DIAGNOSIS AND EPIDEMIOLOGY OF ZOONOTIC NONTUBERCULOUS *MYCOBACTERIA* AMONG DROMEDARY CAMELS AND HOUSEHOLD MEMBERS IN SAMBURU COUNTY, KENYA

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DECLARATION

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

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DEDICATION

To my parents, family, relatives, and friends.

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ABBREVIATIONS AND ACRONYMS

AFB: Acid fast bacilli

BCG: Bacille Calmette-Guérin

BTB: Bovine tuberculosis

CDC: Center for disease control

DNA: Deoxyribonucleic Acid

ELISA: Enzyme-Linked Immunosorbent Assay

EPTB: Extra Pulmonary Tuberculosis

IFN-γ: Interferon gamma

IGRA: Interferon gamma Release Assay

IUATLD: International Union Against TB and Lung Disease (The Union)

KWS: Kenya wildlife service

L-J: Lowenstein-Jensen

MDR: Multidrug-resistant

MIC: Minimum Inhibitory Concentration

MOTT: Mycobacteria other than *M. tuberculosis.*

MTB: *Mycobacterium tuberculosis*

MTC: *Mycobacterium* tuberculosis complex

NTM: Nontuberculous Mycobacteria

PCR: polymerase chain reaction

PPD: Purified protein derivative

PPDa: Avian purified protein derivative

PPDb: Bovine purified protein derivative

RD: Region of difference

RR: Resistant Ratio

SICTT: Single intradermal comparative tuberculosis test

TB: Tuberculosis

TB-LAM: Tuberculosis lipoarabinomannan

TB-LAMP: Tuberculosis Loop-Mediated Isothermal Amplification

TST: Classic tuberculin skin test

OIE: World organization for animal health

WHO: World health organization

XDR: Extremely drug-resistant

ZN: Ziehl-Neelsen

DEFINITION OF OPERATIONAL TERMS

Drug resistant TB:	TB that does not respond to at least one of the main anti-TB drugs.
MDR-TB:	TB that does not respond to at least two of the most powerful first line anti-TB drugs, Isoniazid and Rifampicin.
Non-tuberculous TB:	Tuberculosis infection with mycobacteria other than the MTC.
Tuberculosis Disease:	A chronic bacterial infection caused by *Mycobacterium tuberculosis*, usually characterized pathologically by the formation of granulomas. The most common site of infection is the lung, but other organs may be involved.
Tuberculosis Infection:	A camel is considered to be infected if it tests positive to confirmatory tests, which can be either bacteriological or molecular techniques.
XDR-TB:	Extensively drug-resistant tuberculosis, defined as MDR-TB plus resistance to at least one fluoroquinolone and a second-line injectable.
Zoonotic tuberculosis:	A form of tuberculosis that can be transmitted from vertebrate animals to humans and vice-versa.

ABSTRACT

Zoonotic nontuberculous *Mycobacteria* (NTM) cross infect a wide range of domestic animals, wildlife and man causing various diseases. Despite the public health implications associated with mycobacterial infection and the existing close interaction of pastoralists and camels, information on diagnosis and epidemiology of zoonotic NTM amongst camels and closely associated community members is scanty. The present study was a one-health approach study involving diagnosis and epidemiology of zoonotic NTM infection among dromedary camels and associated household members in Samburu East sub-county. The study was cross sectional covering camels slaughtered at county abattoirs as well as household members and camels. Abattoirs sampled included Isiolo and Athi-River camel abattoirs, where all sampled camels were confirmed to have come from Samburu east. Household camel sampling strategy involved stratification of the study area to Wamba and Waso rearing areas. Sampling at abattoirs was consecutive for camels identified to have TB-like lesions during meat inspection. People presumed to have TB from amongst the sampled households were requested to provide a sputum sample. Screening of lactating camels for Mycobacteria infection was done using single intradermal comparative tuberculin test (SICTT) in a consecutive sample of 612 lactating camels from 83 households. A consolidated milk sample from all four mammary quarters was collected from 238 tuberculin test reactive camels. Post mortem (PM) examination was conducted on 1600 camels originating from the study area. A semi-structured questionnaire was administered to collect data on risk factors from 83 respondents. All milk, sputum and tissue samples were analysed at Kenya Medical Research Institute (KEMRI)/Centre for Respiratory Disease Research (CRDR) enhanced BSL2 laboratory using mycobacteriology, molecular speciation using GenoType® Mycobacterium line probe assay (HAINLifescience), drug susceptibility testing, 16S rDNA sequencing and phylogenetic analysis. This study was conducted between April, 2017 and December, 2018. Results indicated that the proportion of *M. bovis* and *M. avium* reactors was estimated at 6.05% and 39.38% respectively. Out of 238 milk samples, 57 (23.95%) had culture positive acid fast bacilli (AFB). Out of 132 suspected lesions on PM, 27/1600 1.69% (binomial 95%, CI: 1.11%-2.45%) were AFB positive on culture. Of the 48 sputum samples, 7 were AFB culture positive. The NTMs were observed to occur widely in various samples analysed, including post mortem lesions: *M. fortuitum* 17/27 (62.96%), *M. scrofulaceum* 3/27 (11%), *M. szulgai* 2/27 (7.4%); camel milk samples: *M. szulgai* 20/57 (35.09%), *M. monacense* 5/57 (8.77%), *M. litorale* 4/57 (7.02%), *M. fortuitum* 3/57 (5.26%), *M. lehmannii* 3/57 (5.26%), *M. elephantis* 3/57 (5.26%), *M. duvalii* 3/57 (5.26%); and in Human sputum samples: *M. fortuitum* 1/48 (2.08%), *M. szulgai* 2/48 (4.16%), and *M. litorale* 1/48 (2.08%) among others. These findings demonstrate high levels of infection with NTM in both humans and camels. Camel post mortem analyses revealed bronchial, mediastinal lymph nodes and lung lobes were most affected tissues. Several NTMs of medical importance with varying level of commonly used TB drugs sensitivity. The pattern of resistance to first-line TB drugs was as follows: Isoniazid (100%), rifampicin (97.9%) and ethambutol (65.3%). All NTM species isolated were highly resistant to first-line TB drugs. Camel breed, age, production system, origin of new introductions, migration and herd size were identified as risk factors for infection in camels ($p<0.05$). Surveillance and notification systems for NTMs including specific immunological test for NTMs diagnoses are needed. The public health significance of NTM in camels and humans needs further unravelling.

CHAPTER ONE: INTRODUCTION

1.1 Background information

The atypical or nontuberculous *Mycobacteria* (NTM) also refered to as *Mycobacteria* other than tuberculosis complex (MOTT) are mostly ubiquitous free living saprophytes (Tan *et al*., 2018). They have been isolated from water, soil, livestock, wild animals, milk, meat products as well as humans. The isolation of this group of *Mycobacteria* in body secretions was previously considered as due to contamination however; recently they have been linked to disease (Drummond and Kasperbauer, 2019). In humans, the main syndromes associated with these *Mycobacteria* are progressive pulmonary disease, superficial lymphadenitis of cervical lymphnodes, and disseminated disease (Sousa *et al*., 2015). In addition, NTM infection is associated with the presence of other underlying lung diseases such as cystic fibrosis and chronic obstructive pulmonary disease (COPD) (Jones *et al*., 2018; Gardner *et al*., 2019). The pulmonary form is often associated with *M. avium* complex, *M. kansasii* and *M. abscessus* (Johnson and Odell, 2014).

The NTM of public health interest are genomically classified under the following main complexes: *Mycobacterium avium, M. terrae, M. abscessus, M. celatum, M. fortuitum, M. Kansasii, M. simiae, and M. smegmatis* complexes (Fedrizzi *et al*., 2017; Haig *et al*., 2018). *Mycobacteria* in general are classified into five monophyletic clades: Tuberculosis-Simiae; Terrae; Triviale; Fortuitum-Vaccae; and Abscessus-Chelonae clades; however, new classification has been proposed (Gupta *et al*., 2018). They belong to the genus *Mycobacterium* of the family *Mycobacteriaceae,* order

Mycobacteriales which includes, *M. tuberculosis*, the cause of tuberculosis (TB) and *M. leprae*, the cause of leprosy (Gupta, 2019). At the time of writing, the genus *Mycobacterium* consisted of 199 species of which 186 are NTM (http://www.bacterio.net/mycobacterium.html;https://www.namesforlife.com/10.1 601 /tx. 6310). They are non-motile and non-spore forming acid-fast rods of various sizes. Members of this genus possess a high proportion of lipids in their cell wall that make them resistant to host defenses, hence, resulting in a slow chronic disease. They are distinguishable by their ability to synthesise mycolic acid present in their cell wall that imparts resistance to host cell defences, virulence, pathogenicity and acid fast properties (Daffe *et al*., 2017).

The species of NTM are geographically diverse with *M. avium* complex being the most frequently isolated followed by rapidly growing *Mycobacteria* such as *M. fortuitum* and *M. abscessus*, however information from Africa is limited (Honda *et al*., 2018). Nontuberculous *Mycobacteria* are notconsidered notifiable by most countrypublic health authorities, hence, mandatory reporting is lacking and global robust statistics of NTM are limited (Winthrop *et al*., 2017). In a laboratory network study involving 62 centers in 30 countries across six continents, 91 NTM species were identified, most frequently *M. avium* complex, *M. gordonae*, *M. xenopi*, *M.fortuitum* complex, *M. abscessus*, and *M. kansasii* (Hoefsloot *et al*., 2013). The epidemiological study by Kham-ngam *et al*. (2018) in Northeast Thailand, found 150 cases of true NTM infection among 530 suspect cases. In the epidemiological study by Smith *et al*. (2016), a NTM prevalence of 15.9/100,000 among residents of Central North Carolina, USA was

reported. In a 5-year database study in the United States, 0.13% of patients had at least a NTM positive culture with heterogenous species distribution of *M. avium*, *M. abscessus*, and *M. fortuitum* (Spaulding *et al*., 2017).

In livestock, NTM are mostly of production and economic importance due to losses arising from misdiagnosis with other notifiable *Mycobacteria* diseases (Biet and Boschiroli, 2014). In addition, several NTM isolated from livestock are potentially zoonotic (Mbugi *et al*., 2012; Mengistu and Enquselassie, 2014; Thoen *et al*., 2014). However, information on the zoonotic significance of NTM, diagnostic implications in both humans and animals, as well as impact on management of human TB in developing countries is scanty (Hoza *et al*., 2016; Gcebe and Hlokwe, 2017). Some of the important NTM species isolated from animals include; *M. avium intracellulare* complex (*M. avium* subspecies *avium*, *M. avium paratuberculosis*), *M. marinum*, *M. shimoidei*, *M. chelonae*, *M. septicum*, *M. porcinum*, *M. lentiflavum*, *M. fortuitum*, *M. peregrinum*, *M. gordonae*, *M. neoaurum*, *M. parafortuitum*, *M. moriokaense*, *M. confluentis* (Katale *et al*., 2014; Batista *et al*., 2017; Nuru *et al*., 2017; Gcebe *et al*., 2018). The members of this genus that have been isolated from tissue lesions and milk of camelids include: *M. tuberculosis*, *M. bovis*, *M. pinnipedii*, *M. caprae and M. microti*, as well as, atypical *Mycobacteria* such as *M. Kansasii* (Garcia-Bocanegra *et al*., 2010; Wernery and Kinne, 2012; Beyi *et al*., 2014; OIE, 2019). Until recently, the focus has been on identifying known zoonotic *Mycobacteria* such as *M. bovis*, however; not much is known about the epidemiology and implications of the other potentially

zoonotic *Mycobacteria* including *M. avium intracellulare* complex and other NTMs (Gcebe and Hlokwe, 2017).

Protective immunity against mycobacterial infections is dependent on the activation of a cell mediated immunity. The next defensive step is formation of tuberculoid granulomas around the organisms and the containment of the infection (Palmer *et al*., 2015). These nodular lesions form an accumulation of activated T- lymphocytes, neutrophils, multinucleated Langhans giant cells and epithelioid macrophages, which create a microenvironment that limits replication and the spread of the *Mycobacterium* (Miranda*et al*., 2012; Palmer *et al*., 2015; Pai *et al*., 2016). This environment destroys macrophages and produces early solid necrosis at the center of the lesion; however, the bacilli are able to adapt to survive (Pai *et al*., 2016). Within 2 to 3 weeks, it progresses to caseous necrosis. Lesions undergo fibrosis and calcification, successfully controlling the infection so that the bacilli are contained in the dormant, healed lesions. Alternatively, lesions progress to primary progressive tuberculosis and likely extrapulmonary tuberculosis if immunity fails (Shah *et al*., 2017).

The zoonotic importance of *Mycobacteria* has been documented and is attributed to the sociocultural practice of consumption of raw milk as well as the close human – livestock contact amongst most pastoral communities (Fujiwara and Olea Popelka, 2016). Consumption of camel milk is on an upward trend due to the recognition of its properties as a natural health product as well as nutrition for children especially in drier months which are now being exacerbated by climate change (Dietz *et al*., 2014).

According to the Global TB report of 2017, there were an estimated 147,000 incident cases of zoonotic TB globally. The species of zoonotic importance include *M. tuberculosis, M. bovis, M. caprae, M. chelonae, M. fortuitum, M. marinum, M. scrofulaceum, M. xenopi, M. simiae, M. szulgai, M. kansasii, M. leprae* and *M. avium* (Muller *et al*., 2013; Forbes *et al*., 2018). Globally, zoonotic TB is attributed predominantly to *M. bovis* with cattle being the reservoir (Muller *et al*., 2013; WHO, 2017). Nevertheless, other *Mycobacteria* species are zoonotic; hence, improved diagnostics, application of highly discriminative genotypic tools such as DNA sequencing are required to unravel further the zoonotic implications NTMinfections (Perez-Lago, 2014; Fedrizzi *et al*., 2017). The zoonotic potential of NTM is greatest in pastoral settings where livestock keeping is integral to socio-economic and cultural activities where human – livestock interactions are common. In addition, the zoonotic importance of NTMs is related to factors such as the high prevalence of zoonotic *Mycobacteria* in livestock, lack of animal TB control, lack of milk pasteurization, drinking raw milk, a high burden of HIV, and the close human-livestock-wildlife interaction (Michel *et al*., 2010; Mwangi *et al*., 2016). Zoonotic transmission may occur through the oral route or through inhalation (Michel *et al*., 2010; Gumi *et al*., 2012a; Scott *et al*., 2016). There is great risk of zoonotic transmission through the practice of drinking infected unpasteurized milk and inadvertently inhaling infected cough spray from infected livestock as well as from occupational exposure (Michel *et al*., 2010; Biffa *et al*., 2011; Awah Ndukum *et al*., 2012; Youssef and Ahmed, 2014).

In some countries, human tuberculosis of zoonotic origin is estimated at between 3-10%; however, it may be under-reported, andpatients with extra-pulmonary disease have increased odds of being diagnosed with zoonotic tuberculosis (Thoen *et al*., 2009; Michel *et al*., 2010; Scott *et al*., 2016; OIE, 2019). In Ethiopia, a survey of human tuberculosis found *M. bovis* to contribute less than 1% to zoonotic TB amongst patients (Berg *et al*., 2015). In a study by Khattak *et al*. (2016) among occupationally exposed groups in Pakistan, 2% of abattoir workers sampled (*n*=141) were found positive for *M. bovis* by PCR. In addition to the public health importance, the zoonotic form of this disease also has economic and food safety significance to the livestock subsector (Ayele *et al*., 2004).

Various risk factors have been shown to be associated with zoonotic TB, for instance in Ethiopia; camel age and breed, abattoir location, geographic origin, and management system have been identified as risk factors for prevalence of tuberculosis-like lesions in camel (Biffa *et al*., 2011). Elsewhere, risk factors have been classified as animal, herd, environment, wildlife, diagnostic test and social level factors with various sub classifications however; certain factors such as herd size and age of the animal have been shown to be consistent in most risk factor studies (Broughan *et al*., 2016).

In epidemiological or immunological studies, diagnosis relies on the deployment of a series of tests which include: Initial fluorescent microscopy (FM) examination, followed by culture on Solid and liquid media, sub culture, confirmation of acid fast bacilli (AFB) using Ziehl-Neelsen (ZN) or Kinyoun stain and final identification of

Mycobacterial species and strains using molecular Assays. There are various types of solid and broth media used in mycobacteriology laboratories; soild media is either egg based or agar based. The various types of broth media are based on Middlebrook 7H9 broth, often incorporating an automated *Mycobacteria* detection system (Forbes *et al*., 2018). The solid media most commonly used is egg yolk based Lowenstein-Jensen (LJ) media, while the liquid (broth) media commonly used isthe Middlebrook 7H9 broth automated mycobacterial systems to increase recovery of *Mycobacteria*. The use of both solid and broth media is recommended by the British Thoracic Society (BTS) andthe American Thoracic Society (ATS)/ Infectious Disease Society of America (IDSA) for the detection of NTM.

The official TB screening method recommended by the World Animal Health Organization (OIE) for camelids traded internationally is the single intradermal comparative tuberculin test (SICTT) at the axilla (Cousins and Florisson, 2005). Some blood-based assays such as the multi-antigen print immunoassay (MAPIA) and the immunochromatographic lateral flow test (Vet TB Stat-Pak®) may have diagnostic benefit in old world camelids (*Camelus dromedarius*). The Gamma interferon test (IFN-γ) is also recommended by OIE as an alternative for internationally traded camelids. Specific diagnostic tests for NTM in camels are lacking and frequently diagnosis is through modification of the algorithm used for *M. tuberculosis* complex *Mycobacteria*. Diagnosis of *Mycobacteria* in humans relies on conventional methods such as Ziehl Neelsen (ZN) staining, fluorescent microscopy, histology, L-J (solid) and MGIT™ (liquid) medium for culture respectively, and the tuberculin skin test to detect delayed

hypersensitivity. Adenosine deaminase assays, PCR, nucleic acid amplification tests (NAAT) (xpertMTB/RIF® and AmplicorPCR®) are also used. Mycobacterial culture remains the Gold standard for confirmation of infection. Molecular techniques which include genus and deletion typing, spoligotyping, IS6110-RFLP (long considered as the 'GOLD' standard technique) and Mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTRs) have varying cost and level of complexity (Ameni *et al*., 2010; Bouakaze *et al*., 2010).

There is an upsurge of drug resistant strains of *M. tuberculosis* in humans and documentation of the current trend is increasingly critical for this and other pathogenic species (Ogaro *et al*., 2012; Dantas *et al*., 2015; Haeli *et al*., 2015; Lisdawati *et al*., 2015; Thoen, 2016). GenoType MTBC (Hain Lifescience, Nehren, Germany) is acommercial DNA-strip assay for differentiating MTBC strains isolated from cultured material (Neonakis *et al*., 2007). GenoType MTBC 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. However, this line probe assay does not differentiate *M. canetti* from *M. tuberculosis* and *M. africanum* type I from *M. pinnipedii* (Bouakaze *et al*., 2010). Elsewhere, an oligonucleotide array based on the internaly transcribed spacer (ITS) sequence for the genotyping of medically important *Mycobacteria* containing *M. tuberculosis* and 19 NTM has been developed (Park *et al*., 2005). This technology permits simultaneous monitoring and analysis of a large number of target genes. The diagnostic tests recommended for detection of NTM include smear microscopy using

fluorochrome technique, ZN or Kinyoun stain; culture techniques on both solid egg-based LJ and broth media such as nonradiometric mycobacteria growth indicator tube (MGIT); conventional biochemical and phenotypic testing based on growth rate and pigmentation; and genotypic methods. These genotypic methods include; commercial molecular probes for some important NTM species, PCR based molecular diagnostic tests such as the Seegene Anyplex MTB/NTM real time detection assay are available and DNA sequence analysis of the highly conserved genes such as 16S rDNA gene that encodes 16S rRNA, heat shock protein 65 (*hsp65*) and *rpoB* (Griffith *et al*., 2007; Perry *et al*., 2014; Kim and Shin, 2017). A diagnostic algorithm combining real-time PCR and line probe assays as well as other techniques for detection of multiple Mycobacterium pathogens have been described (Deggim-Messmer *et al*., 2016; Maurya *et al*., 2017).

The present study aimed at isolation, identification and epidemiology of zoonotic NTM in dromedary camels (*Camelus dromedaries*) and the people closely associated with the livestock such as herders, milkers and other household members. This involved using milking camel herds, closely associated pastoralists andcamels from the study area slaughtered at county slaughterhouses at Isiolo and Athi River.In this study, prevalence of zoonotic NTM in lactating camels, pathological manifestation, molecular characterization of strains, drug resistance and the identified risk factors associated with infection are reported. This was accomplished using tuberculin skin testing, confirmatory culture, GenoType® *Mycobacterium* CM Assay, DNA sequencing, drug sensitivity testing and collection of risk factor data on zoonotic NTM.

The results of this study give an indication of the expected magnitude of NTM of zoonotic importance in camel pastoral communities, as well as the implications for food safety, the economy, and biodiversity. The results of this study are important at policy level in the national and county level public health and livestock sectors in strategic control of *Mycobacteria* diseases including TB. In addition, the knowledge generated complements the global effort by the FAO-WHO-OIE tripartite, the IUATLD, UN-SDGs and the STOP TB partnership to document all forms of tuberculosis.

1.2 Statement of the problem

Samburu East sub-County, an arid and semi arid area in which nomadic pastoralism predominates, relies heavily on camels as the main source of livelihoods and nutrition for the communities. The inherent existing close interaction between humans and camels has implications for zoonotic disease infections. In addition, milk is a possible source of various zoonotic infections due to inadequate milk hygiene practices (Noor *et al*., 2013). *Mycobacteria* infections which include NTM, infect a wide range of domestic, wild animals and man are of zoonotic importance. In addition, recent data indicates that there has been an increase in prevalence of human NTM infections locally and globally (Biondi *et al*., 2017; Desikan *et al*., 2017; Donohue, 2018: Kaguthi *et al*., 2019). Nontuberculous *Mycobacteria* are of veterinary relevance due to productivity and economic losses due to misdiagnosis with tuberculosis due to *M. bovis* (Biet and Boschiroli, 2014). The types of species of NTM are poorly characterized in both humans and camels, while the pathogenesis, transmission dynamics and biology is

unclear. In addition, the gross and histopathological manifestation of NTM infections in camels is not clearly described.

There is growing evidence in recent times of the role of NTMs as true human pathogens, their resistance to antibiotics and the implications for the control and management of human TB cases (Wang *et al*., 2014; Hoza *et al*., 2016; Cheng *et al*., 2017). In addition, evidence from several studies indicates that several species of NTM are transmissible between humans and animals (Agdestein *et al*., 2014; Katale *et al*., 2014; Malama *et al*., 2014). The zoonotic importance of NTM in pastoralist areas is related to the high prevalence of zoonotic *Mycobacteria* in livestock, lack of animal TB control, poor management, lack of milk pasteurization, HIV, and the close human-livestock-wildlife interaction (Kazoora *et al*., 2016; Mwangi *et al*., 2016). In addition, data is scanty on the occurrence of zoonotic NTM in high risk groups such as camel herders, abbatoir workers, animal health professionals and butchers. Despite this, the occurrence, drug resistanceand public health significance of these other *Mycobacteria* is under-documented for endemic areas in sub-Saharan Africaand globally in general. There is lack of information on epidemiology, magnitude, ecology and public health implications of NTM among camels and camel rearing communities.

Nontuberculous *Mycobacteria* infections are difficult to diagnose in both humans and animals and although recommended guidelines for diagnosis and therapy in humans exist, compliance is poor (Griffith *et al*., 2007; Haworth *et al*., 2017). Standardized guidelines for diagnosis, therapy and control protocols for NTM in animals are lacking.

In addition to undermining TB eradication, the lack of epidemiological information on zoonotic NTM in livestock continues to undermine efforts directed at ensuring the availability of safe food and protecting livelihoods.

1.3 Justification of the study

The dromedary camel (*Camelus dromedarius*) constitutes an important source of livelihoods through the provision of live animals for sale as well as food security through nutritional intake of milk and meat for the pastoralist communities of Samburu County. Milk is the most important product and may contribute up to 60 per cent of nutrient intake. Recently, there is a growing demand for camel milk due to interest in bioactive peptides generated from camel milk with beneficial properties for human health (Mati *et. al.*, 2017).

The NTMs are becoming increasingly important globally in humans as true pathogens in both immunocompromised and immnuocompetent individuals. The epidemiology of zoonotic NTM is even more under-appreciated and under- documented in endemic countries (Hoza *et al*., 2016). This is despite the growing evidence base of the role of NTMs as true human pathogens in NTM pulmonary disease, their resistance to first and second line anti-TB drugs and the implications for the management of human TB cases (Kankya *et al*., 2011; Muwonge, 2012; Wang *et al*., 2014; Cheng *et al*., 2017; Nishiuchi *et al*., 2017; Monde *et al*., 2018). The documentation of the epidemiology, magnitude and drug resistance patterns of these *Mycobacteria* in camels and humans in the study area has significance for improving food safety, food security, public health and

economic livelihoods for communities and the livestock industry in this area. Information on the true magnitude of zoonotic NTM in the study area is required as the lack of this knowledge undermines the efforts to eradicate TB.

Nontuberculous *Mycobacteria* are ubiquitous in the environment and due their co-evolution with moulds which are the source of most antibiotics, these *Mycobacteria* are highly resistant to most antibiotics. Standardized diagnosis protocols and therapy regimens in both humans and animals are urgently required. The communities in Samburu County rely heavily on the camel for livelihood and since human TB is a major health challenge in that region it was important to conduct this study in this area. The county is classified as one the poorest counties with a poverty rate of approximately 80%, much higher than the national poverty rate of 45.9% (Samburu County Government, 2018). Tuberculosis is a big challenge; 584 cases were notified in 2016 and the Kenya TB prevalence survey indicated a burden of 1200 cases in the population of approximately 283, 780 (KNBS, 2016; Samburu County Government, 2018) which is higher than the national average.

1.4 Research questions

i. What is the prevalence of zoonotic NTM in lactating camels from Samburu County?
ii. What is the presentation of pathology associated with NTM in camels?
iii. What is the identity of NTM isolates from the study area?
iv. What is the pattern of drug resistance of NTM isolates from the study area?
v. What are the risk factors for zoonotic NTM infection in the study area?

1.5 Hypotheses

i. There is no zoonotic NTM infection in lactating camels in the study area.

ii. Nontuberculous *Mycobacteria* infections do not induce pathological lesions.

iii. No NTM isolates are present in the study area.

iv. All isolates are susceptible to common TB drugs.

v. No risk factors are associated with NTM infection in camels.

1.6 Objectives

1.6.1 Broad objective

The aim of this study was to conduct diagnosis and epidemiology of zoonotic NTM among dromedary camels and household members in Samburu County, Kenya.

1.6.2 Specific objectives

i. To determine the levels of *Mycobacteria* infection in lactating camels using SICTT.

ii. To describe the gross and histopathological manifestation of NTM in camels at post mortem.

iii. To determine the molecular pattern of NTM isolates from camels and humans in the study area.

iv. To determine the pattern of drug susceptibility to first- line TB drugs amongst zoonotic NTM isolates from both humans and camels.

v. To determine the risk factors associated with NTM infection in camels.

1.7 Significance of study

This study is important with regardto the zoonotic significance of NTM due to the integral importance of camel keeping with its inevitable close human-livestock interaction amongst camel rearing communities. The findings of this study improve our understanding of the zoonotic risk attributable to NTM in camels amongst these communities. The research output isalso of value in validation of diagnostic tests for *Mycobacteria* in camels. The information generated will guide policywith regard to control of zoonotic *Mycobacteria*, assuring food security and food safety during development of camel products value chains, as well as in HIV/AIDS and human tuberculosis management and control efforts.

CHAPTER TWO: LITERATURE REVIEW

2.1 *Mycobacteria*

The genus *Mycobacterium* of the family *Mycobacteriaceae* includes gram-positive, catalase positive, non-motile, non-spore forming, slow or rapid growing acid-fast, rod-shaped bacteria of various sizes (Figure 2.1).

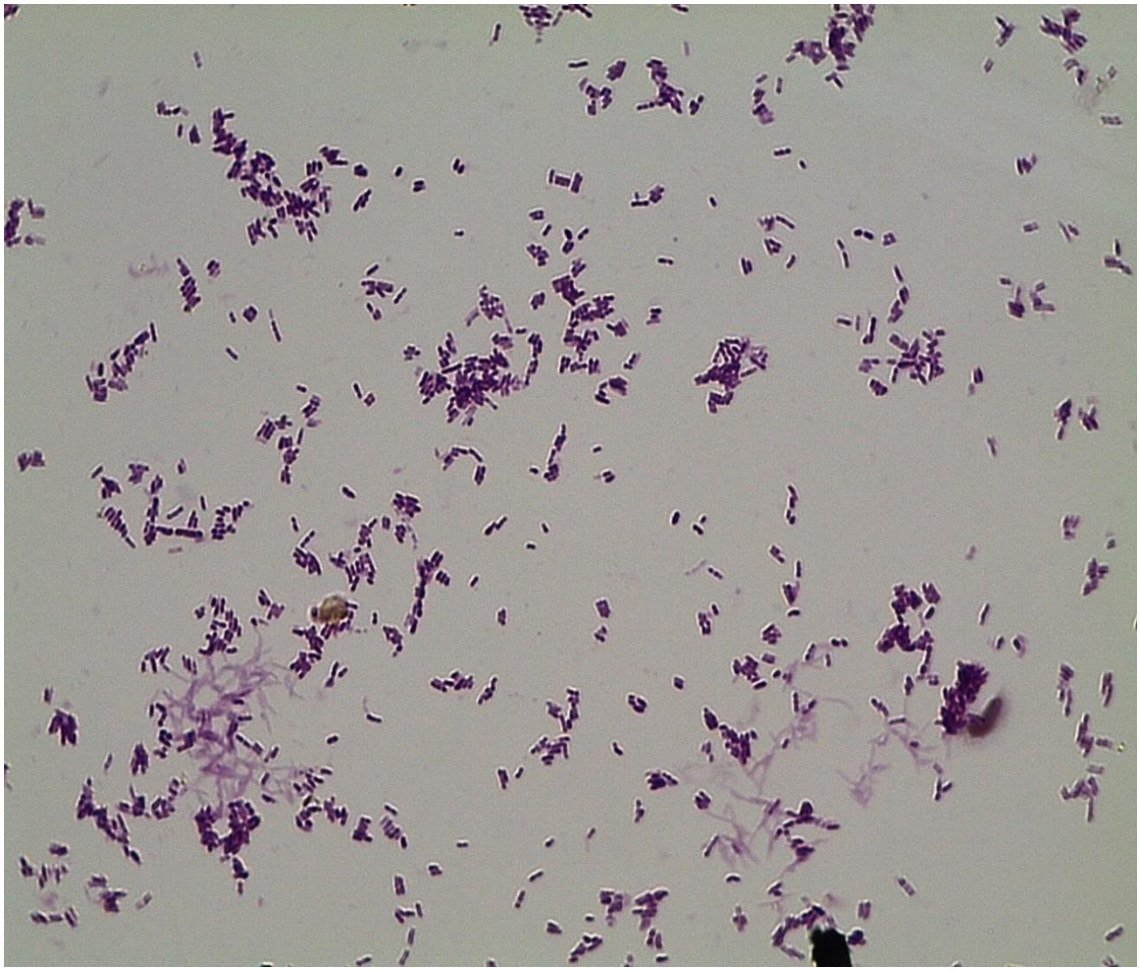

Figure 2.1: *Mycobacterium* on Ziehl-Neelsen (ZN) stain

The colony morphology varies from smooth to rough, and pigmentation ranges from white, orange to pink. The cell walls are thick and consist of peptidoglycans and other

lipids (Alderwick *et al*., 2015; Falkinham III, 2018). The cell walls of *Mycobacteria* species are rich in mycolic acid which resists decolorization with acid alcohol (acid fast), a property shared with bacteria in genera such as *Nocardia*, *Rhodococcus*, *Gordonia*, and *Tsukamurella*. Majority of the known *Mycobacteria* are environmental saprophytes in soil and water, disseminated mainly through aerosols. Members of this genus can be divided into four major groups *M. tuberculosis complex, M. leprae, M. ulcerans* and the NTM (Forbes *et al*., 2018). Clinically, the most important species are *Mycobacterium tuberculosis*, *M. bovis*, *M. africanum* and *M. leprae*. Some of the nontuberculous *Mycobacteria* can be classified according to growth rate and pigment production in Runyon's (1959) scheme of classification (Table 2.1).

Table 2.1: Scheme of classification of nontuberculous mycobacteria (Runyon, 1959)

Slow growing (>7 days)			**Rapid growing** (<7 days) (Group IV)
Photochromogenic (Group I) Form yellow pigment under influence of light	**Scoto-chromogenic (Group II)** Form yellow pigment without light	**Non-chromogenic (Group III)** Don't form yellow pigment	
M. kansasii *M. simiae* *M. szulgai* *M.marinum*	M. gordonae M. scrofulaceum	MAC *M. intracellulare* *M. haemophilum*	*M. fortuitum* *M. abscessus* *M. chelonae* *M. mucogenicum*

The pathogenic species include NTM such as*M. Avium* complex, *M. simiae, M. kansasii, M. haemophillum, M. ulcerans, M. marinum, M. scrofulaceum, M. szulgai, M. elephantis, M. lentiflavum, M. xenopi, M. malmoense, M. celatum, M. genavense, M. fortuitum, M. chelonae*, and *M. abscessus* (Thoen *et al*., 2009; Kazda *et al*., 2009;

Percival and Williams, 2014; Yagi *et al*., 2018). Currently, differentiation of NTM is done using molecular methods such as 16S rRNA gene sequencing and phylogenetics (Forbes *et al*., 2018). Presently, the accepted phylogeny of *Mycobacteria* based on 16S rRNA gene characterization include the following complexes: *M. avium, M. tuberculosis, M. terrae, M. abscessus, M. celatum, M. fortuitum, M. kansasii, M. marinum, M. simiae, and M. smegmmatis* (Fedrizzi *et al*., 2017). The members of the genus *Mycobacterium* may also be categorized into five major clades as follows: 'Tuberculosis-Simiae', 'Terrae', 'Triviale', Fortuitum-Vaccae', and the 'Abscessus-Chelonae' clades, however, a new classification of the genus has been proposed (Gupta *et al*., 2018).

Tuberculosis is caused by mycobacterial species belonging to the *Mycobacterium tuberculosis* complex (MTBC), affecting many vertebrate animals and humans (Kaneene and Thoen, 2004; Radostits *et al*., 2007; Wernery and Kinne, 2012; Thoen *et al*., 2014; WHO, 2017; OIE, 2019). The members of *Mycobacterium* tuberculosis complex (MTBC) include *M. tuberculosis, M. bovis, M. africanum, M. caprae, M. canetti, M. pinnipedii, M. mungi, M. microti, M. orygis,* and *M. suricattae* (Rodriguez-Campos *et al*., 2014; Thoen *et al*., 2014; Bolanos *et al*., 2018). The members of MTBC affect different animal species and humans differently (Thoen *et al*., 2014). The *M. bovis* species can be further divided into *M. bovis* subsp. *bovis*, *M. bovis* subsp. *caprae* and the *M. bovis*-derived BCG vaccine strain, whereas *M. africanum* includes two subtypes, I and II. Infection is a spectrum of disease states, which are classified as latent TB infection (LTBI), asymptomatic and active TB disease (Kiazyk and Ball, 2017).

Tuberculosis disease develops in 5 – 10% of infected non immunocompromised individuals at some point in life and of these, 5% develop disease in 1-2 years of infection (Thoen *et al*., 2014: Lee, 2016). Latent TB infection (LTBI) which is, the persistent immune response to stimulation with *M. tuberculosis* antigens without any clinical signs, radiographical or mycobateriological evidence for active TB, ensues in the remaining 90% (WHO, 2018a). The prevalence of LTBI is highest in low- or-middle-income countries, where it is estimated at 51.5% compared to approximately 33% globally (Kiazyk and Ball, 2017). Groups at high and moderate risk of progression from LTBI to active TB include HIV – infected persons, contacts less than 2 years old, patients on immunosuppresants, silicosis, adolescents, and chronic renal failure/diabetes mellitus patients (Lee, 2016; WHO, 2018). Those at slightly increased risk include alcohol abusers, cigarette smokers, underweight and malnourished individuals (Kiazyk and Ball, 2017). Active TB is characterized by cough with sputum and sometimes with blood, chest pains, weakness, weight loss, fever, and night sweats (Churchyard *et al*., 2017; WHO, 2017b). Most of Human TB cases are mostly attributed to *M. tuberculosis*, *M. africanum*, and rarely to *M. bovis* and *M. caprae* (Pai *et al*., 2016; WHO, 2017a).

