2008; OIE, 2009). The tuberculous granuloma usually has a yellowish white appearance and is caseous, caseo-calcareous or calcified in consistency. Others may take the form of thin-walled abscesses with little calcification and containing purulent material (Whipple *et al.*, 1996; Anonymous, 2009b; OIE, 2009). The caseous centre is usually dry, firm and covered with a fibrous connective capsule of varying thickness (OIE, 2009). Lesion size ranges from small enough to be missed by unaided eye to the involvement of the greater part of an organ (Anonymous, 2009b; OIE, 2009). Serial sectioning of organs and tissues may be required to detect the small lesions contained within the tissue (OIE, 2009). During pathological processes, *Mycobacteria* are present in tuberculous tissue and in various body fluids, secretions and excretions such as milk, blood, sputum, bronchio-alveolar lavages, cerebrospinal fluid and semen (Ayele *et al.*, 2005) and care should be taken when handling such tissue and fluids.

Microscopically, granulomas are composed of aggregates of epithelioid cells surrounded by a rim of lymphocytes (Akhtar and Mana, 2004). The epithelioid cells are highly activated macrophages which are characterized by abundant slightly eosinophilic granular cytoplasm and a relatively small usually eccentric nucleus. A striking morphological feature of granulomas is the presence of multinucleated giant cells which are formed by the fusion of

several epithelioid cells. Some have nuclei evenly dispersed through the cytoplasm (foreign body giant cells) and others have the nuclei disposed in a horse-shoe arrangement (Langhans giant cells) (Akhtar and Mana, 2004). The granulomas are surrounded by a zone of lymphocytic cells which include the natural killer cells, cytotoxic T cells and type I and II T helper cells among others (Dannenberg, 1999). In caseating granulomas, the epithelioid cells form a palisading arrangement around the caseating centre. Histologically, caseous necrosis consists of amorphous, coarsely granular, acellular debris (Dlip, 1997).

2.7 DIAGNOSIS.

Bovine tuberculosis can be diagnosed in live animals (OIE, 2009) and also during post mortem examination of a carcass (Corner, 1994; OIE 2009). Clinical signs, tuberculin skin test and sample collection such as milk, nasal swabs and blood can be used to diagnose tuberculosis in live animals. At post mortem, pathological lesions and acid fast staining are important in preliminary diagnosis of tuberculosis while culture and DNA analysis are important for confirmation purposes.

2.7.1 Diagnosis in live animals

2.7.1.1 Clinical Signs

The signs of tuberculosis in cattle usually vary with the distribution of tubercles in the body (Thoen *et al.*, 1981). Many cattle with bovine tuberculosis are clinically normal, but a

capricious appetite, fluctuating temperature and progressive emaciation unassociated with other signs should arouse suspicion of tuberculosis (Ayele *et al.*, 2004). Pulmonary involvement is eventually characterised by chronic moist cough that is worse in the morning, during cold weather or exercise, and may have dyspnea or tachypnea (anonymous, 2009b). Affected animals are docile and sluggish, but the eyes remain bright and alert (Radostits *et al.*, 2000). When investigating bovine tuberculosis, herd history and thorough clinical examination requiring palpation of all superficial lymph nodes, the udder in females and percussion and auscultation of the pulmonary area are important (OIE, 2009).

2.7.1.2 Tuberculin skin test

According to OIE, (2009), the tuberculin skin test (TST), which was developed by Koch in 1890 is universally recognised and is generally used for preliminary diagnosis in bovine tuberculosis control programmes and represents the OIE's recommended test for international trade. However, TST is a poor test for identifying individual infected animals (Dawson and Trapp, 2004). Tuberculin skin tests include the single intradermal tuberculin test (SITT) and comparative cervical test (CCT).

2.7.1.3 Milk test

M. bovis is excreted in the milk of about 1-2% of infected cattle (Francis, 1947 cited by O'Reilly and Daborn, 1995). This implies that the use of milk for diagnosis of bovine tuberculosis will miss out 98-99% infected cattle and it is limited to lactating females only,

leaving out males, dry female and calves. Mycobacterial DNA extraction in milk and further detection of the bacterium by polymerase chain reaction (PCR) has also been used for diagnosis of *M. bovis* (Zanini *et al.*, 1998).

2.7.1.4 Nasal swabs DNA analysis

PCR assay has successfully been used to detect *M. bovis* in nasal exudates of naturally infected cattle (Vitale *et al.*, 1998; Tejada *et al.*, 2006; Figueiredo, *et al.*, 2010). Indeed, Vitale *et al.* (1998), reported high specificity and positive predictive value in the detection of MTBC in nasal swabs by PCR. However this method is limited to animals shedding *M. bovis* in the respiratory system.

2.7.2 Diagnosis during Post Mortem examination

2.7.2.1 Detection of tuberculous lesions

Post-mortem examination and culture are effective procedures for the diagnosis of bovine tuberculosis (Corner, 1994). However, post mortem meat inspection surveillance for detection of tuberculous lesions in particular depends on the work load, time and diligence of the meat inspector (Corner *et al.*, 1990). The sensitivity of post mortem procedures to detect bovine tuberculosis is also affected by the presence of non-tuberculous parasitic granulomas, bacterial or mycotic pyogranulomas and bacterial abscesses which may be indistinguishable macroscopically from tuberculous granulomas (Liebana *et al.*, 2008; OIE, 2009). However, careful examination of as few as 6 pairs of lymph nodes, the lungs and the mesenteric lymph

nodes can result in 95% of cattle with macroscopic lesions being identified (Corner, 1994). Lesions suspected to be tuberculous at necropsy should be submitted to the laboratory for bacteriological and histological examination (Corner, 1994).

2.7.2.2 Acid-fast staining technique

Demonstration of acid-fast bacilli (AFB) in a smear made from a clinical specimen provides a preliminary diagnosis of mycobacterial disease, while the isolation of mycobacteria on culture provides a definite diagnosis of tuberculosis or disease due to mycobacteria other than *M. tuberculosis* complex (MOTT) (Siddiqi and Rusch-Gerdes, 2006). The acid-fast stain developed in 1882 by Paul Ehrlich and later improved by Ziehl and Neelsen is based on the ability of *Mycobacteria* to retain the primary dye even when decolourized by a powerful solvent such as acid-alcohol (Vasanthakumari, 2007). Most other bacteria are easily decolourized by acid-alcohol. The sensitivity of acid fast smear for specimen from extra-pulmonary tuberculosis and disease caused by MOTT is lower than from sputa (Anonymous, 1998).

2.7.2.3 Histopathological analysis

Histopathological features of granulomas show caseous necrosis with or without calcification surrounded by macrophages, lymphocytes, plasma cells, neutrophils, epithelioid cells and Langhan's giant cells and enclosed partially or completely by a thin capsule (Katia *et al.*, 2008). However chronic granulomas can also be found in various conditions and diseases

such as mycotic and bacterial pyogranulomas (Liebana *et al.*, 2008). Although tuberculous granulomas in cattle often cover large areas of histological sections and typically contain only small number of acid-fast bacilli (Liebana *et al.*, 2008; OIE, 2009); the absence of acid-fast organisms does not exclude tuberculosis in lymphadenitis of unknown aetiology (OIE, 2009). A sample is considered as positive only if it consists of granulomatous inflammation associated with central necrosis and with no evidence of other non-mycobacterial aetiologies (Fitzgerald *et al.*, 2000).

2.7.2.4 Culture and biochemical test

Culture and biochemical tests are considered the gold standard for detection of *Mycobacteria* (Kent and Kubica, 1985; Parthiban *et al.*, 2007). However, isolation of pathogenic *Mycobacteria* and their identification based on phenotypic manifestations are laborious, cumbersome and time consuming (Soini and Musser, 2001; Hosek *et al.*, 2006). Moreover, the phenotype of *Mycobacterial* culture is not stable, but demonstrates a striking variability depending on the cultivation conditions (Kirschner and Bottger, 1998; Soni and Musser, 2001). Samples for culture are decontaminated to inactivate other bacteria that might be present in the sample to avoid their faster growth and media nutrient exhaustion which renders *M. bovis* (and other slow growing pathogenic *Mycobacteria*) growth unfeasible (Anonymous, 1998). The best way to detect *M. bovis* in tissue specimens is by inoculating the samples on Lowenstein-Jensen (L-J) and Stonebrink Leslie solid culture media (Wayne and Kubica, 1986). Liquid culture systems such as Bactec are also used where growth is assessed by radiometric or fluorometric means (OIE, 2009). Characteristic growth patterns and colonial

morphology can provide a presumptive diagnosis of *M. bovis*; however every isolate needs to be confirmed (OIE, 2009). *M. bovis* has sparse thin growth on glycerol-containing media while colonies on egg-based solid media with pyruvate are small, moist, smooth, rounded with irregular edges (Kent and Kubica, 1985; Murray *et al.*, 1990), off-white (buff) and break up easily (OIE, 2009). *Mycobacterium bovis* is sensitive to TCH (thiophen-2-carboxylic acid hydrazide) and INH (isonicotinic acid hydrazide). Niacin production and nitrate reduction are negative for *M. bovis* while these are positive for *M. tuberculosis*. In the amidase test, *M. bovis* is positive for urease and negative for nicotinamidase and pyrazinamidase (OIE, 2009).

2.7.3.5 Molecular Diagnosis

Different molecular tools have been developed to differentiate between *Mycobacterial* isolates (Oloya *et al.*, 2007) and for direct detection of mycobacteria from clinical samples (Neonakis *et al.*, 2008). Notable is spacer oligonucleotide typing (Spoligotyping) and Restriction Fragment Length Polymorphism (RFLP) based molecular techniques (Haddad *et al.*, 2004). Spoligotyping is designed to detect the presence or absence of unique spacers within the direct repeat (DR) locus of members of the MTBC, among them the *M. bovis*. It is known to distinguish between phenotypically different strains and has been successfully used to type *M. bovis* isolates from different African countries such as Cameroon (Njanpop-Lafourcade *et al.*, 2001), Nigeria (Cadmus *et al.*, 2006), Chad (Diguimbaye-Djaibe *et al.*, 2006), Tanzania (Kazwala *et al.*, 2006), Madagascar (Rasolofo *et al.*, 2006), Uganda (Oloya *et al.*, 2007), Mali (Muller *et al.*, 2008), Zambia (Munyeme *et al.*, 2009) and Ethiopia (Biffa

et al., 2010b). Spoligotyping has been performed on *M. bovis* isolates obtained from different animals to show the interaction between species hence it is an important molecular epidemiological tool (Oloya *et al.*, 2007).

A number of PCR-based protocols have been developed for the detection of *Mycobacteria* belonging to the *Mycobacterium tuberculosis* complex (Yuni and Tooru, 2007). These protocols include Gen-Probe TB complex DNA probe or PCR targeting 16S-23S rRNA, the insertion sequences *IS6110-IS1081*, genes coding for *Mycobacterium tuberculosis* complex proteins such as MPB70 and the 38kda antigen b (OIE, 2009). The PCR based sequencing methodology is considered the gold standard molecular identification of mycobacteria (Neonakis *et al.*, 2008). One of the most successful molecular methods for identification of bacteria is the DNA probe technology (Palomino *et al.*, 2007). The AccuProbe (GEN-Probe, San Diego, California, USA) is based on this technology and it is used by the majority of clinical mycobacteriology laboratories. The DNA probes, which are single-stranded DNA oligonucleotides labelled with acridinum ester that are complementary to the target, the ribosomal RNA are used (Neonakis *et al.*, 2008).