Substantial genetic and virulence heterogeneity exists among various *Mycobacteria* as well as genetic predisposition amongst individuals. The genetic basis of virulence is the RD1 genes that encode the five bacterial export systems (ESX-1 to 5), which mediate delivery of bacterial products into the macrophages as well as macrophage escape (Pai *et al*., 2016).These export systems are heterogeneously distributed across species members of NTMs. The other virulence factors include proline-glutamate/proline-

proline-glutamate (PE/PPE), mammalian cell entry (Mce) protein family, Sec-dependent general secretion system and the Twin-arginine translocase (Tat) export system. The Mce protein family (Mce 1 – 9) facilitate invasion and persistence in macrophages and they are distinctly distributed amongst the complexes (Fedrizzi *et al*., 2017). In addition to *M. tuberculosis* complex, the RD1 (Ex-3) is conserved in the NTM complex groups such as *M. kansasii* and *M. marinum* (van Ingen *et al*., 2009). The organisms may be transmitted through inhalation by aerosolized droplets of exudates containing bacilli or by ingestion of food and water contaminated with urine, fecal material, or exudates from diseased animals containing the tubercle bacilli. In animals, other possible routes of infection include formites, congenital as well as consumption of milk by offspring of infected animals (Cooper *et al*., 2011).

2.1.1 Zoonotic *Mycobacteria*

Zoonotic TBprincipally due to *M. bovis* is is not only considered as a neglected zoonotic disease (NZD), it is one of the neglected tropical diseases (NTDs) and is a disease of major public health concern (FAO-IUATLD-OIE-WHO, 2017; WHO, 2017a). Zoonoses are defined as diseases naturally transmitted from vertebrate animals to humans and vice-versa (Reverse zoonoses). In general, zoonotic TB is among NZDs affecting mainly the poor and marginalized communities disproportionately. Okello *et al*. (2014) have further described NZDs as politically neglected endemic zoonotic diseases, which are under-reported and inadequately prioritized in many developing countries. Zoonotic TB is caused by *M. bovis* or *M. caprae*; however, *M. bovis*is the most common zoonotic disease transmitted from animals to humans than zoonotic TB

caused by other zoonotic members of *M. Tuberculosis* complex (MTC) species (Bapat *et al*., 2017; Olea-Popelka *et al*., 2017). Tuberculosis due to *M. bovis* in humans is often associated with manifestation in sites other than the lungs (extrapulmonary) that may include the gastrointestinal tract and lymph nodes of the neck (FAO-IUATLD-OIE-WHO, 2017). Zoonotic TB has high economic impact due to costly eradication programs in livestock and trade barriers. It also has serious consequences for movement of animals and their products, biodiversity, public health and the livelihoods of camel rearing communities (Jemal, 2016). In sub-Saharan Africa, the interface between wildlife, livestock, and the HIV/AIDS epidemic has resulted in a cycle of infection and reinfection (Hardin *et al*., 2011). *Mycobacterium bovis* is a zoonotic disease of cattle, it has a wide host range and readily spills over to humans and a variety of domestic and wild animals (Fitzgerald and Kaneen, 2012). *Mycobacterium tuberculosis* mainly infects humans, non human primates and guinea pigs, while cattle, rabbits, cats and wild hoof stock are susceptible to *M. bovis*. Swine and dogs are equally susceptible to both *M. tuberculosis* and *M. bovis* (Thoen *et al*., 2014).

Non-tuberculous *Mycobacteria* (NTM) include all *Mycobacteria* species except those that cause TB or leprosy (Fedrizzi *et al*., 2017). They are also known as, *Mycobacteria* other than tubercle bacilli (MOTT), atypical *Mycobacteria* or potentially pathogenic environmental *Mycobacteria* (Tyring *et al*., 2017). Currently, there are 186 different species of NTM and information on their clinical significance remains scanty despite being linked with chronic disease and immunosuppression in humans and animals (Monde, 2018). The NTM have been comprehensively catalogued online and an

updated list is available at http://www.bacterio.cict.fr/m/mycobacterium.html as well as at http://www.namesforlife.com. Previously, the NTM were frequently isolated from immunocompromised humans and they included *Mycobacteria* of animal origin mainly, *M. avium* and *M. intracellulare*. However, immunocompetent individuals may be infected (Kaneen and Thoen, 2004). The epidemiology of zoonotic disease due to NTM is under-appreciated and under-documented in countries of sub-Saharan Africa (Hoza *et al*., 2016). This is despite the growing evidence-base in recent times of the role of NTMs as true human pathogens, their resistance to first and second line TB drugs and the implications for the management of human TB cases (Wang *et al*., 2014; Cheng *et al*., 2017). These atypical *Mycobacteria* in animals are of public health interest due to their zoonotic implications, for instance *M. avium- M. intracellulare* complex, *M. chelonae, M. marinum, M. ulcerans, M. fortuitum, M. kansasii, M. gordonae, M. scrofulaceum, M. abscessus* and *M. peregrinum*, are either true or opportunistic pathogens of humans (Shah *et al*., 2017; Tyring *et al*., 2017; Gcebe *et al*., 2018).

The other species of zoonotic importance include *M. tuberculosis, M. africanum*, *M. xenopi*, *M. simiae*, *M. szulgai*, *M. leprae* and *M. avium* among others (Kazda *et al*., 2009). The NTM may infect both immune competent or compromised individuals with one of the clinically important outcome being NTM lung infection (Shah *et al*., 2017). The important sources of infection for humans include cough aerosols and droplets containing infective bacilli or ingestion of food and water contaminated by sputum, faeces, uterine excretions, urine, semen, and milk containing the tubercle bacilli (Kazda *et al*., 2009). In various studies globally, the prevalence of *M. bovis* in milk at

culturehas been shown to be low; however, NTM such as *M. flavescens, M. terrae, M. smegmatis, M. fortuitum, M. gordonae, M. kansasii, M. agri, M. haemophilum, M. genevanse, M. szulgai, M. intracellulare, M. duvalii, M. immunogenum, M. lentiflavum, M. mucogenicum, M. novocastrense, M. parafortuitum,* and *M. simae* have been shown to predominate (Bolanos *et al*., 2018).

2.1.2 Tuberculosis in camels

The important species in animals include *M. tuberculosis, M. bovis, M. paratuberculosis* and *M. avium* and suceptibilty of different host species depends on exposure route, virulence and dose (Thoen *et al*., 2014). The species members of MTBC that have been isolated from tissue lesions and milkof camelids include: *M. tuberculosis, M. bovis, M. pinnipedii, M. caprae and M. microti* (Garcia-Bocanegra *et al*., 2010; OIE, 2019). Atypical *Mycobacteria* such as *M. kansasii, M. aquae, M. chelonae, M. fortitum, M. marinum, M. scrofulaceum, M. xenopi, M. simiae, M. szulgai, M. kansasii, M. leprae,M. avium* and *M. smagmatis* have also been isolated as causative agents of camel TB (Mamo *et al*., 2011; Muller *et al*., 2013). In addition, reverse zoonosis involving infection with *M. tuberculosis* has been reported in camels (Zerom *et. al.,* 2013; Thoen *et al*., 2014). The frequent routes of transmission of tuberculosis between animals are respiratory through close proximity and gastrointestinal tract due to contaminated water, feed and pasture (Kaneen and Thoen, 2004). The disease in animal populations may occur as outbreaks or endemic infections and human to livestock transmission through genitourinary tuberculosis has been documented (Good and Duignan, 2011).

2.1.3 Epidemiology of zoonotic *Mycobacteria*

According to the WHO global TB report 2017, new human cases of zoonotic TB globally were estimated at 147,000, with 12,500 deaths (FAO-IUATLD-OIE-WHO, 2017; WHO, 2017b). Diagnostic limitations are contributing to the continued under-estimation of the true dimension of zoonotic TB. In addition, apart from cattle and *M. bovis*, other animal species and MTBC such as *M. caprae* can contribute to zoonotic TB (Perez-Lago, 2014).The WHO has classified TB due to *M. bovis* as one of the neglected zoonotic diseases and despite a renewed focus on this disease, documentation remains scanty. The levels of *M. bovis* in humans have a wide variation and are estimated at between 0.4-10% based on sputum samples, despite its extrapulmonary preponderance (Malama *et al*., 2013). It is mostly important in developing countries, where it is associated with cervical lymphadenopathy, intestinal and chronic skin TB, while in developed countries cases are mostly pulmonary, gastrointestinal or cervical lymphnode involvement (Cosivi *et al*., 1998; Ayele *et al*., 2004). Humans are highly susceptible to *M. tuberculosis* and cases in animals are mostly due to exposure to infected humans, while many mammalian species are susceptible to *M. bovis* (Kaneen and Thoen, 2004).

Several wildlife species such as the brushtail possum, badger, bison, African buffalo and, the white tailed deer have been identified as wildlife reservoir hosts of *M. bovis* (Palmer, 2013). The members of the *M. tuberculosis* Complex (MTC) which cause tuberculosis in camelids are mainly *M. bovis* and *M. tuberculosis.* Camelids are mainly considered as spill-over hosts although they possibly play a role in interspecies transmission of tuberculosis (Pesciaroli *et. al.*, 2014). An abattoir based epidemiological

study of tuberculosis in dromedaries in eastern Ethiopia in which 293 dromedaries were examined, found a prevalence of tuberculosis compatible lesions of 12.3%, in which *M. tuberculosis* was isolated in 13.6% of those having *Mycobacteria* isolates (61%) (Zerom *et. al.*, 2013). In Ethiopia, Mamo *et al*. (2011) investigated the pathology of camel tuberculosis and characterized causative agents. The prevalence of camel TB was 10.04% based on pathology. Tropism of tuberculous lesions was significantly different among lymph nodes and lung lobes. The results also showed that the majority of tuberculous lesions were as a result of *Mycobacteria* other than *M. tuberculosis* complex. However, Gumi *et al*. (2012b) detected low levels of prevalence of TB amongst camels with an individual animal prevalence of 0.4% in southeast Ethiopia. A recent study by Mwangi *et al*. (2016) found both atypical and typical *Mycobacteria* including MTB complex using GenoType® CM line probe assay in raw and fermented camel milk; however, these findings remain inconclusive without confirmatory culture results.

In Tanzania, Durnez *et al*. (2009) conducted a study to determine the prevalence of *M. bovis* infection as well as atypical mycobacterioses in different cattle herd management systems around Morogoro. In milk taken from tuberculin positive animals, total prevalences of 2.5 and 10.1% were found for *M. bovis* infection and atypical mycobacterioses respectively. A similar study by Kazwala *et al*. (1998) in the southern highlands of Tanzania to determine secretion of *Mycobacterium* species in milk of indigenous cows found 3.9% of the milk samples were positive for *Mycobacteria*. However, most of the isolates were atypical *Mycobacteria*, only two isolates were

confirmed as *M. bovis.* In developing countries, factors such as the integral position of livestock in society, close human-livestock interaction, inadequate food hygiene, HIV co-infection, poverty, malnutrition, social determinants in health, socio-economic status and access to health serve to magnify the zoonotic potential of *Mycobacteria* in humans in most high burden countries (Cosivi *et al*., 1998; Michel *et al*., 2010; Müller *et al*., 2013; Kipruto *et al*., 2015; WHO, 2016). The true incidence of zoonotic TB remains uncertain; however, the magnitude varies as follows among assessed groups; USA (1.4%-45%), Mexico (28%), Nigeria (15%), Tanzania (15%), Ethiopia (17%), India (9%) and Turkey (5%) (Olea-Popelka *et al*., 2017).

The zoonotic importance of *Mycobacteria* in endemic areas is related to factors such as the high prevalence of zoonotic *Mycobacteria* in livestock, lack of animal TB control, the common practice of drinking raw milk, a high burden of HIV, lack of knowledge about the disease among communities and the close human-livestock-wildlife interaction (Michel *et al*., 2010; Mwangi *et al*., 2016; WHO, 2018). The zoonotic potential of *Mycobacteria* is greatest in pastoral settings where livestock keeping is integral to socio-economic and cultural activities where human – livestock interactions are common. In addition, the zoonotic risk exists due to the lack of control programs for tuberculosis in livestock, presence of animal reservoirs, and inadequate pasteurization of dairy products (Michel *et al*., 2010; Mwangi *et al*., 2016). Zoonotic transmission may occur through the oral route or through inhalation; however, zoonotic TB is mostly a food borne illness, hence the oral route is more important (Michel *et al*., 2010; Gumi *et al*., 2012a; Olea-Popelka *et al*., 2017). Great risk of zoonotic transmission specifically

exists through the practice of drinking infected unpasteurized milk and inadvertently inhaling infected cough spray from infected livestock as well as from occupational exposure (Michel *et al*., 2010; Biffa *et al*., 2011; Awah Ndukum *et al*., 2012; Youssef and Ahmed, 2014).

2.2 Pathophysiology and immunopathogenesis of Mycobacterial diseases

The pathogenesis of *M. tuberculosis* infection is widely documented compared to NTM disease mechanism and transmission, which is currently an active area of research (Chalmers *et al*., 2018). Tuberculosis due to *M. tuberculosis* in humans is a pulmonary and systemic disease and infection results when susceptible individuals inhale droplet nuclei containing tubercle bacilli from active or latent TB individuals into the alveoli of the lungs (Miranda *et al*., 2012; Thoen *et al*., 2014; Lee, 2016). The route of entry is the respiratory tract following inhalation and ingestion by alveolar macrophages in the lower respiratory tract through receptor mediated phagocytosis (Thoen *et al*., 2014; Pai *et al*., 2016). In animals, the organisms may be transmitted by aerosolized droplets of exudates containing bacilli or by ingestion of feed and water contaminated with urine, fecal material, or exudates from diseased animals containing the tubercle bacilli. In addition, formites, congenital transmission as well as consumption of milk by offspring of infected animals are possible routes of infection (Cooper *et al*., 2011).

Presence of the pathogen in lung alveoli induces an innate cellular immunity mainly by macrophages initially, which differentiate to multinucleated giant cells and epithelioid macrophages (Marakalala *et al*., 2016). Ingestion by macrophages into the phagosome

enables the host to control infection, but also protects the pathogen from serum immune defences (Thoen *et al*., 2014). The complement system also plays a role in the phagocytosis of the bacteria, the complement protein C3 binds to the cell wall and enhances recognition of the *Mycobacteria* by macrophages (O'gara *et al*., 2013; Pai *et al*., 2016). Macrophages then present mycobacterial antigens on their surface to the T and B cells for the development of acquired immunity. Protective immunity against mycobacteial infections is dependent on the activation of a cell mediated immunity. Inflammatory cytokines which include interleukin 1(IL – 1), IL – 2 and tumour necrosis factor (TNF - α) recruit natural killer T cells, CD4 T cells, CD8 T cells, and gamma delta T cells (Cooper *et al*., 2011; Lyadova and Panteleev, 2015).

The two main outcomes of exposure to *M. tuberculosis* are elimination or persistence of the pathogen, and elimination is the result of innate or adaptive immune response. Fusion of lysosome with the phagosome to form phagolysosomes attempt to destroy the pathogen (Thoen *et al*., 2014). Virulent *M. tuberculosis* and surfactant protein D present in the alveolus may prevent phagosome-lysosome fusion and phagocytosis respectively (Hsieh*et al*., 2018). Persistence results from the pathogen gaining access to lung interstitium via infection of epithelial cells or transmigration of infected macrophages (Ryndak *et al*., 2016). The pathogen can persist in a latent asymptomatic state until reactivated by environmental stress or immunosuppression (Delogu *et al*., 2013; Thoen *et al*., 2014). Infected macrophages release chemokines and cytokines which promote recruitment of other immune cells such as dendritic cells, lymphocytes and more

macrophages to form tubercles or tuberculoid granulomas (Gonzalez-Domingo *et al*., 2016).

The other cells responsible for production of these cytokines are the dendritic cells, epithelioid cells, Langhans cells, and T-lymphocytes (Pai *et al*., 2016). The granuloma (Figure 2.2) is the characteristic pathologic feature of TB disease, it is an immune environment to control the pathogen; however, it also provides a niche for *Mycobacteria* to survive (Pagan and Ramakrishnan, 2015).

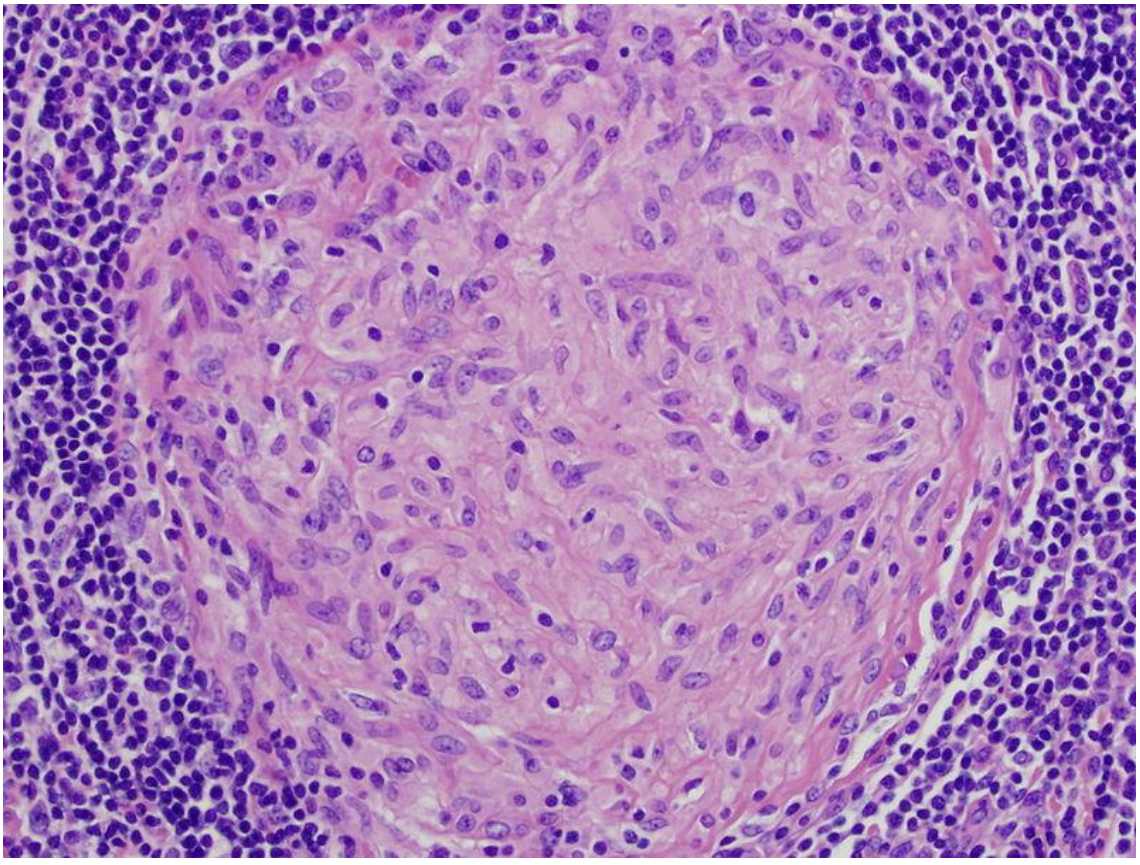

Figure 2.2: Histopathology of a non necrotizing granuloma caused by *Mycobacterium avium* in a lymph node, 20x magnification (Source: By Sanjay Mukhopadhyay - Syracuse, NY, Public Domain, https://commons.wikimedia.org/w/index.php?curid=6608276)

Granulomatous inflammation is a multicellular host response characterized by accumulation of histiocytes (tissue macrophages), the mounting of an innate and a Th1 and Th17 cells– dominant adaptive immune response as well as cytotoxic T effector cells primed by dendritic cells and major histocompatibility (MHC) II (Ehlers and Schaible, 2013; Shah *et al*., 2017).

The organization of the granuloma is spherical with infected macrophages, epithelioid histiocytes in the middle surrounded by a rim of lymphocytes and plasma cells with or without a necrotic center (Miranda *et al*., 2012; Ehlers and Schaible, 2013; Marakalala *et al*., 2016; Shah *et al*., 2017). Complement activated histiocytes, chemokines and cytokines from Th1 recruit more macrophages (Shah *et al*., 2017). Therefore, the center contains pro-inflammatory components while the surrounding components are anti-inflammatory $CD4^+$, $CD8^+$, and γδ T-Cells (Marakalala *et al*., 2016). Macrophages may fuse to multinucleated giant cells (Langhans giant cells) or foamy cells and B-lymphocytes gather into follicular structures surrounding the granuloma (Miranda *et al*., 2012). Later, the granuloma undergoes central necrosis to form caseum, followed by fibrosis and calcification, mycobateria lie latent and domant until reactivated (Marakalala *et al*., 2016). Alternatively, the granuloma disintegrates through liquefaction resulting in cavitation as well as allowing spread of bacteria to distant tissues and organs if immunity fails (Thoen *et al*., 2014). Histologically, granulomas of mycobacterial origin are heterogenous and pathologic NTM produce granulomas that are histologically indistinct from those by members of MTBC (Shah *et al*., 2017).

The pathogen may disseminate to the apical lung lobes, regional lymph nodes, kidneys, brain, and bones (Qian *et al*., 2018). Progression from LTBI to active disease likely depends on genetic susceptibility as well as on the balance between pro- and anti-inflammatory cytokines such as TNF-α, INF-γ, and IL-10 (Lee 2016; Kiazyk and Ball, 2017). The types of granulomas may include caseous, non-necrotizing, necrotic, diffuse, suppurative, histiocytic responses and completely fibrotic granulomas and can be confused with those by other aetiology (Guler *et al*., 2012; Shah *et al*., 2017). In humans, as the cellular processes occur, tuberculosis may develop differently depending on the immune status. The stages may include, latency, primary disease, primary progressive disease, and extrapulmonary disease or Latent TB infection, subclinical TB disease and, active TB disease (Pai *et al*., 2016). Amongst those with latent TB, nearly half show negative TST results due to elimination of the infection by the immune responses (Nayak and Achariya, 2012).

Lesions are often found in the lung and associated lymphnodes, liver and kidneys in cattle, sheep and goats. In horses, the lesions are often in the liver and mesenteric lymph nodes, rarely in the spleen and kidneys; and in the lungs in case of *M. bovis*. In swine, the lesions are associated with lymph nodes and the gastrointestinal tract with *M. tuberculosis*; *M. bovis* is associated with lesions in the lungs, liver and spleen in this species. Lesions in dogs are often found in lungs, liver and kidneys as well as in the pleura and peritoneum. Gross lesions may be extensive, involving whole organs or cavities, they usually have an appearance of yellowish caseous necrotic areas in nodules of firm white to light gray fibrous tissue, some lesions are observed to be purulent.

Microscopic examination of tubercles in the lungs and other organs usually reveals granulomatous lesions composed of caseous central areas of necrosis bordered by epithelioid cells, multinucleated giant cells, an accumulation of lymphocytes, a few granulocytes, CD4+ T cells but also CD8+ T cells and B cells and a capsule composed of fibrous connective tissue. Various proinflammatory and inhibitory cytokines and chemokines, in addition to adhesion molecules, play key rolesin the formation of granulomas (Thoen *et al*., 2009).

2.3 Clinical signs of tuberculosis and other clinically important mycobacterioses

In animals, the signs of tuberculosis usually vary with the distribution of tubercles in the body. The clinical evidence of disease in chronic cases may not be manifested until the terminal stages of the disease. Enlarged superficial lymph nodes, emaciation despite good nutrition, dyspnoea, a low grade fever, anorexia and general weakness are diagnostic (Kaneen and Thoen, 2004). In animals, cases with the progressive pulmonary form of the disease may show dyspnoea with an associated cough. The clinical signs of tuberculosis in camelids may include respiratory distress, anorexia, enlargement of superficial lymph nodes, wasting, recumbency and eventually death. Clinical signs are often associated with extensive respiratory pathologyand sometimes death occurs with no previous clinical observations (Thoen *et al*., 2009; Wernery and Kinne, 2012).

In humans, the common symptoms of active lung TB include, cough with sputum and sometimes with blood, chest pains, weakness, weightloss, fever, and night sweats (Churchyard *et al*., 2017; WHO, 2017b). However, upto 50% of culture positive active

TB cases lack a productive cough and 25% have no symptoms (Pai *et al*., 2016). The TB disease continuum consists of those able to eliminate infection with innate or acquired immunity, latent TB infection (LTBI), subclinical TB disease, and active TB disease (Drain *et al*., 2018). Clinical categorization is normally either LTBI, which is the asymptomatic and non-transmissible state or active TB, which is transmissible and usually culture positive (Jilani and Siddiqui, 2019). Subclinical TB refers to that state in which culture positive is asymptomatic (Dowdy *et al*., 2013). It is estimated that without treatment between 3-5% of LTBI will develop the disease within 2 years of infection (Thoen *et al*., 2014).

Many of the NTM are currently recognized as important human pathogens causing pulmonary, extrapulmonary, soft tissue infections, cutaneous ulcers, lymphadenitis, joint and disseminated infections (Bi *et al*., 2015; Pang *et al*., 2015). There is a large variety in diseases caused by NTM, most of which are associated with disorders such as bronchiectasis, silicosis, cystic fibrosis, chronic obstructive pulmonary disease and immunosuppression (Deggim-Messmer *et al*., 2016). Of greatest interest is the TB-like pulmonary disease, NTM-pulmonary disease (NTM-PD), commonly misdiagnosed and as TB, hence therapeutically challenging (Wu *et al*., 2018). *Mycobacterium kansasii, M. abscessus, M. szulgai, M. gordonae* and *M. simiae* are frequently isolated from pulmonary disease cases. *Mycobacterium scrofulaceum* and *M. avium* are associated with cervical lymphadenitis (scrofula) as well as pulmonary disease. *Mycobacterium haemophilum* has been associated with joint infections. *Mycobacterium marinum* causes a warty skin infection known as ‘fish tank’ or ‘swimming pool’ granulomas while *M.*

ulcerans causes cutaneous ulceration. *Mycobacterium avium*, *M. avium intracellulare* complex (MAC) and *M. genavense* are important opportunists in AIDS patients, causing lymphadenitis, pulmonary and disseminated disease (Percival and Williams, 2014).

2.4 Molecular epidemiology of *Mycobacteria* of zoonotic importance

Molecular epidemiology provides the predictive and quantifiable power to differentiate species and strains of *Mycobacteria*, and confirm their transmission patterns and clusters of infection in populations (Thompson *et al*., 1998; Mathema *et al*., 2006; Nadin-Davis, 2013). Molecular epidemiology studies to address phylogenetic questions and transmission dynamics have been conducted with various results. Strain typing including the application of next generation sequencing is widely used to resolve outbreaks of *M. tuberculosis* (Roetzer *et al*., 2013). Most recently in a study in Mexico, involving cattle (n=155) and human (n=17) sample isolates, two predominant spoligotype patterns were identified in both cattle and humans using whole genome sequencing, spoligotype and SNP analysis (Sandoval-Azuara *et al*., 2017). In their study, Ereqat *et al*. (2013) were able to identify and characterize a new genotype of *M. bovis* from apparently healthy animals using IS6110-PCR and spoligotyping. In that study, real time PCR with high resolution melt curve analysis was employed to differentiate *M. tuberculosis* and *M. bovis*. In addition, the IS6110-PCR findings were confirmed by RFLP analysis, direct sequencing and deletion typing. In a study by Laniado-Laborin *et al*. (2014), 8 loci of MIRU-VNTR were used to determine prevalence of *M. bovis* in human patients. In that study, 4.5% of clinical samples

analyzed were identified molecularly as *M. bovis*. A study to analyze MTBC isolates from Japanese patients using multiplex PCR with RD based identification detected *M. tuberculosis*, *M. africanum*, and *M. canetti* (Ueyama *et al*., 2014). In that study, 4.5% of cultured samples (*n*=2699) were identified molecularly as *M. bovis*.

In a study by Khattak *et al*. (2016) among occupationally exposed groups in Pakistan, 2% of abattoir workers sampled (*n*=141) were found positive for *M. bovis* by PCR. This group was considered to be particularly at higher risk due to the potential of aerosilization and dispersion of bacilli during skinning and slaughtering.In a study by Ben Kahla *et al*. (2011) involving raw milk samples (n=306) from 102 SCITT positive cows from farms in Tunisia, found 5 cows and one human cutaneous lesion with *M. bovis*. The spoligotype patterns of the six isolates in that study strongly suggested occupational exposure. A similar study by Franco *et al*. (2013) involving individual and bulk milk samples (n=300) from farms and informal markets in Sao Paulo, Brazil, identified 15 unique mycobacterial species, one of which belonged to *M. bovis* subsp. *bovis*. Hussein and Mahrous (2016) have isolated and characterized MTBC from raw milk in some dairy farms in Egypt, and out of 9 colonies examined using multiplex PCR, 5 were *M. bovis*, while 3 were *M. tuberculosis*, and 1 MOTT.

Reverse zoonosis is also possible where livestock act as source of infection for humans as demonstrated by the study by BhanuRekha *et al*. (2015). In that study, among 181 bovine milk samples, one sample was culture positive for *M. tuberculosis*. In the abattoir based epidemiological study of tuberculosis in dromedaries in eastern Ethiopia,

characterization of isolates using PCR revealed that 13.6% of the isolates were *M. tuberculosis*. On further spoligotyping, one of the isolates was found to be spoligotype international type (SIT) 21 in addition to two new isolates not previously reported to the Spoligotype International Type VNTR International Type (SITVIT) database (Zerom *et al*., 2013).

Due to the recently emerging importance of NTM, 16S rRNA as well as other genes including *hsp*65, *dnaJ*, 32-kDa gene, *rec*A, *rpo*B, *sod*A, and 16S-23S spacer regions are being applied in molecular diagnostic identification using DNA sequencing (Turenne *et al*. 2001). In a recent study, Fedrizzi *et al*. (2017) have genomically characterized NTM using whole genome sequencing and found concordance with sequencing phylogeny based on 16S rRNA gene. In their study, Monde *et al*. (2018) used 16S-23S internal transcribed spacer (ITS) region sequencing to charterize NTM from human sputum samples and various water sources. In that study, *M. arupense* was the most common isolate from human sputum, while *M. gordonae, M. fortuitum and M. avium* were most prevalent in water samples. In a PCR based molecular epidemiological study of NTM from clinical and environmental sources in Iran, the most frequently identified species were *M. farcinogens, M. fortuitum, M. senegalense, M. kansasii, M. simiae* (Velayati *et al*., 2014). The study by Okoi *et al*. (2017) shows molecular diversity inNTM distribution in sub Saharan Africa, with predominance of MAC in the west and south; *M. kansasii* and *M. scrofulaceum* in the south and; the rapidly growing NTM in Eastern Africa. Similarly, Couto *et al*. (2009) identified predominantly MAC among NTM isolates from clinical samples of hospital patients in 12 Lisbon hospitals, Portugal.

Studies in Ethiopia indicate high levels of infection with NTM in slaughter camels though speciation is lacking (Mamo *et al*., 2011; Gumi *et al*., 2012b).

2.5 Risk factors associated with zoonotic mycobacterial infection

Transmission and infection with NTMs in humans is dependant on the biology of the *Mycobacterium*, host and environmental factors (Honda *et al*., 2015). Host factors such as pre-existing lung disease, previous TB/ mycobacteria infections, thoracic skeletal abnormalities, rheumatoid arthritis, neoplasms, immunity status and immunomodulatory drugs interact with environmental factors such as warmth and humidity to influence disease risk (Prevots and Marras, 2015; Kham-ngam *et al*., 2018; Szturmowicz *et al*., 2018). There is however, need to document factors and practices associated with NTM zoonotic infection.

The most common route of transmission of zoonotic *Mycobacteria* is attributed to unpasteurized milk and untreated animal products, however, airborne infections and direct contact with infected animals is possible (FAO-IUATLD-OIE-WHO, 2017). Recently, in a study by Scott *et al*. (2016), data showed that infants and children below 14 years, foreign-born patients, Hispanics, females, and patients residing in US-Mexico border counties had higher prevalence of *M. bovis*. The same study also showed that exclusively extrapulmonary disease or disease that was both pulmonary and extrapulmonary were associated with a higher prevalence of *M. bovis*. In addition, data from this study suggests that airborne transmission accounts for a substantial proportion of *M. bovis* cases. A recent study in Pakistan among occupationally exposed groups has

shown that long duration of work as an abbatoir worker (>15 years) was significantly associated with occurrence of zoonotic TB (Khattak *et al*., 2016). In the study, it was also found that the level of knowledge of signs and symptoms of TB were very poor, with only 1% of respondents (*n*=141) indicating knowledge. Misdiagnosis, drug resistance, lack of surveillance may also be important factors as they contribute to persistence and maintenance of the zoonotic TB (Gcebe and Hlokwe, 2017).

A study in south-central Spain by Martinez-Lopez *et al***.** (2014) revealed that risk for TB in cattle herds is related to factors such as TB persistence on the farm, extensive production systems, large farm size in terms of numbers and proximity to fenced farming estates with wild ungulates. A cross sectional study by Ibrahim *et al*. (2016) in Gombe state, Northeastern Nigeria to evaluate prevalence and risk factors of bovine tuberculosis showed that there is a low level of awareness of the zoonotic importance of tuberculosis. Season of the year has been shown to be a significant explanatory variable for the occurrence of tuberculous lesions in a study by Saidu *et al*. (2017). In that study, multivariable logistic regression analysis additionally identified age, breed, abattoir location, geographic origin, herd management system to be risk factors for prevalence of tuberculosis-like lesions in cattle. In yet another study in Ethiopia, Mamo *et al*. (2013) estimated the prevalence of BTB in cattle and assessed associated risk factors for infection under pastoral and agro-pastoral systems. In bivariate analysis, prevalence was found to be significantly associated with study districts, herd size, sex and age of cattle. In multivariable logistic regression, statistical significance was maintained with study district, age and herd size. In a study by Durnez *et al.* (2009) more *M. bovis* infections

were found in the extensive management system raising the level of risk in these type of herd management systems. Risk factors in camel may be categorised as either household, animal, herd, diagnostic, environment, management, social and wildlife level factors similar to those associated with bovine TB infection. Certain factors such as herd size seem consistent in most risk factor studies (Broughan *et al*., 2016).

2.6 Diagnosis of *Mycobacteria* infections

Due to the increasing importance of distinguishing infection with NTM, the diagnostic strategy currently adopted is sequential testing to detect TB followed by testing for NTMs (Sarro *et al*., 2018). The diagnostic tests recommended include smear microscopy using fluorochrome technique, ZN or Kinyoun stain; culture techniques on both selective solid egg-based (Lowenstein –Jensen) LJ and non selective broth media such as nonradiometric *Mycobacteria* growth indicator tube (MGIT); conventional biochemical and phenotypic testing based on growth rate and pigmentation; and genotypic methods such as commercial molecular probes for some important NTM species, PCR based molecular diagnostic tests such as the Seegene Anyplex MTB/NTM real time detection assay are available and DNA sequence analysis of the highly conserved 16S rDNA gene that encodes 16S rRNA (Griffith *et al*., 2007; Perry *et al*., 2014). The study by Deggim-Messmer*et al*. (2016), describes a diagnostic algorithm combining real-time PCR and line probe assays for detection of multiple *Mycobacterium* pathogens. The methods commonly used for diagnosis of clinical mycobacterial infections including NTM infections and the limitations of the available techniques have been reviewed (Maurya *et al*., 2017; Sarro *et al*., 2018).

Diagnosis of mycobacterial infections depends on purpose, stage of disease, age of the patient, co-morbidity and in mostof these cases the diagnostic algorithm relies on a series of tests (Pai *et al.*, 2016). Diagnosis involves History, observation of pathognomonic signs and symptoms followed by tests which include: Tuberculin skin tests (TST) and interferon gamma release assays(IGRA); antigen based serology; chest X-ray or imaging; sputum ZN staining and FM examination; culture on LJ media (Solid) or MGIT (BACTEC) media (Liquid); and mycobacterial species and strains typing using molecular assays which employ PCR and nucleic acid amplification (Xpert®MTB/RIF, HAIN line probe assays and Amplicor PCR®) (Patel *et al*., 2014; Purohit and Mustafa, 2015; Pai *et al*., 2016) .

2.6.1 Tuberculin test

The tuberculin test, which has been in use since the early 1900s is one of the diagnostic tests for tuberculosis recommended by Office International des Epizooties (OIE) for screening purposes in camels traded internationally (OIE, 2019). The basis of this test is the detection of a delayed Th1 type cellular immune response to tuberculosis infection (Cousins and Florisson, 2005; Lyadova and Panteleev, 2015; Yu *et al*., 2017). This test detects early immunological changes due to infection with *M. bovis* from 21 and 50 days post infection, however, in later stages there is anergy (Broughan *et al*., 2016). The single intradermal comparative tuberculin test (SICTT) is one of the ways in which this test can be applied, the others are caudal fold test (CFT) and single intradermal test (SIT), both use only bovine PPD (Good and Duignan, 2011; Cousins and Florisson, 2005). The official TB screening method recommended by OIE for camelids traded

internationally is the single intradermal comparative tuberculin test (SICTT), despite its limited sensitivity.

The single intradermal comparative tuberculin test (SICTT) measures delayed hypersensitivity response to *M. bovis* and *M. avium* purified protein derivative (PPD) and it is usually injected at the axilla, however, this test is yet to be validated in camelids (Cousins and Florisson, 2005; OIE, 2019). The technique involves the intradermal injection of *M. bovis* and *M. avium* purified protein derivatives (PPD) followed by measurement of an induration three days later due to delayed type hypersensitivity. The sensitivity and specificity of SICTT have been estimated at 81% (95%, CI: 70%-89%) and 99.98% (99.979%-99.987%), respectively (Goodchild *et al*., 2015; Karolemeas *et al*., 2012). Therefore, SICTT is not perfect and often ancillary tests such as the interferon gamma assay, post mortem meat inspection or epidemiological principles are required to arrive at a conclusion (Clegg *et al*., 2018; Zarden *et al*., 2016). *Mycobacterium tuberculosis, M. avium* complex, *M. fortuitum*, *M. avium* sub species *paratuberculosis*, and *M. kansasii*, may cause responses to *M. bovis* PPD. The comparative intradermal tuberculin test with bovine and avian tuberculin is used mainly to differentiate between animals infected with *M. bovis* and those sensitized to tuberculin due to exposure to other mycobacteria or related genera (OIE, 2018).

The decision to use either the single or comparative test depends on the prevalence of TB infection and on the level of environmental exposure to other sensitizing organisms. Single intradermal comparative tuberculin test remains as the primary tool for TB

screening; however, it is prone to false positives in the range of 10 – 20% most probably due to concurrent NTM infections, hence generally it is unsuitable for *M. bovis* screening (Michel, 2018). Various host and environmental factors such as age, breed, season, sex, concurrent parasitic burden, vaccination status and, husbandry may affect response to PPDs (Chambers, 2013). The other factors that result in variation in reactivity include conditions associated with farm/herd, test level factors, conflict of interest of the tester, duration after previous testing, and immunosuppression due to factors such as comorbidity (Broughan *et al*., 2016).

2.6.2 Culture

Culture – based methods are the current reference 'Gold' standard and take up to 12 weeks to provide results, while Xpert®MTB/RIF (Cepheid, USA) is the only rapid molecular test recommended by WHO as an initial diagnostic. Culture for between 3 to 8 weeks is still considered to be the gold standard method (Kaneen and Thoen, 2004). The other molecular technologies endorsed by WHO include Line probe assays (Hain Lifescience, Germany) for detection of TB as well as resistance and TB LAMP (Eiken, Japan) for detection of TB. Other non molecular technologies endorsed by WHO include: Alere determine TB-LAM (Alere, USA); IGRA for latent TB detection (Oxford immunotech, UK and Qiagen, USA); commercial liquid culture systems and rapid speciation; culture based phenotypic drug sensitivity testing (DST) using 1% critical proportion in LJ and MGIT media; and Light and LED microscopy (WHO, 2017a). Tuberculin skin test (TST) and interferon gamma release assays (IGRA) have low predictive value due to existence of T cell memory in absence of infection (Pai *et*

al., 2016). These methods are either direct or indirect and have varying sensitivity/ specificity, advantages and limitations including cost which might be prohibitive (Cosivi *et al*., 1998; Patel *et al*., 2014; Purohit and Mustafa, 2015).

2.6.3 Polymerase chain reaction

Many polymerase chain reaction (PCR) tests are based on amplification of *IS6110*, an insertion element believed to be restricted to members of MTBC; these tests are especially useful for diagnosis of extra pulmonary TB wherescantybacilliare expected in clinical sputum samples (Kulkarni *et al*., 2012). Despite the limitations of sputum microscopy developed over 100 years ago, application is still widespread in many low income settings (Pai *et al*., 2016; WHO, 2017a). Xpert MTB/RIF® (Cepheid Inc. Sunnyvale, California, USA) is now conditionally recommended by WHO for first line diagnosis of active TB disease, TB lymphadenitis and TB meningitis in adults and children (WHO, 2013). In HIV positive individuals, it is strongly recommended that the Xpert MTB/RIF be used as an initial diagnostic test (WHO, 2013). The other technology endorsed by WHO for use in HIV people is the Alere Determine TB-LAM (Alere, USA) (WHO, 2017a). Currently, the goal is to replace sputum microscopy and traditional drug susceptibility tests with rapid, low cost, molecular techniques at primary care level (Pai *et al*., 2016). Tests for diagnosis of LTBI are TST and IGRA that measure memory T-cell response (WHO, 2017a). None of the diagnostic tools available are predictive of the TB progression inlatently infected individuals, therefore, the identification of reliably predictive biomarkers remains the ultimate goal (Pai *et al*.,

2016). The GeneXpert Omni® which is a close to care platform instrument for TB diagnosis is in the final stages of development (WHO, 2017a).

2.6.4 Genotyping

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 (Hain Lifescience, Nehren, Germany) is a recently developed commercial DNA-strip assay for differentiating MTBC strains isolated from cultured material (Neonakis *et al*., 2007). GenoType MTBC 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. However, this line probe assay does not differentiate *M. canetti* from *M. tuberculosis* and *M. africanum* type I from *M. pinnipedii* (Bouakaze *et al*., 2010). Elsewhere, an oligonucleotide array based on the internaly transcribed spacer (ITS) sequence for the genotyping of medically important *Mycobacteria* containing *M. tuberculosis* and 19 NTM has been developed (Park *et al*., 2008). This technology permits simultaneous monitoring and analysis of a large number of target genes.

Genotyping of isolates to address evolutionary questions, phylogenetics, transmission dynamics and epidemiology may be performed using either Restricted Fragment Length Polymorphism (RFLP), Spoligotyping (Spacer Oligonucleotide Typing), or Mycobacterial Interspersed Repetitive Units – Variable Numbers of Tandem Repeats (MIRU-VNTR) (Scott *et al*., 2016; Sandoval-Azuara *et al*., 2017). Insertion sequence

(IS) 6110 RFLP, the previous gold standard for genotyping MTBC isolates, has been extensively used for TB epidemiological studies because of its high discriminatory power (DP). However, the method is labor-intensive, time consuming and is limited by the need for a large quantity of pure genomic DNA and its inability to discriminate between strains with low IS copy numbers. MIRU-VNTR is a PCR-based typing method where 24 loci containing variable numbers of tandem repeat DNA elements called mycobacterial interspersed repetitive units (MIRU) are analyzed (Jonsson *et al*., 2014). A comparison of these methods by Jonsson *et al*. (2014) revealed that spoligotyping alone had the lowest discriminatory power (DP), while a combination of all three methods had the highest. MIRU-VNTR alone had a higher DP than RFLP alone but when each method was combined with spoligotyping, RFLP performed slightly better. Even though less isolates were clustered by MIRU-VNTR, the clusters were larger. A single nucleotide polymorphism (SNP) typing based strategy based on SNaPshot minisequencing has been developed to distinguish members of MTBC to the species level and *M. tuberculosis* lineages (Bouakaze *et al*., 2010). Currently, most diagnostics in development are for molecular detection of TB and drug resistance (WHO, 2017a).

2.6.5 Serology

Some of the blood-based assays have low sensitivity but others such as the multi-antigen print immunoassay (MAPIA) and the immunochromatographic lateral flow test (Vet TB Stat-Pak®) may have diagnostic benefit in old world camelids (*Camelus dromedarius*). The Gamma interferon test (IFN-γ) which is also recommended by OIE

as an alternative for internationally traded camelids and the lymphocyte transformation assay measure immune responses of circulating lymphocytes. Bovigam® an IFN-γ, is unsuitable for camelids, while other tests such as fluorescent pollarization test are still under-investigation (Dean *et al*., 2009; Twomey *et al*., 2010; Werney and Kinne, 2012; OIE, 2019). A new commercial enzyme linked assay (ELISA) that detects antibody to *M. bovis* antigens MPB83 and MPB70 in infected cattle has been developed and evaluated in cattle but not in camel (Waters *et al*., 2011). Buddle *et al*. (2013), have evaluated the IDEXX ELISA in detection of *M. bovis* using milk samples and found that it may be of use in countries with advanced control programs. However, non-specificity and highly variable sensitivity were noted as issues that might reduceadoption of IDEXX ELISA. A simple rapid method using a bacteriophage – based method in combination with PCR, previously used to detect paratuberculosis has been adapted successfully to detect MTBC in peripheral blood mononuclear cells (Swift *et al*., 2016). Elsewhere, Casal *et al*. (2017) conducted a study to evaluate the sensitivity of SICTT, two interferon gamma assays, and three different antibody detection techniques for bovine tuberculosis (bTB) diagnosis in cattle. In the study, it was determined that the interpretation in parallel of cellular and antibody detection techniques reached the highest sensitivity. Hence, these finding suggest that the use of diagnostic tests detecting both cellular and humoral responses should be considered in the control of TB in high prevalence settings.

2.6.6 Human TB diagnosis in Kenya

In Kenya, the diagnosis of human tuberculosis is by sputum smear microscopy, radiography and GeneXpertMTB/RIF® (Cepheid, USA), while extra pulmonary TB is based on clinical presentation and subjecting specimens to bacteriology (DLTLD, 2013). The TB diagnosis policy is to collect and examine sputum specimens from TB cases with signs and symptoms, and treatment may be commenced for those smear negative with clinical diagnostic criteria or abnormal chest x – ray (Kipruto, *et al*., 2015). The Kenya National TBControl Program maintains a surveillance system and database. This surveillance system (TIBU) is an electronic case based system where information flows from TB facility, to county TB coordinators, to the national database and reporting to WHO is done periodically. Despite the disease by *M. bovis* and *M. tuberculosis* being clinically indistinguishable and requiring distinct interventions to interrupt transmission, there is no attempt at differentiating causative agents (Kipruto, *et al*., 2015).

2.7 Control and treatment of mycobacterial diseases

The treatment of NTM is often associated with long duration of treatment,poor tolerability, significant side effects, toxicity, poor treatment outcomes due to innate resistance of NTM; difficulties in measuring anti-NTM antibiotic activity and the choice of drug is challenging (Soni *et al*., 2016; Falkinham III, 2018). In addition, nontuberculous *Mycobacteria* therapy has the risk of drug interactions. Moreover, the diagnostic criteria commonly applied lacks coordination and poor compliance exists for the diagnostic and treatment guidelines recommended by the American Thoracic

Society (ATS), the British Thoracic Society (BTS) and the Infectious Diseases Society of America (IDSA). Treatment of NTM is often general despite variability in drug susceptibility and regimens exist only for a few common pathogens (Wu *et al*., 2018). The decision to treat NTM infections requires a careful evaluation of risk benefit analysis and the greatest challenge is the varied and complex drug regimens.

The major drug families used in regimens for NTM therapy include carbapenems, ethambutol, isoniazid, aminoglycosides, tetracyclines, glycylcyclines, macrolides, ketolides, oxazolidinones, fluoroquinolones, rifamycins and sulfonamides (Brown-Elliot *et al*., 2012). Drug therapy consists of treatment with standard anti-TB drugs such as rifampicin, ethambutol and isoniazid in combination with macrolides such as clarithromycin and azithromycin for MAC, *M. kansasii, M. malmoense* and *M. xenopi*. For other NTM such as *M. abscessus* complex, therapy is customized based on drug susceptibility results using a combination of other compounds such as intravenous amikacin, tigecycline and imipenem; and oral macrolides such as clarithromycin and azithromycin (Chalmers *et al*., 2018). The NTM drug development pipeline lacks activity as compared to TB drugs due to various challenges (Falkinham III, 2018). Nontuberculous mycobacteria drugs at various phases of development include clofazimine, linezolid, inhaled nitric oxide, aerosolized amikacin, bedaquiline, β-lactams (cefoxitin, imipenem, avibactam), rifabutin, thiacetazone derivatives, piperidinol-based compound 1(PIPD1) and indole-2-carboxamides (Wu *et al*., 2018).

2.7.1 Treatment in man

Accurate and timely diagnosis and treatment of all forms of TB limits deaths due to TB, propagation of the disease as well as pathogen resistance (WHO, 2017a). Without treatment, the general prognosis is a ten year case fatality rate of 70%. The WHO recommended regimens for MDR and XDR-TB have cure rates of 50% and 20% respectively. Treatment for individuals with subclinical or active TB involves an intensive phase with four drugs followed by a longer maintenance phase with two drugs. It is recommended that patients with drug susceptible TB be treated with a six month regimen of two months Isoniazid, Rifampicin, pyrazinamide and ethambutol, followed by 4 months of isoniazid and rifampicin (2HRZE/4HR) (WHO, 2010). The WHO recommended treatment for MDR-TB and RR-TB is with a second line regimen of a fluoroquinolone (Moxifloxacin, levofloxacin, gatifloxacin) and a second line injectable (amikacin, capreomycin, or kanamycin) and two or more core second line agents (Ethionamide, prothionamide, cycloserine, terizidone, clofazime or linezolid (WHO, 2017a). The regimen can be strenghthened by addition of first-line drugs (Isoniazid or Ethambutol). This regimen is 9-12 months in selected patients or an alternative longer regime of more than 20 months using pyrazinamide and four second line drugs. Currently, therapy for active drug resistant TB is based on poor evidence base, drugs of uncertain efficacy, high toxicity, and poor adherence and treatment outcomes.

Extensively drug resistant TB has been reported in India, China, South Africa, Russia, and countries in Eastern Europe and the treatment relies on two effective drugs

(Bedaquiline and Delamanid) or surgery (Pai *et al*., 2016). The interim solutions for MDR-TB are use of intermittent regimens, existing anti-TB drugs, higher doses, and re-purposed drugs such as Rifapentine, Fluoroquinolones, linezolid, and carbapenems (Kurz *et al*., 2016). There are probably 17 drugs in various phases of trials for treatment of drug susceptible, drug resistant, and latent TB infections. Of these, eight are new compounds such as delpazolid and sutezolid, while seven are repurposed drugs such as clofazimine, linezolid, moxifloxacin, nitazoxanide, rifampicin, and rifapentine. These drugs mostly belong to three chemical classes oxazolidinones, nitroimidazoles, and fluoroquinolones. The phase III trials of these drugs focuses on combination of new and repurposed drugs with the aim of obtaining regimens that are shorter, simpler, of increased efficacy and reduced toxicity (Marcus, 2018). Bedaquiline and delamanid have received conditional regulatory approval based on phase IIb results (WHO, 2017a). Currently, there are an estimated 12 possible candidates for TB vaccines in development; however, a new TB vaccine is yet to be developed. These vaccine candidates include those to be used to prevent development of TB as well as to improve outcomes of treatment (WHO, 2017a; Voss *et al*., 2018).

2.7.2 Control of TB in livestock

In livestock, control is mainly aimed at eradication or reduction of incidence of *M. bovis* to low threat levels through a sustained test and cull program (Good and Duignan, 2011). Countries such as Australia that are bovine TB free were successful due to implementation of sustained skin testing and culling, postmortem surveillance, epidemiological risk assessment, removal of maintenance hosts and strict movement

control. In addition, prevention of spread to humans through public awareness, improved food hygiene, pasteurization of milk and professional public health service arethe main goals (Fitzgerald and Kaneen, 2012). The various strategies that exist for control include test and slaughter policies, slaughterhouse surveillance-and- trace back, vaccination, culling, restriction of movement, legislation and, breeding for genetic resistance (le Roex *et al*., 2013). Typically, treatment in livestock is controversial and rarely done due to fears of drug resistance and residues except maybe in zoo animals (Kaneen and Thoen, 2004; Anaelom, *et al*., 2010). Vaccination may be recommended in areas without control programs or for feral reservoirs in control areas as it might interfere with diagnostic tests. In Africa, a majority of livestock and human populations are resident in countries with inadequate control measures (Cosivi *et al*., 1998).

2.8 Drug resistance in *Mycobacteria* of zoonotic importance

The target of antimycobacterial drugs is proteins and organelles through inhibition of cell wall, protein and nucleic acid synthesis (Brown-Elliot *et al*., 2012). Among the zoonotic *Mycobacteria*, *M. bovis* is known to be resistant to pyrazinamide, while the NTMs isolated from human respiratory samples are extensively resistant to first and second line drugs used to treat drug susceptible TB (Wang *et al*., 2014). Pyrazinamide (PZA) is a standard first and second line agentfor drug susceptible TB and MDR-TB, resistance is conferred by mutation of the *pncA* gene that encodes pyrazinamidase (Sangstake *et al*., 2017). In contrast to INH and RIF resistance, PZA resistance mutations are diverse. Hence, accurate diagnosis as well as drug susceptibility testing

are prerequisites for the effective management of zoonotic TB caused by these other *Mycobacteria*.

Since the first TB therapy in 1948, strain resistance has occurred within a few years of introduction of every new drug, and resistance is the result of spontaneous genetic mutations, selection resulting from suboptimal chemotherapy, and high transmission (Hu *et al*., 2010; Smith *et al*., 2013; Pai *et al*., 2016). For instance, resistance to isoniazid (INH) is mediated by mutations in different hotspot genes in *M. tuberculosis* that include *KatG*, and the *inhA* genes, while Rifampicin (RIF) resistance is mediated by the *rpoB* gene (Hu *et al*., 2010). Resistance to INH is mainly attributed to mutation in the *KatG* gene, with majority of resistant isolates containing the *KatG -Ser315Thr* nucleotide substitution, while RIF resistance is due to single nucleotide substitutions in the *rpoB-S531L* gene (Hu *et al*., 2010; Sengstake *et al*., 2017). According to the global TB report of 2017, 601,000 (range, 541,000-664,000) people developed anti-TB drug resistance, and among these 492,820 people developed multidrug resistant TB (MDR-TB) and an additional 108,180 had rifampicin-resistant TB (RR-TB) worldwide. Globally, drug resistance is much higher (>20%) in the former Soviet Union, China, and India where totally drug resistant strains have been identified. These coutries contribute approximately 45% to global burden (Pai *et al*., 2016; Ding *et al*., 2017; WHO, 2017a). The three categories used for global surveillance and treatment include RR-TB, MDR-TB, and XDR-TB (WHO 2011; WHO, 2017a). Rifampicin resistant TB (RR-TB) is TB resistant to Rifampicin, MDR-TB is TB resistant to both Rifampicin and Isoniazid, and

XDR-TB is MDR-TB plus resistance to at least one fluoroquinolone and any of the three second line injectable aminoglycosides (Pai *et al*., 2016).

The new compounds with conditional recommendation from WHO to strengthen MDR-TB treatment regimens include Bedaquiline and Delamanid (WHO, 2017a). Evidence from molecular studies indicates that drug resistance is mostly transmitted rather than acquired and the *KatG315Thr* mutation may be a possible risk factor for progression from INH mono resistance to MDR-TB (Hu *et al*., 2010; Pai *et al*., 2016). The most important causal pathway of MDR/RR-TB among retreatment cases is most probably due to transmission of resistant strains, while the failure to diagnose MDR/RR-TB initially is the cause of high rates. Importantly though, there exists heterogeneity in the causal pathways globally. The pathways are divided evenly in Africa between resistance amplification and inappropriate therapy pathways. Hence, the need for context specific solutions to limit MDR/RR-TB, for instance, due to the high rates of re-infection in Lesotho, South Africa and Swaziland, it might be appropriate to focus on limiting case numbers (Ragonnet *et al*., 2017).

Drug resistance patterns can be determined using phenotypic (conventional/microbiological/biochemical) methods such as absolute concentration, resistance ratio and the proportional method. Molecular (genotypic) based methods using PCR amplification and direct DNA sequencing with appropriate primers as well as hybridization with probes are used to detect resistance conferring genetic mutations (Pai *et al*., 2016; WHO, 2017a). The Clinical and Laboratory Standard Institute (CLSI)

recommends the broth microdilution as the gold standard for drug susceptibility testing of NTMs (CLSI, 2011; Brown-Elliot *et al*., 2012). The technology most widely available and in use currently for *M. tuberculosis* is Xpert MTB/RIF; however, culture-based phenotypic drug susceptibility tests (DST) remain in clinical use as the reference method (Githui, 2002; Hu *et al*., 2010; Pai *et al*., 2016; WHO, 2017a). For XDR/TB, molecular line probe assays (LPA) are the only rapid molecular tests reccomended by WHO (WHO, 2017a). The WHO has also approved the use of loop-mediated isothermal amplification and molecular line probe assays for diagnosis of TB and drug susceptibility testing. Drug resistant *M. tuberculosis* in humans is a global epidemic which threatens efforts to eradicate TB, anddocumentation is also scanty for the case of other *Mycobacteria* circulating in livestock in endemic areas (Ogaro *et al*., 2012; Dantas *et al*., 2015; Haeli *et al*., 2015; Lisdawati *et al*., 2015; Ragonnet *et al*., 2017). The treatment outcomes of MDR-TB and XDR-TB are generally poor globally (52%). Moreover, treatment takes long between 12-24 months and it has toxic side effects (Alene *et al*., 2017).

2.9 Future perspectives and knowledge gaps addressed by the current study

Currently, epidemiological knowledge on NTM is scanty and prevalence of zoonotic NTM of camel origin is unknown, especially in the face of widespread isolation of NTM in humans, livestock and the environment. In addition, information on transmission and pathogenesis of these other types of zoonotic mycobateria is scanty. Current diagnostic tests are imperfect due to interference from cross reactivity between various species of *Mycobacteria* which, results in misdiagnosis and drug resistance.

Infections with other *Mycobacteria* are possible confounders in TB diagnosis due to cross reactivity, especially with the current diagnostic limitations in highly endemic countries. These other *Mycobacteria* are also potentially pathogenic in both immunocompromised and immunocompetent individuals. The current study sought to address the knowledge gap including types of species present, prevalence of zoonotic NTMs in camel, characterization of other potentially zoonotic *Mycobacteria* of camel origin, drug resistance of isolates, and assessment of risk factors likely associated with zoonotic infection.

Apart from *M. tuberculosis* and *M. bovis* the biology of other types of potentially pathogenic *Mycobacteria* is poorly understood. The true significance of the isolation of high levels of NTM in both humans and livestock is not well understood. There is continued absence of established monitoring and surveillance systems for NTMs in both humans and livestock in Kenya and standardized diagnostsic protocols and treatment regimens are lacking.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study area

This study was carried out in Samburu East Sub-County of Samburu County, Kenya. Samburu County is bordered by; Turkana County to the west; The tip of Lake Turkana to the northwest; Marsabit County to the north and northeast; Isiolo County to the south east and; Laikipia and Baringo Counties to the south west. The study area consists of four administrative wards, namely; Wamba North, Wamba East, Wamba West, and Waso (Figure 3.1). Wamba and Archers Post are the two urban centers within the study area with approximately 8,918 and 8,958 inhabitants respectively (Samburu County CIDP, 2018).

The dromedary camel (*Camelus dromedarius*) constitutes an important part of livelihoods, economy and food security. The camel population is approximately 20,000, and the camel breeds in this study area are predominantly Somali, Turkana and Rendile breeds and camel milk is the most important product and may contribute up to 60 per cent of nutrient intake. Average daily camel milk yield has been reported at between 3-40 liters per day (Musinga *et al*., 2008). The Somali breed and its mixes predominate and they are mainly kept for milk. Turkana and the Rendile breeds are mostly for meat. There is one major livestock market day in the study area at Lolkuniyani market, in Wamba every Thursday. Most camels are trekked to the market from the area around Wamba and Sereolipi and some are trucked in from Moyale and Marsabit. Camels are rarely slaughtered in the study area due to low demand for camel meat (Samburu County Government, 2018).

The area is predominantly semi arid, characterized by hot and dry weather most of the year. Nomadic pastoral production predominates, characterized by extensive livestock grazing of mainly cattle, camels, sheep, and goats. Tourism is a major source of income and most of the study area is composed of wildlife protection areas such as Samburu national reserve and several community wildlife conservancies. These areas are inhabited by abundant and highly diverse wildlife and it is also home to a number of rare wildlife species. The county is classified as one the poorest counties with a poverty rate of approximately 80%, much higher than the national poverty rate of 45.9%. Tuberculosis is a big challenge as 584 cases were notified in 2016 and the Kenya TB prevalence survey indicated a burden of 1200 cases in a population of approximately 283, 780 (KNBS, 2016; Samburu County Government, 2018).

Post mortem inspection was done on slaughtered camels at Isiolo abattoir and at the Athi River slaughter house, where all sampled camels were confirmed to have come from Samburu east. All samples collected were processed at the Kenya Medical Research Institute – Center for Respiratory Diseases Research (KEMRI – CRDR) enhanced biosafety level 2 laboratory (BSL2) and histopathology was done at the Institute of Primate Research – National Museums of Kenya (IPR-NMK).

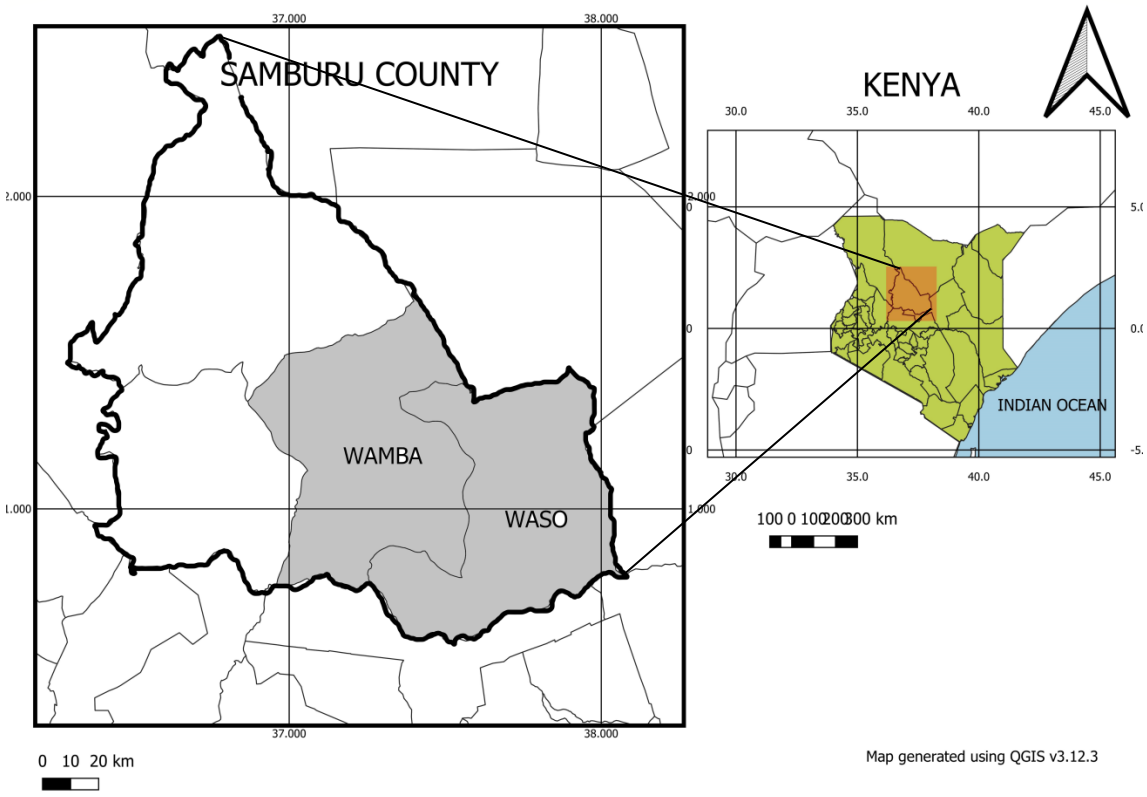

Figure 3.1: A map of Samburushowing the location of the study area (Samburu East) the shaded area

3.2 Study design

A cross sectional study was conducted involving two main populations: one composed of lactating camels and household members from participating *manyatta* households; and the other composed of camels confirmed to have originated from the study area and slaughtered at Isiolo and Athi River county slaughterhouses. For the purpose of sampling camels and household members, the study area was stratified into the two camel rearing areas of Waso and Wamba and the sampling unit was the individual households within *manyattas*. This area is predominantly inhabited by the Samburu people and covers approximately 10,049.7 km^2 with a population of 77, 994 (KNBS, 2019). The villages visited in Waso region included Lerata, Ndonyo Lekita, Ntilal,

Ntabasy, Kirish, Laresoro, Leparo, Loipusakini, Losupulai, Ntilal, Sirata, Noltinga, Lekipelen and Sereolipi. The villages visited inWamba region included Lpus Lelui, Lpashie, Lelui Westgate, Nasunyai and Kiltamanya. Study *manyattas* and participating households (n=83) with their respective camel herds were selected purposively from a sampling frame depending on accessibility and on whether they were consenting. The sampling frame of households was generated with assistance from the ward administrators and county government veterinary officers during a preliminary visit to the study area. The households were uniquely identified using numbers, and the numbers of milking camels within each household was recorded. The milking camels were selected consecutively as the number of milking camels obtainable in the duration of the study were approximately equal to the targeted sample size.

It was also determined during the prior visit to the study area that camels are rarely slaughtered within the study areaand that the major slaughter destination for camels from the study area is Isiolo and Athi River county slaughterhouses. Therefore, for the purpose of characterizing the pathology in slaughtered camels, consecutive sampling of each camel slaughtered at these slaughter points was carried out. This was due to the fact that the required sample size of 1600 was significantly more than what could have been obtained in the available time using random methods. Each slaughtered camel originating from the study area was examined at post mortem and the procedure involved observation, photography and collection of suspect lesions.

For the purpose of collection of data on risk factors, questionnaires were administered to each of the participating households. Symptomatic human subjects were identified from among members of participating households using the case definition for tuberculosis suspects (WHO, 2010). Only those human subjects who were symptomatic and suspect for tuberculosis were requested to provide a sputum sample. The suspect human subjects were enrolled consecutively as encountered, and only sampled after consent was given. Confirmed positive cases were referred to tuberculosis treatment centers at the local health facilities. The human subjects in this study such as household members, herders, milkers, and others who had closely interacted with the camels were considered as any other risk factor used to explain the pattern of zoonotic mycobacterial infections observed in the area. The duration of the slaughterhouse sampling was from April, 2017 to August, 2017, while sampling amongst *Manyatta* camels and pastoralists was between October, 2017 and December, 2018. All samples were collected and transported in cool boxes to KEMRI-CRDR enhanced biosafety level 2 laboratory for mycobacteriology.

3.3 Inclusion criteria

The study subjects included in this study were members of selected pastoralist households from the study area who were adequately informed and consented, their milking camels, and camels from the study area slaughtered at Isiolo and Athi River slaughter houses.

3.4 Exclusion criteria

The following were not included in the study: Pastoralists and lactating camels not from within the study area, camels for slaughter not from the study area, and pastoral dropouts (those forced out of the pastoralist way of life to other forms of livelihoods such as sedentary farming). In addition, urban dwellers who have no contact with camels and who do not habitually consume camel milk or meat, as well as non-consenting households from within the study area during the study period were excluded.

3.5 Sample size determination

3.5.1 *Manyatta* camels sample size

A *manyatta* is a temporary Samburu settlement or encampment consisting of several sticks, mud and cow dung huts and thorn enclosures (Figure 3.2) for livestock established by a family or clan to protect inhabitants and livestock from wild carnivores.The sample size was computed using the method of Thrusfield (2005), using the following formula;

$$n = \frac{Z^2_{\frac{\alpha}{2}} p(1-p)}{d^2}$$

Where n = sample size, $Z_{\alpha/2}$ = normal deviate (1.96) at 5% level of significance, p = estimated prevalence and, and d= precision of the estimate not exceeding 20% of expected p (WHO, 2009). For the first objective, a purposive sample size of 600

lactating camels was calculated, assuming a 50% NTM prevalence rate and precision (*d*) set at 4%.

Figure 3.2: Camels in a thorn enclosure at a *Manyatta*

3.5.2 Slaughter house camel sample size

A sample size of 1600 camels slaughtered was computed using the method of Thrusfield (2005), assuming a 10% prevalence rate of NTM (Beyi *et al*., 2014) and precision (*d*) set at 1.5%.

3.5.3 Household sample size

A total of 60 households were targeted, calculated as follows:

$$\text{Number of households (HH)} = \frac{calculated\ sample\ size\ of\ milking\ camels}{(percent\ lactating \times average\ herd\ size)} = 60$$

Where, calculated sample size of milking camels was 600 camels, percent lactating was set at 20% (Range: 18-35%) and average herd size at 50 (Range: 30-500) (Musinga *et al.,* 2008). At least 60 households were targeted for administration of a questionnaire to collect risk factor data.

3.6 Definition of people presumed to have tuberculosis

A human TB suspect was defined as any individual with a TB symptom (WHO, 2010). The cardinal symptomsof TB considered were: current cough of any duration or severity, haemoptysis any time in the previous year, reports of fever, night sweats and reported weight loss. Cough was defined as acute, if less than 2 weeks and chronic otherwise.

3.7 Data collection

3.7.1 Tuberculin skin testing and collection of camel milk samples

Tuberculin skin testing of camels was conducted in the two regions of Wamba and Waso in the study area between 20th October, 2017 and 31st of December, 2018. Eighty three (83) households were visited, 68 in Waso and 15 from Wamba. Tuberculin skin testing was conducted by a veterinary surgeon assisted by a veterinary paraprofessional on 612 camels using the single intradermal comparative tuberculin test (SICTT) as described (De la Rua Domenech *et al*., 2006; Wernery and Kinne, 2012; Beyi *et al*., 2014; OIE, 2018). Of these, 91 were from Wamba and 521 were from Waso. The test

was performed at the axilla site, with 0.1 ml (3,000 IU/ml) of bovine tuberculin PPD, *M. bovis*, strain AN5 (Prionics Lelystad BV, The Netherlands) on the left and 0.1 ml (2,500 IU/ml) of avian tuberculinPPD, *M. avium* subsp. *avium*, strain D4ER (Prionics Lelystad BV, The Netherlands) on the right (Figure 3.3). Skin induration was read using Vanier calipers 72 hours after administration of tuberculin test and the relative difference in thickness between these sites used to interpret test results. The reaction at each site was derived as the difference in skin thickness before injection and 72 hours after injection.