Line probe assays uses the reverse hybridization technology with differently specific DNA-probes immobilized in parallel lines on a paper strip (Palomino *et al.*, 2007). The target DNA is PCR amplified using biotinylated primers and finally incubated with the strip for hybridization (Palomino *et al.*, 2007; Neonakis *et al.*, 2008). Once the hybridization has been carried out under highly stringent conditions and the unbound amplicons have been washed out, the hybridized probe is revealed as a coloured band, developed following the addition of

a streptavidin-labeled enzyme and a chromogenic substrate. The specificity of the hybridized line-probe is inferred by the position of the coloured bands on the strip. Two commercial methods are available, INNO-LiPA MYCOBACTERIA (Innogenetics, Ghent, Belgium) and GenoType Mycobacterium (Hain, Germany).

GenoType Mycobacterium (Hain Lifescience, Nehren, Germany)

The GenoType MTBC is based on the *gyrB* gene sequence polymorphism and RD1 deletion of *M. bovis* BCG (Richter *et al.*, 2003). Eleven probes are present on the strip: one is aimed at the 23S ribosomal DNA, nine at four regions of the *gyrB* gene and one at the flanking regions of RD1 (Palomino *et al.*, 2007). The 23S rDNA specific probe is used to confirm the isolate as belonging to the *M. tuberculosis* complex (MTBC) while the nine probes aim at different regions of the *gyrB* gene differentiate *M. tuberculosis*, *M. africanum* type 1, *M. bovis*, *M. caprae* and *M. microti* and RD1 differentiate *M. bovis* from *M. bovis* BCG (Palomino *et al.*, 2007). The identification is not based on the specificity of a single line but on the different combinations of multiple bands characterizing each species. Richter *et al.* (2003), evaluated the ability of the GenoType MTBC to differentiate *Mycobacterium tuberculosis* complex species and demonstrated that all the MTBC species can be unambiguously identified, with the exception of *M. tuberculosis*, *M. africanum* subtype II and *M. canetti* that have identical hybridization patterns. The GenoType assays are rapid, easy-to-perform and easy-to-interpret (Gitti *et al.*, 2006). According to Franco-Alvarez de Luna *et al.* (2006), the assay is useful, reliable and rapid, with sensitivity and specificity of 92% and 100%, respectively.

Although these DNA tests are more reliable and much more rapid than the traditional identification methods, tissue samples are still required and the tests are still limited to the post-mortem diagnosis of the infection, despite some studies regarding their use on milk, lymph node aspirates and nasal swab samples (Fabrizio *et al.*, 1998; Zanini *et al.*, 1998; Figueiredo *et al.*, 2008; Figueiredo *et al.*, 2010).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 SETTING/ STUDY SITES

The study comprised of abattoir meat inspection, laboratory analysis and a ten years retrospective analysis of bovine tuberculosis reports from the Department of Veterinary services, meat inspectorate division. Meat inspection was conducted at the Kenya Meat Commission, (KMC), Athi River and Njiru abattoirs which are two of the ten main abattoirs that supply inspected beef to over 3.2 million inhabitants of Nairobi city. The abattoirs were selected based on their through put, organization and source of slaughter cattle. KMC abattoir is a state owned export slaughterhouse while Njiru abattoir is a privately owned local slaughterhouse. Laboratory analysis was carried out at the Central Tuberculosis Reference Laboratory (CTRL) and the Department of Veterinary Pathology, Microbiology and Parasitology, University of Nairobi. Retrospective analysis was conducted by examination of national meat inspection reports from the Department of Veterinary services, meat inspectorate division.

3.2. ABATTOIR MEAT INSPECTION

3.2.1 Study population and Sample size

Sampling was conducted between July and November 2009. The animals examined were randomly picked during meat inspection and demographic data on area of origin, sex and breed was recorded. No ethical clearance was required for this study because confiscation of diseased organs and carcasses is part of routine meat inspection procedure.

The sample size required for calculating the prevalence at 95% confidence interval was determined by using the following formula (Daniel, 1999, cited by Naing *et al.*, 2006):

$$n = Z^2 P(1-P)/d^2$$

n = sample size,

Z = Z statistic for a level of confidence,

P = expected prevalence or proportion (in proportion of one; if 2%, P = 0.02)

d = precision (in proportion of one; if 5%, d = 0.05).

Since the prevalence of bovine tuberculosis in Kenya is unknown, it was assumed that it would be similar to those found in neighbouring countries where similar studies have been conducted. Therefore 2% prevalence was assumed.

$$n = Z^2 P (1-P)/d^2$$

$$Z = 1.96 \text{ [95\% CI]}$$

$$P = 0.02$$

$$d = 0.01 \text{ [} d = 1/2P \text{ where } P < 0.1, \text{ Naing } et al., 2006 \text{]}$$

$$n = 1.96^2 \times 0.02(1-0.02)/0.01^2$$

$$= 753 \text{ (However the number of animals examined was 929).}$$

3.2.2 Post mortem examination

Routine post mortem meat inspection was performed as stipulated in the Meat Control Act, Chapter 356 (Cap 356, 1972). In the head, parotid, sub-maxillary and medial retropharyngeal lymph nodes were incised and examined. In the thoracic cavity, the pleura was examined, the apical, left bronchial and posterior mediastinal lymph nodes were incised and examined while the lung tissue was palpated, incised and examined. In the abdominal organs, the spleen, liver and portal lymph nodes were palpated, incised and examined while the kidneys were de-capsulated and examined. Mesenteric lymph nodes were also randomly palpated, incised and examined. In the carcass, prescapular, superficial inguinal (males)/ supramammary (females) and the iliac lymph nodes were palpated, incised and examined. Any suspected tuberculous lesions with yellowish appearance, caseous, purulent, caseo-calcareous or calcified in consistency were collected. Type of organ or tissue in which the lesion was located was recorded as well as the nature of the gross pathological lesion. Observation of localised tuberculous lesion in the various parts of the carcass led to partial condemnation of affected parts while generalised infection led to total-condemnation.

3.2.3 Sample collection, transport and storage

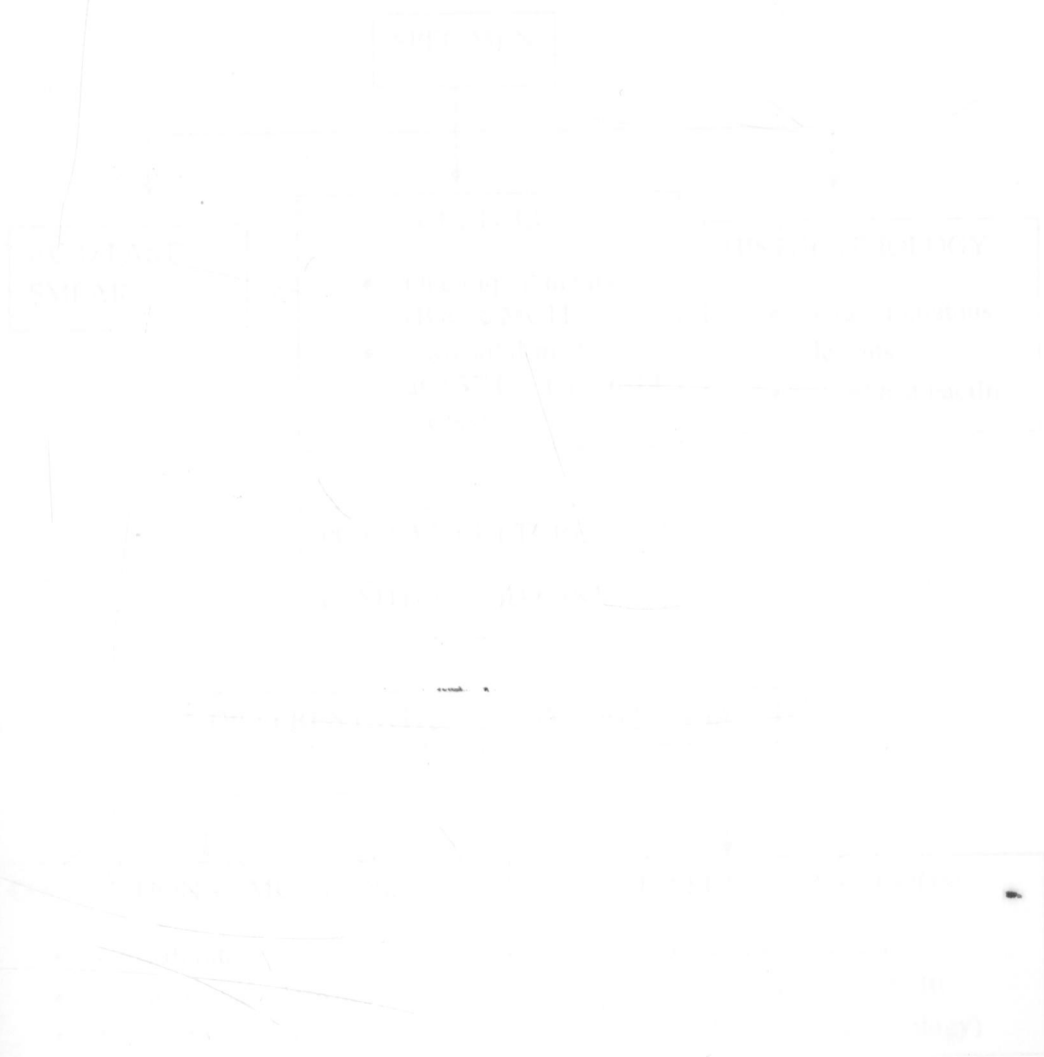
Samples of affected lymph nodes and other tissues were collected individually into sterile plastic bags, labelled, secured to prevent any leakage and placed into a cooler-box containing ice packs for transportation to the laboratory. In the laboratory fat and connective tissue were trimmed off using sterile surgical blade for each sample. All specimens were split into two parts depending on the size and consistency. One portion was placed in 100ml plastic containers and fixed in 10% buffered formalin for use in histological examination. The other

portion was placed in sterile bijoux bottles for use in microbiological analysis. Specimens for histology were stored at room temperature while those for microbiological analysis were preserved at -20°C .

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portion was placed in sterile bijoux bottles for use in microbiological analysis. Specimens for histology were stored at room temperature while those for microbiological analysis were preserved at -20°C .

Handling of Mycobacterial specimens for laboratory diagnosis



3.3 LABORATORY ANALYSIS

Laboratory analysis was conducted using the methods summarised in Figure 2.