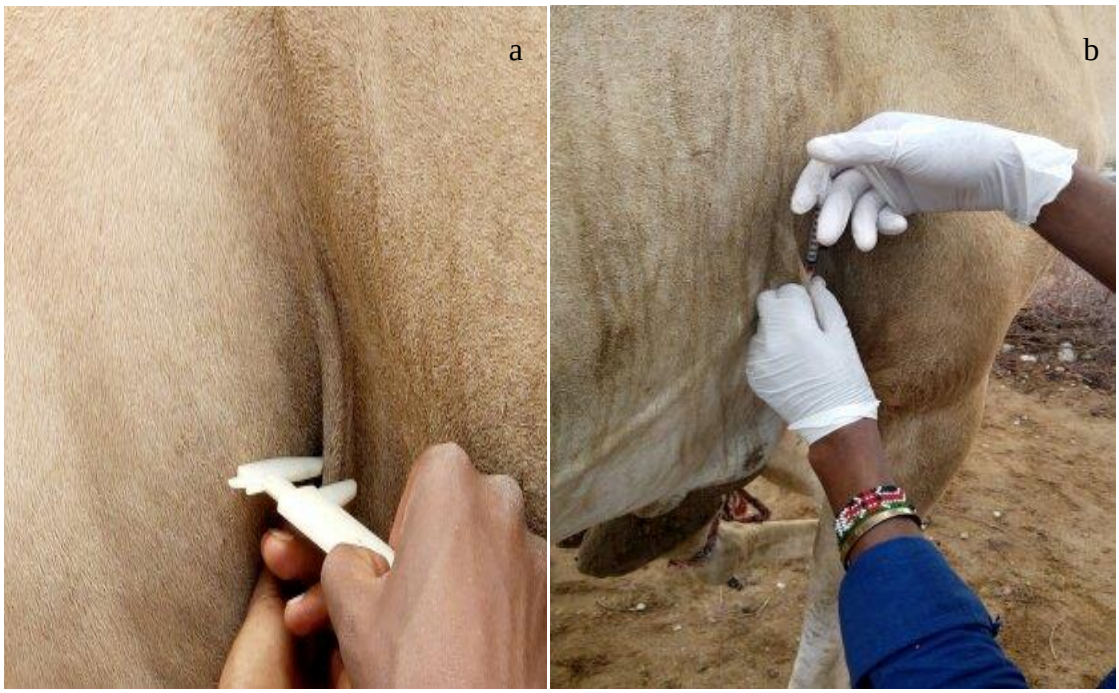

Figure 3.3**: Measurement of skin thickness before intradermal injection (a), and intradermal injection of *Mycobacterium avium* purified protein derivative (PPD) at axilla site on the right (b)**

Interpretation was as recommended by OIE (2018) to determine positive reactors to *M. bovis* infection. Briefly, the variance in the measurement of the skin thickness before and after 72 hours of PPD injection was determined as the final record of induration

size at injection site. A positive result was recorded if the increase in skin thickness at the bovine tuberculin PPD (PPD-B) injection site was more than 4mm greater than the reaction at avian tuberculin PPD (PPD-A) injection site; inconclusive if PPD-B reaction was greater than that at the PPD-A site with a difference of less than 4 mm; and negative if the skin swelling at PPD-B injection site was less than or equal to the size of the skin reaction at the PPD-A injection site. Positive reactors to *M. avium* PPD were determined as described by Gumi *et al*. (2012b). Briefly, a positive result for NTM infection was recorded if an increase in skin fold thickness with visible reaction at the PPD-A injection site was more than 1mm greater than reaction at PPD-B injection site.

Composite milk samples, 30-40ml, were collected in sterile 50ml centrifuge tubes by hand milking from those camels classified as positive reactors and inconclusivefor confirmatory culture. Prior to collecting milk samples, the teats were thoroughly cleaned with surgical spirit and the first 10-20ml of milk discarded. Techniques that reduce contamination between samples, between study subjects as well as the use of personal protective equipment (PPE) to minimize possible infection of person collecting sample were employed. Samples were packed according to standard protocols and transported in cool boxes on ice according to international guidelines (Stinson *et al*., 2014). The milk samples were transported on ice in cool boxes at +2 to +8°C to the KEMRI/CRDR laboratories and submitted within 3 days for mycobacteriology and molecular typing. Data consisting of unique sample identification, village, unique household identification, and collection date was collected in a dedicated '*camel milk*

sample form' (Appendix VI). All data were then transferred to a customized electronic database in a password protected computer.

3.7.2 Investigation of the pathology of *Mycobacteria* in slaughtered camels

Suspect tuberculous lesions were collected from camels slaughtered at the Athi River and Isiolo county slaughter houses between April 2017 and March 2018. During this period, post mortem examination was conducted on 269 camels at Athi River slaughter house and 1331 at Isiolo slaughter house comprising of 84.97% males (Figure 3.4). Post mortem examination was conducted on a total of 1600 camels consecutively to characterize the pathology associated with camel tuberculosis. Post-mortem inspection involved examination of the mandibular, retropharyngeal, bronchial, mediastinal, mesenteric and hepatic lymph nodes, as well as the lungs, liver, small intestines and kidneys. Identification of lesions was based on gross detection of typical tubercles, yellowish caseated granulomatous lesions or calcifications in these organs through careful visual examination, palpation, incision and photography.

Figure 3.4: **Post mortem examination of camels slaughtered at Isiolo County slaughter slab**

Suspect tuberculous lesions from slaughter camels were collected in sterile centrifuge (Falcon) tubes with about 5 ml of 0.9% normal saline solution and deep frozen meanwhile prior to transportation on ice in cool boxes at +2 to +8°C to the KEMRI/CRDR laboratories. The lesions for histology were preserved and fixed in 10% formalin before transported to the Institute of Primate Research- National Museums of Kenya (IPR-NMK) laboratories. Due to the nature of slaughter at the slaughter houses, it was often difficult to avoid contamination of tissues, however, samples were packed separately and labeled using a unique identification for the slaughter house and the individual camel. The age of slaughtered camels was determined using dentition, where:

the presence of permanent front teeth indicated that the camel was at least four years old; the presence of the permanent canine teeth indicated that the camel was at least seven years old; and a full set of permanent teeth and the extent to which they were worn off was used to estimate age onwards (Bello *et al*., 2013). Personal protective equipment (PPE) were used to minimize possible infection of person collecting sample. Meat inspection was conducted through observation, photography and collection of suspect lesions by trained meat inspectorsguided by the abattoir daily recording form (Appendix VII), and raw data consisting of unique sample identification, village, unique household identification, collection date and sample type was collected in a dedicated '*slaughter house sample form*' (Appendix VIII). All data were then transferred to a customized electronic database in a password protected computer.

3.7.3 Human sputum samples

The sputum samples were collected from participating households between 20th October, 2017 and 31st of December, 2018. In total, there were 48 sputum samples, 9 from Wamba and 39 from Waso. A spot and morning sputum sample from any symptomatic household member were collected by community health workers trained for this purpose. Using personal protective equipment (PPE) and techniques that reduce contamination between samples and study subjects, a minimum of 15ml of sputum was collected from every symptomatic individual. Briefly, the identified symptomatic household member was asked to rinse their mouth with plain water, next, they were instructed to take at least three deep breaths followed by a forced deep cough. The expectoration of a good sputum sample consisting of thick secretions from the lungs was aimed for. The samples were collected in 50ml centrifuge tubes with tight fitting

screw-on cap and transported within three dayson ice in cool boxes at +2 to +8°C to the KEMRI-CRDR laboratories for analysis. Data consisting of unique sample identification, village, unique household identification, collection date and sample type were collected in a dedicated '*sputum sample form*' (Appendix IX). All data were then transferred to a customized electronic database in a password protected computer.

3.7.4 Determination of risk factors

The categorization of risk factors was as follows: Household; herd; management; and wildlife level factors. Risk factors data was collected in questionnaires between 20th October, 2017 and 30th of March, 2018. A standard semi structured questionnaire about knowledge of zoonotic TB, practices that predispose to infection with zoonotic TB, camel herd level factors and wildlife interactions was administered via personal interview to 83 respondents by trained enumerators (Figure 3.5). The questionnaire was used to record the respondents personal information; location; household information; camel meat and milk consumption practices; livestock management practices; and wildlife interactions (Appendix X). The questionnaire data was then transferred to a customized electronic database in a password protected computer.

Figure 3.5: An enumerator administering the questionnaire to collect social and herd level risk factor data

3.8 Clinical and laboratory methods

3.8.1 Mycobacteriology

Mycobacteriological culture and isolation from tuberculous lesions, camel milk and human sputum was carried out according to KEMRI-CRDR TB research laboratorystandard operating procedures (SOPs) with a few modifications. External quality assurance was provided by a WHO Supranational Reference Laboratory network in the UK and Belgium.For the tissue samples from the slaughter houses, the initial processing involved cutting into cubes approximately 2mm x 2mm, followed by maceration using a blender in normal saline (KEMRI, 2015).

Prior to fluorescent microscopy and culture, all the samples were decontaminated according to KEMRI-CRDR TB research laboratory SOP. Decontamination was with

N-acetyl-L-cysteine (Nalc-liquefying agent) and, an alkali (2–4% sodium hydroxide with sodium citrate) as the decontaminating agents, allowing strictly 15 minutes of contact time. Equal volume of *N*-acetyl-L-cysteine (NALC)-sodium hydroxide mixture was added to the sample which were then shaken intermittently during the 15 minutes incubation time at room temperature and then neutralized with phosphate buffered saline (PBS), pH 6.8, prior to centrifugation. For camel milk samples, the suspension was centrifuged twice for 15 minutes each at 3000rpm (1008*g*) at 4°C and both the sediment and the top fat layer were retained for fluorescent microscopic (FM) examination and culture after being reconstituted with 2 ml of PBS, pH 6.8. For tissue samples, centrifugation was done once for 15 minutes at 3000rpm (1008*g*) at 4°C, the supernatant discarded, and the sediment reconstituted with 2 ml of PBS, pH 6.8 and used for FM examination and culture.

All samples were subjected to FM examination according to KEMRI-CRDR TB research laboratory SOP, where a drop of sample was added to a labeled slide and spread to an area approximately 2cm x 2cm which was left to dry on a slide warmer followed by heat fixing on the warmer at 65°C - 75°C for at least 2 hours. Preparation for FM examination involved: Staining with Auramine-O-Rhodamine B solution (1.5g Auramine O and 0.75g Rhodamine B in 75mL glycerol, 10mL phenol crystals, and 50mL distilled water) for 15 minutes; rinsing with dechlorinated water; decolorizing for 2-3 minutes; washing with distilled water; destaining with acid alcohol (0.5ml concentrated hydrochloric acid to 70% ethanol) for 2 minutes; rinsing thoroughly with distilled water; counter staining for 2 minutes with potassium permanganate; and then

examining under a fluorescence microscope using the 20x or 40x objective for screening and the 100x oil immersion objective to observe cell morphology.

All samples regardless of FM examination outcome were cultured on both solid and broth mediaaccording to KEMRI-CRDR TB research laboratory SOP. Löwenstein-Jensen (LJ) solid media which is specific for the isolation of *Mycobacteria*, and the modified middlebrook 7H9 broth in the *Mycobacteria* growth indicator tubes (MGIT; BD Sparks, MD, USA), a non-specific nutrient-rich liquid media. For culture on solid media, 0.1 ml of inoculums suspension from each sample was inoculated onto two slants of Löwenstein-Jensen (LJ) medium, one containing glycerol and the other containing pyruvate as a source of energy. Cultures were incubated aerobically at 37°C for 8 weeks with weekly observation for growth of colonies (Figure 3.6). Milk samples were additionally cultured at 42°C for the same duration for isolation of *Mycobacterium avium* complex (MAC). For culture in liquid media, individually wrapped, sterile, 3 ml plastic pipettes were used for inoculating the MGIT media with 0.5 mL of decontaminated sample inoculum followed by incubation at 37°C examined daily in a 365nanoMeter (nM) wavelength UV light source that detects fluorescence, for upto 8 weeks of innoculation. Positive control and negative control tubes were used and bacterial growth was also identified as non-homogenous turbidity of flakes (Figure 3.7). Smears of the content of positive cultures (LJ slants and MGIT tubes) were confirmed for acid fast bacilli with Ziehl- Nelseen (ZN) staining (Figure 3.8). This was followed by sub-culturing of content of cultures (LJ slants and MGIT tubes) that were positive on ZN on LJ media, and growth was considered true positive isolates. The isolates were

heat killed at 95°C for 15 minutes and then stored at (-20°C) for molecular typing analysis (KEMRI, 2015).

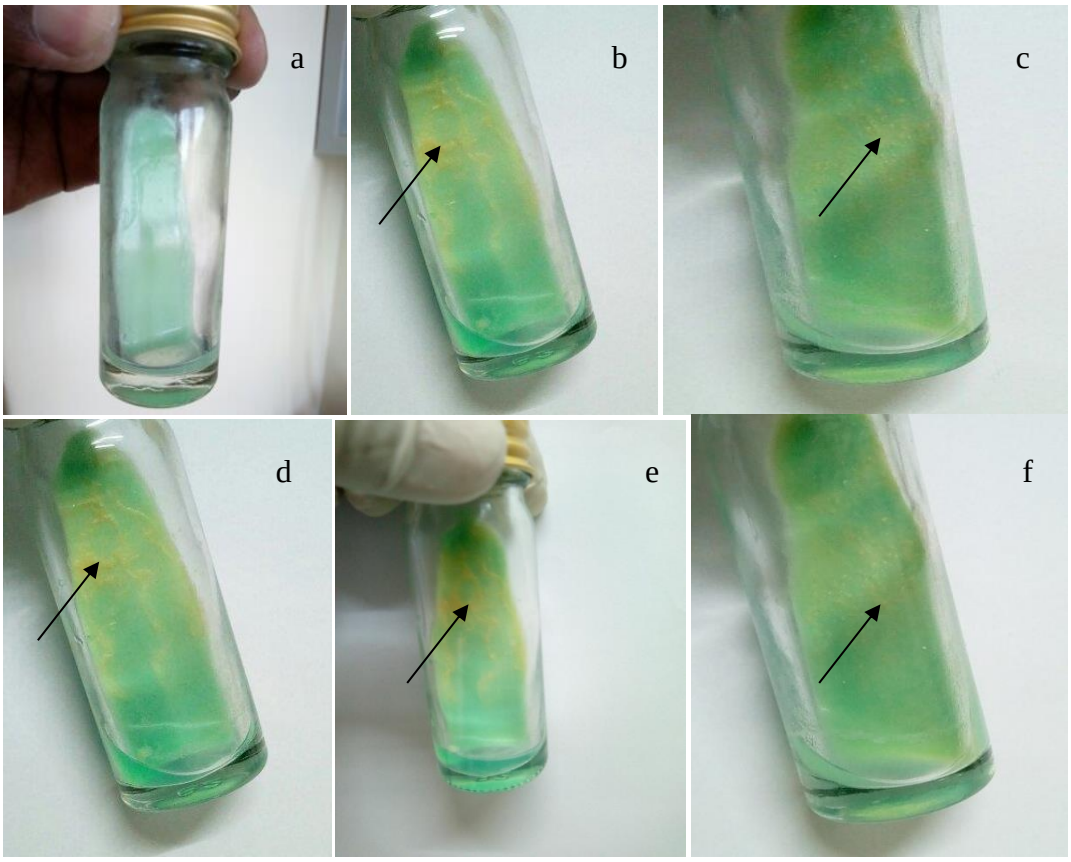

Figure 3.6: Slants of LJ solid media showing (a) No growth and (b, c, d, e, f) different patterns of growth (Black arrows) of some selected samples (No. 299 and 332) showing the buff white colonies characteristic of *Mycobacteria*

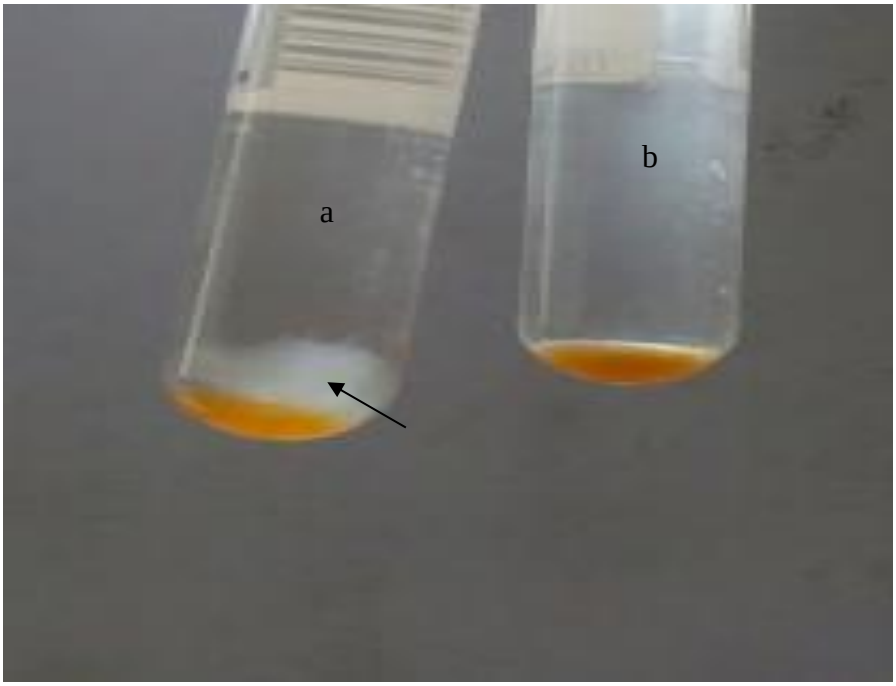

Figure 3.7: *Mycobacteria* growth indicator tubes (MGIT) tubes, (a) is a positive tube with the characteristic 'cotton' or 'cloudy' appearance (Black arrow) of the growth close to the bottom of the tube and (b) is a negative tube with no growth

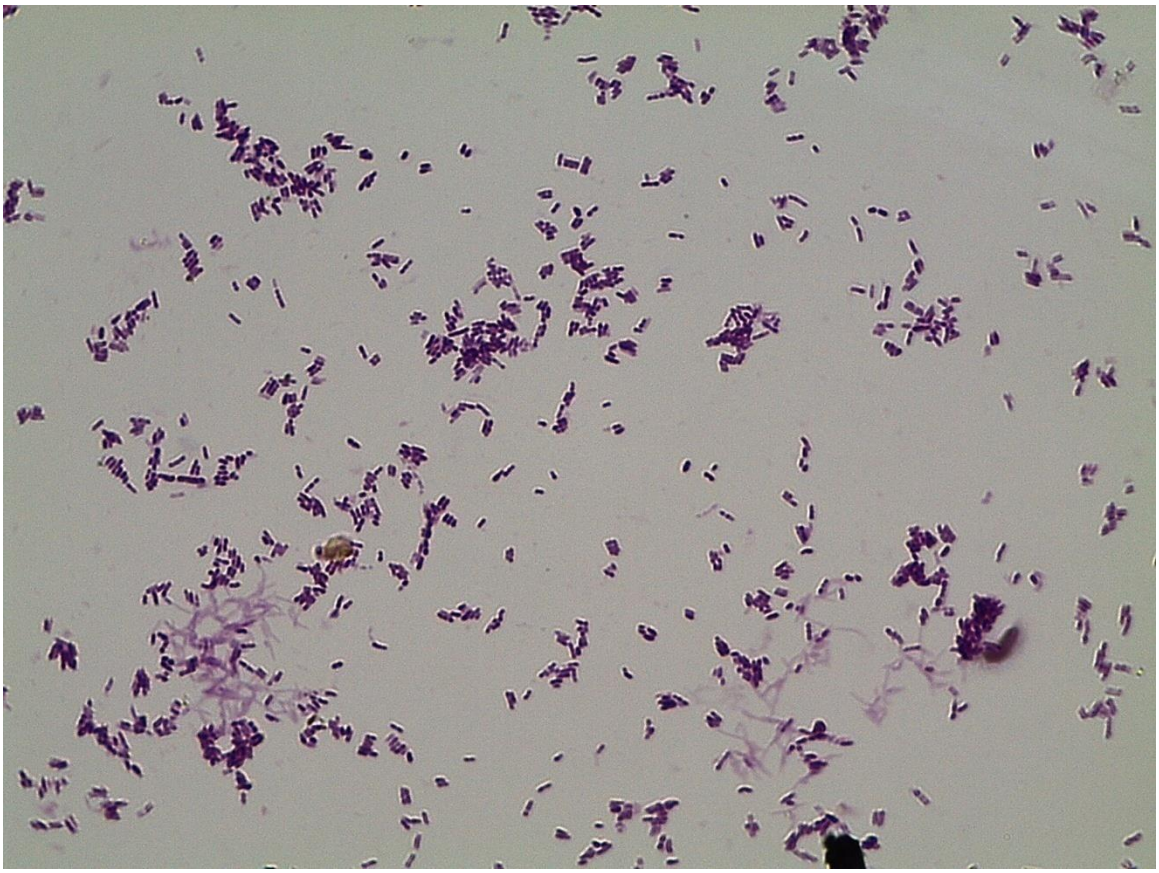

Figure 3.8: Acid fast bacilli (AFB) in Ziehl-Neelsen (ZN) stain

3.8.2 Histopathology

Suspect lesions were processed for histopathology according to IPR-NMK pathology laboratory SOP. Briefly, tissue specimens of suspected lesions were fixed in 10% formalin as a phosphate buffered solution and processed for embedding in paraffin - based wax. The embedded wax block was sectioned at 2µ thick and stained with haematoxylin and eosin (H&E) and examined at microscopy at 20x and 40x to determine cell morphology. For the detection of acid fat bacilli (AFB), sections were stained with ZN.

3.8.3 Immunochromatography to differentiate between MTBC and NTM

Capilia Neo TB® test was conducted according to manufacturer's instructions. This test detects MPB64 antigens produced only by species members of *M. tuberculosis* complex. Briefly, specimen isolates grown on LJ and Mycobacterial growth indicator tubes (MGIT™; Becton, Dickinson and company, NJ, USA) were suspended in 0.2ml of extraction buffer supplied with the kit. Approximately 80-100µl of resulting mixture was placed in the specimen area of test plate and read after 15 minutes. A positive liquid culture of reference strain *M. tuberculosis* H37RV was used as positive control and phosphate-buffered saline (PBS) containing 0.1% (w/v) Tween 80 was used as negative control. A reaction was produced and positive test results visualized by purple red lines at both the test (T) and control (C) in the reading area (Figure 3.9).

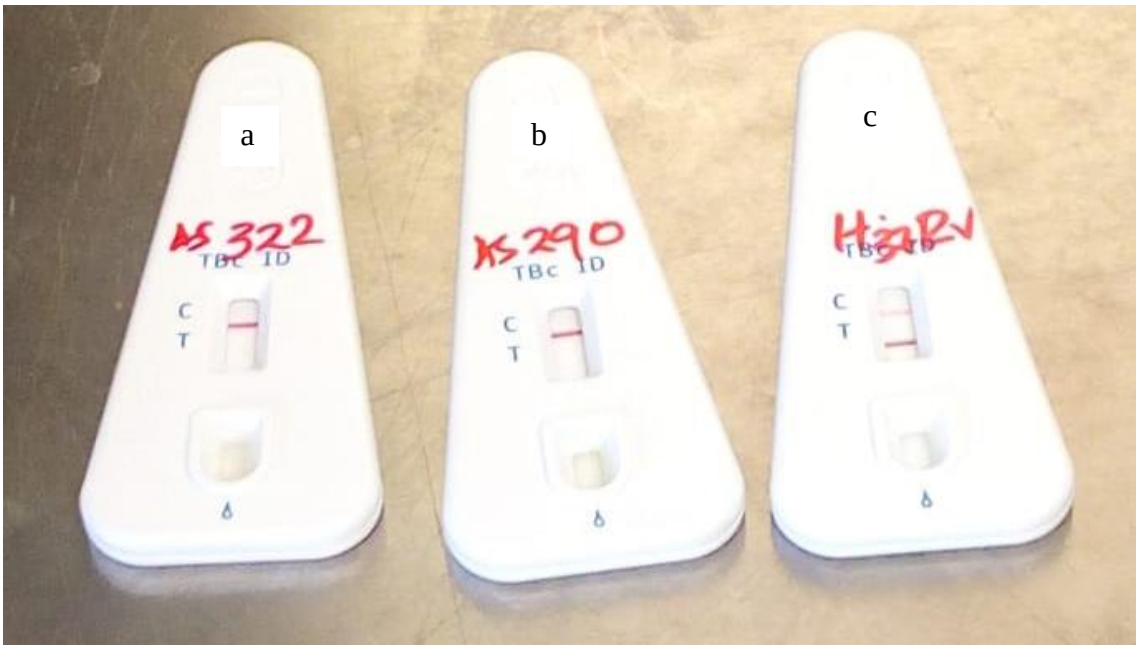

Figure 3.9: Immunochromatography test plates, (a) and (b) are negative while (c) *M. tuberculosis* H37RV (Right) was used as a positive control

3.8.4 GeneXpertMTB/RIF® diagnosis

GeneXpert diagnosis was conducted for all samples according to the manufacturer's instructions. The assay uses filter based sample processing or PCR cartridge for detection of *M. tuberculosis* and rifampicin resistance. Briefly, samples were initially decontaminated using NALC-NaOH, allowing strictly 15 minutes of contact time followed by addition of phosphate-buffered saline (PBS). This was followed by centrifugation at 3000*g* for 15 minutes, after which supernatant was poured off and followed by resuspension in 1-2ml of PBS. For camel milk, both cream and deposit was used, into which, 1.4ml of sample reagent was added. This mixture was vortexed for 30 seconds and incubated at room temperature for 15 *min* as per manufacturer's instructions. Thereafter, 2ml of mixture was transferred to GeneXpert MTB/RIF®cartridge. The cartridge was loaded into GeneXpert instrument and results reported as positive or negative for *M. tuberculosis* and rifampicin resistance reported as susceptible or resistant.

3.8.5 *Mycobateria* speciation using GenoType®*Mycobacterium* CM line probe assay

Molecular species typing was conducted using the GenoType®*Mycobacterium* CM line probe assay (HAIN Lifescience GmbH, Nehren, Germany) at an enhanced BSL 2 laboratory under biosafety cabinet according to KEMRI/CRDR TB research laboratory SOP (KEMRI, 2015). The line probe assay differentiates the genus *M. tuberculosis* complex from *M. avium* complex, *M. intracellulare* and other mycobacterial species. Heat killed AFB culture positive samples were used as source of DNA template. This was accomplished by transferring asmallamount of culture growth into micro centrifuge

tubes containing 300 µl of distilled water using a 1 µl loop. The bacteria were then incubated in the micro-centrifuge tubes at 95°C for 5 minutes in genolyse reagent. This were then spinned down for 5 minutes at 3000rpm (1008*g*) and then 5µl of supernatant transferred to a new tube to be used for PCR. The PCR reaction mix containing Primer-Nucleotide-dye mix (PNM), PCR buffer, magnesium chloride, sterile distilled water, and Hot start Taq was constituted. The 1 hour and 50 minutes HAIN x 30 PCR reaction wasusedand it consisted of the followingcycles:95°C for 15 minutes; 10 cycles of 95°C for 30 seconds and 58°C for 2 minutes; 20 cycles of 95°C for 25 seconds, 53°C for 40 seconds and 70°C for 40 seconds; 70°C for 8 minutes; and finally held at 4°C. The development of the strips was done as follows for each strip, in each of the wells used in the plastic tray provided with the kit, 20µl of amplified sample was added to 20 µl of denaturation solution which were mixed well and incubated at room temperature for 5 minutes. This was then followed by the addition of 1 ml of pre-warmed hybridization buffer to this mixture, to which a strip was added and completely immersed with the coated side facing up. The tray was then incubated in a Twincubator® for 30 minutes at 45°C, after which, the hybridization buffer was completely aspirated and 1 ml of stringent wash solution added to each strip followed by incubation for 15 minutes at 45°C in a shaking Twincubator®.

The stringent wash solution was then completely removed followed by washing of each strip with 1 ml of rinse solution for 1 minute on Twincubator®. This was then followed by the addition of 1 ml of diluted conjugate to each strip, which was then incubated for 30 minutes at room temperature on Twincubator®. The remaining solution was then

completely removed and each strip washed twice, first for 1 minute with1 ml of rinse solution and then with 1 ml of distilled water on Twincubator®. This was followed by addition of 1 ml of diluted substrate to each strip, which was incubated at room temperature. The strips were finally removed from the wells after rinsing twice with distilled water and then fixed on the GenoType®*Mycobacterium* Reporting and identification Form (Appendix XI), where results were interpreted using a guiding template. *Mycobacterium tuberculosis* H37 RVATCC# 27294 and *M. bovis* BCG P3 were used as the positive controls, while double distilled water was used as the negative control.

3.8.6 Identification of unknown *Mycobacteria* using partial DNA sequencing of 16SrRNA gene

The strain type diversity of unknown *Mycobacteria* isolates from camels and humans in the study area were determined through *16S rRNA* gene sequencing as described by de Zwaan *et al*. 2014 and Fedrizzi *et al*. 2017. This was accomplished by initial AFB identification using ZN and Capilia TB-Neo® kit rapid assay as either MTC or NTM followed by GenoType®*Mycobacterium* CM assay. Genomic DNA extraction was done using Quick-DNA™ Miniprep Plus Kit (ZYMO RESEARCH CORP.), followed by amplification of the 16S rRNA genes using PCR. The PCR mixture consisted of 5 µlPCR buffer, 0.5 µl dNTPs, 2µl $MgCl_2$, 0.5µl Taq DNA polymerase and 0.5 µl of 8 FPLand 1492 primer, 13 µl of distilled water and 1µ of isolate DNA. The PCR was conducted as follows: 1 cycle at 94 °C for 5 minutes, 30 cycles of 1 minute each at 94, 55, and 72°C, a final extension at 72°C for 10 minutes followed by holding at 4°C.

Gelelectrophoresis of PCR products was conducted to confirm successful applification of the correct band. Finally, partial sequencing of *16S rRNA* gene using the forward primer 8FPL (5′ AGT TTG ATC CTG GCT CAG 3′) (Turenne *et al*., 2001). Sequencing was done using ABI3500XL genetic analyzer (Applied Biosystems, Foster city, CA) and the BrilliantDye® Terminator v3.1 cycle sequencing ready reaction kit (NimaGen BV. The Netherlands) according to manufacturers instructions at Inqaba Biotech, South Africa. The sequencing output was analyzed using ABIPrism® 310 Genetic Analyzer and accompanying computer software (Applied Biosystems, Foster city, CA). The obtained NTM sequences were viewed using Snapgene® viewer, edited using BioEdit® sequence alignment editorand compared with reference strains in the 16S rRNA and the nucleotide collection databases in GenBank® (https://www.ncbi.nlm.nih.gov/ genbank/) using the Basic Local Alignment Search Tool (BLAST 2.9.0) algorithm (Zhang *et al*., 2000) (available at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi.) with mycobacterium taxid 1763 as reference organism. The strain name with the highest expected (E) -value and identity hit and score was selected and used to identify each of the unknown NTM. An identity hit of 100% was aimed for; however, a cut-off of ≥ 98% identity was used. In addition, the NTM sequences were compared with the sequences of reference strains identified using GenoType® *Mycobacterium* CM assay.

Prior to phylogenetic analysis, multiple sequence alignment was conduced using the MUCSLE algorithm (Edgar, 2004) in MEGA version X (Kumar *et al*., 2018). The phylogenetic tree was estimated using the maximum likelihood method with the

Kimura-2 parameter substitution model and the discrete gamma distribution with invariant sites (K2+G+I). This model was selected prior to tree estimation by testing the aligned sequence data to find the best fitting substitution model in MEGA version X. The reference strains used included *M. szulgai* strain ATCC 35799 (Accession no. 118584.1), *M. fortuitum* DSM 46621 ATCC 6841(Accession no. 114893.1), *M. tuberculosis* variant *bovis* strain CIP 105234 (Accession no. 114677.1) and *M. tuberculosis* H37Rv (Accession no. 102810.2). The reliability of the tree was estimated using bootstrap method with 500 bootstrap replicates and a cutoff of ≥ 70% bootstrap percentages for each node was used (Pattengale, 2010; Hall, 2013). The final tree was presented in the newick tree format in MEGA version X (Kumar *et al*., 2018). The tree was rooted using the reference strain *Enterococcus faecium* (282_8FPL), which had the longest branch indicating high amount of sequence divergence (Baum, 2008; Hall, 2013).

3.8.7 Drug sensitivity testing (DST) using resistant ratio (RR) method on LJ solid media

Drug resistance patterns of isolates from the study area were determined using the resistant ratio method on L-J solid media according to KEMRI/CRDR TB research laboratory SOP for drug susceptibility testing by resistant ratio method and the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2011; KEMRI, 2015). Drug susceptibility testing was done on the first-line TB drugs isoniazid (H), ethambutol (E), and rifampicin (R). The method involved microdilution of LJ media starting with the highest to the lowestdrug concentration by serially diluting with

appropriate diluent. In addition to a drug free negative control tube containing the respective sample, the dilutions included the Minimum Inhibitory Concentration (MIC) of the positive control (Figure 3.10) and all greater dilutions. The specific dilutions in micro grams per milliliter (μg/ml) were isoniazid (0.2μg/ml and 1μg/ml), rifampicin (16μg/ml, 32μg/ml, and 64μg/ml), and ethambutol (1μg/ml, 2μg/ml, 4μg/mL, 5.6μg/ml, and 8μg/ml) (Figure 3.11). This was accomplished by inoculating slopes of L-J containing the respective drug dilutions with standard inoculate prepared from AFB positive sub-cultures of mycobacteria isolates, followed by incubation at 37°C and reading at week 4 (28 days). Inoculation of the middle of the slopes of L-J containing the respective drug dilutions with standard inoculate was done starting from the lowest to highest drug concentration.

Figure 3.10: **Drug susceptibility testing control rack for calibration of the resistant ratio.** *Mycobacterium tuberculosis* H37Rv (Rows 3 and 4 on the Right) was used as susceptible control anda known resistant strain 1369 (Rows 1 and 2 on the Left) used as resistant control. The dilutions used were: isoniazid (H) (0.025 μg/ml, 0.05 μg/ml, 0.1 μg/ml, 0.2 μg/ml, 1 μg/ml); rifampicin (R) (4 μg/ml, 8 μg/ml, 16 μg/ml, 32 μg/ml, 64 μg/ml); and ethambutol (E) (1 μg/ml, 2 μg/ml, 4 μg/ml, 5.6 μg/ml, 8 μg/ml). The minimum inhibitory concentration (MIC) for the susceptible control was0.2 μg/ml, 16 μg/ml, and 1 μg/ml (White block arrows) for isoniazid, rifampicin, and ethambutol respectively. The resistant ratio method was used, which involved microdilution

Figure 3.11: **Drug susceptibility testing samples rack showing (From left) thedrug free negative control tube containing the respective sample (Row 1); respective drug dilutions for isoniazid (H) 0.2*μ*g/mL and 1*μ*g/mL (Row 2 and 3); rifampicin (R) 16*μ*g/mL, 32*μ*g/mL, and 64*μ*g/mL(Rows 4, 5 and 6); and ethambutal (E)) 1*μ*g/mL, 2*μ*g/mL, 4*μ*g/mL, 5.6*μ*g/mL, and 8*μ*g/mL (Rows 7, 8, 9, 10 and 11).**The starting dilution for each drug was the minimum inhibitory concentration (MIC) of the suceptible control strain, *M. tuberculosis* H37 Rv. The resistant ratio method was used, which involved microdilution

Briefly, standard inoculate were prepared by picking a standard quantity ($1/8^{th}$) of culture growth using standard wire loops (Gauge 22) and diluting in 1 ml of sterile distilled water containing standard (diameter, 3-4mm) glass beads. This was followed by vortexing to disentangle clumps of bacteria to make a homogenous mycobacteria suspension of turbidity equivalent to 0.5 McFarland, with an approximate concentration of 1.5×10^8 colony forming units (CFU)/ml. *Mycobacteria tuberculosis* H37RV and a known MDR strain, EQA 1369, were used as the sensitive and resistant quality control strains, respectively. The drug dilutions used for DST of isolates were determined based on the minimum inhibitory concentration (MIC) of the positive sensitive control strain.

External quality assurance (EQA) was provided by a WHO Supranational Reference Laboratory network in the UK and Belgium. In addition to a drug free tube, the dilutions included the MIC of the positive control and all greater dilutions. Growth of more than twenty colonies was considered significant resistance at a given dilution, while inhibition of visible growth to less than twenty colonies was considered the susceptibility break point MIC.

Drug sensitivity testing of the strains was repeated twice. Results were recorded in the form of a resistant ratio (RR) defined as the (MIC) of the test organism divided by MIC of the control strain.Confluent growth at all dilutions or a resistance ratio greater than 4 was considered resistant. Resistance to one anti-TB drug was considered mono-resistant TB, MDR-TB when resistance to both isoniazid and rifampicin was observed according to WHO guidelines for treatment of TB.

3.9 Data handling and analysis

3.9.1 Determination of positivity using SICTT, post mortem examination and mycobacteriology

Data from the comparative tuberculin skin testing, sample collection forms and laboratory findings on FM examination andculture on LJ and MGIT and ZN were entered in a customized electronic database (MySQL). Summary spreadsheets generated from this database were then exported to Microsoft Excel (Microsoft Corporation) spreadsheets and data analysis was performed in STATA version 9.0, where proportions, confidence intervals and standard errors were computed using STATA's immediate commands *cii*. Two sample tests of equality of proportions and means with p-values were computed using STATA's immediate commands, *bitesti*, *prtesti* and *ttest* respectively. In two sample tests of equality of proportions and means the null hypothesis was that the difference of proportions or means was equal to zero, and differences were considered significant at a p-value of 0.05 or less.

3.9.2 Molecular characterization and phylogenetic analysis

Data from confirmation of the genus of each observed colony by ZN staining combined with results obtained from the GenoType *Mycobacterium* CM®line probe assay (HAIN Lifescience GmbH, Nehren, Germany) was used to report the identity of isolates. The species and strain type of unknown isolates was determined using 16S rRNA gene sequencing and identification using homologous sequences in the GenBank® database available at: https://www.ncbi.nlm.nih.gov/genbank/. In addition, the relative

proportions of the isolates were reportedby study location (Ward) and sample type. Phylogenetic analysis was used to determine clustering of isolates.

3.9.3 Drug susceptibility testing

Data obtained from the results of the resistance ratio was used to report drug resistance forcultured isolates. Simple proportions, percentages, frequency and distribution of the resistance and susceptibility of each NTM species as well as species-drug combinations tested were reported for each sample type and overall for human sputum, camel milk and tissue samples. The RR, MIC_{50} and MIC_{90} values were calculated as the ratio of MIC of test over control MIC, drug concentrations at which 50% and 90% of isolates showed no growth, respectively.

3.9.4 Statistical analysis of datafor risk factors associated with zoonotic *Mycobacteria* infection

The information from the questionnaires was initially entered into a custom database; tables were then generated and exported into Stata®9 (StataCorp, College station, Texas, USA) where frequency tabulation (descriptive statistics) was done. Chi-square was used to test for unconditional associations between possible risk factors and SICTT result (Household / herd infection status). The main factors that were assessed using bivariate analysis include: Respondents age, education and location; History of tuberculosis in the household; camel - human interactions; nature of camel products consumption; wildlife interactions; animal health; herd management; and camel movement and migration.

The questionnaire data among households were translated into explanatory variables and combined with the tuberculin skin data in Stata®9 (StataCorp, College station, Texas, USA). The unit of statistical analysis was the individual camel. Binomial logistic regression was used to determine associations between explanatory variables and the outcome variable, laboratory culture result. Explanatory variables were initially explored through summarization, tabulation and chi-square or Fishers exact tests of association with the outcome variable. A liberal p-value (0.15) was used to determine variables to included in the model. Correlations between explanatory variables was used to determine highly correlated variables (>0.5), hence potential confounders and a decision to retain or remove was made based on a causal diagram (Figure 3.12). A backwards elimination logistic regression was conducted to arrive at the most parsimonious model, a threshold p-value of 0.05 was used to retain significant variables. In addition, model diagnostics included checking specific error using *linktest* command, Hosmer and Lemeshow goodness-of-fit test, and eliminating multicollinearity. A backwards elimination logistic regression was conducted to arrive at the most parsimonious model, and a threshold p-value of 0.05 was used to retain significant variables.

3.10 Ethical considerations

Research authorization was given by Kenyatta University Graduate school. Ethical approval was given by KEMRI, Scientific and Ethical review Unit (SERU) protocol number**:** KEMRI/SERU/CRDR/021/3428 as well as KEMRI, Animal Care and Use Committee (ACUC), Ref. No. KEMRI/ACUC/01.04.17. Consent of the study

participants was sought according to the KEMRI-SERU guidelines using consent form (Appendix X), while all manipulations involving the selected camels were done according to the KEMRI-ACUC guidelines. Research approval and permits were granted by the National Commission for Science Technology and Innovation, Permit No. NACOSTI/P/17/63884/15855.

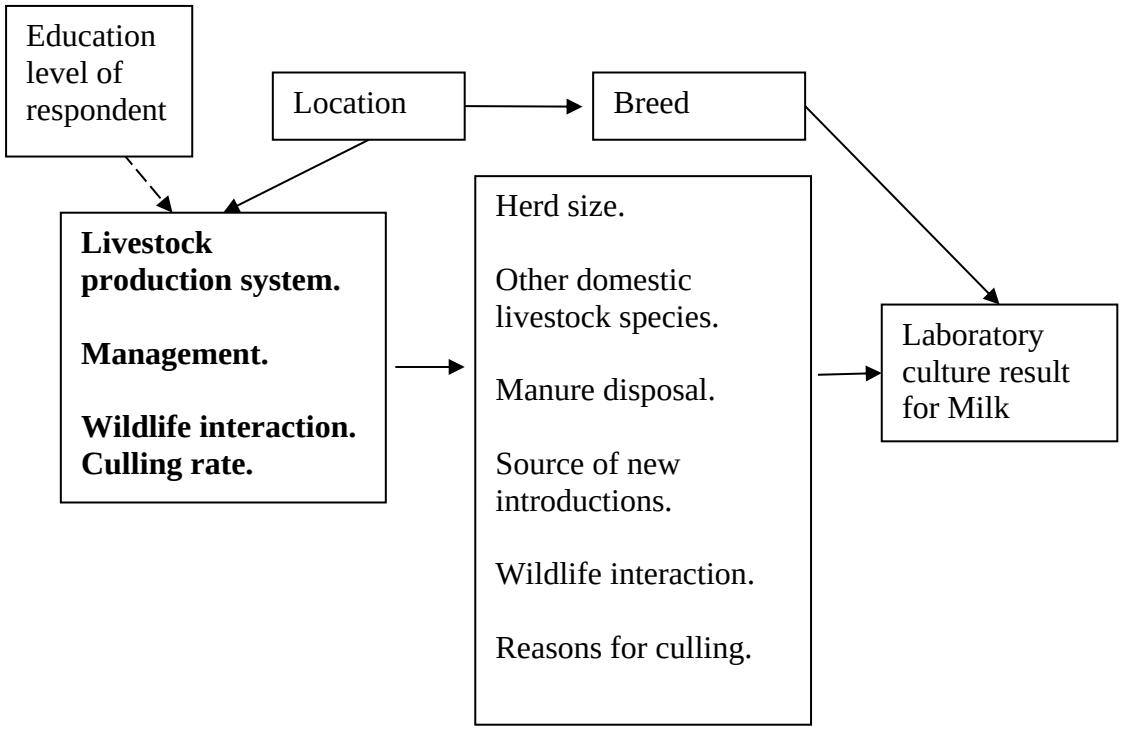

Figure 3.12: The plausible causal relationships between identified risk factors and the result of laboratory culture of camel milk sample

CHAPTER FOUR: RESULTS

4.1 *Mycobacteria* infection in lactating camels

Tuberculin skin testing to detect *Mycobacteria* infection of camels was conducted in the two regions of the study area between 20th October 2017 and 30th of December 2018. A total of 16 villages were visited and these were 13 (81.3%) in Waso and 3 (18.7%) in Wamba. A total number of 427 (69.77%) of the camels tested were Somali breed followed by Rendile breed (97; 15.85%), Turkana breed (84; 13.73%) and indeterminate breeds (4; 0.65%). Thirty seven (6.05%) out of 612 camels were positive for *M. bovis* infection while 241 (39.38%) reacted positive to *M. avium* PPD (Table 4.1). Twenty two (5.15%) out of 427 Somali breeds, 5/97 (5.15%) Rendile breeds and 10/84 (11.9%) Turkana breeds were positive for *M. bovis*. The positivity per region for *M. bovis* was 28/521 (5.37%) for Waso area and, 9/91 (9.89%) for Wamba area. A two sample test of equality (H_o: proportion 1-proportion 2=0) of proportions found positive for *M. bovis* in the two regions showed that these proportions were not statistically different (P=0.0954); however, the proportions seen among the regions varied from each other with a range of 4.52 %.

Table 4.1: Single intradermal comparative tuberculin test (SICTT) results in lactating dromedary camels in Samburu East, Kenya

Region	Village	Number of Herds	Tested camels (%)	*M. bovis* positive(%)	*M. avium* positive(%)
Waso east	Ntabasy	1	15 (2.45)	2 (5.4)	3 (1.25)
	Kirish	3	9 (1.47)	2 (5.4)	5 (2.07)
	Laresoro	6	37 (6.06)	3 (8.1)	3 (1.25)
	Leparo	2	18 (2.95)	1 (2.7)	8 (3.32)
	Lerata	5	18 (2.95)	4 (10.8)	4 (1.66)
	Loipusakini	7	67 (10.97)	0 (0.00)	43 (17.84)
	Losupulai	7	44 (7.2)	1 (2.7)	23 (9.54)
	Ndonyo lekita	1	7 (1.15)	2 (5.4)	2 (0.83)
	Ntilal	8	54 (8.84)	4 (10.8)	26 (10.79)
	Sirata	4	19 (3.11)	0 (0.00)	11 (4.56)
	Noltinga	3	26 (4.26)	0 (0.00)	5 (2.07)
	Lekipelen	1	11 (1.8)	0 (0.00)	0 (0.00)
	Serolopi	20	196 (32.03)	9(24.3)	76 (31.54)
Wamba west	Lpus lelui	7	20 (3.27)	3 (8.1)	11 (4.56)
Wamba east	Lpashie /Kiltamanya	7	56 (9.17)	6 (16.2)	12 (4.98)
	Lelui westgate/ Nasunyai	7	15 (2.45)	0 (0.00)	9 (3.73)
Total	16	83	612	37 (6.05)	241 (39.38)

PPD: Purified protein derivative of Mycobacteria, a - *Mycobacterium avium*, b - *Mycobaterium bovis*
PPD_a: Variance of measurement at *M. avium* PPD injection site, before injection and after 72 hours
PPD_b: Variance of measurement at *M. bovis*PPD injection site, before injection and after 72 hours
M. bovis positive: PPD_b-$PPD_a \geq 4$mm, 37/612 (6.05%); *M. avium* positive: PPD_a-PPD_b>1mm, 241/612 (39.38%)

One hundred and seventy (39.8%) out of 427 Somali breeds, 42/97 (43.3%) Rendile breeds and 29/84 (34.52%) Turkana breeds were positive for *M. avium*. The Somali breed comprised, 170/241 (70.54%), Rendile, 42/241 (17.43%) and Turkana, 29/241 (12.03%) of the *M. avium* positive cases. Two hundred and nine (40.12%) out of 521 in Waso and 32/91 (35.17%) in Wamba were positive for *M. avium*. A two sample test of equality (H_o: proportion 1-proportion 2=0) of the proportionsfound positive for *M. avium* in the two regions showed that the proportions were not statistically different (p=0.3716).Of those positive for *M. avium*, Waso had 209/241 (86.72%) and Wamba 32/241 (13.28%). Plotting of the differences between the variance of measurements of skin thickness at the PPDa and PPDb injection sites with the cut-off point set at >1mm shows the distribution of test results for NTM infection and the position of the cut-off point (Figure 4.1).

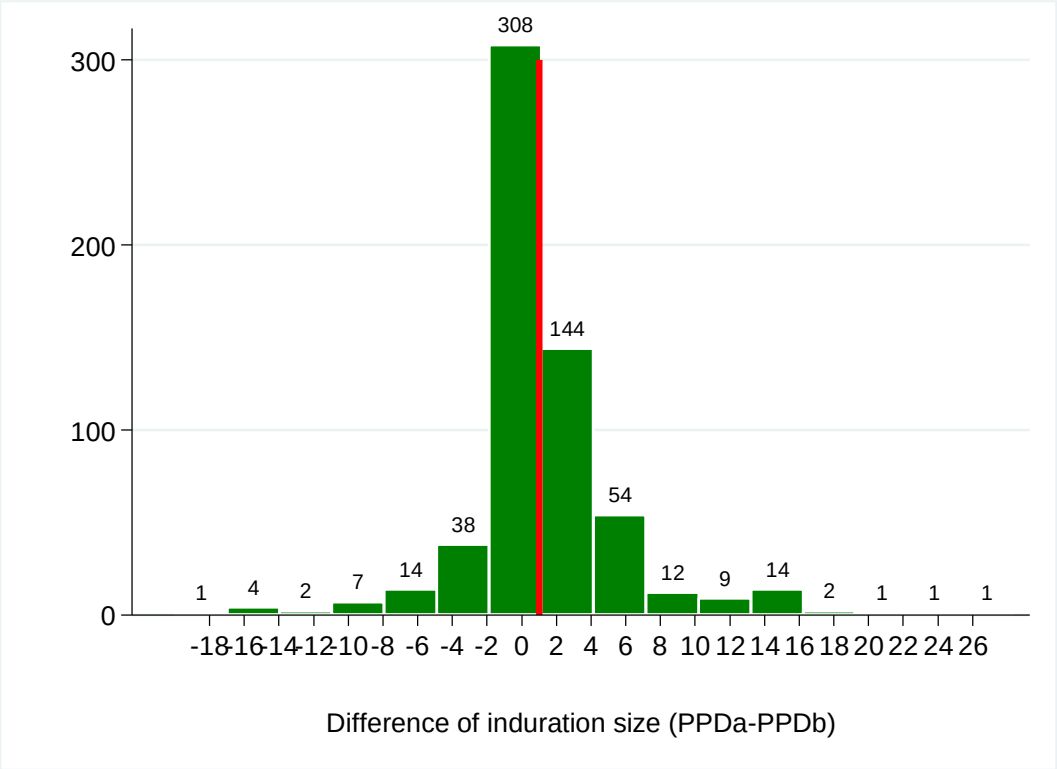

Figure 4.1: Test results for NTM infection in camels (n=612) screened using SICTT and showing the cut-off point (>1mm) (Red line). The difference between the variance of measurements of skin thickness at the *M. avium* purified protein derivative (PPDa) and *M. bovis* purified protein derivative (PPDb) injection sites ((Av72-Av0)-(Bov72-Bov0)) were plotted against frequency (Number). The variances were computed as difference between skin measurements before and after 72 hours at the PPDb and PPDa

Plotting of the differences between the variance of measurements of skin thickness at the PPDb and PPDa injection sites with the cut-off point set at >4mm shows the distribution of test results for *M. bovis* infection and the position of the cut-off point (Figure 4.2).

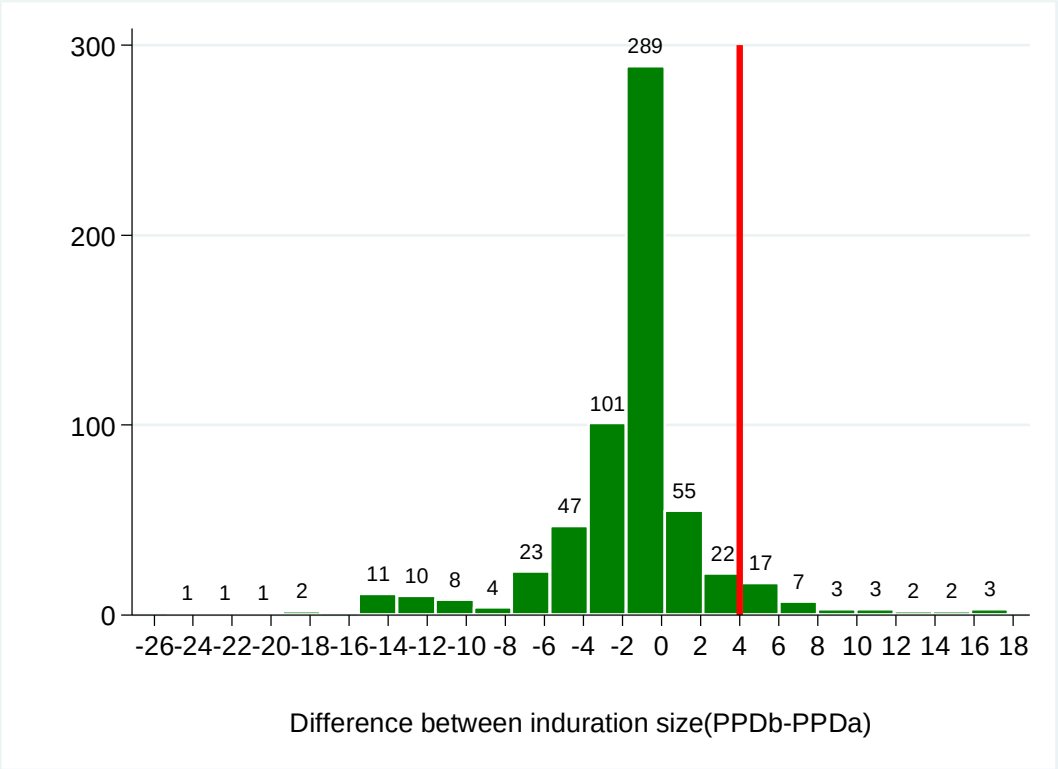

Figure 4.2: Test results for *M. bovis* infection in camels (n=612) screened using SICTT and showing the cut-off point (>4mm) (Red line). The difference between the variance of measurements of skin thickness at the *M. bovis* purified protein derivative (PPDb) and *M. avium* purified protein derivative (PPDa) injection sites ((Bov72-Bov0) - (Av72-Av0)) were plotted against frequency (Number). The variances were computed as difference between skin measurements before and after 72 hours at the PPDb and PPDa injection sites

Plotting using an additional third variable, 'difference between variance of induration size at PPDb and PPDa' against variance of induration size at PPDb and PPDa injections sites showed a clustered distribution pattern of induration sizes recorded from both sites (Figure 4.3).

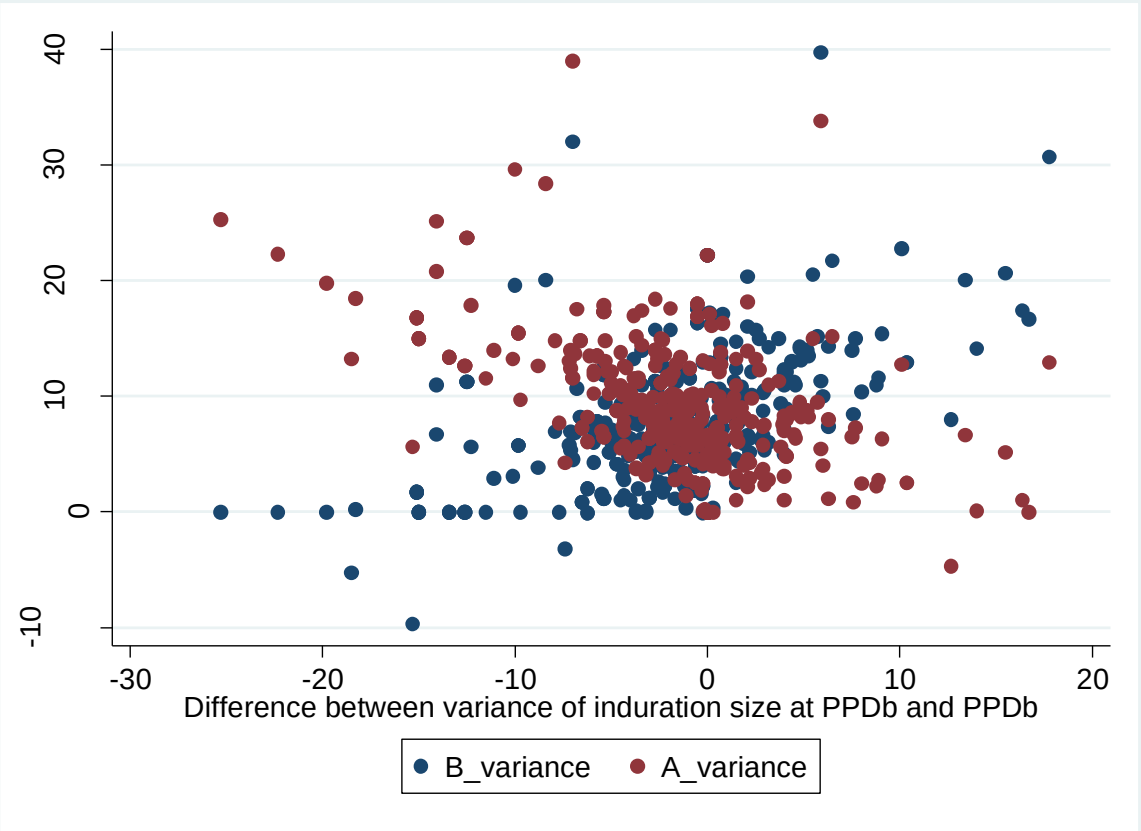

Figure 4.3: Comparison of variance of skin induration sizes at PPDa and PPDb injection sites. The variance of induration sizes at both sites show a clusterd distribution. A two-way scatter plot was used to plot the differences of variances of measurements at *M. bovis* purified protein derivative (PPDb) and *M. avium* purified protein derivative (PPDa) injection sites against variance of induration sizes at PPDa and overlaid on a similar plot against variance of induration sizes at PPDb sites. Skin thickness (mm) was measured before injection and at 72 hours post injection. The variance was computed as difference between skin measurements before and after 72 hours at both the PPDb (Bov72-Bov0) and PPDa (Av72-Av0)

A strong positive linear relationship with a correlation coefficient of (*r)*=0.7676 was determined from a correlation matrix of variance of measurements at the bovine PPD and avian PPD injection sites after 72 hours (Appendix XIII). The mean PPDb induration size after 72 hours was 12.86mm (SD=4.07) while that for PPDa was slightly higher being 13.94mm (SD=4.08) (Table 4.2).

Table 4.2: Skin size induration after 72 hours at the *M. bovis* purified protein derivative (PPDb) and *M. avium* PPD (PPDa) injection site by region

	PPDb				PPDa			
Region	**Mean (mm)**	**Median**	**Standard deviation**	**Range**	**Mean (mm)**	**Median**	**Standard deviation**	**Range**
Waso	12.8	12.55	(3.86)	0 - 22	13.90	13.95	(3.85)	0 – 23.8
Wamba	13.26	12.4	(5.30)	4.5 - 27	14.18	12.9	(5.46)	4.5 29.3
Overall	12.86	12.5	4.07	0 - 27	13.94	13.7	4.08	0 – 29.3

However, a test of difference of means with the null hypothesis that the difference between the means equals to zero, showed the difference between the means at the injection sites and by region to be statistically significant ($p<0.05$). Altering the choice of diagnostic cut-off from standard interpretation (>4mm) to severe interpretation (>2mm) increased the *M. bovis* positivity to 63/612 (10.29%) and reduced those classified as inconclusive (PPD_b-PPD_a= between 1 to 2mm) to 17/612 (2.78%). Correction for false positives (20%) gave positivity of 4.74% and 8.17% at standard and severe interpretation respectively. A t-test of the two corrected proportions at standard and severe interpretation indicated that their difference was significant (P=0.0146).

4.2 Pathologic manifestation of NTM infection in slaughtered camels

4.2.1 Prevalence of gross pathology suspect NTM lesions at post mortem meat inspection

A total of 1600 camel were screened at post mortem, and of these 269 (16.8%) were slaughtered at Athi river and 1331 (83.2%) at Isiolo county slaughter houses, respectively. Following post mortem inspection in this study, 132/1600,(8.25%) (Binomial 95% CI: 6.95% – 9.71%) suspect TB lesions were found in camels originating from the study area slaughtered at both slaughter houses. Of these, 111 (84.09%) were males and 21(15.91%) were females. The average age was 6.4 (+/-0.97) with a range of 4 to 9 years. Ninety four (71.76%) of the camels with lesions were of the Somali breeds followed by Turkana breed (16.03%), Somali-Rendile crosses (11.45%) and Rendile (0.76%). The highest proportion of the suspect lesions was obtained from mediastinal and bronchial lymph nodes (59.5%), followed by retropharyngeal lymph nodes (12.2%) and medial lung lobes (10.7%). Annual prevalence of suspect TB lesions based on meat inspection of 168 out of 8395 (2%) and 99 out of 3285 (3%) were computed from the records of the respective County Veterinary Departments of Machakos and Isiolo respectively.

Out of those total number of suspect lesions collected at post mortem, culture (LJ and MGIT) results found 27/1600, (1.69%) (Binomial 95% CI: 1.11%-2.45%) to have AFB. The difference between the proportion of suspect lesions identified and that of culture positives from these was significant ($p< 0.001$). The proportion of suspect tuberculous lesions observed from this study was also significantly different ($p< 0.001$) from annual

prevalence of suspect TB lesions calculated from the respective County Veterinary data. Most of the lesions identified were either nodular, caseous or purulent masses involving part or the whole lung lobes (Figure 4.4).

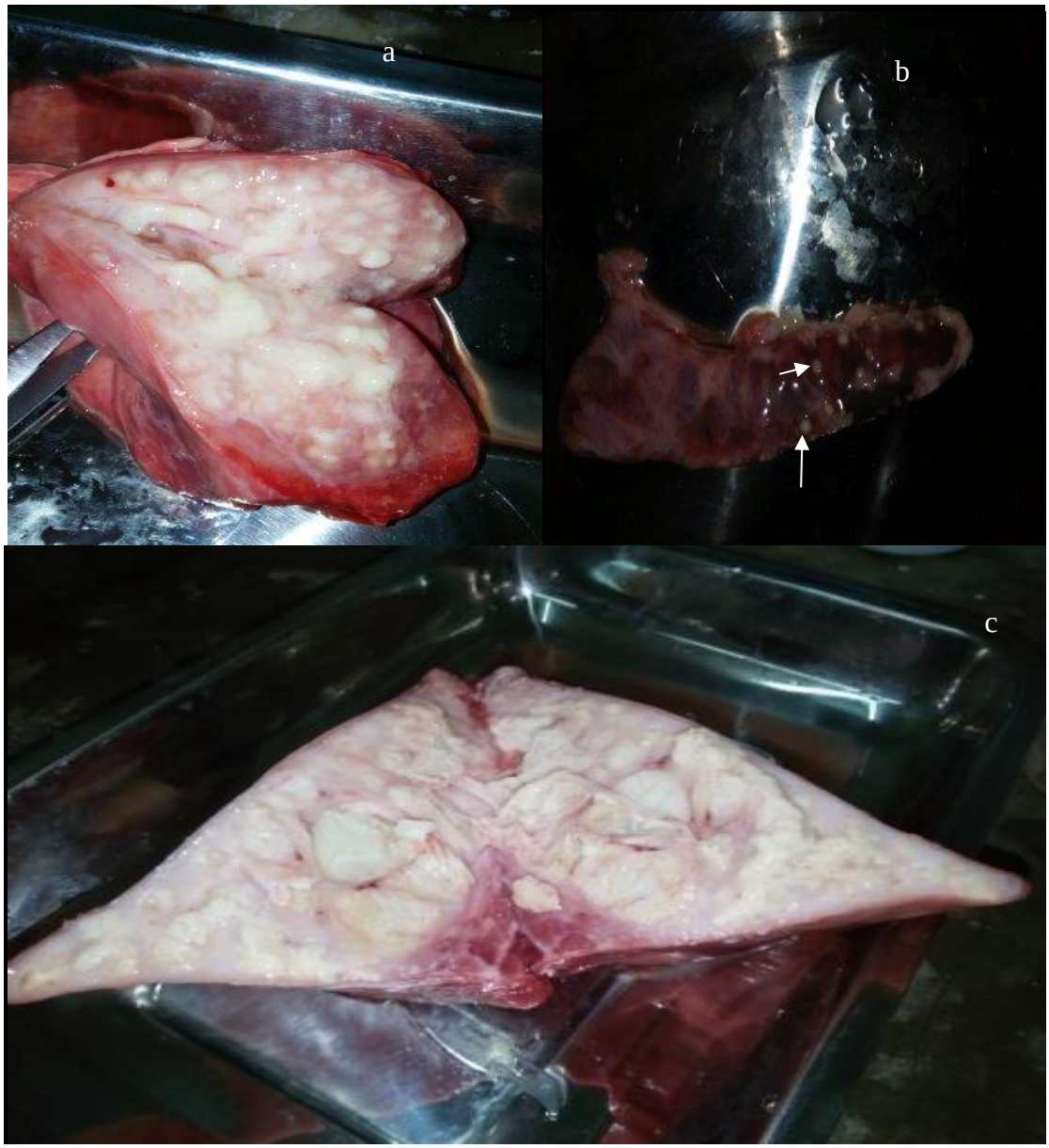

Figure 4.4: The gross pathology of selected suspect NTM lesions at post mortem. An incised purulent mass involving a whole lung lobe (a), nodular lesions (white arrows) in a mediastinal lymphnode (b), and an incised lung lobe with caseous masses (c)

There was a higher proportion of culture positive compared to culture negative suspect TB lesions from mediastinal and bronchial lymph nodes (Figure 4.5).

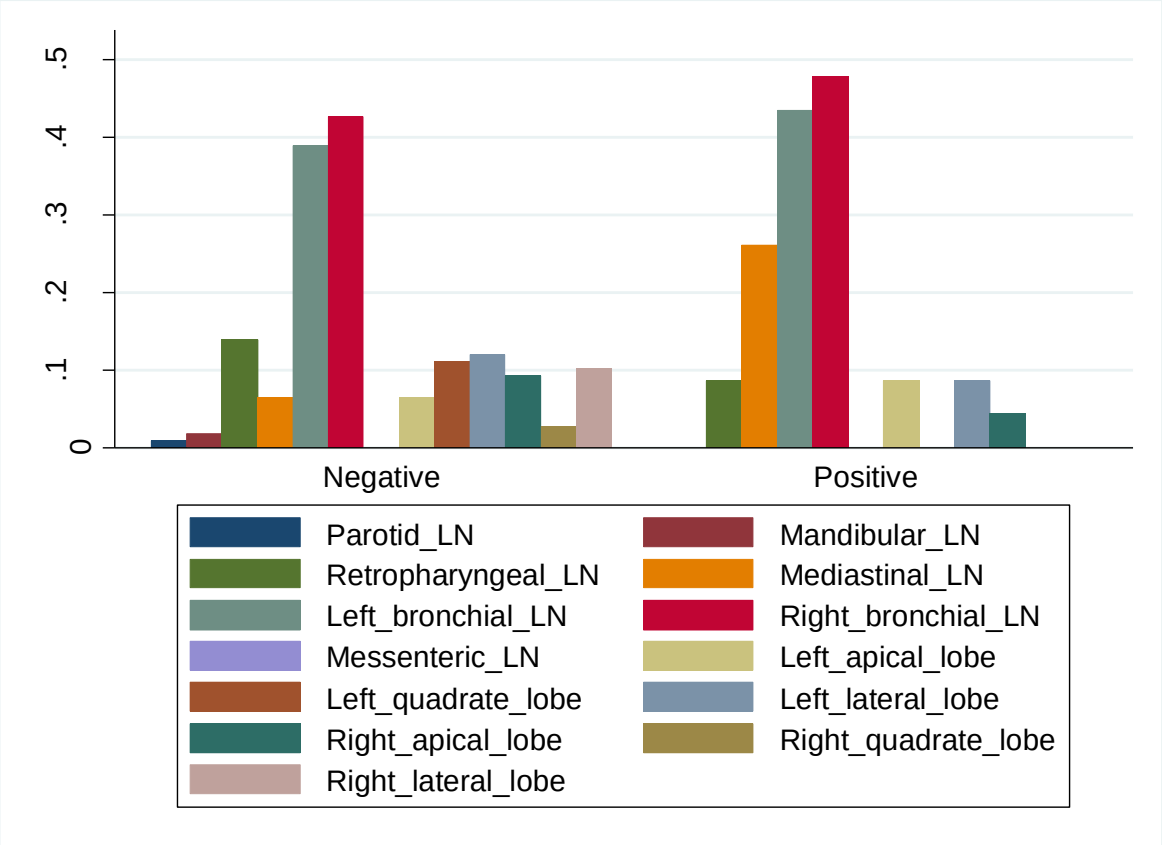

Figure 4.5: Distribution of proportion of sample types (n=131) among culture negative and positive samples using a two-way bar graph. There was a higher proportion of culture positive lesions among the mediastinal and bronchial (Orange, grey and red bars) lymph nodes

4.2.2 Histopathology of suspect NTM lesions

Histopatholoy of formalin fixed paraffin wax- based sections showed necrosis of the center of the lesion as well as surrounding lung tissue, fibrous connective tissue proliferation surrounded by infiltration of large numbers of lymphocytes (Figure 4.6).

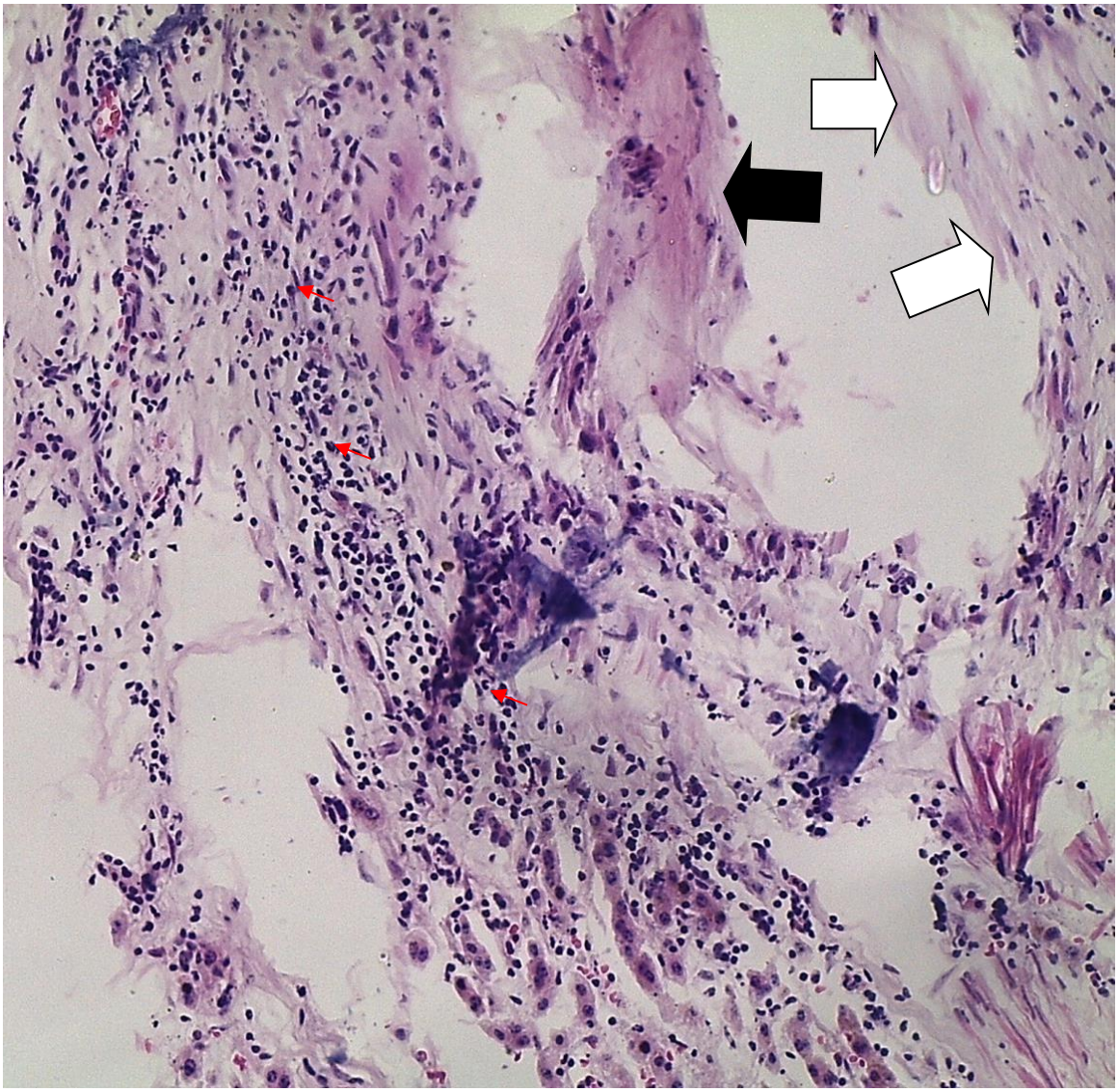

Figure 4.6: A section of the edge of a granulomatous lesion from the lung. The slide was prepared from formalin fixed paraffin- based sections and shows central necrosis (Big Black arrow), fibrous connective tissue proliferation (Big white arrows) and lymphocyte infiltarion (Red arrows) forming a cuff around the necrosed area. Magnification 40x Haematoxylin and Eosin (H&E)

At the margins of the central necrotic areas, fibrous connective tissue proliferation was evident with clusters of elongated epithelioid cells (Figure 4.7).