Handling of Mycobacterial specimens for laboratory diagnosis

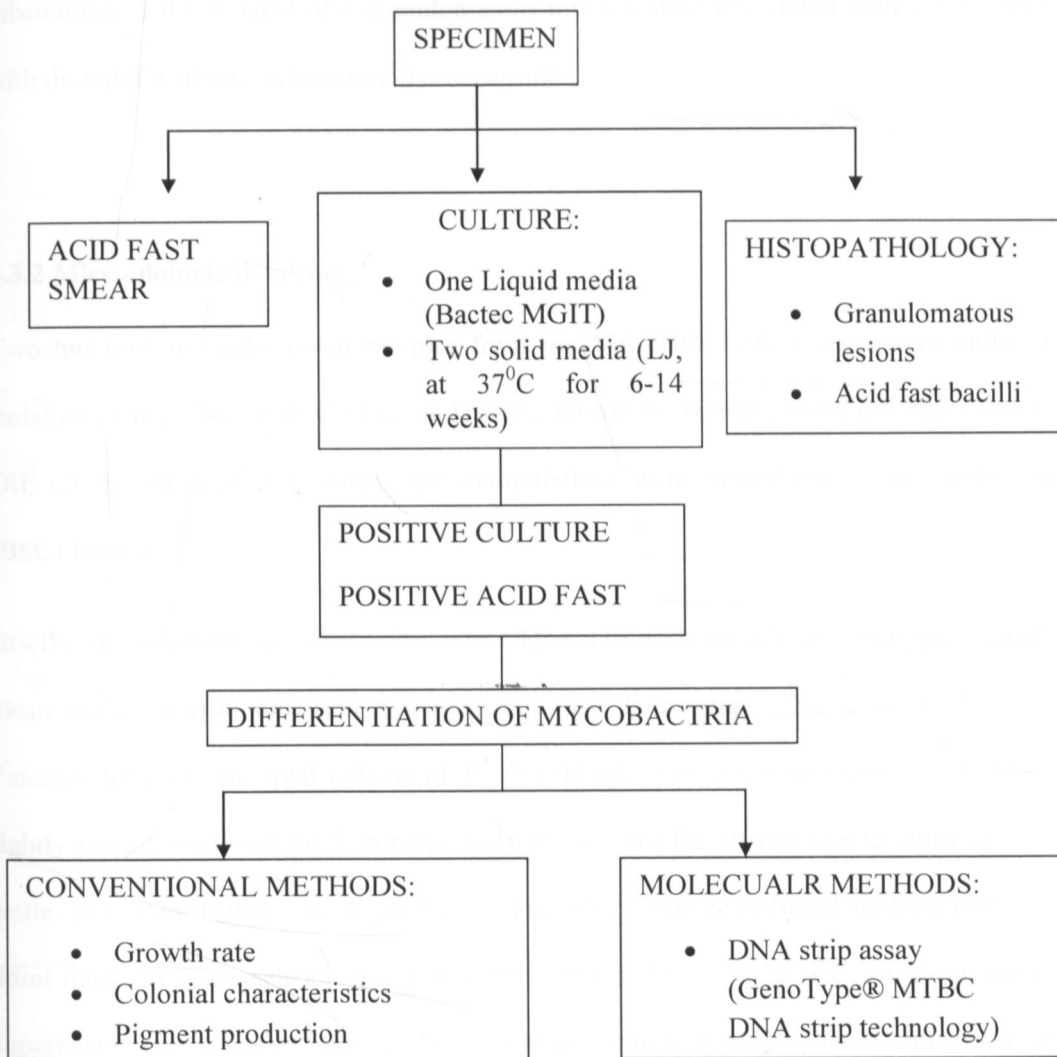


Figure 2: Algorithm for handling of Mycobacterial specimens. Adopted from Neonakis *et al.*, 2008, with modification.

3.3.1 Histopathological examination

Tissue samples were embedded in paraffin wax, sectioned at 5µm, and stained with hematoxylin-eosin by use of standard procedures (Bolin *et al.*, 1997; Varello *et al.*, 2008). Slides were evaluated microscopically at increasing magnifications. A sample was considered tuberculous if it consisted of a granulomatous inflammation associated with central necrosis with or without fibrous connective tissue capsule.

3.3.2 Microbiological culture

Two hundred and fifty seven samples from the 176 cattle with lesions were cultured for isolation of mycobacterial. Culture of *Mycobacterium bovis* was carried out according to the OIE (2009) standard procedures. All manipulations were carried out in bio-safety cabinet (BSC) level II.

Briefly 5ml of sterile distilled water was added into each sample and then transferred into clean sterile Griffith tube and homogenized. The homogenate was transferred into 50ml Falcon® tube and an equal volume of 4% NaOH added for decontamination. The tube was tightly closed, vortexed for 5 minutes and left to stand for 10 minutes to allow aerosols to settle. Decontamination was stopped by adding 6.8 phosphate buffered solution (PBS) to the 45ml mark. The suspension was then centrifuged at 3150 rpm at 4°C for 20 minutes. The supernatant was discarded into a plastic container containing 5% phenol and the pellet re-suspended in 1ml PBS by vortexing until the entire pellet dissolved. Five hundred (500) µl of the suspension was inoculated to 7ml BBL™ MGIT™ (Mycobacterium growth indicator tubes) containing OADC enrichment and PANTA antibiotic mixer and incubated in

BACTEC™ MGIT™ 960 Mycobacteria detection system at 37°C for a period of 7-42 days. Two hundred (200) µl of the suspension was also inoculated into one tube of Lowenstein-Jensen (L-J) media containing 0.4% pyruvate and another containing 0.75% glycerol. The tubes were incubated at 37°C for up to 12 weeks with weekly observations for any discernable growth. Smears were prepared with one drop of the sediment after centrifugation of the homogenized suspensions for detection of AFB by microscopy.

The cultures were considered as *M. bovis* if they grew slowly, showed small colonies and the growth was enhanced by pyruvate and not glycerol. All isolates grown in BBL™ MGIT™ tubes and Lowenstein-Jensen (LJ) media were tested for mycobacterial by acid fast staining.

3.3.3 Ziehl-Neelsen (ZN) staining

Tissue sections, direct smears and culture smears were stained by the standard Z-N staining technique. Smears were prepared by placing a loopful of the suspension on a labelled clean grease free slide and spread as thinly as possible (2cm by 1 cm) and left overnight under ultra violet light to inactivate viable mycobacteria. Z-N staining procedures were then carried out as described in the World Health Organization manual (Anonymous, 1998) as briefly described here. Labelled tissue section slides or smears were placed on a slide-rack and heat fixed for about 5 seconds. The slides were then flooded with strong carbol fuchsin and heated until they began to steam and left to stain for 5 minutes. Each slide was then rinsed separately in running tap water to remove excess stain. The slides were then replaced on the slide-rack and flooded with 25% sulphuric acid for 3 minutes to de-stain and then rinsed gently using running tap water. The slides were again placed on the slide-rack and flooded with the counter

stain, 0.3% methylene blue for 1 minute followed by washing gently in running tap water. The slides were left to dry in the open air. Each slide was examined carefully under oil immersion (X1000 magnification). The samples were considered positive when one or more acid-fast bacteria were detected (OIE, 2009).

3.3.4 Molecular analysis

GenoType® MTBC DNA strip technology was used (HAIN Diagnostica, Nehren, Germany). The test is based on a specific reverse hybridization step of the amplicon against a fixed DNA of different Mycobacterium species on a blotting membrane. The procedure involves isolating DNA from cultured material, multiplex amplification with biotinylated primers and reverse hybridization of the single-stranded, biotin-labeled amplicons to membrane-bound probes (Neonakis *et al.*, 2007).

DNA extraction

A loopful of bacteria was collected from the LJ slant and suspended in 300µl of molecular grade water in labelled 1.5ml sterile microcentrifuge tubes and then tightly closed. The manipulations were carried out in a BSC II cabinet. The tubes were fixed in a floater and incubated for 20 minutes in boiling water (95⁰C) to inactivate the mycobacteria. After inactivating the mycobacteria, the tubes were incubated in ultrasonic bath for 15 minutes to break the mycobacterial cell wall to release DNA. The tubes were then centrifuged at 14000 rpm for 5 minutes at room temperature and the supernatant containing DNA was transferred into fresh tubes and stored at 4⁰ C until they were used.

Amplification

These operations were carried out in a DNA clean room. 35 μ l Primer nucleotide mix (PNM), 5 μ l PCR buffer, 2 μ l $MgCl_2$, 0.2 μ l Taq (HotStar Taq, Qiagen) and 3 μ l molecular grade water per sample was measured into 1.5 ml sterile microcentrifuge tube to make the master mix aliquot and gently mixed. PCR tubes were labelled and 45 μ l aliquots of the mixture dispensed into each tube. All the tubes were capped and put in a PCR tube rack. The tubes were transferred to the DNA addition area. Working on each sample at a time, 5 μ l aliquot of the corresponding DNA template was carefully transferred to the amplification mixture and the tube recapped. The loaded PCR tubes were then transferred to the automated PCR amplification and detection room.

The thermocycler was turned on and the line probe programme selected for culture samples (Hot30 programme) which has the following amplification profile:

95 ⁰ C	5 minutes	
95 ⁰ C	30 seconds	} 10 cycles
58 ⁰ C	2 minutes	
95 ⁰ C	25 seconds	} 20 cycles
58 ⁰ C	40 seconds	
70 ⁰ C	40 seconds	
70 ⁰ C	8 minutes	

The program ran for 2.5 hours. The PCR tubes were removed, placed on a rack and transferred to hybridization area.

Hybridization and evaluation

Twenty μl aliquot of denaturation solution (DEN) was dispensed into one corner of the required number of test wells in a plastic reaction tray. Twenty μl aliquot of the amplified product was added to the DEN solution and gently mixed. The mixture was incubated at room temperature for 5 minutes. While the tray contents were incubating, the MTBC strips were removed from the kit and labelled at the coloured stripe end with a fine permanent pen. One thousand μl of hybridization buffer (HYB) was added into each well and allowed to mix with the DEN-amplified mixture. Using a pair of forceps, each numbered test strip was loaded into its corresponding well and fully submerged. The loaded reaction tray was placed on a TwinCubator and incubated for 30 minutes at 45°C . HYB buffer was removed by inverting the tray. 1000 μl stringent wash solution (STR) was added into each, placed on the TwinCubator and incubated for 15 minutes at 45°C . STR was removed by inverting the tray and placing it on absorbent paper towel.

One thousand μl of rinsing solution (RIN) was added into each well and incubated for 1 minute then discarded by inverting the reaction tray. One thousand μl of diluted conjugate (CON) was added into each well and incubated at room temperature for 30 minutes in the TwinCubator. CON solution is discarded by inverting the reaction tray followed by two successive rinsing using RIN solution each for 1 minute and one rinse using distilled water for

one minute. One thousand μ l of diluted substrate was added into each well and incubated in a dark place for 3-20 minutes and then discarded by inverting the reaction tray and washing with distilled water. The developed strips were pasted in the designated fields by aligning the conjugate control (CC) and universal control (UC) bands with the respective lines on the evaluation sheet (Appendix 4).

3.4 REVIEW OF SECONDARY DATA

A ten year retrospective analysis of bovine tuberculosis in Kenya was done by analysing the annual meat inspection reports from the Department of Veterinary service, Meat inspectorate division. Data for years 1997-2005 and 2007 was analysed and collated.