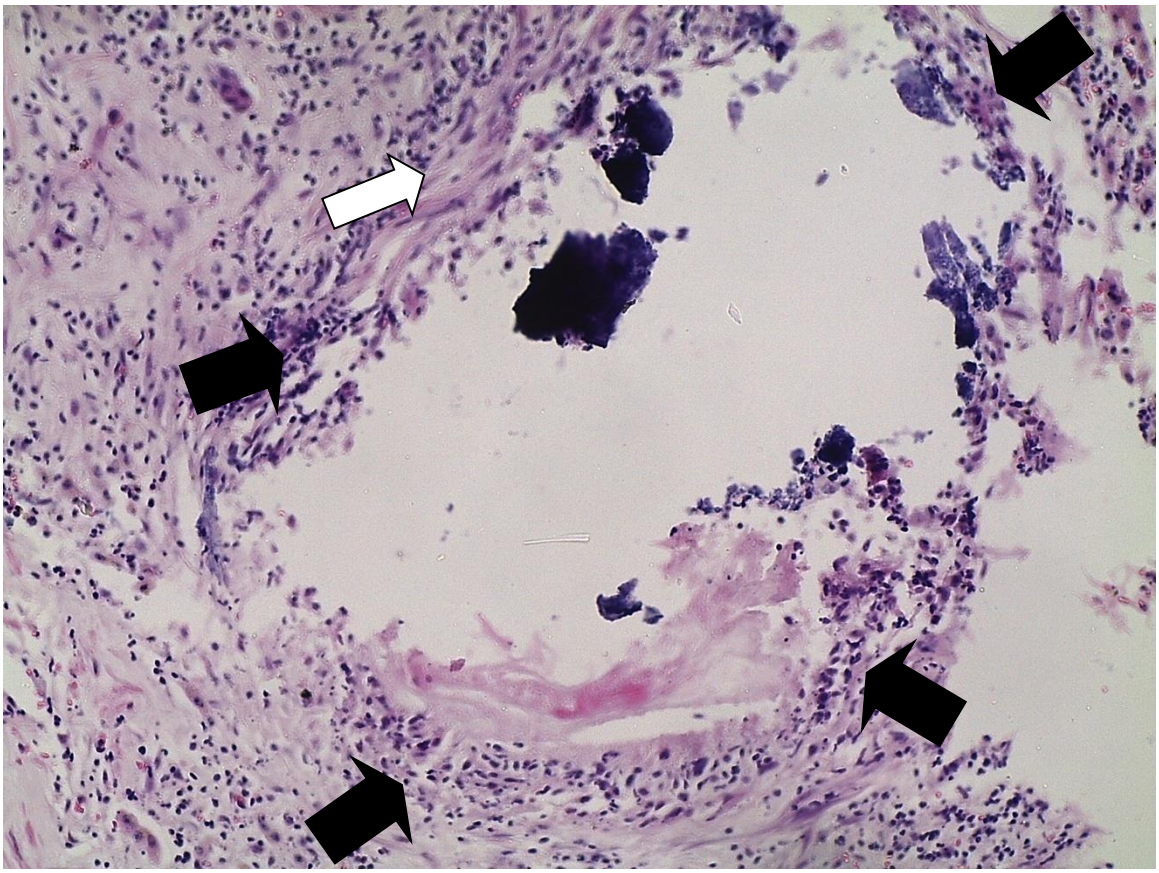

Figure 4.7: A section of an encapsulated granuloma from the lung. The slide was prepared from formalin fixed paraffin- based sections and shows fibrous connective tissue proliferation made of elongated epitheliod cells (Big white arrow) and mononuclear cell infiltration around the necrotic area (Big black arrows). Magnification 40x H&E

4.3 Molecular identification of zoonotic nontuberculous *Mycobacteria* isolates from tissue lesions, camel milk, and human sputum

4.3.1 Molecular speciation using GenoType® *Myobacterium* CM line probe assay

After LJ and MGIT culture, 131 lesionswere successfully cultured, out of which 27 (20.6%) of the samples were positive for AFB species. The culture positivity was higher with MGIT (n=25, 19%) than LJ (n=9, 6.9%). Species identified included *M. fortuitum* (17) isolated from retropharyngeal lymph nodes, mediastinal and bronchial lymph nodes, Left lateral lymph nodes and medial lung lobes; *M. scrofulaceum* (3) from mandibular lymph nodes, mediastinal and bronchial lymph nodes; *M. szulgai* (2) from mediastinal and bronchial lymph nodes; *M. marinum* (1) from retropharyngeal lymph nodes; *M. gordonae* (1) from mediastinal and bronchial lymph nodes and; *M. intracellulare* (1) from mediastinal and bronchial lymph nodes. Two unknown Mycobacterial species were isolated from mediastinal and bronchial lymph nodes. The highest proportion of the culture positive lesions (LJ; 6/78 and MGIT; 19/78) were identified from the bronchial and mediastinal lymph nodes (Table 4.3).

Table 4.3: Species of *Mycobacteria* isolated from suspect tissue lesions collected at post mortem inspection. Genotype *Mycobacterium*®CM assay was used for speciation

Source	Number of lesions (%)	Culture positive Number (%)			Molecular speciation using HAIN						
		LJ	MGIT	Total	*M. fortuitium*	*M. szulgai*	*M. scrofulaceum*	*M. marinum*	*M. gordonae*	*M. intracellulare*	Unknown AFB *spp.*
Mandibular Lymph nodes	1(0.76)	1	0	1	-	-	1	-	-	-	-
Parotid Lymph nodes	1(0.76)	0	0	0	-	-	-	-	-	-	
Retropharyngeal Lymph nodes	16(12.21)	0	2	2	1	-	-	1	-	-	-
Mediastinal and bronchial Lymph nodes	78(59.54)	6*	19*	19	11	2	2	-	1	1	2
Mesenteric Lymph nodes	0	0	0	0	-	-	-	-	-	-	-
Left lateral lymph nodes	9(6.87)	0	2	2	2	-	-	-	-	-	-
Medial lung lobes	14(10.69)	1*	2*	2	2	-	-	-	-	-	-
Right lung lobes	2(1.53)	0	0	0	-	-	-	-	-	-	-
Caudate lung lobes	1(0.76)	1	0	1	1	-	-	-	-	-	-
Quadrate lung lobes	9(6.87)	0	0	0	-	-	-	-	-	-	-
Total	131	9 (6.9)	25(19)	27(20.6)	17	2	3	1	1	1	2

*Figure include samples that were positive on both LJ and MGIT, total culture positive were 27/131 (20.6%)

The species isolated from the Somali breed included *M. fortuitum* (12), *M. gordonae* (1), *M. marinum* (1), *M. scrofulaceum* (1), *M. szulgai* (2), and two unknown mycobacteria *spp*. In the Turkana breed, *M. fortuitum* (3), *M. scrofulaceum* (1), and *M. intracellulare* (1) were isolated while those identified in the cross breeds were *M. fortuitum* (2) and *M. scrofulaceum* (1) (Table 4.4). The distribution of *Mycobacterium* species among the different camel breeds were not statistically different (P= 0.695).

Table 4.4: Distribution of identified *Mycobacteria* species among camel breeds

Breed	***M. fortuitum***	***M. gordonae***	***M. marinum***	***M. scrofulaceum***	***M. spp***	***M. szulgai***	***M. intracellulare***	**Total**
Somali	12	1	1	1	2	2	0	19
Turkana	3	0	0	1	0	0	1	5
Crosses	2	0	0	1	0	0	0	3
Rendile	-	-	-	-	-	-	-	-
Total	17	1	1	3	2	2	1	27

Amongst the sexes, *M. fortuitum* (13), *M. gordonae* (1), *M. marinum* (1), *M. scrofulaceum* (3), *M. szulgai* (2), *M. intracellulare* (1) and *M. spp.* (2) were isolated from males, while only *M. fortuitum* (4) were from females (Table 4.5). The distribution of *Mycobacterium* species among sex was not statistically different (P=0.838).

Table 4.5: Distribution of identified *Mycobacteria* species among sex of camels

Sex	*M. fortuitum*	*M. gordonae*	*M. marinum*	*M. scrofulaceum*	*M. Spp*	*M. szulgai*	*M. intracellulare*	Total
Male	13	1	1	3	2	2	1	23
female	4	0	0	0	0	0	0	4
Total	17	1	1	3	2	2	1	27

Mycobacterium fortuitum (10), *M. gordonae* (1), *M. marinum* (1), *M. scrofulaceum* (2), *M. szulgai* (1), *M. intracellulare* (1) and one unknown *M. spp.* were isolated from camels less than or equal to 6 years old, while *M. fortuitum* (7), *M. scrofulaceum* (1), *M. szulgai* (1) and one *M. spp.* were from camels greater than or equal to 7 years old (Table 4.6). There difference between the age categories was not statistically significant (P=0.901).

Table 4.6: Distribution of identified species of *Mycobacteria* by age group of camels

Age	*M. fortuitum*	*M. Gordonae*	*M. Marinum*	*M. Scrofulaceum*	*M. Spp*	*M. Szulgai*	*M. Intracellulare*	Total
≤6 years	10	1	1	2	1	1	1	17
≥7 years	7	0	0	1	1	1	0	10
Total	17	1	1	3	2	2	1	27

Following screening of lactating camels using SICTT, 238 composite milk samples were obtained from skin test reactive camels as well as those classified as inconclusive for confirmatory mycobacteriology, culture and speciation. One hundred and sixty seven (167) (69.73%) of the camels sampled were of the Somali breed followed by the Rendile breed (38; 15.88%) and the Turkana breed (33; 13.75%). After Lowenstein-Jensen (LJ) and mycobateria growth indicator tube (MGIT) culture, all of the culture growth confirmed as acid fast bacilli (AFB) after eight weeks, were 57 (23.95%). Waso Eastregion with 37/57 (64.91%) accounted for the largest proportion of those that were culture positive. At least a culture positive sample was obtained from 10/16 villages visited; the highest culture positives were recorded for Sereolipi village where 14/57 (24.56%) culture positive milk samples showed presence of AFB. There were three samples positive on both LJ and MGIT, one each from Kiltamanya, lelui/westgate and Sirata villages. The species identified from culture positive milk samples using GenoType® *Mycobacterium* CM assay were *M. fortuitum* (1) and *M. szulgai* (20) (Table4.7). The distribution of Mycobacterium species among regions was not statistically different (P = 0.108).

Table 4.7: Acid fast bacilli (AFB) species identified in camel milk samples by region

Region (ward)	Village	Number of samples (%)	Culture Positive, LJ/MGIT (%)	Identified species													
				M. fortuitum	*M. szulgai*	*M.elephantis*	*M. monacense*	*M.litorale*	*M.lehmannii*	*M.arcueilense*	*M. duvalii*	*M. lentiflavum*	*M. brasiliensis*	*M. flavescens*	*Nocardia spp.*	Other AFB *spp*	Uncharacterized AFB
Sere olipi	Sere olipi	45 (18.9)	14(25.45)*	-	1*	2	1	1	3	-	2	1	1	-	-	2	-
Wamba East	Kiltamanya/ Lpashie	15 (6.3)	4 (7.2)*	-	4*	-	-	-	-	-	-	-	-	-	-	-	-
Wamba West	Lelui westgate/ Lpus lelui	31 (13.0)	10(18.18)*	-	9*	1	-	-	-	-	-	-	-	-	-	-	-
	Lelui westgate/ Nasunyai	6 (2.52)	6(7.27)	2	-	-	-	-	-	1	-	-	-	-	-	2	1
Waso East	Kirish	4 (1.68)	0 (0.00)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Laresoro	6 (2.52)	2 (3.63)	-	-	-	1	-	-	-	1	-	-	-	-	-	-
	Lepuro	5 (2.1)	0 (0.00)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Lerata	24 (10.08)	3 (5.45)	-	-	-	-	1	-	-	-	-	-	-	-	-	2
	Loipusakini	20 (8.4)	7 (12.72)	-	-	-	1	1	-	-	-	-	-	1	1	3	
	Ndonyo lekita	5 (2.1)	0 (0.00)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ntilal	26 (10.92)	1 (1.81)	-	-	-	1	-	-	-	-	-	-	-	-	-	-
	Sirata	40 (16.8)	9 (16.36)*	1*	5*	-	1	1	-	-	-	-	-	-	-	-	1
	lekipelen	11 (4.62)	1 (1.81)	-	1*	-	-	-	-	-	-	-	-	-	-	-	-
Total	n=12	n=238	n=57 (23.95)	3	20*	3	5	4	3	1	3	1	1	1	1	7	4

Identification was done using GenoType *Mycobacterium* CM assay and 16S rRNA partial sequencing. The asterisk (*) denotes those samples identified using the GenoType® *Mycobacterium* CM assay

Symptomatic humans were encountered in 8/16 villages visited in the course of the study, the majority of whom were from Lerata and Sereolipi in the Waso region. Out of a total of 48 symptomatic humans from whom sputum samples were obtained, 7 (14.58%) were found positive on culture showing presence of AFB after 8 weeks. From the results of speciation using GenoType® *Mycobacterium* CM line probe assay, it was shown that most of the isolates cultured were NTM (Appendix XII). The species identified from positive cultures of human sputum include *M. fortuitum* (1), *M. litorale* (1) and *M. szulgai* (2) (Table 4.8). GeneXpert MTB/RIF® was conducted for all AFB obtained from culture growth to detect *M. tuberculosis* and rifampicin resistance where all were found negative. The results for the Capilia–Neo TB®rapid immunochromatographic test used to distinguish between members MTB complex and NTM indicated that all the AFB isolates were NTM.

Table 4.8: Acid fast bacilli (AFB) species identified in human sputum samples by region and village

Region (Ward)	Village	Number Sampled (%)	Culture Positive (%)	Identified species					
				M. fortuitum	*M. szulgai*	*M. litorale*	*Paenibacillus Spp.*	*Kocuria marina*	Unknown species
Wamba east	Kiltamanya	4 (8.33)	1	-	1	-	-	-	-
Wamba west	Lelui/ westgate	5 (10.42)	1	1	-	-	-	-	-
Waso east	Lepuro	1 (2.08)	0	-	-	-	-	-	-
	Lerata	12 (25)	0	-	-	-	-	-	-
	Donyo lekita	2 (4.17)	0	-	-	-	-	-	-
	Ntilal	8 (16.67)	0	-	-	-	-	-	-
	Sirata	1 (2.08)	1	-	-	-	-	1	-
Sereolipi	Sereolipi	15 (31.25)	4	-	1	1	1	-	1
	n=8	n=48	7 (14.58)	1*	2*	1	1	1	1

Molecular identification was done using GenoType® *Mycobacterium* CM assay and 16S rRNA sequencing.The asterisk (*) denotes those samples identified using GenoType® *Mycobacterium* CM assay.

4.3.2 *Mycobacteria* strains

Out of the 42 AFB that remained unknown after GenoType® *Mycobacterium* CM assay, a total of 36 were successfully sequenced using 16S rRNA partial sequencing. A total number of 10 additional NTM species were identified from human sputum, camel milk and tissue lesion samples from slaughtered camels at PM. From camel milk the following nine additional species were identified: *M. monacense* (5), *M. litorale* (4), *M. lehmannii* (3), *M. elephantis* (3), *M. duvalii* (3), *M. arcueilense* (1), *M.lentiflavum* (1), *M. brasiliensis* (1) and *M. flavescens* (1). Two additional isolates of *M. fortuitum* were identified from camel milk. The other AFB species identified from milk samples include *Nocardia spp*. (1), *Enterococcus spp*. (1), *Roseomonas spp*. (1), *Paenibacillus spp*. (3),*Cellulosimicrobium* (1) and *Bosea spp*. (1).*Mycobacterium litorale* (1), *Kocuria marina* (1), *Paenibacillus spp*. (1) were identified from human sputum samples (Table 4.9).

Table 4.9: *Mycobacteria* species identified using BLAST search tool in GenBank database by region and source

Source	Region	Village	Species homologue in GenBank, Accession number (Tip name in phylogenetic tree)	No.
Milk	Waso east	Sereolipi	*M. monacense,* NR041723.1 (386_8FPL)	1
			M. duvalii NR026073.1 (385_8FPL, 383_8FPL)	2
			M. lehmanii NR159196.1 (418_8FPL)	1
			M. elephantis, NR025296.1 (398_8FPL)	1
			M.brasiliensis EU165538.1 (370_8FPL)	1
			Paenibacilus spp. CCOS10, MK005262.1(401_8FPL)	1
			M. litorale, NR117568.1 (392_8FPL)	1
			M. lehmannii, NR159196.1 (371_8FPL)	1
			Roseomonas fluminis, NR159916.1 (390_8FPL)	1
			M. lentiflavum, NR041898.1 (404_8FPL)	1
			M. elephantis, NR025296.1 (412_8FPL)	1
			M. lehmannii NR159196.1 (377_8FPL)	1
		Loipusakini	*M. monacense*, NR041723.1 (279_8FL)	1
			M. litorale, NR117568.1 (277_8FPL)	1
			M. flavescens AF480579.1 (291_8FPL)	1
			Nocardia brevicatena, NR117404.1 (278_8FPL)	1
			Enterococcus faecium NR113904.1 (282_8FPL)	1
			Paenibacillus sp. NR042947.1 (290_8FPL)	1
			Paenibacillus sp. NR025739.1 (284_8FPL)	1
		Laresoro	*M. monacense* NR041723.1 (295_8FPL)	1
			M. duvalii, NR026073.1 (297_8FPL)	1
		Sirata	*M. monacense*, NR041723.1 (243_8FPL)	1
			M. litorale, NR117568.1 (261_8FPL)	1
		Ntilal	*M. monacense*, NR041723.1 (190_8FPL)	1
		Lerata	*M. litorale* NR117568.1 (157_8FPL)	1
	Wamba	Lpus Lelui	*M. elephantis* NR025296.1, MH581231.1 (332_8FPL)	1
		Nasunyai	*M. fortuitum* NR104775.1 (448_8FPL, 434_8FPL)	2
			M. arcueilense NR151954.1 (435_8FPL)	1
			Bosea sp. NR114668.1(429_8FPL)	1
			Cellulosimicrobium funkei NR042937.1 (446_8FPL)	1
Lesions	Abattoir	Athi River	*M. kumamotonense*, KX954384.1 (041_8FPL)	1
Human sputum	Waso East/ Sereolipi	Sereolipi	*Paenibacillus spp.* MK055262 (360_8FPL)	1
		Sereolipi	*M. litorale* NR117568.1 (353_8FPL)	1
		Sirata	*Kocuria marina*, NR025723.1 (246_8FPL)	1
Total				36

A *Mycobacteria* strain similar to *M. kumamotonense* (91.45% similarity) was identified from tissue lesions. Six AFB isolates from milk (4), tissues (1) and sputum (1) remain unknown. The level of similarity of the isolates in this study with homologues in the GenBank® database (https://www.ncbi.nlm.nih.gov/genbank/) varied from 82.34% to 98.8%. Of the NTMs identified using 16S rRNA gene sequencing, 18/26 (69.23%) showed >97% similarity in identity to homologous sequences in GenBank database (Table 4.10). The expected value (E-value) in GenBank BLAST for all sequences obtained in this study were 0.0, meaning that the database matches were not very likely by chance as the E-value was close to zero.

Table 4.10: Percent identity groups of species of *Mycobacteria* identified using the basic local alignment serach tool (BLAST) in GenBank® database

Percent level of identity	Species identification, Accession number (Tip name on phylogenetic tree)	No.
>98% identity{	*M. lehmannii*, NR159196.1(377_8FPL)	1
	M. lehmannii (371_8FPL)	1
	M. lentiflavum, NR041898.1 (404_8FPL)	1
	Enterococcus faecium, NR113904.1 (282_FPL)	1
97% identity{	*M. duvalii*, NR026073.1(297_8FPL, 383_8FPL, 385_8FPL)	3
	M. elephantis, AJ536100.1, NR025296.1, NR025296.1(398_8FPL,332_8FPL)	2
	M. arcueilense (435_8FPL)	1
	M. fortuitum, NR104775.1(434c_8FPL)	1
	M. fortuitum,NR104775.1 (448_8FPL)	1
	M. litorale, NR117568.1 (392_8FPL, 353_8FPL, 261_8FPL, 157_8FPL)	4
	M. monacense, NR041723.1 (295_8FPL, 279_8FPL, 190_8FPL)	3
	Paenibacillus sp., MK005262.1 (401_8FPL)	1
	Kocuria marina, NR025723.1 (246_8FPL)	1
	Nocardia sp., NR117404.1 (278_8FPL)	1
	Bosea sp., NR114668.1 (429_8FPL)	1
	Celluosimicrobium sp., NR042937.1 (446_8FPL)	1
96% identity{	*M. litorale* (277_8FPL)	1
	M. lehmannii NR159196.1 (418_8FPL)	1
	M. flavescens, AF480579.1 (291_8FPL)	1
	Paenibacillus sp., MK005262.1 (360_8FPL)	1
95% identity**{**	*M. monacense*, NR041723.1 (386_8FPL, 243_8FPL)	2
	M. elephantis, NR025296.1 (412_8FPL)	1
<95% identity{	*M. brasiliensis* EU165538.1 (370_8FPL)	1
	M. kumamotonense, KX954384.1 (041_8FPL)	1
	Paenibacillus sp., NR042947.1 (290_8FPL), NR025739.1 (284_8FPL)	2
	Roseomonas fluminis, NR159916.1 (390_8FPL)	1
Total		36

Note: The percent identity was obtained from the computer generated results output of the basic alignment search tool (BLAST) which was used to search the GenBank® 16S rRNA reference sequences database for homologous sequences

Based on the most common recent ancestry, a reliability of>70% bootstrap value threshold and bootstrap replicates set at 500 (Baum, 2008; pattengale, 2010; Hall, 2013), the percentage bootstrap values determined high support for grouping of isolates in this study into five major groups: Group I, 98% bootstrap support (*M. lehmannii* and *M. flavescens*); Group II, 97% bootstrap support (*M. elephantis*); Group III, 83% bootstrap support (*M. elephantis*); Group IV, 98% bootstrap support (*M. duvalii*); and Group V, 77% bootstrap support (*M. arcuilense* and *M. fortuitum*). The other monophyletic taxa determined but not supported by a >70% bootstrap value include; *M. brasiliensis* (39%), *M. kumamotonense* (24%), *M. litorale* (46%), and *M. monacense* (39%). *Mycobacterium lentiflavum* was the only taxa that clustered with the reference strains but with low percent bootstrap support. The reference sequences obtained from GenBank® used during the estimation of the phylogenetic tree cladogram included NR 118584.1 *M. szulgai* strain ATCC 35799, NR 114677.1 *M. tuberculosis* variant *bovis* strain CIP 105234, NR 102810.2 *M. tuberculosis* H37Rv, and NR 114893.1 *M. fortuitum subsp. Fortuitum* DSM 46621 ATCC 6841 (Figure 4.8).

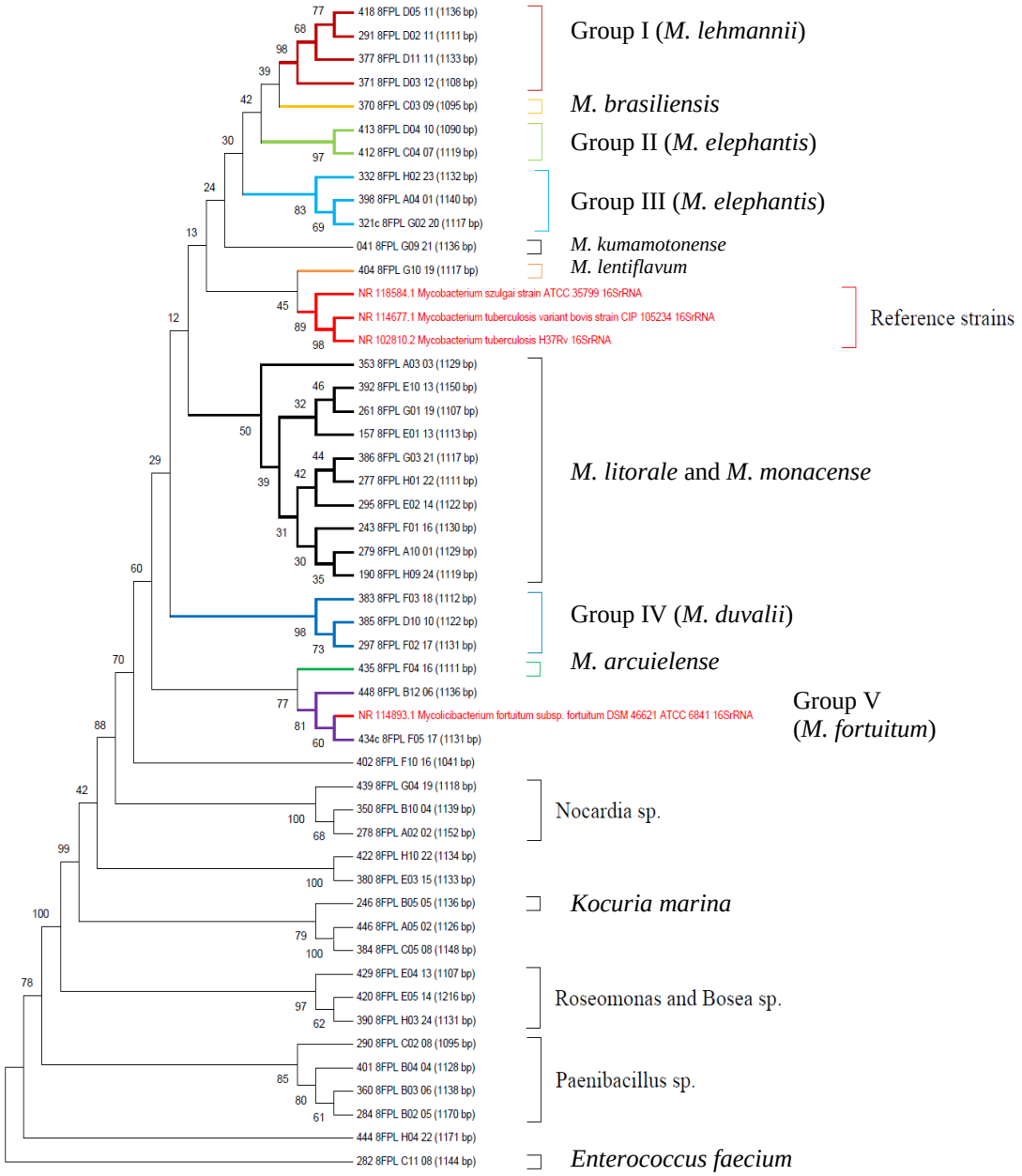

Figure 4.8: Evolutionary history dendrogram showing relationship between NTM isolates with reference sequences from GenBank database. This evolutionary history cladogram was inferred using Maximum Likelihood method based on Kimura-2 parameter model, with discrete gamma distribution (+G = 0.9364) and rate variation (+I, 0.00% sites). The tree with highest Log Likelihood (-6577.72) is shown. The percentage of the trees in which associated taxa clustered together is shown next to each branch. The bootstrap concensus tree inferred from 500 replicates is shown. The reference strains are highlighted in red. The tree was rooted using *Enterococcus faecium*. Taxa clustered in groups supported by >70 bootstrap values and common ancestry are shown using color coding and group number (I-V), taxa name is annotated otherwise. Other species identified incude *Nocrdia sp.*, *Bosea sp.*, *Cellulosimicrobium funkei*, *Paenibacillus sp.*, *Roseomonas fluminis* and *Kocuria marina*. Analysis was performed in MEGA X software

4.4 Drug susceptibility of NTM isolates

After testing for drug susceptibility, all (15/15) were found to be resistant to isoniazid, while 14 (93%) and 11 (73%) were resistant to rifampicin and ethambutol respectively. Of these, 14/15 (93%) were resistant to both isoniazid and rifampicin while, 11/15 (73.3%) were resistant to all three first-line TB drugs and none of the isolates were mono-resistant to rifampicin (Table 4.11). Among the three first-line TB drugs, no isolate was susceptible to isoniazid (0/15), while 1 (6.7%) and 4 (26.7%) isolates were susceptible to rifampicin and ethambutol respectively.

Table 4.11: The number and percentage of *in vitro* susceptibility to first-line TB drugs of non-tuberculous *Mycobacteria* isolates from TB suspect camel tissue lesions

Species isolate	Total Number of isolates	Total Number tested	Resistance No. (%)			Susceptible No. (%)			Resistance categories (WHO, 2017)	
			INH	RIF	EMB	INH	RIF	EMB	RR-TB	MDR-TB No. (%)
M. fortuitum	17	13	13	12	11	-	1	2	-	12
M. scrofulaceum	3	0	-	-	-	-	-	-	-	-
M. szulgai	2	0	-	-	-	-	-	-	-	-
M. spp	1	0	-	-	-	-	-	-	-	-
M. kumamotonense	1	1	1	1	-	-	-	1	-	1
M. gordonae	1	1	1	1	-	-	-	1	-	1
M. intracellulare	1	0	-	-	-	-	-	-	-	-
M. marinum	1	0	-	-	-	-	-	-	-	-
Total	27	15	15(100)	14(93)	11(73)	-	1(6.7)	4(26.7)	-	14(93)

INH: Isoniazid
RIF: Rifampicin
EMB: Ethambutol
RR-TB: Rifampicin resistance
MDR-TB: Resistance to both Rifampicin and Isoniazid

Of the 36 isolates obtained from camel milk samples, 9 (25%) were *M. szulgai*, 5 (13.89) *M. monacense*, 3 (8.33%) *M. duvalii*, 2 (5.56%) *M. fortuitum*, 3 (8.33%) *M. elephantis*,1 (2.78%) *M. arcueilense*, 3 (8.33%) *M. lehmannii*, 2 (5.56%) *M. litorale*, 1 (2.78%) *M. lentiflavum*, 1 (2.78%) *M. brasiliensis*, 2 (5.56%) *paenibacillus spp*., 1 (2.78%) *Methylbacterium / Bosea sp.*, 1 (2.78%) *Cellulosimicrobium*, 1 (2.78%) *Roseomonas sp.* and 1 unknown AFB *sp*. After drug susceptibility testing, all 36 isolates were resistant to both isoniazid and rifampicin while, 21/36 (58.3%) were resistant to ethambutol. Fifteen (41.7%) of the isolates were susceptible to ethambutol, of which 4/15 (26.67%) were *M. szulgai*, 3/15(20%) *M. elephantis*, 2/15 (13.33%) *M. duvalii*, 1/15 (6.67%) *M. lehmannii*, 1/15 (6.67%) *M. brasiliensis*,1/15 (6.67%) *M. litorale* and 1 unknown AFB *sp.* (Table 4.12).

Table 4.12: Number and percentage of in vitro susceptibility to first-line TB drugs of nontuberculous mycobacteria (NTM) isolates from camel milk samples

Species isolate	Total Number of NTM isolates	Total Number tested	Resistance No. (%)			Susceptible No. (%)			Resistance categories (WHO, 2017)	
			INH	RIF	EMB	INH	RIF	EMB	RR-TB	MDR-TB (%)
M. fortuitum	3	2	2 (100)	2(100)	2(100)	-	-	-	-	2
M. szulgai	20	9	9(100)	9(100)	5(55.6)	-	-	4(44.4)	-	9
M. monacense	5	5	5(100)	5(100)	5(100)	-	-	-	-	5
M. lehmanni	3	3	3(100)	3(100)	2(66.67)	-	-	1(100)	-	3
M. elephantis	3	3	3(100)	3(100)	-	-	-	3(100)	-	3
M. litorale	4	2	2(100)	2(100)	1(100)	-	-	1(100)	-	2
M. duvalii	3	3	3(100)	3(100)	1 (33.3)	-	-	2 (66.67)	-	3
M.brasiliensis	1	1	1(100)	1(100)	-	-	-	1(100)	-	1
M. arcueilense	1	1	1(100)	1(100)	1(100)	-	-	-	-	1
M. lentiflavum	1	1	1(100)	1(100)	1(100)	-	-	-	-	1
M. flavescens	1	-	-	-	-	-	-	-	-	-
Other AFB *sp.*	8	5	5(100)	5(100)	3(60)	-	-	2(40)	-	5
Unknown	4	1	1(100)	1(100)	-	-	-	1(100)	-	1
Total	57	36	36(100)	36(100)	21(58.3)	-	-	15(41.67)	-	36(100)

INH: Isoniazid **RIF:** Rifampicin **EMB:** Ethambutol **RR-TB:** Rifampicin resistance**MDR-TB:** Resistance to both Rifampicin and Isoniazid

A total number of seven isolates from human sputum samples were tested for drug susceptibility: 1 (14.29%) *M. fortuitum*, 2 (28.57%) *M. szulgai*, 1 (14.29%) *M. litorale*, 11 (14.29%) *Paenibacillus sp*., 1 (14.29%) *Kocuria marina* and 1 (14.29%) unknown AFB species. Six of the seven isolates (85.7%) were resistant to isoniazid and rifampicin, while another 6/7 (85.7%) were resistant to ethambutol. One *M. litorale* isolate was susceptible to ethambutol however, all NTM isolates were resistant to both isoniazid and rifampicin (Table 4.13).

Table 4.13: Number and percentage of *in vitro* susceptibility to first-line TB drugs of nontuberculous mycobacteria (NTM) isolates from human sputum samples

Species isolate	Total Number of NTM isolates	Total Number tested	Resistance No. (%)			Susceptible No. (%)			Resistance categories (WHO, 2017)	
			INH	RIF	EMB	INH	RIF	EMB	RR-TB	MDR-TB (%)
M. fortuitum	1	1	1	1	1	-	-	-	-	1
M. szulgai	2	2	2 (100)	2 (100)	2 (100)	-	-	-	-	2
M. litorale	1	1	1(100)	1(100)	-			1(100)		1
Other AFB sp.	2	2	2(100)	1(50)	2(100)	-	1(50)	-		1
Uncharacterized	1	1	1 (100)	1 (100)	1 (100)	-	-	-	-	1
	7	7	7(100)	6(85.7)	6(85.7)	-	-	1(20)	-	6(85.7)

INH: Isoniazid **RIF:** Rifampicin **EMB:** Ethambutol **RR-TB:** Rifampicin resistance **MDR-TB:** Resistance to both Rifampicin and Isoniazid

Rifampicin and ethambutol showed a range of activity, from susceptibility to resistance: (RR: 1- >4) and (RR: 1 - >8) respectively, while isoniazid had no activity (RR > 5) against the isolates (Table 4.14).

Table 4.14: Resistance ratio (RR) and MIC (µg/mL) of the first-line TB drugs tested on nontuberculous Mycobacteria isolates from TB suspect camel tissue, camel milk and human sputum samples

Anti- TB drug	Susceptibility RR (MIC)	Intermediate susceptibility	Resistance RR (MIC)
Rifampicin	1 (16)	32	> 4 (>64)
Isoniazid	-	-	> 5 (>1)
Ethambutol	1 (1)	2 – 5.6	> 8 (>8)

The MIC_{50} and MIC_{90} values of isoniazid and rifampicin was >1µg/mL and > 64 µg/mL respectively, for all NTM species tested. The MIC_{50} and MIC_{90} values of ethambutol was > 8 µg/mL for *M. fortuitum*, *M. szulgai*, *M. elephantis*, *M. litorale*, *M. lehmannii*, *M. arcueilense*, *M. monacense*, unknown AFB*spp*. and 1 µg/mL for *M. gordonae* (Table 4.15).

Table 4.15: The MIC range, MIC50, MIC90 of the first-line TB drugs against NTM isolates

Drugs	*M. fortuitum*			*M. gordonae*			*M. szulgai*			*Other M. spp*		
	MIC range	**MIC_{50}**	**MIC_{90}**	**MIC range**	**MIC_{50}**	**MIC_{90}**	**MIC range**	**MIC_{50}**	**MIC_{90}**	**MIC range**	**MIC_{50}**	**MIC_{90}**
INH	>1	>1	>1	>1	>1	>1	> 1	> 1	> 1	>1	>1	>1
RIF	16 - >64	>64	>64	>64	>64	>64	> 64	> 64	> 64	16 - >64	>64	>64
EMB	1 - >8	>8	>8	1	1	1	1 - >8	>8	>8	1 - >8	>8	>8

INH: Isoniazid
RIF: Rifampicin
EMB: Ethambutol
MIC_{50}: Drug concentration at which 50% of isolates showed no growth
MIC_{90}: Drug concentration at which 90% of isolates showed no growth

4.5 Effect of assessed risk factors on *M. avium* PPD and camel milk culture result

Guided by the bivariate analysis (Table 4.16), the following explanatory variables were included in the logistic regression analysis with culture result as the outcome variable: Ward, village, breed, presence of other domestic species, livestock production system, management practices, sheltering, manure disposal, origin of new introductions, camel movement, culling rate, reasons for culling and herd size.

Table 4.16: Associations of the culture result of camel milk with identified personal information of respondents and herd level risk factors

Variable	Details	Milk culture result		Chi-square*	Fischer's Exact*	P-value (χ^2)
		AFB	Negative			
Administrative Ward	Waso east and Sereolipi	23	466	17.1554	0.001	0.0001
	Wamba east	8	63			
	Wamba west	5	15			
Breed	Somali	20	389	14.7530	0.002	0.001
	Rendile	3	84			
	Turkana	13	71			
Other domestic species	Small stock and cattle	23	150	8.6440	0.013	0.013
	Small stock, Cattle and donkeys	11	161			
	Small stock only	1	51			
Origin of new introductions	Archers post	8	160	14.3141	0.005	0.003
	Lolkuniyani Livestock	20	1676			
	Garissa and Isiolo	2	24			
	No new introductions	5	11			
Culling rate	0	4	110	30.6930	0.001	0.001
	1	3	63			
	2	4	51			
	3	10	25			
	4	3	13			
	5	2	10			
Reason for culling	No culling	6	114	12.3137	0.041	0.031
	Age, young males, mismothering	2	3			
	Age	10	81			
	Age, debilitation and injuries	7	69			
	Age and injuries	6	30			

*Chi-square and Fisher's exact tests were used to determine associations

The explanatory variables that remained in the logistic regression model after backwards elimination included breed, manure disposal, presence of other domestic species and origin of new introductions (Table 4.17). The Turkana breed of camel was found to be three times (OR=3.4) more likely to test AFB positive on culture compared to the Somali breed. Additionally, households that reportedly introduced replacement camels from neighboring counties and those using intraherd replacements were twice and thrice likely to have milk samples testing positive for AFB on culture respectively, compared to those which sourced for new introductions from within the county.

Table 4.17: Strength of associations between culture result of milk samples and identified risk factors at herd level

Variable	Details	Odds ratios (OR)	95% CI of OR
Breed	Somali*	1	-
	Rendile	0.5	0.1 -1.9
	Turkana	**3.4**	**1.2 - 9.3**
Manure disposal	Manure heaps and burning*	1	-
	Manure heaps, burning and other methods such as moving to a new site	**2.5**	**0.8 -7.3**
	Manure heaps only	**0.9**	**0.2 - 2.9**
Other domestic livestock species	Small stock and cattle*	-	-
	Small stock, cattle and donkeys	0.5	0.1 -1.3
	Small stock only	0.1	0.01 - 1.3
Origin of new introductions	Archers post*	-	-
	Lolkuniyani livestock market, Wamba	**0.8**	**0.2 -2.6**
	Garissa and Isiolo	**2.1**	**0.3 -12.3**
	No new introductions brought	**3.2**	**0.7 -14.7**

*Reference comparison group

Logistic regression modeling was used to generate odds ratios and confidence interval of odds. Model Log likelihood = -106.0983 (n=397); LR $\chi^2(9)=24.62$; P-value=0.0034; and Pseudo $R^2= 0.1040$.

Bivariate analysis using chi- square and Fishers exact indicated that the following explanatory variables were significantly associated with *M. avium* positivity: human sputum culture positivity, herd size, origin of new introductions, manure disposal, livestock production system, other domestic species, and camel movement (Table 4.18).

Table 4.18: Associations of *M. avium* positivity with household and husbandry variables

No.	Category	Variable	levels	*M. avium* SICTT result +	-	χ^2	Fischers Exact	P-value (χ^2)
1.	Household level factors	Location (Ward)	Waso east and Sereolipi	209	312	5.02	0.083	0.081
			Wamba east	21	50			
			Wamba west	11	9			
2.	Herd level factors	Breed	Somali	170	261	1.4546	0.492	0.483
			Rendile	42	55			
			Turkana	29	55			
		Herd size	Less than 50	182	323	13.4936	0.000	0.000
			Above 50	59	48			
3.	Management factors	Origin of new introductions	Archers post	120	199	15.1469	0.002	0.002
			Lolkuniyani livestock market, Wamba	82	148			
			Outside the county (Garissa/Isiolo)	29	18			
			From within herd	10	6			
		Manure disposal	Manure heaps	107	117	17.2677	0.001	0.002
			Manure heaps and burning	112	230			

Table 4.18: Associations of *M. avium* positivity with household and husbandry variables. Continued

No.	Category	Variable	levels	*M. avium* SICTT result +	-	χ^2	Fischers Exact	P-value (χ^2)
			Manure heaps, burning and shifting	18	23			
			Burning	2	0			
			Shifting	2	1			
		Livestock production system	Pastoral: camel, cattle and small stock	142	173	9.2797	0.01	0.01
			Pastoral: camel and cattle	8	21			
			Pastoral: came and small stock	91	177			
		Other domestic species	Small stock and cattle	91	118	16.4466	0.001	0.001
			Small stock, cattle and donkeys	108	218			
			Small stock only	40	30			
			Camels only	2	5			
		System	Extensive	239	369	0.1903	0.514	0.663
			Intensive	2	2			
		Sheltering	Permanent	6	4	1.8109	0.154	0.178
			Traditional *bomas*	235	367			
		Camel movement	Between 50-100 kM	24	71	9.3867	0.001	0.002

Table 4.18: Associations of *M. avium* positivity with household and husbandry variables. Continued

No.	Category	Variable	levels	*M. avium* SICTT result +	-	χ^2	Fischers Exact	P-value (χ^2)
			Less than 50 kM	217	300			
		Movement reason	Pasture	2	0	5.3347	0.055	0.069
			Pasture and water	127	220			
			Pasture, water and market	112	151			
		Movement destination	Within County	165	231	2.6379	0.259	0.267
			Within and neighboring County	73	136			
			Neighboring County	3	4			
		Sputum lab result	AFB positive	1	12	10.1232	0.003	0.006
			AFB negative	240	359			

*Fishers exact was used to compute associations if any of the expected values in any of the cells was less than five, otherwise Chi-square was used.

The best fitting (final) logistic regression model included the following variables: herd size, livestock production system, presence of other domestic species, and camel movement distance. The odds ratios of the levels of these variables were as follows: for herd size it was shown that herds with more than 50 camels were 1.91 times more likely to have camels positive for *M. avium* on SICTT compared to those from herds with less than 50 camels; for type of livestock production system, it was shown that camels from the mixed camel, cattle and small stock systems were 1.96 times more likely to turn positive for *M. avium* on SICTT compared to those from the mixed camel and small stock systems; for presence of other types of domestic species, it was shown that camels were over four times (OR=4.6) more likely to turn positive for *M. avium* on SICTT where camels are kept together with small stock only compared to where camels were kept together with small stock, cattle and donkeys; for distance of camel movement to pasture and water, it was shown that camels from herds whose movement was less than 50 kM were 1.74 times more likely to turn positive compared to those from herds whose movement was between 50 -100 kM (Table 4.19).

Table 4.19: Strength of associations between *M. avium* positivity with household and husbandry variables

No.	Category	Variable	Levels	Odds ratios (OR)*	95% CI of OR*	P -value
1.	Herd	Herd size	Below 50	1	-	
			Above 50	1.91	1.21 – 3.02	0.05
2.	Management	Camel movement distance	50 – 100 kM	1	-	-
			Less than 50 kM	1.75	1.03 – 2.98	0.04
		Livestock production system	Pastoral: camel and small stock	1	-	-
			Pastoral: camel and cattle	0.34	0.13 – 0.90	0.029
			Pastoral: camel, cattle and small stock	1.97	1.29 – 3.00	0.002
		Other domestic species	Small stock, cattle and donkeys	1	-	-
			Small stock and cattle	0.93	0.60 – 1.41	0.719
			Small stock only	4.65	2.53 – 8.55	0.000

*A logistic regression model was used to generate odds ratios and 95% confidence interval of odds.
Model Log likelihood = -373.05842 (n=612); LR $\chi^2(6) = 49.75$; p-value=0.0000; and Pseudo $R^2 = 0.0606$.

Bivariate and multivariate analysis of culture positivity of tissue lesions as the outcome variable with age, breed, and sex as risk factors found only the 6-7 age category to have higher odds (OR=2.5) of culture positivity (Table 4.20).

Table 4.20: Association of culture positivity of tissue lesions with age, sex and breeds of camels

			Bivariate				Multivariate			
	Positive (n=27)	Negative (n=104)		95% CI				95% CI		
Variables	n(%)	n(%)	OR	Lower	Upper	p value	AOR	Lower	Upper	p value
Age										
4-5'	3	18	ref.	ref.	ref.	ref.	ref	ref	ref	ref
6-7'	23	70	2	0.5	7.3	0.31	2.5	0.58	11.25	0.218
8-9'	1	16	0.4	0.04	4	0.42	0.49	0.04	5.7	0.57
Sex										
Male	23	87	ref.	ref.	ref.	ref.	ref	ref	ref	ref
Female	4	17	0.89	0.27	2.9	0.847	0.86	0.25	2.97	0.82
Breed										
Somali	20	74	ref.	ref.	ref.	ref.	ref	ref	ref	ref
Turkana	5	16	1.16	0.38	3.54	0.799	1.62	0.44	5.96	0.47
Crosses	2	13	0.57	0.12	2.73	0.481	0.56	0.11	2.77	0.48
Rendile	0	1								

4.6 Knowledge, attitude and practices of the sampled population

4.6.1 General information

Information gathered on the knowledge, attitudes and practices indicated that the average household size was 15 (Range: 5-38) while the mean age of respondents was 45 years, most of whom were male 65/83 (78.31%). Of these, household heads were 58/83 (69.88%), herders 1/83 (1.2%), other household members 9/83 (10.84%) and wife 15/83 (18.08%). A majority 78/83 (93.98%) of respondents had no formal education (Table 4.21). The average number of bulls per household herd was one (Range: 0 – 1); mean number of breeding females was 13 (Range: 1 – 87); mean number of juvenile females was 5 (Range: 1 -22); and the average number of calves was 6 (Range: 1 – 31).

Table 4.21: Demographic information of role of respondents, gender and education

Attribute/ Knowledge / practice	Level	Number (%)
Sex	Male	65 (78.31)
	Female	12 (21.69)
Role in the household	Heads	58 (69.88)
	Herders	1 (1.2)
	Members	9 (10.84)
	Wife	15 (18.08)
Education	Secondary	
	Primary	
	None	(70%)
Age	<40 years	25 (30.12)
	Above 40 years	58 (69.88)
Household size	<15	48 (57.83)
	Above 15	35 (42.17)
Herd size	>50	11(12.96)
	<50	72 (87.04)
Awareness of zoonotic infection	Yes	75 (90.36%)
	No	8 (9.64)
Handling of camels	Yes	83 (100)
	No	-
Living in close proximity with camels	Yes	82 (98.8)
	No	1 (1.2)
Consumption of raw camel milk	Yes	83 (100)
	No	-
Veterinary care	Self	82 (98.8)
	Other	1 (1.2)
Wildlife interaction	High	82 (98.8)
	Low	1 (1.2)

4.6.2 Awareness of Tuberculosis and other zoonotic mycobacteria

A majority 75/83 (90.36%) of those interviewed were aware of the possibility of being infected with zoonotic mycobacteria from camels. However, only 1/83 (1.2%) of the respondents could correctly identify the most important routes of transmission as through inhalation and ingestion, although a large majority felt that ingestion 61 (73.49%) was the most likely route. Of all the respondents, a large proportion (87%) reported members of their households as having had BCG vaccination, while those who reported the occurrence of a TB case in the household were in the minority (9%).

4.6.3 Interactions with camels

All of the respondents 83/83 (100%) indicated that they handled camels and lived 82/83 (98.8%) in close proximity with their herd.

4.6.4 Camel product consumption information

All the respondents indicated that they consumed camel meat and milk; however, while meat was mostly consumed cooked 61(73.49%), unequivocally 83/83 (100%), the response by all respondents was that fresh and fermented milk was consumed raw.

4.6.5 Herd level and management information

The majority of respondents indicated that the main management practice was extensive production with vaccinations, treatment and tick control done rarely 82/83 (98.8%). Sheltering of camels was mostly using traditional *bomas* 82/83 (98.8%) situated very close to mud and stick houses.

4.6.6 Animal health information

The ranking of the most common diseases in camels indicated that lymphadenopathy (26.25%), orf (26.25%), pneumonia (12.5%) and camel pox (11.25%) were the most common. The other less common camel diseases were mange (8.75%), trypanosomiasis (7.5%), haemorrhagic septicaemia (3.75%), anthrax (1.25%) and mastitis (1.25%). Regarding veterinary care, due to inadequate accessibility, an overwhelming majority 82/83 (98.8%) indicated that they personally provided veterinary care for their livestock, such as administration of drugs after consultation with County veterinary personell or community animal health workers. The treatments comprised mostly of deworming and tick control (60.29%) and treatment of other specific diseases (27.22%). In all the manipulations, it was unequivocal that all respondents did not use personal protective equipments (PPEs).

4.6.7 Household income and marketing information

A majority (93.3%) of the respondents indicated that their average monthly income was below Kshs. 50,000. The source of income was mostly from the sale of livestock and livestock products (98.2%), which in most cases were live animals (99.51%).

4.6.8 Wildlife interaction

A large majority of respondents,82/83(98.8%), reported that there existed a high level of interaction between livestock and wildlife which possed a great risk of disease transmission to camels.

CHAPTER FIVE: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 *Mycobacteria* infection in lactating dromedary camels

In the present study, the overall proportion of camels positive for *M. avium* and *M. bovis* infection as determined using SICTT had varying similarity with results obtained in similar studies among camel rearing communities in Eritrea, Kenya and Ethiopia (Gumi *et al*., 2012 b; Beyi *et al*., 2014; Ghebremariam *et al*., 2018; Lamuka *et al*., 2018). The overall proportion that was found positive to *M. avium* PPDin the present study was much greater (39.38%) compared to results from similar studies which found proportions of between 10% and 18.18% (Gumi *et al*., 2012b; Lamuka *et al*., 2018). There is a possibility that the infection rate is much lower in these other regions due to various factors such as differences in geography and breed predisposition, in addition, the study design, study population and procedure were varied.

The reported *M. bovis* positivity proportion in the present study may be much lower than what would have otherwise been reported, considering that some of the other studies used a lower diagnostic cut-off point (>2mm) (Whelan *et al*., 2010; Clegg *et al*., 2018; Awah-Ndukum *et al*., 2012; Ghebremariam *et al*., 2018) compared to the >4mm used in this study, which is recommended by the OIE for camels traded internationally (OIE, 2018). In a previous similar study, prevalence of *M. bovis* of 6% in camels in Ethiopia based on SICTT was estimated by Beyi *et al*. (2014). In yet another recent study involving 195 camels in Eritrea animal and herd prevalence of *M. bovis* in camels was found to be 1.5% and 2.9% respectively (Ghebremariam *et al*., 2018). The *M. bovis*

positivity on SICTT reported in camels in the present study is similar to prevalence levels estimated in cattle (4-6%) in a similar geographical location of Laikipia County (Mugambi *et al*., 2016).

The difference in induration from the two studies at the *M. avium* PPD site could be due to several host, environmental and procedural factors (Chambers, 2013; Tschopp and Aseffa, 2016). In similar studiesby Beyi *et al*. (2014) and Ghebremariam *et al*. (2018) sample size and study design was varied and hence results are not comparable; Age of camels in our study was generally higher as only the lactating camels which tend to be older (>6 years) were considered while similar studies considered a wider range of camel of varying ages; Breeds considered in our study were Somali, Rendile and Turkana which may be different genetically from those in Ethiopia and Eritrea; with regard to sex, our study considered lactating camels only which have a unique physiological and immunity status, while similar studies looked at both male and female camels. In all of these studies, despite the low sensitivity of tuberculin skin testing, it was concluded that the camels were infected with *Mycobacteria* without confirmatory isolation of the causative agent (Jibril *et al*., 2016). Currently, the screening tests for *Mycobacteria* recommended by the Office International des Epizooties (OIE) are specifically for detection of *M. bovis* and are not appropriate for detection of other *Mycobacteria* species. Rapid, highly sensitive and affordable field screening tests for NTM in livestock are nonexistent, biomarkers of *Mycobacteria* in milk are lacking, and molecular typing is unavailable (Carruth *et al*., 2016).

The reactions to both the avian and bovine tuberculin were moderate; however, a strong correlation was observed between the induration sizes at the bovine and avian PPD injection sites, an indication that possibly both PPDs antigens were probably targeting related antigen epitopes. However, there were few in which specific epitopes were targeted and these constituted some of the clear-cut positive reactors. This observed general tendency of the bovine and avian PPD to correlate implies an existing high level of cross reactivity from environmental and nontuberculous *Mycobacteria* (Jenkins *et al*., 2018). The recommended OIE cut-off for *M. bovis* positivity is >4 mm, however, this can be manipulated to increase sensitivity (Ngandolo *et al*., 2009; Goodchild *et al*., 2015).The interpretation as recommended by the OIE (OIE, 2018) is that the variance in the measurement of the skin thickness before and after 72 hours of PPD injection was determined as the final record of induration size at injection site. A positive result was recorded if the increase in skin thickness at the bovine tuberculin PPD (PPD-B) injection site was more than 4mm greater than the reaction at avian tuberculin PPD (PPD-A) injection site; inconclusive if PPD-B reaction was greater than that at thePPD-A site with a difference of less than 4 mm; and negative if the skin swelling at PPD-B injection site was less than or equal to the size of the skin reaction at the PPD-A injection site. Positive reactors to *M. avium* PPD were determined as described by Gumi *et al*. (2012b). Briefly, a positive result for NTM infection was recorded if an increase in skin fold thickness with visible reaction at the PPD-A injection site was more than 1mm greater than reaction at PPD-B injection site.

5.1.2 Pathologic manifestation of NTM lesions from slaughtered camels

In the present study, there was higher proportion of suspect granulomatous lesions from mediastinal and bronchial lymph nodes among those that were culture positive possibly an indication of the importance of the respiratory route of infection regardless of the mycobacterium type. This distribution is similar to what has been described by Michel (2018), where granulomatous lesions were found to predominate in lungs, mediastinal, retropharyngeal and mesenteric lymph nodes. In another study by Ahmad *et al*. (2019), three different pathological patterns in camels with suspect granulomatous lesions were described, namely pulmonary, abdominal and disseminated forms. In that study, a much higher proportion (33.5%) of suspect granulomatous lesions was reported. In yet another study, Beyi *et al*. (2014) estimated a prevalence of 8.3% of granulomatous lesions in Ethiopian camels out of which 18.18% (6/33) showed mycobacterial growth on culture. The distribution of granulomatous lesions in the present study corresponds to the distribution recorded previously (Mamo *et al*., 2011; Zerom *et al*., 2013; Beyi *et al*., 2014), where most lesions were found in the lungs and associated lymph nodes (57.7%) followed by lymph nodes of the head (27.2%). In the study by Mamo *et al*. (2011), tropism of tuberculous lesions was significantly different among lymph nodes and lung lobes. The distribution observed in the present study is possibly a confirmation that inhalation may be the main route of mycobacteria infection in camels.

The annual prevalence of granulomatous lesions based on retrospective meat inspection records at county level was low compared to the results in the present prospective study. Interestingly, the proportion of tuberculous lesions calculated using the combined meat

inspection data (2.29%) from county meat inspection records was almost similar to the proportion of lesions that were culture positive (1.69%) in the present study. This is an indication that granulomatous lesions due to NTM detected are being misdiagnosed as tuberculous lesions.Similar abbatoir based studies in Ethiopia found prevalence of tuberculosis compatible lesions of 10.04% (Mamo *et al*., 2011) and 12.3% (Zerom *et al*., 2013). In the study by Mamo *et al*. (2011), it was found that the majority, if not all the of, tuberculous lesions observed were as a result of Mycobacteria other than *Mycobacteria tuberculosis* complex. Similarly, Gumi *et al*. (2012b) detected low levels of prevalence of TB due to *M. bovis* amongst camels with an individual animal prevalence of 0.4% in Southeast Ethiopia, and Kasaye *et al*. (2013) found < 1% *M. bovis* prevalence in camels. This may be confirmation of what Michel (2018) described as the possibility of overestimation of TB due to *M. bovis* when basing on observation of lesions at post mortem only. The use of post mortem inspection of carcasses to estimate specificity is imperfect, although it can be used to confirm presence of infection with *M. bovis* (Goodchild *et al*., 2015).

In the present study, none of the samples were positive on initial FM microscopy; the capilia Neo-TB® immunochromatography and GeneXpert® were negative indicating that all isolates were NTM. The lack of growth at 42°C was an indication that there were no members of *Mycobacterium avium* complex isolated, which are mainly implicated in the causation of cases of NTM pulmonary disease in HIV and other immune suppressive diseases in humans. The negative Capilia test was an indication that none of the isolates in the present study were members of *Mycobacterium*

tuberculosis complex. The poor culture yield from tissue lesions is mostly associated with first and foremost the low sensitivity of post-mortem inspection which may result in misclassification of parasitic granulomas and abscesses. The other factors include excessive decontamination, non-viable calcified granulomas, and inappropriate handling of lesions (Beyi *et al*., 2014).

The histopathological findings from the present study confirm the difficulty in distinguishing between the pathology caused by infection with various *Mycobacteria*, since the histopathological presentation observed was similar to that of granulomatous reactions (Uma and Varshney, 2011; Menin *et al*, 2013). Distinguishing disease due to NTM and that caused by members of the MTB complex based on clinical presentation, smear microscopy, radiography and histopathology is difficult, definitive diagnosis is by culture and isolation of NTM (Kwon and Koh, 2014).

5.1.3 Molecular identity of NTM isolates from camel milk, human sputum and tissue lesions

The present study describes for the first time the isolation and identification of NTM species of public health importance from both camel milk and sputum samples of associated household members presumed to have TB in Kenya. Nontuberculous mycobacteria have previously been isolated elsewhere globally in humans and from samples obtained from various species of livestock, livestock products and the environment (Kankya *et al*., 2011; Gumi *et al*., 2012a; Muwonge *et al*., 2012; Agdestein *et al*., 2014; Katale *et al*., 2014; Jerker *et al*., 2014; Monde *et al*., 2018). In

the present study, a variety of NTM were identified and the most predominant species identified in both camels and humans were categorised in two main clades; the 'Tuberculosis-simiae' and Fortuitum-Vaccae'; and a third minor clade, the 'Terrae' clade. This was in contrast to the findings of Gumi *et al*. (2012a) in South East Ethiopia, where a variety of *Mycobacteria* including *M. tuberculosis, M. bovis* and NTM were isolated from human sputum, fine needle aspirate and tissue lesions. In the present study, the proportion that was found culture positive with NTM isolates in people was much lower than the levels reported elsewhere where human pulmonary infections with NTM of environmental and livestock origin have been estimated at upto 20% (Kankya *et al*., 2010). As in the study by Mamo *et al*. (2011), it was found that the majority if not all theof tuberculous lesions observed were as a result of mycobacteria other than *Mycobacteria tuberculosis* complex.

In tuberculosis-simiae clade *M. szulgai* was the most common and with the highest NTM isolates in both humans and camels. At the time of writing this thesis, there is no documentation where *M. szulgai* has been isolated in camel milk. However it has been previously isolated in bovine milk in cattle in Brazil in a study done by Bolanos *et al*. (2018). *Mycobacterium szulgai* is a potential human and animal pathogen with manifestation similar to active TB clinically and on radiography (Kim *et al*., 2014a; Kim *et al*., 2014b; Gcebe *et al*., 2018). There were several species in the fortuitum-vaccae clade with *M. fortuitum* being the most common isolate of medical importance, in both study populations. There is no study at the time of writing this thesis which has documented the presence of *M. fortuitum* in camel milk. *Mycobacterium fortuitum*

causes transcient infection or colonization in patients with underlying lung disease and the major presenting symptom is usually cough and sputum production (Park *et al*., 2008). Although underlying lung disease was not the focus of our study, the isolation of *M. fortuitum* from sputum of people presumed to have TB may be an indication of such a condition.

It has been indicated that in humans the syndromes caused by NTM include progressive NTM pulmonary disease, superficial lymphadenitis, cutaneous disease and disseminated disease. Pulmonary NTM disease cases have been identified among patients with chronic obstructive pulmonary disease, asthma, cystic fibrosis, suspected pulmonary TB and chronic TB (Nyamogoba *et al*., 2012; Chanda-Kapata *et al*., 2015; Shao *et al*., 2015; Hoza *et al*., 2016; Cadmus *et al*., 2016; Gardner *et al*., 2019). In TB high burden countries, the public health significance of this is that the existing diagnostic procedures that rely on clinical presentation and detection of AFB on microscopy often categorize NTM pulmonary disease patients as suspect TB cases (Maiga *et al*., 2012). The implications of this misdiagnosis may be reflected in treatment failure, drug resistance and even country TB statistics which are probably confounded by a substantial number of pulmonary NTM disease cases (Badoum *et al*., 2011; Maiga *et al*., 2012; Shahraki *et al*., 2015).

A study by Chinombe *et al*. (2016) in Zimbabwe isolated several NTM species including *M. lentiflavum, M. brasiliensis, M. elephantis* and *M. fortuitum* from sputum samples. In that study, sampling of the environment and livestock was not done. It is

noteworthy to mention that in the present study, similar isolates were identified from camel milk.Nontuberculous mycobacteria are an important cause of disseminated infection and disease in people living with HIV. A study in Southeast Asia to determine NTM mycobacterial disease in HIV patients found *M. fortuitum* causing pulmonary disease among other species (McCarthy *et al*., 2012). In the same study, *M. szulgai* was isolated from HIV patients. Despite that in our study the HIV component was not investigated, the isolation of *M. fortuitum* is a reflection of the importance of the NTMs isolated in our study which should be investigated in future studies.

Few studies have focused on camel milk as a potential source of nontuberculous mycobacterial infection. A study conducted in humans, camels, cattle and goats in South East Ethiopia by Gumi *et al*. (2012b), found *M. tuberculosis*, *M. bovis* and NTMs which were not characterised. Ethiopia borders the study area in northern Kenya, where there is free movement of people and animals; it is most likely that some of the uncharacterised NTM isolates could beof medical importance. Additionally, camel milk which was used in our study was not investigated in the study by Gumi *et al*. (2012b). The choice of camel milk in our study was due to the fact that milk is a major component of dietary intake in the study population. Therefore, isolation of NTM is an indicator of the need for urgent public health intervention. A recent study done in Kenya by Mwangi *et al*. (2016) determined presence of *Mycobacteria* species in raw and fermented camel milk including NTM using Genotype®*Mycobacterium* CM/AS assay. However, the genotypic aspects of the test renders the viability of the *Mycobacteria* detected unreliable without culture results. Most studies carried out in

camels elsewhere have focused on post mortem lesions, yet camel meat consumption is not as widespread compared to camel milk (Mamo *et al*., 2011; Zerom *et al*., 2013; Beyi *et al*., 2014; Ahmad *et al*., 2019). Taking into consideration of its nutritional value the consumption of camel milk is increasingly becoming popular globally, thus the need to closely monitor the public health aspects of this product.

The present study used a combination of several methods for isolation and identification of NTMs. This increased the opportunity to isolate and identify different species from both camels and humans. The proportion of culture positive was much higher compared to findings of other studies which used only solid media with culture positivity ranging between 5.1% to 8.4% (Aydin *et al*., 2012; Katale *et al*., 2014; Bolanos *et al*., 2018). In our study, using both the creamy and pellet layers of milk samples increased the chance to isolate various NTMs. This approach was similar to the study by Aydin *et al*. (2012) where the creamy and pellet layers were cultured on LJ and where *M. fortuitum* and *M. szulgai* were detected from raw cattle milk samples. *Mycobacterium fortuitum* has also been isolated in a study by Sevilla *et al*. (2017) using cattle milk and milk products purchased at Spanish super markets, however it was not indicated whether different milk layers were used.

The use of 16S rRNA partial sequencing with commercial kits in our study, improved the results of NTM identification. Similar findings were found in a study by Joao *et al*. (2014). The percent similarities of the majority of 16S rRNA gene sequences of isolates obtained in our study with those in GenBank public database was high (>97%). This

was consistent with the findings by Bolanos *et al*. (2018) and Kim and Shin (2018). Due to the limited availability of sequencing facilities in many settings most studies may be missing valuable information on identity of NTMs for management of human and animal health. The clinical and laboratory standards instituteguidelines on interpretive criteria for identification of bacteria and fungi using 16S rRNA sequencing recommends a threshold of $\geq$ 99% homologywith a reference of high quality and a minimum of >0.8% distance to the nearest alternative in order to assign species identity (CLSI, 2018). Basedon these guidelines, a majority of the isolates in the present study may be considered as novel species.

5.1.4 Drug sensitivity of Mycobacteria isolates

Isolation of NTM is on the increase worldwide and multidrug resistance of NTM is recognized as a major challenge globally and therapy is not standardized (Tsai *et al*., 2011; Shiau *et al*., 2016; Sengupta *et al*., 2017; Bonnet *et al*., 2017). The increase in reports of NTM are related to immunosuppression due to disease or therapy induced, an aging population, increase in susceptibility, and improved diagnostic methods (Chalmers *et al*., 2018). In the present study, various mycobacteria species were obtained from tissue lesions at post mortem meat inspection, camel milk and sputum samples of closely associated household members. Drug susceptibility against a panel of the three first-line TB drugs showed high resistance. This concurred with the results of Wang *et al*. (2014) in Northern China, where among three first-line TB drugs, ethambutol was shown to be the most useful against NTM. In contrast, the study by Wang *et al*. (2014) tested against a wider panel including seven second-line TB drugs

and most of the NTM isolates showed extensive drug resistance. In another study in China, isolates which included *M. abscessus* and *M. fortuitum* exhibited great antibiotic resistance, while complete inhibition of growth of *M. intracellulare* required higher drug concentrations (Pang *et al*., 2015). In that study, isolates had low resistance to amikacin, linezolid, and tigecycline; and high resistance to first-line TB drugs as well as amoxicillin-clavulanic acid, rifapentine, dapsone, thioacetazone and pasiniazid.

In the present study, multidrug resistance to both isoniazid and rifampicin was high in NTM from tissue samples. Among the three first-line drugs tested, ethambutol exhibited a varied level of susceptibility in NTM from different sample types and monoresistance to rifampicin was high. Ethambutol might still be useful in regimens against some species of NTM in combination with aminoglycosides and macrolides (Litimov *et al*., 2018). Multi drug resistant NTM is a major public health issue globally where, resistance of isolates to several drugs including first and second-line TB drugs, macrolides and aminoglycosides has been demonstarted (Litvinov *et al*., 2018).

The public health significance of the isolates tested are varied, *M. fortuitum*, a rapidly growing *Mycobacterium*, is one of the most common causes of extrapulmonary disease in soft tissues and *M. szulgai* causes pulmonary disease similar to TB (Pang *et al*., 2015). Finally, NTM constitute a major burden to the logistics, infrastructure and finances of public health programs due to treatment failure, repeat hospital visits and misdiagnosis (Desikan *et al*., 2017).

5.1.5 Risk factors associated with NTM infection in camels

Several potential risk factors associated with camel tuberculin skin test positivity and camel milk culture positivity were documented in our study. Those associated with tuberculin test result included herd size, source of new camel introductions, manure disposal, camel production system, presence of other domestic animal species, and distance of camel movement.

In our study it was found that herd size had a positive correlation with *M. avium* reactivity. These findings are in agreement with those of the studies done by Lamuka *et al*. (2018) and Mekonnen *et al*. (2019). In their studies in Ethiopia, Mekonnen and team found higher odds of *M. avium* positivity among dairy cattle herds with herd size greater than 20. In that study, new cattle introductions into the herd were found to be associated with higher odds of *M. avium* positivity. In other studies by Gumi *et al*. (2012b) and Jibril *et al*. (2016) in Ethiopia, herd size, presence of other livestock species, annual migration dynamics, recent introductions of new animals into the herd, age, sex and body condition were considered as possible risk factors for SICTT positivity but no associations were found. Those associated with culture positivity included administrative ward, breed, presence of other domestic animal species, origin of new introductions and culling rate. Significant associations were found with breed of camel and source of new introductions of camels into the herd. Documented studies on mycobacteria in camels have considered tuberculin skin test results and pathology as definitive diagnosis. The present study is the first to document risk factors forcamel milk culture positivity. In the present study, those households that reportedly sourced

new introductions had low levels of culture positive camel milk samples. Similar findings were reported by Clegg *et al*. (2018) in cattle herds in Ireland, where it was shown that herds in which more animals were introduced were less likely to experience severe episodes of bovine tuberculosis. There is need to determine the underlying reasons for this phenomenon in future studies.

The most common practices identified among the household members that may predispose to zoonotic infections between humans and camels included; consumption of raw milk, living in close proximity to animal shelters and handling of camels without personal protective clothing (PPE). These findings are in agreement with a review done by Tschopp and Aseffa, (2016), where it was indicated that living in close proximity with livestock and frequent consumption of raw animal products were considered as practices predisposing to zoonotic risk. As in a study by Ghebremariam *et al*. (2018) in Eritrea, consumption of raw milk, lack of zoonotic transmission awareness, low levels of education, introduction of new animals and migration over large distances were found as common events that predispose to human-animal and animal-animal infection.In the present study, awareness of TB was high; however, awareness of zoonotic importance of mycobacteria was low similar to the finding of Beyi *et al*. (2014) and Lamuka *et al*. (2018). Sharing of living area with camels and consumption of raw camel milk were common practices similar to the findings in the studies by Boukary *et al*. (2012) and Ghabremariam *et al*. (2018). In our study a much higher proportion (48/83) of households interviewed indicated the presence of respiratory disease symptomatic of TB compared to a study where 2.3% was reported

(Ghebremariam *et al*., 2018). This could be due to difference in sample size as well as stigma attached to being presumed to have TB which was also encountered in our study.

5.2 Conclusions

The following conclusions were made from this study:

i. This study has demonstrated high levels of reaction to *M. avium* (39.38%) and *M. bovis* (6.7%) using SICTT, indicating infection of lactating camels with both typical and atypical *Mycobacteria*.

ii. The histopathological manifestation of pathological tissue lesions associated with NTM infection was similar to a granulomatous reaction. Most of these lesions were observed in the right, left bronchial lymph nodes and the mediastinal lymph nodes (59.54%). The lesions were associated with NTM and camel TB may be overestimated due to misclassification.

iii. A variety of NTM species isolates of medical importance are here described for the first time in various pathological lesions in camels, camel milk and sputum of closely associated household members. These species include: *M. szulgai, M. fortuitum, M. scrofulaceum, M. marinum* and *M. intracellulare*. Most of these isolates in slaughtered camels were obtained from the bronchial and mediastinal lymph nodes and lung lobes. In addition, the data shows NTM of zoonotic importance (*M. szulgai*, *M. fortuitum*, *M. litorale*) exist in camel milk and sputum of camel handlers, implying possible cross transmission between camels and humans. Nontuberculous mycobacteria in two main clades: Fortuitum-

vaccae and Tuberculosis-Simiae were determined using 16S rRNA sequencing and phylogenetic analysis.

iv. Strain isolates were highly resistant to all the first – line TB drugs (isoniazid, Rifampicin and Ethambutol). There was variation in susceptibility to ethambutol with a wide range in resistance ratio (RR) (1 - >8) reported; high RR (>4)were considered resistant. Isolates from all types of samples showed total resistance to isoniazid at a maximum drug concentration of 1 μg/mL.

v. It was determined that herd size, camel movement distance, the type of camel production system, and the presence of other domestic species were risk factors for *M. avium* skin test positivity following SICTT. Breed, method of manure disposal and the origin of replacement stock were found to be risk factors for culture positivity of milk samples. Finally, age of camels was found to be a risk factor for culture positivity of post mortem tissue lesions.

5.3 Recommendations

i. This study demonstrated high levels of infection with NTM in camels. There is need for monitoring using surveillance and notification systems at local and national level. This should involve; skin testing, movement control and involvement of a one-health approach strategy.

ii. Evidence based approaches for distinction of disease caused by NTM and other *M. tuberculosis* complex mycobacteria are urgently required to reduce the level of false positives at post mortem.

iii. Several medically important NTM were demonstrated in camels, tissues and closely associated household members. It is recommended that awareness creation and education of pastoralists onTB, other mycobacterial zoonoses and camel milk hygiene be done. In addition, people with NTM infections should be monitored and given appropriate treatment.

iv. High levels of resistance of NTM isolates to commonly used first- line TB drugs were demonstrated. It is recommended that routine drug susceptibility testing of *Mycobacteria* isolates from symptomatic community members from the study area be conducted prior to therapeutic intervention.

v. There is need to educate pastoralists on management practices that minimize risk factors for infection with NTM.