3.5 STATISTICAL EVALUATIONS OF RESULT

Statistical analysis was undertaken in Stata (Stata/IC 11.0 for Windows, Stata Corp, College Station, TX) using *ci* command in Stata with the binomial exact confidence interval and the Statistics calculator (www.statpac.com/statistics-calculator/free-version.htm). Microsoft office excel 2007 was used to draw the charts. Prevalence was determined as a proportion of the positive cases out of total carcasses examined expressed in percentage.

CHAPTER FOUR

4.0 RESULTS

4.1 ABATTOIR MEAT INSPECTION

4.1.1 Source of slaughter cattle

The slaughter cattle came from 18/47 (38.3%) Districts (Figure 3) although the source of 71 (1.4%) could not be ascertained. These districts cover approximately 452, 000 km², which represent about 77.4% (452,00km²/ 584,000 km²) of the total land size of Kenya. These are the ASAL areas where pastoralism, ranching and small scale agriculture-livestock farming is practised. All slaughtered cattle were Zebu breed.

4.1.2 Examination of slaughter cattle

A total of 3308 (66.4%) females and 1676 (33.6%) males were slaughtered in the two abattoirs during the study period. However, the investigator examined 929 (18.6%) cattle for tuberculous lesions; 704 in KMC and 225 in Njiru abattoir. These comprised of 666 females and 263 males. A hundred and seventy six (176) cattle had lesions consistent with bovine tuberculosis (Figure 4) accounting for a prevalence rate of 18.95% (95% CI: 16.43-21.48). The lesions were detected in 122/666 (18.32%) and 54/263(20.53%) females and males respectively. Statistically there was no significant difference between prevalence of bovine tuberculosis in males and females ($p>0.05$). The lesions were either localised (173/176 or 98.3%) or generalised (3/176 or 1.70%). In both slaughterhouses, old multi-parous females with poor body condition had more lesions than young adults with good body condition.

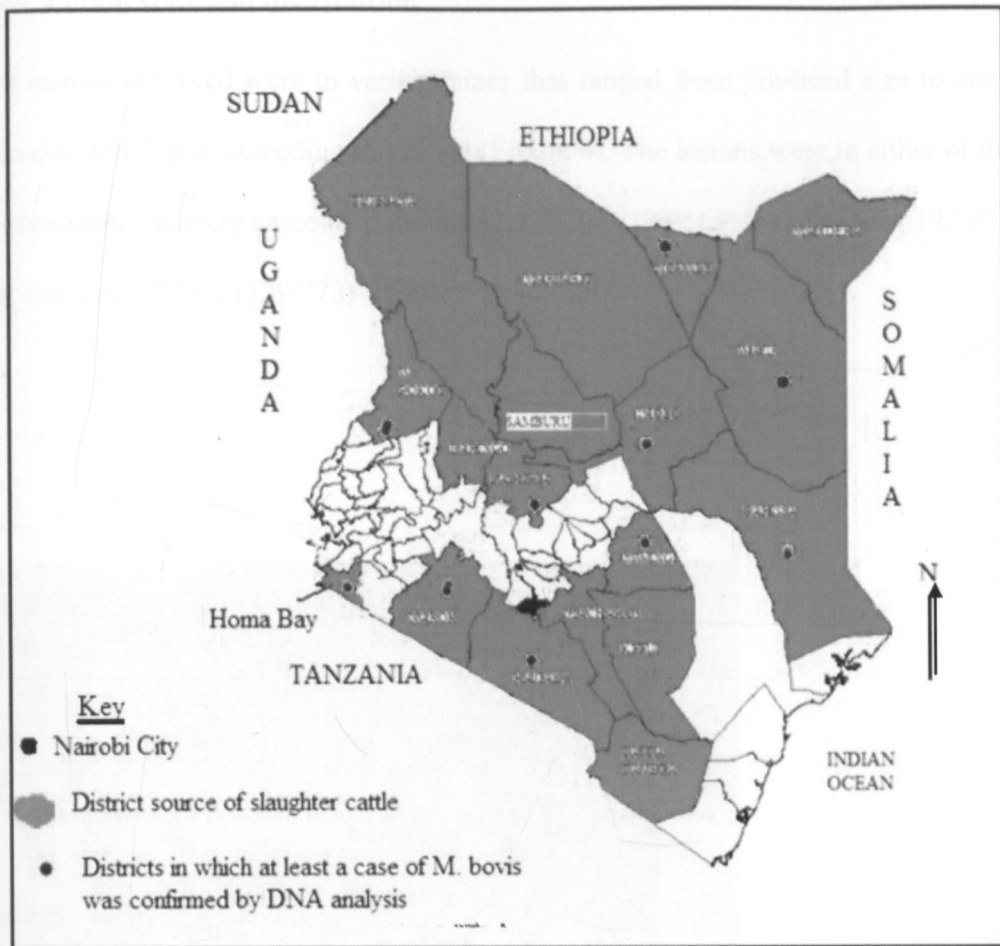


Figure 3: Map of Kenya showing sources of cattle examined for bovine tuberculosis at Kenya Meat Commission (KMC) and Njiru abattoirs, Nairobi, July- November 2009.

4.1.3. Lesion state and distribution

The lesions observed were in various sizes that ranged from pin-head size to about 3cm in diameter with a few exceeding this range (Figure 4). The lesions were in either of three stages of consistency namely caseous/ purulent 32.97% (90/273), caseo-calcareous 19.78% (54/273) and calcified 47.25% (129/273) (Figure 5).

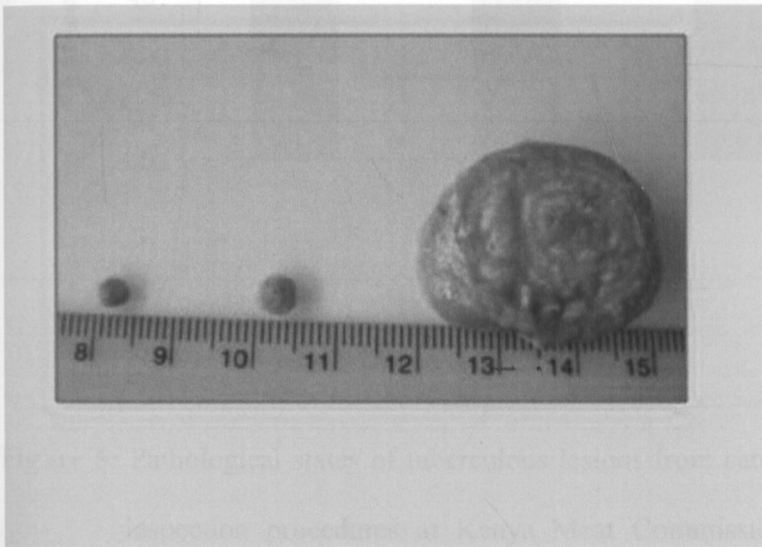


Figure 4: Tuberculous lesions of varying sizes (4-30 mm) from bovine lymph nodes observed during routine meat inspection at Kenya Meat Commission (KMC) and Njiru abattoirs, Nairobi in July-November 2009.

A total of 211 lesions were observed in the 176 cattle. The lesions were distributed as follows:

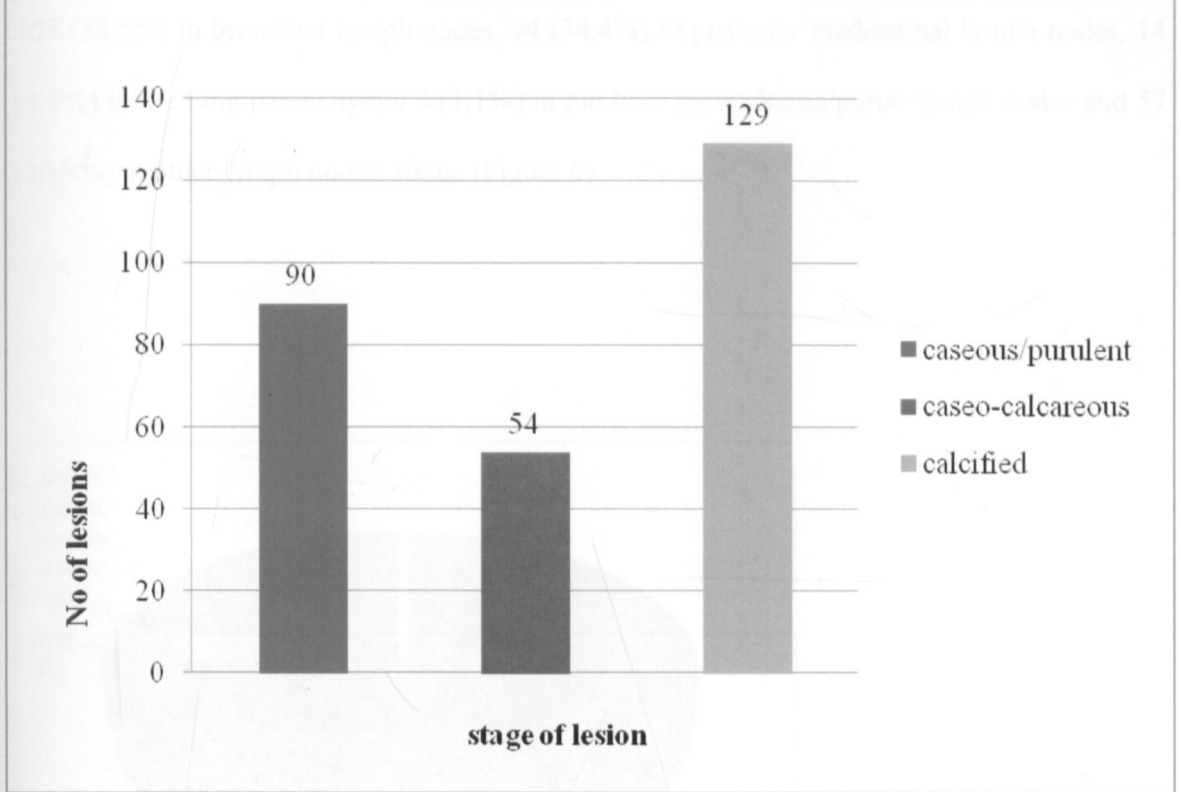


Figure 5: Pathological states of tuberculous lesions from cattle collected during routine meat inspection procedures at Kenya Meat Commission (KMC) and Njiru abattoirs, Nairobi in July-November 2009.

A total of 273 lesions were observed in the 176 cattle. The lesions were distributed as follows: 105 (38.5%) in bronchial lymph nodes, 94 (34.4%) in posterior mediastinal lymph nodes, 14 (5.1%) in the lung parenchyma, 3 (1.1%) in the liver parenchyma/portal lymph nodes and 57 (20.9%) in other lymph nodes/ tissue (Figure 6).

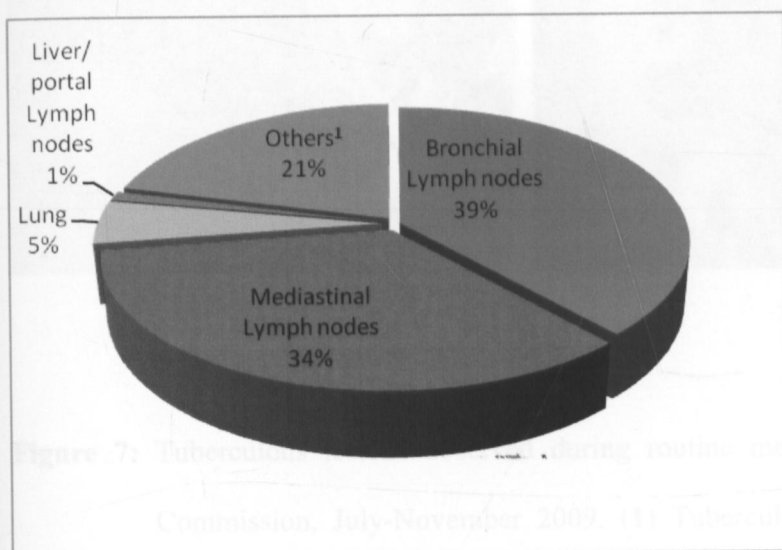


Figure 6: Distribution of tuberculous lesions in cattle examined at Kenya Meat Commission (KMC) and Njiru abattoirs, Nairobi, July-November 2009. Others¹- all other lymph nodes and tissue routinely examined during meat inspection.

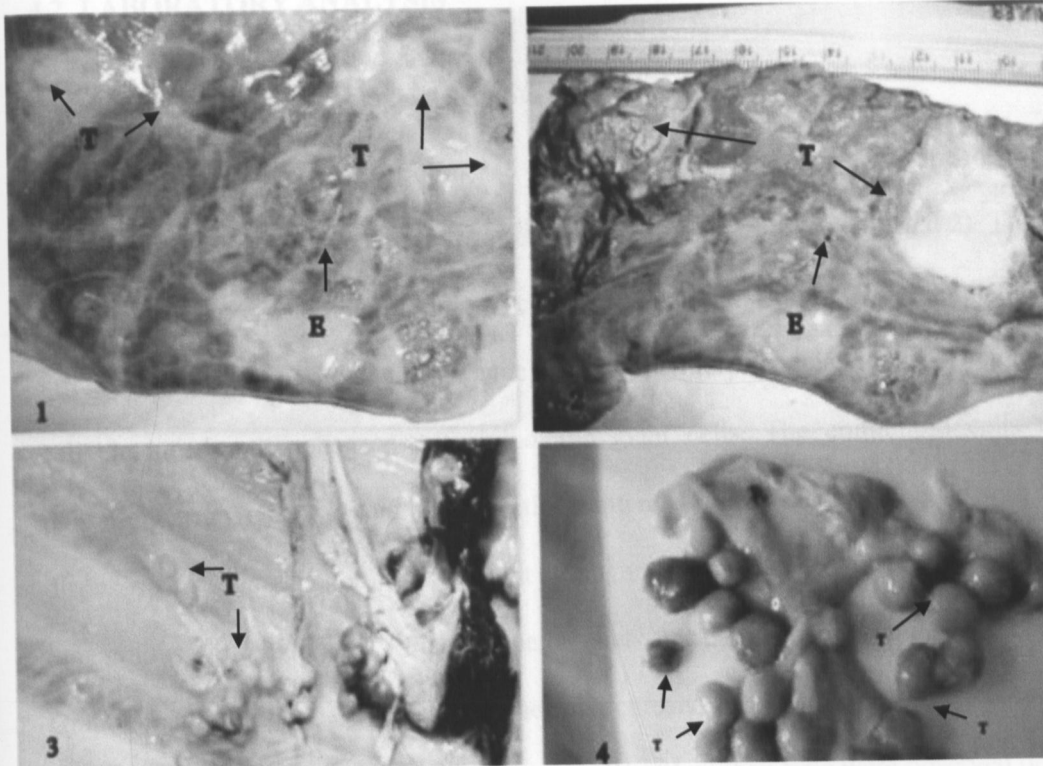


Figure 7: Tuberculous lesions observed during routine meat inspection at Kenya Meat Commission, July-November 2009. (1) Tuberculous lesions in the lungs of a bovine showing multiple tubercles (T) Note the yellowish colour the lesion material and also the emphysema (E). (2) Incised lungs lesions showing tubercles (T) and emphysema (E). (3) Multiple tubercles (T) along the ribs of a bovine carcass. (4) Multiple tubercles (T) attached to the diaphragm of a bovine carcass.

4.2. LABORATORY ANALYSIS

4.2.1 Examination of histological sections

Tuberculous granulomas were composed of caseous necrotic centre surrounded by a zone of inflammatory cells followed by fibrous tissue capsule (Figure 8: plates 1 and 2). The caseous necrotic centre consisted of amorphous, coarsely granular, acellular, pink staining debris. The inflammatory cell comprised of mononuclear and polymorphonuclear cells. The mononuclear cells included macrophages, epithelioid and giant cells. The epithelioid cells formed a palisading arrangement around the caseating centre. Multinucleated giant cells with the nuclei disposed in a horse-shoe arrangement (Langhans giant cells) were observed in some tuberculous granulomas. Plasma cells and occasional neutrophils were observed. Granulomatous lesions typical of bovine tuberculosis, manifesting granulomas with central necrosis surrounded by multinucleated giant cells of Langhan type were observed only in 4/36 (11.1%) tissue sections.

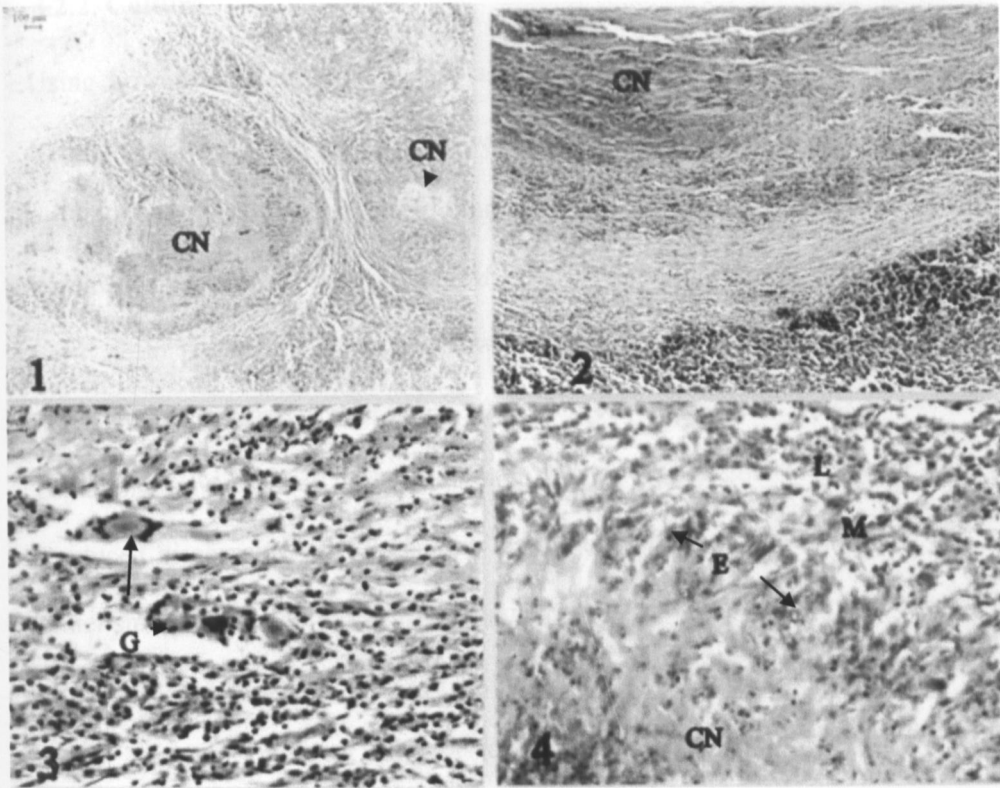


Figure 8: (1) Photomicrograph section of a bovine lymph node showing caseous necrosis (CN). Note the rounded, focal nature of these granulomas at low power magnification, HE, X40. (2) A higher magnification (X100) of the tuberculous lesion showing an amorphous region of caseous necrosis (CN) surrounded by blue ring of epithelioid and macrophage cells. (3) A higher magnification (X400) of the tuberculous lesion showing two giant cells of the Langhans type (G). (4) This photomicrograph shows elongated epithelioid cells (E) forming a ring around pink, amorphous area of caseous necrosis (CN). This zone is in turn bordered by macrophages, lymphocytes, plasma cells and neutrophils.

4.2.2. Culture

Using Lowenstein-Jensen (L-J) media, observable mycobacterial colonies suggestive of the MTBC were first noted on the sixth week after incubation. However some did not show any growth until the fourteenth week although majority showed growth between the eighth and tenth week of incubation. Growth of *M. bovis* was promoted with the addition of 0.4% sodium pyruvate to L-J media. *M. bovis* growth produced white, moist, small slightly rough friable non-pigmented colonies after about 10 weeks (6-14 weeks). *M. tuberculosis* grew well in L-J containing glycerol and produced rough large colonies that were hard to break. MOTT produced discernable growth as early as the second week of incubation. The colonies were either smooth or rough with yellow pigmentation. In total, growth suggestive of Mycobacteria was confirmed by acid fast test in sixty four out of the one hundred and seventy six lesioned cattle. Caseous/purulent and caseo-calcareous lesions yielded 62/64 (96.87%) of the *Mycobacterial* positive culture while 2/64 (3.13%) calcified lesions yielded positive *Mycobacterial* culture. Growth in BBL MGIT tubes was characterised by high contamination with some tubes being positive within a day. On Z-N staining, these tubes were negative for acid fast bacilli. Tubes that produced acid fast positive cultures showed growth from third day of incubation. Mycobacterial growth was characterised by a non-homogenous turbidity with flakes appearing as small grains.

4.2.3 Ziehl-Neelsen (Z-N) staining.

Three out of thirty six tissue sections stained with Ziehl-Neelsen had acid fast bacilli. The number of acid-fast bacilli seen was extremely low, ranging from two to five (2-5) per slide. The acid fast bacilli were seen within the cytoplasm of multinucleate giant cells and 1 within macrophage cytoplasm (Figure 12). However since not all tissue collected from the 176 cattle was prepared for histology, the results could not be used for calculation of the prevalence of bovine tuberculosis. Direct Ziehl-Neelsen staining had 104/257 (40.5 %) acid fast positive samples. These comprised of 31/94 (33.0%) bronchial lymph nodes, 37/94 (39.4%) posterior mediastinal lymph nodes, 5/14 (35.7%) lung tissue and 31/55 (56.4%) other lymph nodes and tissue. In total 63/176 [35.80; 95%: (28.64-42.96)] cattle with lesions were acid fast positive on direct microscopy (Table 1). The prevalence using direct Z-N staining technique was 6.78% (95% CI: 5.16-8.40). Sensitivity of Z-N staining technique using DNA analysis as the gold test was found to be 28.57% [95% CI: 18.24-41.54] and Specificity as 99.11% [95% CI: 94.45-99.95]. The acid fast smear positive from culture (L-J and MGIT tubes) was 64/176 (36.36%). The prevalence using culture-Z-N staining was found to be 6.89% [95% CI: 5.26-8.52]. There was no significant difference between direct Z-N and culture acid fast positive, ($p>0.05$).

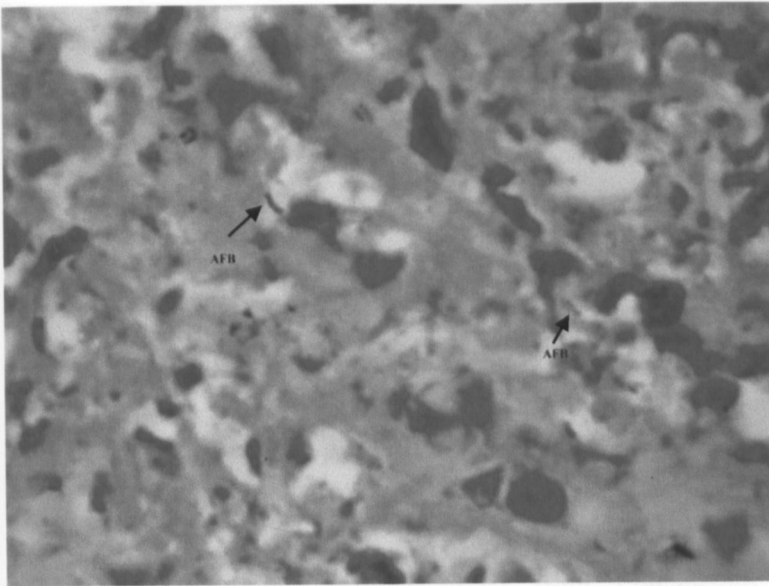


Figure 9: Photomicrograph of a section of tuberculous lesion in a bovine lymph node showing red acid fast bacilli (AFB). (Ziehl-Neelsen (Z-N) stain, X1000)

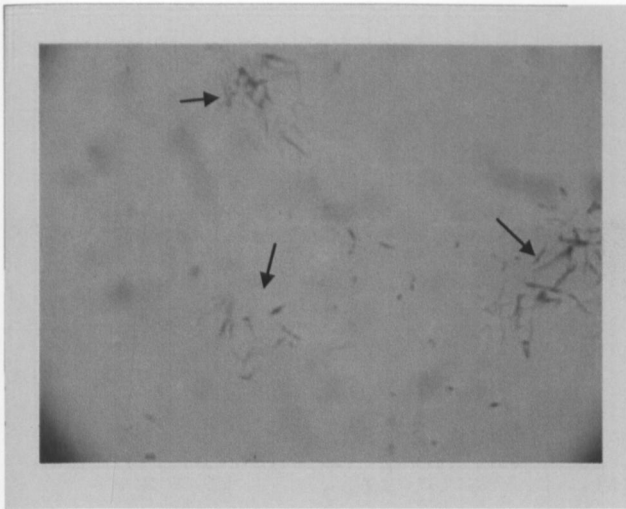


Figure 10: Acid fast stained smear from Lowenstein-Jensen media. Note the red straight/curved slender bacilli (→) forming linear clumps, a characteristic of tubercle bacilli, X1000.

4.2.4 DNA analysis

The identification of the mycobacterial species is based on the different combinations of multiple bands characterizing each species. Using the evaluation sheet, the banding pattern revealed that proportion of *M. bovis* was 19/64 (32.8%), *M. tuberculosis* was 2/64 (3.1%) and MOTT was 43/64 (67.2%). *M. bovis* was confirmed in 10/18 of the districts from where the cattle originated (Figure 3). The two *M. tuberculosis* cases were isolated from two cattle that came from Moyale District. No attempt was made to identify the mycobacterial species that comprised the MOTT isolates. Using DNA analysis, the prevalence of *M. bovis* was found to be 2.05% (95% CI: 1.13-2.96).

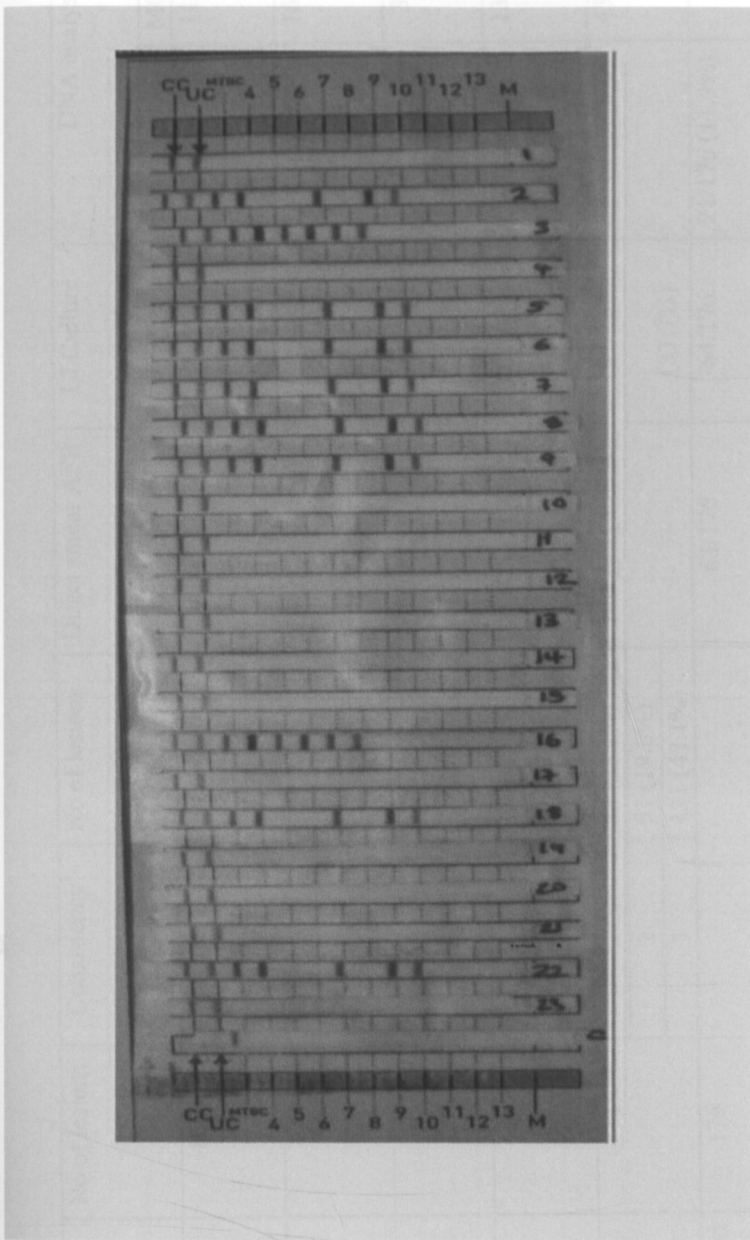


Figure 11: DNA analysis of mycobacterial culture from L-J media. Note the banding pattern: *M. bovis* samples are 2, 5,6,7,8,9,18 and 22; *M. tuberculosis* samples are 3 and 16 while all the rest were MOTT positive and C is control. Genotype MTBC 96, VER 1.X was used

Table 1: Examination of the tissue samples using Ziehl-Neelsen (Z-N) staining, culture and DNA analysis

Location of Tissue	No of lesions	Consistency ³	No of lesions	Direct smear AFB positive	LJ Culture AFB positive	DNA analysis ⁴	
						MTBC	MOTT
Bronchial Ln ¹	105	1	30(31.9%)	31/94 (33.0%)	25/94 (26.6%)	4/25 (16.0%)	11/25 (44.0%)
		2	15 (16.0%)				
		3	49 (52.1%)				
Mediastinal Ln ¹	94	1	24 (25.5%)	37/94 (39.4%)	41/94 (43.6%)	12/41 (29.3%)	16/41 (39.0%)
		2	23 (24.5%)				
		3	47 (50.0%)				
Lung tissue	14	1	8 (57.1%)	5/14 (35.7%)	7/14 (50.0%)	4/7 (57.1%)	3/7 (42.9%)
		2	3 (21.4%)				
		3	3 (21.4%)				
Other tissue ²	60	1	23 (41.8%)	31/55 (56.4%)	24/55 (43.6%)	1/24 (4.2%)	13/24 (54.2%)
		2	10 (18.2%)				
		3	22 (40.0%)				
Total lesions	273	1	85 (33.1%)	104/257 (40.5%)	97/257 (37.7%)	21/64 (32.8%)	43/64 (67.2%)
		2	51 (19.8%)				
		3	121 (47.1%)				
Total lesioned cattle	176			63/176 (35.8%)	64/176 (36.4%)	21/176 (11.9%)	43/176 (24.4%)

Ln¹ - Lymph node; other tissues² - any other tissue/lymph nodes other than those listed above; consistency³: 1 (caseous or purulent), 2 (caseo-calcareous), 3 (calcified); DNA analysis⁴ - MTBC positive isolates and MOTT positive isolates. GenoType specific for simultaneous detection of *Mycobacterium*, MTBC and species within MTBC was used. PCR was carried out on acid fast positive cultures only.

4.3 SECONDARY DATA

Review of secondary data from the annual meat inspection reports from the Department of Veterinary Service, meat inspectorate division revealed that bovine tuberculosis in slaughter cattle was reported only once in 2007 but no isolation was attempted. Out of 489,529 cattle slaughtered in that year, 45 (0.009%; 95% CI: 0.006-0.012) were found with generalised tuberculous lesions. Ziehl-Neelsen staining technique confirmed acid fast bacilli suggestive of *Mycobacterium*. However no further tests were carried out to confirm *M. bovis*. The ten year review gave mycobacterial prevalence of 0.001% (45/4172647) among the slaughter cattle. Tuberculous lesions were not reported in any other meat animal species during the ten years reviewed.

CHAPTER FIVE

5.1 DISCUSSION

This study comprised of routine abattoir meat inspection, laboratory analysis and review of the national annual meat inspection reports over a period of ten years. In Kenya, routine abattoir inspection for any infection, including bovine tuberculosis, applies the method prescribed in the Meat Control Act (Cap.356, 1972). Briefly it involves visual examination, palpation and incision of intact organs like the lungs, liver and the drainage lymph nodes. In the carcass, prescapular, supra-mammary/superficial inguinal, deep inguinal, ischiatic and iliac lymph nodes are routinely palpated and incised. The pleura are visually examined. If tuberculous lesions are detected in one of these tissues, other lymph nodes not routinely examined are incised. Should miliary tuberculous lesions in various parts of the carcass (lung, intestine, liver and multiple lymph nodes) be observed, the whole carcass is condemned, while condemnation of organs is undertaken if localised tuberculous lesions are observed in organs and their associated lymph nodes.

The two abattoirs in which the investigation was conducted receive their slaughter stock from all parts of the country and provide part of the daily beef requirements to over 3.2 million residents of Nairobi City. During the study period, the two abattoirs received cattle from 18/47 districts covering 77.4% of Kenya's land size (Appendix 2). Although *M. bovis* was confirmed by DNA analysis in 10/18 (55.56%) of the sampled districts, the number of cattle involved in some of the districts was too small and therefore the absence of a confirmed case does not rule out the presence of the disease in those areas. Furthermore, the source of 5/19 (26.32%) confirmed *M. bovis* cases could not be ascertained and some could have come from

the districts with no confirmed cases (Appendix 3). Even in those districts where *M. bovis* was confirmed the sample size was too small to give accurate district prevalence (Appendix 3). From the results, it is evident that bovine tuberculosis due to *M. bovis* is wide spread in the pastoral and agro-pastoral areas of Kenya where beef production is practiced, zebu cattle being the predominate breed. Similar findings have been reported in Tanzania (Kazwala *et al.*, 2001), Uganda (Oloya *et al.*, 2006), Ethiopia (Biffa *et al.*, 2010a), and many African countries (Cleaveland *et al.*, 2007). The ASAL areas have poor infrastructure, veterinarians are scarce and drought is a frequent phenomena. Also livestock theft is a cultural practice among the pastoralist, both within and outside Kenya and this further aggravate the problem. This implies that control of livestock movement is not feasible as livestock move extensively in the expansive area and across borders in search of pastures and water. These factors are conducive for the spread of bovine tuberculosis within the domestic animals, domestic animals and wildlife and from cattle to humans.

Using routine abattoir meat inspection, granulomatous lesions suggestive of bovine tuberculosis were detected in 18.95% [95% CI: (16.43-21.48)] of the slaughtered cattle. These findings, though slightly lower, are comparable with those reported in Tanzania where granulomatous lesions suggestive of bovine tuberculosis were reported in 19.8% (1504/7589) of slaughtered cattle (Cleaveland *et al.*, 2007). Igbokwe *et al.* (2001), reported in North-eastern Nigeria abattoirs prevalence ranging between 2.8-20% which compare with prevalence of this study. However the findings in this study are higher than those reported in Zambia where a similar study reported 14.1% prevalence (Munyeme *et al.*, 2009). According to Thoen *et al.* (2006), in areas where disease control program is absent, as high as 40%

prevalence of tuberculosis can occur in slaughter cattle in public abattoirs. Uncontrolled cross-border movement of livestock from Somali, Ethiopia, Sudan and Tanzania (Aklilu *et al.*, 2002) and Uganda (Michael *et al.*, 2008) form an important source of livestock slaughtered in Nairobi and other parts of Kenya. Since *M. bovis* has been confirmed in all these countries, and that cattle movements are a significant predictor of the distribution of bovine tuberculosis (Gilbert *et al.*, 2005), it can be postulated that the prevalence of bovine tuberculosis among slaughter cattle in Kenya is not significantly different from what has been reported in Ethiopia, Sudan, Uganda, Tanzania and Somalia. The sensitivity of routine abattoir inspection is considered low (Asseged *et al.*, 2004; Teklu *et al.*, 2004; Biffa *et al.*, 2010a) and a recent study in Ethiopia has shown that routine abattoir inspection failed to detect 72.0% of lesioned carcasses identified by detailed abattoir inspection (Biffa *et al.*, 2010a). Therefore in view of absence of bovine tuberculosis surveillance and control strategies in Kenya, the 18.95% abattoir meat inspection prevalence may be an under-estimation of the true prevalence of infection among slaughter cattle in Kenya.

There was no significant difference in prevalence of bovine tuberculosis in males and females ($p>0.05$). This agrees with the findings reported in Ethiopia by Teklu *et al.* (2004). This implies that males and females are equally susceptible to *M. bovis* infection. In this study, old multi-parous females with poor body condition had more lesions than young adults with good body condition. Biffa *et al.* (2010a) and Ndukum *et al.* (2010) reported similar findings in Ethiopia and Cameroon respectively. Advance in age and poor body conditions suppress immunity to diseases. This may explain why more lesions were observed in old cattle with poor body condition and in particular multi-parous females. This finding is significant since

zebu cattle contribute about 40% of the total cattle milk and the bulk of beef consumed in Kenya (Anonymous, 2010). Consumption of milk contaminated by *M. bovis* and close association between animals and humans has long been regarded as the principal mode of bovine tuberculosis transmission from animals to humans (Daborn and Grange, 1993). Most pastoralists consume a diet largely consisting of milk which may be consumed fresh, directly after being drawn from the cow or the milk may be soured. *M. bovis* has been found to survive in soured milk for up to 14 days (Minja *et al.*, 1998). Pastoralists also have the tradition of eating certain parts of freshly slaughtered animals raw. These cultural practices are important predisposing factor to *M. bovis* infection. Although studies conducted in 1958 (Sula *et al.*, 1960) and recent studies by Koech (2001) among the Maasai pastoralist in Narok did not yield any *M. bovis* positive cases, human tuberculosis due to *M. bovis* has been reported in neighbouring countries such as Ethiopia (Kidane *et al.*, 2002), Tanzania (Mfinanga *et al.*, 2004) and Uganda (Oloya *et al.*, 2008) where cultural practices are similar to those of the Kenyan pastoralists. It is therefore imperative a deeper investigation into bovine tuberculosis in humans in Kenya be carried out.

Seventy eight percent of the tuberculous lesions were observed predominantly in the lungs and associated lymph nodes, particularly the posterior mediastinal and the bronchial nodes. Similar findings have been reported in Ethiopia where Tsegaye *et al.* (2010), detected tuberculous lesions predominantly in mediastinal and bronchial lymph nodes. The findings are also consistent with those observed by Sahraoui *et al.* (2008), who reported 76.92% of tuberculous lesions in the lungs and thoracic lymph nodes but differ from the findings of Cleaveland *et al.* (2007), who reported 61.3% lesions in the gastrointestinal tract and 35.3% in

lungs and thoracic lymph nodes. However Whipple *et al.* (1996), Asseged *et al.* (2004), Teklu *et al.* (2004), and Ndukum *et al.* (2010), reported that tuberculous lesions were predominately found in the lungs and associated lymph nodes. According to Corner (1994), up to 95% of cattle with visible tuberculous lesions could be identified by examination of the lung and the associated lymph nodes. The present study finding is therefore consistent with studies conducted elsewhere and shows that in most cases transmission of *M. bovis* between cattle is aerogenic (Ayele *et al.*, 2004).

The lesions observed were in various sizes that ranged from pin-head size to about 3cm in diameter with a few exceeding this range (Figure 4). The finding of lesions small enough to be missed during routine meat inspection is significant and emphasis for diligence by the meat inspectors. Low detection rate of tuberculosis infected carcasses implies that such meat is approved as fit for human consumption thus exposing the consumers and all those involved in the beef industry chain to risk of infection. Similar concern has been raised in Ethiopia (Biffa *et al.*, 2010a). Finding of lesions in various pathological stages (caseous or purulent, caseo-calcareous or calcified in consistency) was consistent with findings reported by others (Whipple *et al.*, 1996; OIE, 2009). Caseous/purulent and caseo-calcareous lesions yielded 62/64 (96.87%) of the *Mycobacterial* positive cultures. However only 2/64 (3.13%) calcified lesions yielded *Mycobacterial* positive culture. The presence of calcification denotes that repair has occurred but does not imply sterility of the lesion (Churg *et al.*, 2005) although in completely calcified lesions, tubercle bacilli may be dead and therefore no growth is obtained upon culture (Quinn *et al.*, 1994; Teklu *et al.*, 2004). The stage of calcification is therefore important and calcified tuberculous lesions should also be handled with caution just as caseous/purulent and caseo-calcareous lesions are treated.

Of the 36 tissue samples processed for histological analysis, granulomatous lesions typical to bovine tuberculosis, manifesting granulomas with central necrosis surrounded by multinucleated giant cells of Langhan type, as described by Varello *et al.* (2008), were observed only in 4 (11.1%) tissue sections. In a similar study in Ethiopia, granulomatous lesions typical of bovine tuberculosis were observed in 4.3% tissue sections (Shitaye *et al.*, 2006). Although other diagnostic techniques are often used in diagnosis of bovine tuberculosis, histopathology is a valuable technique since it can characterise lesions unrelated to mycobacterial infection and can produce results within a few days, a major advantage over culture which could take up to 12 weeks. Ziehl-Neelsen staining of the tissue sections yielded 3/36 (8.3%) acid fast bacilli sections. The Z-N low sensitivity has been reported by others (Shitaye *et al.*, 2006; Varello *et al.*, 2008). *M. bovis* are often low in bovine specimens (Quinn *et al.*, 1994). However, lesions determined to be tuberculous upon examination of HE-stained section with no acid fast bacilli may be regarded as suggestive of tuberculosis (Thoen *et al.*, 1995). Berk *et al.* (1996) reported that out of 18 PCR positive samples fixed with formaldehyde solution, Z-N staining was positive only in three samples (16.6%) while Shitaye *et al.* (2006) did not detect any AFB in 69 Z-N stained tissue sections. The findings of this study therefore agree with those reported by others.

Tuberculous lesions confirmed as acid fast positive by direct Ziehl-Neelsen staining yielded 35.80% of the 176 lesioned cattle. This is lower than that reported by Sulieman and Hamid (2002) in Sudan where a similar study revealed 53.3% of the samples as acid fast positive. It is however higher than that reported in Algeria (Sahraoui *et al.*, 2008), Cameroon (Ndukum *et*

al., 2010) and Sudan (Manal *et al.*, 2005). The findings in this study are therefore within what most others have reported in Africa. Although microscopic examination can only detect acid fast bacilli but cannot differentiate between mycobacterial species, it is a valuable tool in preliminary diagnosis of *M. bovis* in granulomatous lesions collected during abattoir meat inspection and can provide results within a short time (Kallenius *et al.*, 1994).

In this study, granulomatous lesions suggestive of bovine tuberculosis caused by non-mycobacterial infections accounted for 64.2%. These findings are however lower than those reported in Sudan (Manal *et al.*, 2005) and Tanzania (Cleaveland *et al.*, 2007) where similar studies revealed 79.04% and 98.17% lesions consistent with bovine tuberculosis in slaughter cattle were caused by non-acid fast organisms respectively. In Ethiopia tubercle-like non-mycobacterial lesions were reported in 74.14% (Biffa *et al.*, 2010a) which is still higher than those reported in this study. Statistically there was a significant difference between the number of non-mycobacterial lesions reported by Manal *et al.* (2005), Cleaveland *et al.* (2007) and Biffa *et al.* (2010a) as compared to those reported in this study, ($p < 0.05$). However, there was no significant difference between prevalence of confirmed *M. bovis* by Manal *et al.* (2005) (13/167) and Biffa *et al.* (2010a) (58/406) and what this study confirmed (19/176), ($p > 0.05$). Liebana *et al.* (2008) found 45% parasitic granulomas, 27% bacterial or mycotic pyogranuloma and 23% bacterial abscesses in lesions that macroscopically are indistinguishable from tuberculous lesions.

Growth in the liquid media (BACTEC MGIT 960) was detected from third day. However there was high rate of contamination of the liquid media making it less ideal for primary

isolation of *M. bovis*. The high rate of contamination could be attributed to the fact that it is a highly rich medium and the fast growing bacteria outgrew the slow growing mycobacteria. Similar findings were reported by Tortoli *et al.* (1999). The liquid media proved to shorten diagnosis period by seven-fold (42/6), despite the disadvantage of high contamination rate. Although growth in L-J media took long compared with liquid media, L-J media has an advantage since a presumptive diagnosis of *M. bovis* can be made from the colonial morphology. The L-J positive culture rate which was 36.36% is lower than what Sahraoui *et al.* (2008) and Ndukum *et al.* (2010) reported but higher than those Biffa *et al.* (2010a) reported. One possible explanation for the low culture rate is that some tissues may contain non-viable or only a few live bacteria that fail to grow. Again in completely calcified lesions, tubercle bacilli may be dead and therefore no growth will be obtained upon culture (Quinn *et al.*, 1994; Teklu *et al.*, 2004). In this study, calcified lesions accounted for 47.25%. Therefore, the prevalence of bovine tuberculosis reported in this study could actually be underestimation of the true prevalence.

Identification of mycobacteria to the species level on the basis of growth rate, phenotypic characteristics and biochemical tests is laborious and extremely time consuming. GenoType MTBC proved to be a rapid, reliable and easy to interpret technique that identified MTBC to the species level. Richter *et al.* (2004) and Neonakis *et al.* (2007) also reported similar findings. MTBC were identified in 21/64 (32.8%) as: 19 *M. bovis* and 2 *M. tuberculosis*. The 2.05% prevalence of *M. bovis* found in this study is comparable with the 2.19% (25/1138) prevalence reported in Ethiopia (Ameni *et al.*, 2010). This is not unexpected since the prevailing conditions and uncontrolled livestock movement across the borders favour spread

of the disease regionally. The GenoType MTBC assay was found to enable identification of *M. tuberculosis* complex species and species differentiation within the complex within five hours. Although direct mycobacterial DNA isolation from the pathological lesions was not attempted, future studies should explore it to enable confirmation within a shorter time. The isolation of two *M. tuberculosis* isolates from mediastinal lymph node and the lungs was a significant finding. The presence of this human pathogen in the lungs of cattle with tuberculous lesions is an indication of the aerogenous transmission. Proximity between farmers and their cattle constitute a high risk of transmission of *M. tuberculosis* from humans to animals. Similar findings have been reported in Burkina Faso (Delafosse *et al.*, 1995), Tanzania (Weinhaupl *et al.*, 2000), Sudan (Sulieman and Hamid, 2002) and by Pavlik (2006). This could be attributed to the increasing animal-human contact due to increased human population and insecurity in some areas where animals are housed in close proximity with human especially at night.

In this study 24.4% of the lesions were caused by mycobacteria other than tuberculosis (MOTT). Studies in Chad and Uganda isolated MOTT from more than 40% of the animals exhibiting granulomatous lesions (Diguimbaye-Djaibe *et al.*, 2006; Oloya *et al.*, 2007) which is almost twice what this study found. Corner (1981) and Jorgensen (1981) reported that certain MOTT can produce in cattle localised lesions which macroscopically and histologically resemble lesions caused by *M. bovis*. This study further gives an insight that other *Mycobacterial* species infect cattle causing lesions that macroscopically resemble those caused by *M. bovis*. Further, this could explain why tuberculin tests give higher prevalence of bovine tuberculosis compared to other diagnostic tests.

In humans, the incidence of individual infections and outbreaks associated with MOTT has risen dramatically over the past decade establishing these organisms as significant human pathogens. The presence of MOTT in slaughter cattle therefore pose a big risk to human health especially immunocompromised individuals, pastoralists, veterinarians, meat inspectors, abattoir workers and other agricultural workers. Ingestion of raw animal products was identified as a risk factor for MOTT adenitis in Tanzania (Mfinanga *et al.*, 2004). *M. fortuitum* and *M. farcinogenes* have been isolated from tuberculous lesions in cattle and human in Chad (Diguimbaye-Djaibe *et al.*, 2006). Although the MOTT species involved in this study were not identified, their presence in meat approved for consumption is an important public health concern.

The ten year secondary data analysis revealed low detection rate of tuberculosis by the meat inspectors. Similar concern had been raised by Piers and Wright (1946 cited by Sula, 1960), who reported that in Kenya very little tuberculosis was reported by Veterinary officers, though post mortem results from selected districts gave an incidence ranging between 0.005% and 4%. Low rate of detection of bovine tuberculosis in abattoirs has been reported in Tanzania (Bakuname, 1994) and Cameroon (Ndikum *et al.*, 2010). Low detection rate of lesioned carcasses by the meat inspectors in Kenya during routine abattoir meat inspection poses great health risk to the consumers. Kazwala *et al.* (2001) reported that TB-contaminated meat poses a great public health danger particularly among pastoral communities who normally consume undercooked meat.

The confirmation of mycobacterial infection in slaughter cattle in this study is significant. A study conducted by Bartos *et al.* (2006) in Czech Republic concluded that dissections of cadavers with undiagnosed mycobacterial infection pose a risk to professionals and students performing post mortem examination. Similarly, veterinarians, meat inspectors and other abattoir workers are at high risk of contracting zoonotic tuberculosis and precautionary measures should be taken during post mortem examination of carcasses. Furthermore the high HIV/AIDS burden and other immuno-suppressing diseases such as diabetes and malnutrition especially among the rural communities in Kenya pose a great danger to human population contracting *M. bovis* and other opportunistic mycobacteria. Bovine tuberculosis in humans should be monitored especially in those who are at high risk of primary infection such as Veterinarian, meat inspectors, abattoir workers and agricultural and to identify any transmission between animals and humans (Gibson *et al.*, 2004).

5.2 CONCLUSION AND RECOMMENDATION

The overall objective of this study was to document the presence of bovine tuberculosis and estimate its prevalence among slaughter cattle in Kenya. The presence of tuberculous lesions in slaughter cattle and confirmation of *M. bovis* by culture and molecular analysis prove that bovine tuberculosis, though a neglected zoonosis, exist among the slaughter cattle in Kenya. Since the sampled cattle came from over 75% of the country, it can be concluded that *M. bovis* is widely distributed in the ASAL areas of Kenya where the bulk of beef cattle are reared. Considering the low sensitivity of meat inspection in detection of tuberculosis and that calcified lesions usually contain non-viable mycobacteria, the true prevalence of *M. bovis* infections could considerably be higher than the 2.05% found in this study. Low rate of detection of tuberculous lesions by meat inspectors imply that tuberculosis infected carcasses are passed undetected and meat approved for human consumption. Continuous education and training of meat inspectors is certainly of major importance. Isolation of *M. tuberculosis*, a human pathogen, shows that man is increasingly becoming a risk to cattle.

Recommendations

The low rate of detection of bovine tuberculosis lesions by meat inspectors during routine meat inspection procedures is a problem that urgently needs to be addressed. Refresher courses and further training for meat inspectors and close supervision by veterinary officers is deemed necessary to address this problem.

Further research on the importance of bovine tuberculosis in Kenya need be carried out through a jointly planned veterinary and medical programme. Also the Veterinary and Medical institutions in Kenya and in the neighbouring countries should collaborate and design a feasible bovine tuberculosis control programme to reduce zoonotic threat of the disease regionally since slaughter animals move freely across the porous international boundaries.

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APPENDICES

Appendix 1: Detection of tuberculous lesions in animals slaughtered in Kenya (1997-2007)

YEAR	CATTLE		SHEEP		GOATS		PIGS		CAMELS	
	No	TB ¹	No	TB ¹	No	TB ¹	No	TB ¹	No	TB ¹
1997	391229	0	157999	0	206074	0	93912	0	1551	0
1998	383038	0	129524	0	153926	0	87438	0	1153	0
1999	383088	0	129524	0	153986	0	85438	0	1357	0
2000	363183	0	128390	0	153806	0	85345	0	1726	0
2001	381393	0	172594	0	178173	0	93484	0	1806	0
2002	402463	0	198928	0	195479	0	76999	0	1778	0
2003	469631	0	249337	0	291193	0	80094	0	1763	0
2004	485853	0	299764	0	332208	0	95511	0	1763	0
2005	423240	0	281021	0	367480	0	91305	0	1465	0
2007	489529	45	358794	0	413542	0	93564	0	3426	0
TOTAL	4172647	45	2105875	0	2445867	0	883090	0	17788	0

TB¹ -number of animals with tuberculous lesions; data for 2006 were not available.

Source of data: Department of Veterinary service, Meat inspectorate division.

Appendix 2: Summary of origin of cattle slaughtered and examined for TB lesions at Kenya Meat Commission (KMC) and Njiru abattoirs, July-November 2009.

District of Origin	No. Slaughtered		Total	No. Inspected		Total	No. With lesions		Total
	Males	Females		Males	Females		Males	Females	
Baringo	37	141	178	3	5	8	0	1	1
Garissa	55	90	145	11	24	35	4	10	14
Homa Bay	58	30	88	7	0	7	1	0	1
Isiolo	257	532	789	45	86	131	3	12	15
Kajiado	359	843	1202	59	231	290	9	14	23
Kitui	13	39	51	3	10	13	0	2	2
Laikipia	86	393	479	16	34	50	7	11	18
Machakos	67	124	191	5	41	46	0	2	2
Mandera	9	6	15	5	2	7	1	1	2
Marsabit	195	92	287	13	10	23	1	0	1
Moyale	203	307	510	61	97	158	16	32	48
Mwingi	154	291	445	11	38	49	1	4	5
Narok	31	80	111	3	13	16	1	4	5
Pokot	34	83	117	7	23	30	0	7	7
Samburu	29	135	164	2	7	9	1	2	3
Taita Taveta	25	20	45	5	3	8	0	0	0
Turkana	5	37	42	0	5	5	0	2	2
Wajir	32	21	53	1	4	5	1	0	1
Unknown	27	44	71	6	33	39	8	18	26
Total	1676	3308	4984	263	666	929	54	122	176
%	33.6	66.4	100	28.3	71.7	100	30.7	69.3	100

Appendix 3: Districts where at least a case of *M. bovis* was confirmed by DNA analysis (Genotype® MTBC 96, VER 1.X)

District of origin	No of cattle:				%		<i>M. bovis</i>
	Slaughtered	Examined	With lesion	Examined	With lesions		
Kajiado	1202	290	23	24.1	7.9	2	
Narok	111	16	5	14.4	31.3	1	
Laikipia	479	50	18	10.4	36.0	1	
Garissa	145	35	14	24.1	40.0	1	
Wajir	53	5	1	9.4	20.0	1	
Moyale	510	158	48	31.0	30.4	4	
Isiolo	789	131	15	16.6	11.5	1	
Mwingi	445	49	5	11.0	10.2	1	
Homa Bay	88	7	1	8.0	14.3	1	
West Pokot	117	30	7	25.6	23.3	1	
Unknown	71	39	26	54.9	66.7	5	
Total	4010	802	163	20.0	20.3	19	

Appendix 4: Evaluation sheet for members of the MTBC (Genotype® MTBC)