5.4 Suggestions for further studies

i. There is need for establishment of a catalogue of specific tests for NTMs and the standardization of tuberculin tests according to environment and other variable factors.

ii. Further studies are needed to develop of evidence-based methods for detection of NTM infection.

iii. Further studies to characterize and determine the public health significance of some of the isolated NTMs are required.These future studies should incorporate the one-health concept.

iv. There is need to establish a standard therapeutic regimen for NTMs in both humans and animals.

v. Further research is required to determine the reasons underlying the association between the risk factors identified and *M. avium* skin positivity in camels.

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APPENDICES

Appendix I: Research approval- Kenyatta University, Graduate School

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 810901 Ext. 57530

Internal Memo

FROM: Dean, Graduate School

DATE: 4th November, 2015

TO: Mr. Lucas L. A. Asaava
C/o Zoological Sciences Dept.
Kenyatta University

REF: I84/29435/14

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

This is to inform you that Graduate School Board at its meeting of 4th November, 2015 approved your Research Proposal for the Ph.D. Degree, entitled "Molecular Epidemiology of Mycobacteria Tuberculosis Complex and Risk Factors Associated with Infection amongst Camels and Pastoralists in Samburu, Kenya".

You may now proceed with your Data collection, subject to Director General, National Commission for Science, Technology & Innovation

As you embark on your data collection, please note that you will be required to submit to Graduate School completed supervision Tracking Forms per semester. The form has been developed to replace the progress Report Forms. The Supervision Tracking Forms are available at the University's Website under Graduate School webpage downloads.

By copy of this letter, the Registrar (Academic) is hereby requested to grant you substantive registration for ~~your Ph.D.~~ studies.

Thank you.

KENYATTA UNIVERSITY OFFICE OF DEAN 04 NOV 2015 GRADUATE SCHOOL P.O. BOX 43844-00100 NAIROBI

JULIA GITU
FOR: DEAN, GRADUATE SCHOOL

c.c. Registrar (Academic) Att. Mr. Likam
Chairman, Zoological Sciences Department

Supervisors:

1. Prof. Michael M. Gicheru
   C/o Zoological Sciences Dept.
   KENYATTA UNIVERSITY

2. Dr. Willie Githui
   KEMRI
   National Tuberculosis Reference Laboratory
   C/o Zoological Sciences Dept.
   KENYATTA UNIVERSITY

IG/cao

Appendix II: Ethical approval

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
E-mail: director@kemri.org, info@kemri.org, Website. www.kemri.org

KEMRI/RES/7/3/1 **March 31, 2017**

TO: **LUCAS ASAAVA,**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CRDR,**
NAIROBI

05 Apr 2017

Dear Sir,

RE: PROTOCOL NO. KEMRI/SERU/CRDR/021/3428 (*RESUBMISSION OF INITIAL SUBMISSION*): MOLECULAR EPIDEMIOLOGY OF MYCOBACTERIUM TUBERCULOSIS AND RISK FACTORS ASSOCIATED WITH ZOONOTIC INFECTION IN CAMELS IN SAMBURU, KENYA_ (*VERSION 3.0 DATED 6TH JANUARY, 2017)*

Reference is made to your letter dated 16th March, 2017. The KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on the same day.

This is to inform you that the Committee noted that the issues raised during the **260th meeting of the KEMRI/Ethics Review Committee (ERC)** held on 14th February, 2017 have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **31st March, 2017** for a period of one year. Please note that authorization to conduct this study will automatically expire on **March 30, 2018.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **February 16, 2018**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study

Yours faithfully,

FOR: **DR. EVANS AMUKOYE,**
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT

Appendix III: Animal care and use approval

KENYA MEDICAL RESEARCH INSTITUTE

Centre for Virus Research P.O. Box 54628 - 00200, NAIROBI - Kenya
Tel: (254) (020) 2722541, 2713349 0722-205901, 0733-400003, Fax: (254) (020) 2726115
E-mail: cvr@kemri.org

KEMRI/ACUC/ 01.04.17 **20th April 2017**

Asaava Lucas
Kenyatta University

Lucas,

RE: Animal use approval for "Molecular epidemiology of mycobacterium tuberculosis and factors associated with zoonotic infection in camels in Samburu, Kenya" Protocol

The KEMRI ACUC committee acknowledges the submission of the above mentioned protocol for review. It has been determined that that post mortem sampling of the representative numbers of camels and collection of milk from selected animals is justified in achieving the study objectives.

It has also been noted that permission to collect specimens has been granted by the Department of Veterinary services and the committee is in receipt of letters confirming this.

Approval is granted for a period of three years starting from when the SERU approval will be obtained. The committee expects the study to provide an annual report on the progress of animal use simultaneously with the annual continuing review report to SERU.

The committee expects you to adhere to all the animal handling procedures as described in the protocol.

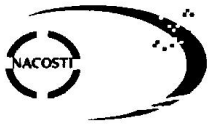
The committee wishes you all the best in your work.

Yours sincerely,

Dr. Konongoi Limbaso
Chairperson KEMRI ACUC

KENYA MEDICAL RESEARCH INSTITUTE
★ 20 APR 2017 ★
ANIMAL CARE AND USE COMMITTEE
Signature: .....

Appendix IV: Research Authorization - NACOSTI



NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY AND INNOVATION

Telephone:+254-20-2213471,
2241349,3310571,2219420
Fax:+254-20-318245,318249
Email:dg@nacosti.go.ke
Website: www.nacosti.go.ke
when replying please quote

9th Floor, Utalii House
Uhuru Highway
P.O. Box 30623-00100
NAIROBI-KENYA

Ref: No.
NACOSTI/P/17/63884/15855

Date:
8th March, 2017

Dr. Lucas Luvai, Azaale Asaava
Kenyatta University
P.O. Box 43844-00100
NAIROBI.

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on ***"Molecular epidemiology of mycobacteria tuberculosis complex and risk factors associated with infection amongst camels and Pastoralists in Samburu, Kenya,"*** I am pleased to inform you that you have been authorized to undertake research in **Isiolo, Machakos and Samburu Counties** for the period ending **8th March, 2018.**

You are advised to report to **the County Commissioners and the County Directors of Education, Isiolo, Machakos and Samburu Counties** before embarking on the research project.

On completion of the research, you are expected to submit **two hard copies and one soft copy in pdf** of the research report/thesis to our office.

DR. STEPHEN K. KIBIRU, PhD.
FOR: DIRECTOR-GENERAL/CEO

Copy to:

The County Commissioner
Isiolo County.

The County Director of Education
Isiolo County.

Appendix V: Research permit

THIS IS TO CERTIFY THAT:
DR. LUCAS LUVAI, AZAALE ASAAVA
of KENYATTA UNIVERSITY, 43844-100 NAIROBI,has been permitted to conduct research in *Isiolo , Samburu , Machakos Counties*

on the topic: *MOLECULAR EPIDEMIOLOGY OF MYCOBACTERIA TUBERCULOSIS COMPLEX AND RISK FACTORS ASSOCIATED WITH INFECTION AMONGST CAMELS AND PASTORALISTS IN SAMBURU, KENYA.*

for the period ending:
1st April,2017

.....
Applicant's Signature

Permit No : NACOSTI/P/16/63884/9145
Date Of Issue : 9th June,2016
Fee Recieved :Ksh 2000

[redacted]

.....
Director General
National Commission for Science, Technology & Innovation

THIS IS TO CERTIFY THAT:
DR. LUCAS LUVAI, AZAALE ASAAVA
of KENYATTA UNIVERSITY, 43844-100 NAIROBI,has been permitted to conduct research in *Isiolo , Machakos , Samburu Counties*

on the topic: *MOLECULAR EPIDEMIOLOGY OF MYCOBACTERIA TUBERCULOSIS COMPLEX AND RISK FACTORS ASSOCIATED WITH INFECTION AMONGST CAMELS AND PASTORALISTS IN SAMBURU, KENYA.*

for the period ending:
8th March,2018

.....
Applicant's Signature

Permit No : NACOSTI/P/17/63884/15855
Date Of Issue : 8th March,2017
Fee Recieved :Ksh 1000

[redacted]

.....
Director General
National Commission for Science, Technology & Innovation

Appendix VI: Camel milk sample form

RECEPTION
Lab No: _____Sample ID: _____ Unique Household ID: _____Collection date:_____Village:_____ Tuberculin Result :□Positive□Negative□Inconclusive Initials: _____ Date: _____
MICROSCOPY FM
□ Absolute Number (1-10 Bacilli) □ + Scanty (>10 Bacilli) □ 2+ Moderate (up to 100 Bacilli) □ 3+ Heavy Date: Staff Initials:
GENEXPERT
GENEXPERT Result: □Positive □ Negative Rif Resistance:□Rif positive □Rif Negative Date: Staff Initials:

MIGIT									
Workup		Result		Date		Staff Initials	Comments		
MIGIT Result		□Positive □ Negative							
ZN		□Positive □ Negative							
HAIN		□Positive □ Negative							
SPECIATION		□1 □2 □3 □4							
LJ									
Date	W0	W1	W2	W3	W4	W5	W6	W7	W8
LJ P									
LJ G									

SUMMARY OF POSITIVE LJ P				
WORKUP	RESULT	DATE	STAFF INITIALS	COMMENTS
LJ P RESULT	□Positive □ Negative			
ZN	□Positive □ Negative			
HAIN	□Positive □ Negative			
SPECIATION	□1 □2 □3 □4			

SUMMARY OF POSITIVE LJ G				
WORKUP	RESULT	DATE	STAFF INITIALS	COMMENTS
LJ G RESULT	□Positive □ Negative			
ZN	□Positive □ Negative			
HAIN	□Positive □ Negative			
SPECIATION	□1 □2 □3 □4			

DST			
WORKUP	RESULT	DATE	STAFF INITIALS
Isoniazid	□ Sens □ Res		
Rifampicin	□ Sens □ Res		
Ethambutol	□ Sens □ Res		

Appendix VII: Abattoir daily recording form

S/No.	abattoir name, location	Date of slaughter	Individual animal identification number	Body condition, 1 day prior to slaughter	Significant ante mortem findings	Origin	Sex	post mortem findings
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								
17								
18								
19								
20								
21								
22								
23								
24								

Appendix VIII: Slaughterhouse sample form

RECEPTION
Lab No: _____Sample ID: _____
Collection Date:_____ Location: □ Arthiriver □ Isiolo
Sample Type: A)□ Lung Lobes Left (□ Apical □ Cardiac □ Diaphragmatic)
Right (□ Apical □ Cardiac □ Diaphragmatic)
B) □ Lymph Nodes □ Parotid □ Mandibular □ Retropharyngeal □ Bronchial
Initials: _____ Date: _____
MICROSCOPY FM
□ Absolute Number (1-10 Bacilli) □ + Scanty (>10 Bacilli)
□ 2+ Moderate (up to 100 Bacilli) □ 3+ Heavy
Date: Staff Initials:
GENEXPERT
GENEXPERT Result: □ Positive □ Negative Rif Resistance: □ Rif positive □ Rif Negative
Date: Staff Initials:

MIGIT									
Workup		Result		Date		Staff Initials		Comments	
MIGIT Result		□Positive □ Negative							
ZN		□Positive □ Negative							
HAIN		□Positive □ Negative							
SPECIATION		□1 □2 □3 □4							
LJ									
Date	W0	W1	W2	W3	W4	W5	W6	W7	W8
LJ P									
LJ G									

SUMMARY OF POSITIVE LJ P				
WORKUP	RESULT	DATE	STAFF INITIALS	COMMENTS
LJ P RESULT	□Positive □ Negative			
ZN	□Positive □ Negative			
HAIN	□Positive □ Negative			
SPECIATION	□1 □2 □3 □4			

SUMMARY OF POSITIVE LJ G				
WORKUP	RESULT	DATE	STAFF INITIALS	COMMENTS
LJ G RESULT	□Positive □ Negative			
ZN	□Positive □ Negative			
HAIN	□Positive □ Negative			
SPECIATION	□1 □2 □3 □4			

DST			
WORKUP	RESULT	DATE	STAFF INITIALS
Isoniazid	□ Sens □ Res		
Rifampicin	□ Sens □ Res		
Ethambutol	□ Sens □ Res		

Appendix IX: Sputum sample form

RECEPTION
Lab No: _____Sample ID: _____Village: _____ Unique Household ID: _____Collection Date:_____ SAMPLE TYPE: □SPOT□MORNING Initials: _____ Date: _____
MICROSCOPY FM
□ Absolute Number (1-10 Bacilli) □ + Scanty (>10 Bacilli) □ 2+ Moderate (up to 100 Bacilli) □ 3+ Heavy Date: Staff Initials:
GENEXPERT
GENEXPERT Result: □Positive □ Negative Rif Resistance:□Rif positive □Rif Negative Date: Staff Initials:

MIGIT									
Workup		Result		Date		Staff Initials	Comments		
MIGIT Result		□Positive □ Negative							
ZN		□Positive □ Negative							
HAIN		□Positive □ Negative							
SPECIATION		□1 □2 □3 □4							
LJ									
Date	W0	W1	W2	W3	W4	W5	W6	W7	W8
LJ P									
LJ G									

SUMMARY OF POSITIVE LJ P				
WORKUP	RESULT	DATE	STAFF INITIALS	COMMENTS
LJ P RESULT	□Positive □ Negative			
ZN	□Positive □ Negative			
HAIN	□Positive □ Negative			
SPECIATION	□1 □2 □3 □4			

SUMMARY OF POSITIVE LJ G				
WORKUP	RESULT	DATE	STAFF INITIALS	COMMENTS
LJ G RESULT	□Positive □ Negative			
ZN	□Positive □ Negative			
HAIN	□Positive □ Negative			
SPECIATION	□1 □2 □3 □4			

DST			
WORKUP	RESULT	DATE	STAFF INITIALS
Isoniazid	□ Sens □ Res		
Rifampicin	□ Sens □ Res		
Ethambutol	□ Sens □ Res		

Appendix X: Questionnaire

QUESTIONNAIRE

TITLE:*MOLECULAR EPIDEMIOLOGY OF MYCOBACTERIUM TUBERCULOSIS IN CAMEL AND FACTORS ASSOCIATED WITH ZOONOTIC INFECTION IN SAMBURU, KENYA*

RESPONDENT AND HOUSE HOLD DATA (Instructions: Fill required information in the spaces provided):

UNIQUE HH ID:…….NUMBER OF HH MEMBERS:……..

AGE OF RESPONDENT:……GENDER OF RESPONDENT:……..

ROLE OF RESPONDENT IN HH:……EDUCATION LEVEL OF RESPONDENT…….

LOCATION/VILLAGE NAME:……..WARD:……

SUBCOUNTY……

TUBERCULOSIS AWARENESS (Instructions: Tick appropriate choice (s) and fill the responses):

1. (i) Do you know what tuberculosis(TB) is ? [a] Yes [b] No

(ii) If yes in (i) above, state symptoms of its manifestation (List)…… …… (iv) If yes in (c) above, what is the duration of these symptoms?.....

2. Have you and your family had BCG vaccination? [a] Yes [b] No [c] Not sure

3. The cardinal symptoms of tuberculosis are; A productive cough of more than two (2) weeks duration, expulsion of blood by coughing, fever, night sweats and weight loss.Is there reason to believe your household has had previous contact with a tuberculosis case? [a] Yes [b] No

5. What is your personal HIV status [a]Positive [b] Negative [c] Unknown [d] Rather not divulge

6. Are you aware of a risk for zoonotic tuberculosis infection from camels? [a] Yes [b] No

7. In what ways do you suspect zoonotic exposure could occur? [a] Inhalation[b] Ingestion [c]Close contact

[d]Other……..

INTERACTIONS WITH CAMELS (Instructions: Tick appropriately or fill in the response):

8. (i) Are you routinely involved with handling/ restraint of camels? [a] Yes [b] No.

(ii) If yes in (i) above, with what frequency? [a] Occasionally [b] Daily

(iii) If daily in (ii) above, what is the purpose ?

:……..

(iv) Describe the method used in handling or restraint for the purpose above:…….

9. (i) Do you herd camels? [a] Yes [b] No

(ii) If yes in (i) above how often? [a]Daily [b]Occasionally

10. (i) Do you milk camels? [a] Yes [b]No

(ii) If yes above, how often [a]Daily [b] Occasionally

11. (i) Do you share sleeping area/ space with camels [a] Yes [b] No

(ii) If yes, how often [a] Daily [b] Occasionally

HOUSEHOLD INCOME (Instructions: Tick appropriately the choice (s)or fill in the response):

11. Approximate Monthly income (Ksh.) [a]Below 3000 [b] Upto 50,000 [c] Above 50,000

12. (i) Main source of income [a] Employment [b] Sale of Livestock and products [c] Family remittances [d]

Other……

13. (ii) If livestock and products in (i) above, specify which [a]Live Camels [b] Meat [c] Milk [d] hides [e] Other,
specify……
……..
CAMEL PRODUCTS CONSUMPTION (Instructions: Tick appropriately the choice (s)or fill in the response):
14. (i) Do you consume camel meat? [a] Yes [b} No
(ii) If yes above, How do you prepare camel meat for consumption?
…….
(iii) How often? [a] Frequently [b] Occasionally
15.(i) Do you consume fresh camel milk? [a] Yes [b] No
(iii) If yes above, How do you consume fresh camel milk [a] Raw [b] Boiled [c]
Other……..
(iv) How often? [a]Frequently [b] Occasionally [c]
other……..
16. (i) Do you consume fermented camel milk? [a] Yes [b] No
(ii) If yes in (i) above, is the milk boiled before fermentation? [a] Yes [b] No
(iii) How often do you consume fermented camel milk? [a]Frequently [b]Occasionally
[c]Other……

HERD LEVEL FACTORS (Instructions: Tick appropriately the choice (s)or fill in theresponse):
18. Camel Herd size [a] less than 50 [b] 50 and above.
19. Herd composition
[i] Bulls No…….. Age…….
[ii] Breeding Females No…… Age……
[iii] Juvenile females No……. Age……
[iv] Calves No…… Age…….
20. Other domestic species:
[a] Small stock (Goats and Sheep) [b] Cattle [c] Donkeys [d]
Other(s)…….
21. Type of livestock production system:
[a] Pastoral; camel, small stock. [b] Pastoral; camel and cattle. [c] Pastoral; camel, cattle, small stock. [d]Other
(specify)……
……..
22. Management :
[a] Extensive; no *boma*, routine vaccinations, tick control.
[b] Extensive; minimal *boma*, rare vaccinations and treatment, tick control rarely.
[c] Intensive; *bomas*, routine vaccination and treatment, regular tick control frequent .
[d]Other(specify)……
…….
23. Sheltering:
[a] Permanent animal Shelters [b] Traditional *bomas*[c] No elaborate shelters [d]Other
(specify)……
…….
24. If sheltered, how is Manure treated:
[a] Manure heaps[b] Burning [c] Other
(specify)……..
25. (i) Introductions into the herd [a] Purchase [b] Exchange [c] Gifts and Dowry [d] Auctions/market [e] Other(specify)……
…….
(ii) Identify origin
(source)……..

(iv) If auctions, What is the catchment [a] within county [b] neighboring counties [c] Neigbouring countries-Ethiopia, Somalia, Sudan
[d]Other……
26. (i) Movements of camels [a] Less than 50 km[b] Between 50 and 100 km[c] Over 100km
(ii) Reasons for movements [a] pasture [b] water [c] market [d] other
(iii) Destinations [a] within county [b] neighbor county [c] cross border [d] other…….
27.Contact with other herds [a] Watering [b] Pasture [c] Mass treatments [d]Other
(Specify)……
28. (i) Culling rate per year:……(Previous year)
(ii) Reasons for culling [a] age [b]debilitation [c] Injuries [d] Other
(specify)…….

SECTION G: Animal health (Instructions: Tick appropriately the choice (s)or fill in theresponse):
29. Common diseases (List in order of
priority)……
30.Veterinary care [a]self [b]CAHW [c]AHA [d] VET [e] Other
(specify)……..
31. If self, type of treatment [a] Vaccination [b] Deworming [c] Tick control [d] Other
(Specify)……
……
32. (i) During treatments do you use personal protective equipment? [a] yes [b] No
(ii) If yes, what type of protective equipment do you use?
(List)……
……

SECTION H: Marketing (Instructions: Tick appropriately the choice (s)or fill in theresponse):
33. Types of products marketed [a] live animals [b] milk [c] both [d]Other
(Specify)……
……
34. Live animals sales (Number in Previous year):……..
Sex distribution: Males (%)……..price…….Females (%)…….price…….
Age distribution:Adults(%)……Ages.……..price……..
Juveniles(%)…….Ages…….price……
Market destination……
35.Milk Quantity per day……Market destination……..Farm gate price……
36. Types of veterinary screening tests at market:
Liveanimals(List)……
……
Milk(List)……
……
WILD LIFE LEVEL FACTORS (Instructions: Tick appropriately the choice (s)or fill in the response):
37. Wildlife present [a] wild ruminants [b] wild carnivores [c] Rodents (Squirrels) [d] Non human primates
[e]others……
……
38. Potential for interaction between wildlife-livestock [a] high [b] low
39. If high, in (38) above, type of interaction [a] Watering [b] Pasture [c] Predation
[d] Other……
40.Potential of type of interaction to modulate horizontal disease transmission between wildlife-livestock[a] High [b] Negligible

Appendix XI: Genotype Mycobacterium®CM reporting form

Appendix XII: Consent forms

KENYATTA UNIVERSITY
INFORMED CONSENT FORM

Title of Project: **Molecular epidemiology of mycobacterium tuberculosis and factors associated with zoonotic infection in camels in samburu, kenya.**

Principal Investigator: Lucas L. A. Asaava (MSc.)
Participant's Printed Name:_____

***Introduction*:** We invite you / dependant to take part in a research study titled as above which seeks to determine risk factors associated with zoonotic tuberculosis infection between camels and humans, in Samburu County. Taking part in this study is entirely voluntary. We urge you to discuss any questions about this study with our field assistants. If you decide to participate, you must sign this form to show that you want to take part.
***Procedures*:** If you choose to participate, a standard questionnaire will be administered to you, in addition you may be required to give a sputum sample if it is preliminarily determined that you have a condition symptomatic of tuberculosis. For the case of your milking camels that will return a positive TST skin test, a sample of not more than 40ml of milk will be collected for laboratory testing.
***Time duration of the procedures and study*:** If you agree to take part in this study, your involvement will last approximately 20 minutes for the questionnaire. In the event, a sputum sample is required, a standard protocol will be followed, which you will be guided by our field assistants. This is expected to take an additional 15 minutes.
Discomforts and risks:The anticipated risks include nonphysical risks such as potential anxiety related to sensitive nature of some questions; tuberculin skin testing of camels and minor discomfort during the process of obtaining sputum samples. However, all efforts and precautions have been taken to mitigate this.

Potential benefits:The anticipated benefits of this study include estimation of levels of zoonotic tuberculosis in camel milk and meat, and in humans. In addition, those members of participating households suspected to have tuberculosis will have their status confirmed, and commencement of treatment where necessary.

The results of this study will guide future Tuberculosis control efforts. However, there is no direct benefit from being in this research.
***Statement of confidentiality*:** A description of this study will be available on http:///www.nacosti.go.ke, as required by the laws of Kenya. In addition, a description will be available on the Kenyatta University as well as KEMRI websites. These Web sites will not include information that can identify you. At most, the Web sites will include a summary of the results.
Privacy and Confidentiality Measures: Your research records that are reviewed, stored, and analyzed at *Kenyatta University/KEMRI* will be kept in a secured area in hard copy/secured soft copies. Your samples collected for research purposes will be labelled with code number and will be stored at KEMRI biosecurity laboratory in locked freezers with restricted access. For research records and specimens sent to overseas laboratories, you will not be identified by name, ID number, address, or phone number. The records and specimens may include code number and age. Any list that matches your name with the code number will be kept in a locked file in the principal investigators office. In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared. Some of these records could contain information that personally identifies you. Reasonable efforts will be made to keep the personal information in your research record private and confidential but absolute confidentiality cannot be guaranteed.

Research funding: This study and investigators are currently not supported for this research.
Voluntary participation: Taking part in this research study is voluntary. If you choose to take part in this research, your major responsibilities will include responding to the questionnaire and giving a sample when necessary. You do not have to participate in this research. If you choose to take part, you have the right to stop at any time. If you decide not to participate or if you decide to stop taking part in the research at a later date, there will be no penalty or loss of benefits to which you are otherwise entitled. Your investigator may take you out of the research study without your permission. Some possible reasons for this are if you do not meet the inclusion criteria. Also, the investigator may end the research study early.
Contact information for questions or concerns: You have the right to ask any questions you may have about this research. If you have questions, complaints, or concerns or believe you may have developed an injury related to this research, contact the principle investigator at 0724062512. For more information about participation in a research study and about the Scientific and ethics Review Unit (SERU), a group of people who review the research to protect your rights, please visit the KEMRI, SERU Web site at kemri.org. or contact Kenya Medical Research InstituteP.O. Box 54840 00200 Off Mbagathi Road, Nairobi, Kenya. Tel:+254 020 2713349 / +254 020 2722541 / 0722-205901.

Signature and consent/permission to be in the research: Before making the decision regarding enrolment in this research, you should have:

1. Discussed this study with an investigator
2. Reviewed the information in this form
3. Had the opportunity to ask any questions you may have.

Your signature below means that you have received this information, have asked the questions you currently have about the research, and have received answers to those questions. You will receive a copy of the signed and dated form to keep for future reference.

Participant: By signing this consent form, you indicate that you are voluntarily choosing to take part in this research.

_____	_____	_____	_____
Signature of Participant	Date	Time	Printed Name

Participant's Legally Authorized Representative: By signing below, you indicate that you give permission for the participant to take part in this research.

_____	_____	_____	_____
Signature of Participant's Legally Authorized Representative	Date	Time	Printed Name

(The signature of the participant's legally authorized representative is required for people unable to give consent for themselves).

Description of the Legally Authorized Representative's Authority to Act for Participant_____

Person Explaining the Research: Your signature below means that you have explained the research to the participant or participant representative and have answered any questions about the research.

_____	_____	_____	_____
Signature of person who explained this research	Date	Time	Printed Name

INFORMED ASSENT FORM FOR CHILDREN / MINORS

This informed assent form is for children between the ages of 7 and 16 in participating households from the study area, who are suspected to have tuberculosis. An Informed Assent Form doesnot replace a consent form signed by parents or guardians.

Principle Investigator: LUCAS L.A. ASAAVA

Organization: KENYATTA UNIVERSITY

Sponsor: NACOSTI

Name of Project: ***Molecular epidemiology of Mycobacteria tuberculosis and risk factors associated with zoonotic infection in camels in Samburu, Kenya.***

Certificate of Assent

I understand that the research is about finding out if human beings can become infected with tuberculosis through consumption of camel products. I understand that i am being asked to provide a sputum sample only because i am exhibiting signs similar to those of some one suffering from tuberculosis. I understand that this sample will be taken to a special laboratory in Nairobi for testing and that i will be informed of the result.

I have read this information (or had the information read to me) I have had my questions answered and know that I can ask questions later if I have them. I agree to take part in the research.

OR

I do not wish to take part in the research and I have not signed the assent below._____(initialled by child/minor)

Only if child assents:
Print name of child _____
Signature of child: _____
Date:_____
day/month/year

If illiterate:

A literate witness must sign (if possible, this person should be selected by the participant, not be a parent, and should have no connection to the research team). Participants who are illiterate should include their thumb print as well.

I have witnessed the accurate reading of the assent form to the child, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness (not a parent)_____ AND Thumb print of participant
Signature of witness _____
Date _____
Day/month/year

I have accurately read or witnessed the accurate reading of the assent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given assent freely.

Print name of researcher_____
Signature of researcher_____
Date_____
Day/month/year

Statement by the researcher/person taking consent
I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the child understands that the following will be done:

1. A sputum sample will be collected.
2. The result of the sputum sample will be communicated to them.

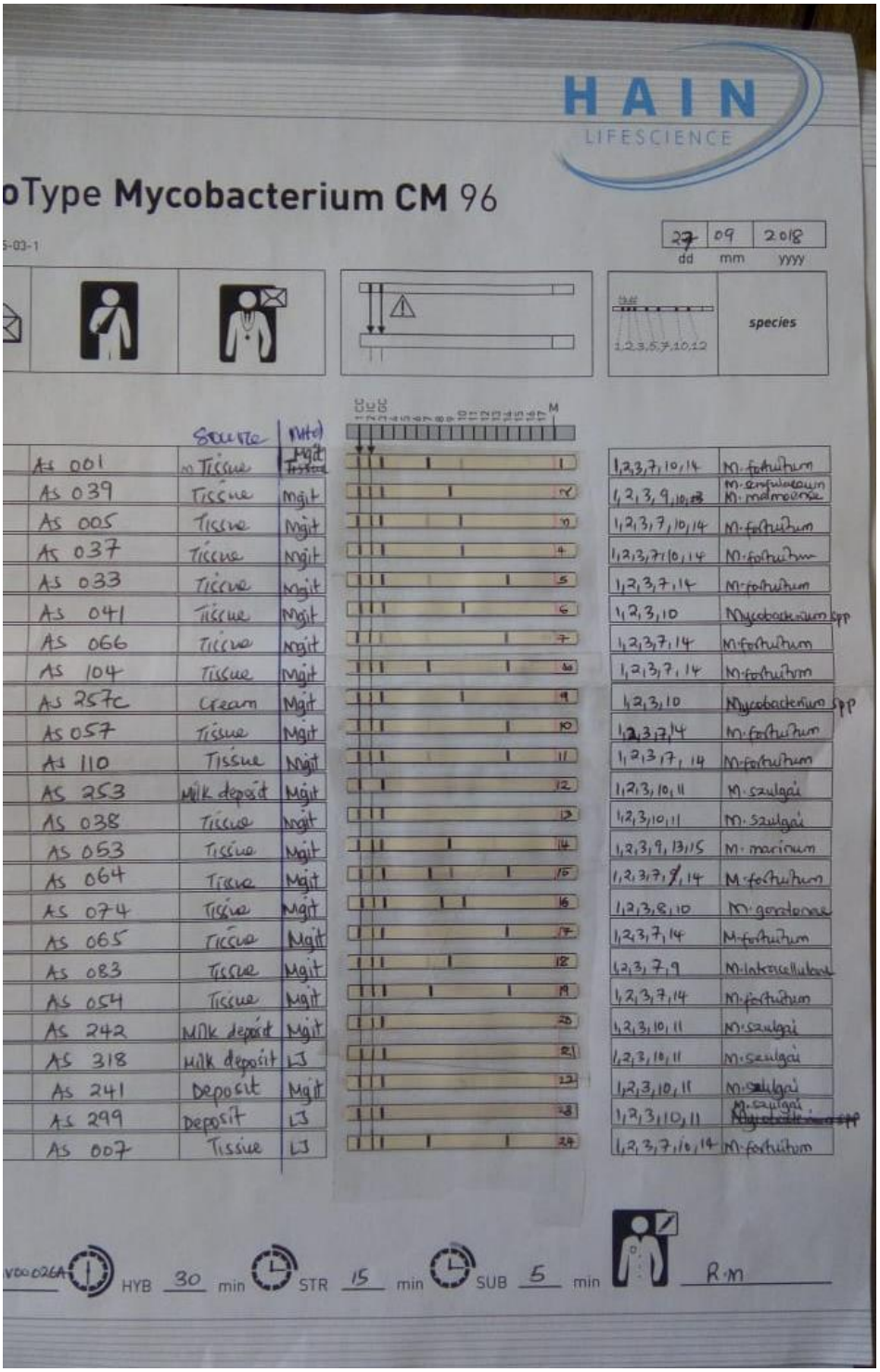
I confirm that the child was given an opportunity to ask questions about the study, and all the questions asked by him/her have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.
A copy of this assent form has been provided to the participant.
Print Name of Researcher/person taking the assent_____ Signature of Researcher /person taking the assent _____
Date _____
Day/month/year
Copy provided to the participant _____(initialed by researcher/assistant)

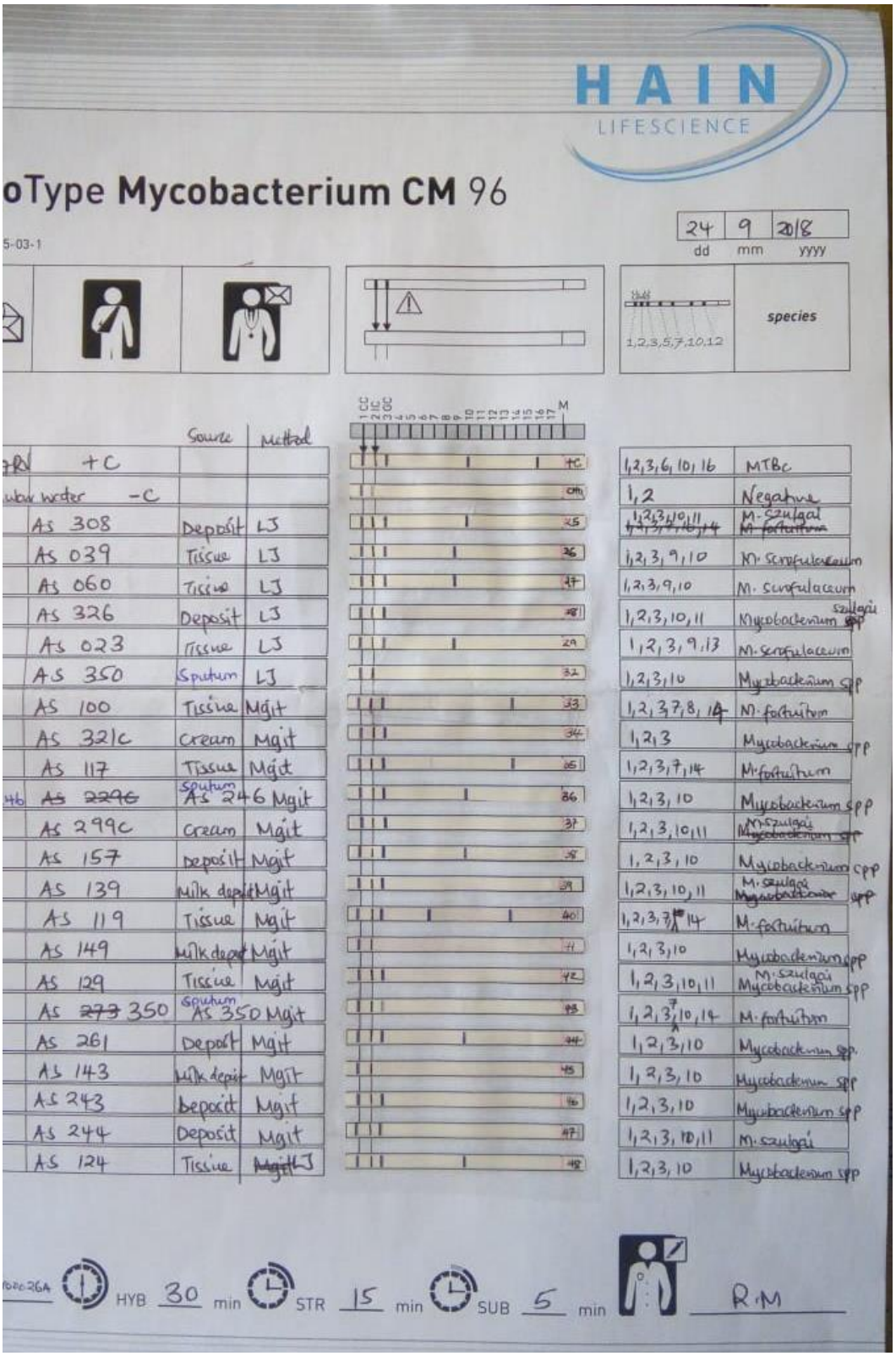
Parent/Guardian has signed an informed consent ___Yes ___No _____(initialed by researcher/assistant)

Appendix XIII: Correlation matrix

	a_variance	b_variance
a_variance	1.0000	
b_variance	0.7676	1.0000

Appendix XIV: Genotype Mycobacterium®CM results




HAIN
LIFESCIENCE
oType Mycobacterium CM 96
5-03-1
24 9 2018
dd mm yyyy
species
1,2,3,5,7,10,12
Source
Method
+C
MTBc
Negative
Deposit LJ
Tissue LJ
Tissue LJ
Deposit LJ
Tissue LJ
Sputum LJ
Tissue Mgit
Cream Mgit
Tissue Mgit
Sputum Mgit
Cream Mgit
Deposit Mgit
Milk depot Mgit
Tissue Mgit
Milk depot Mgit
Tissue Mgit
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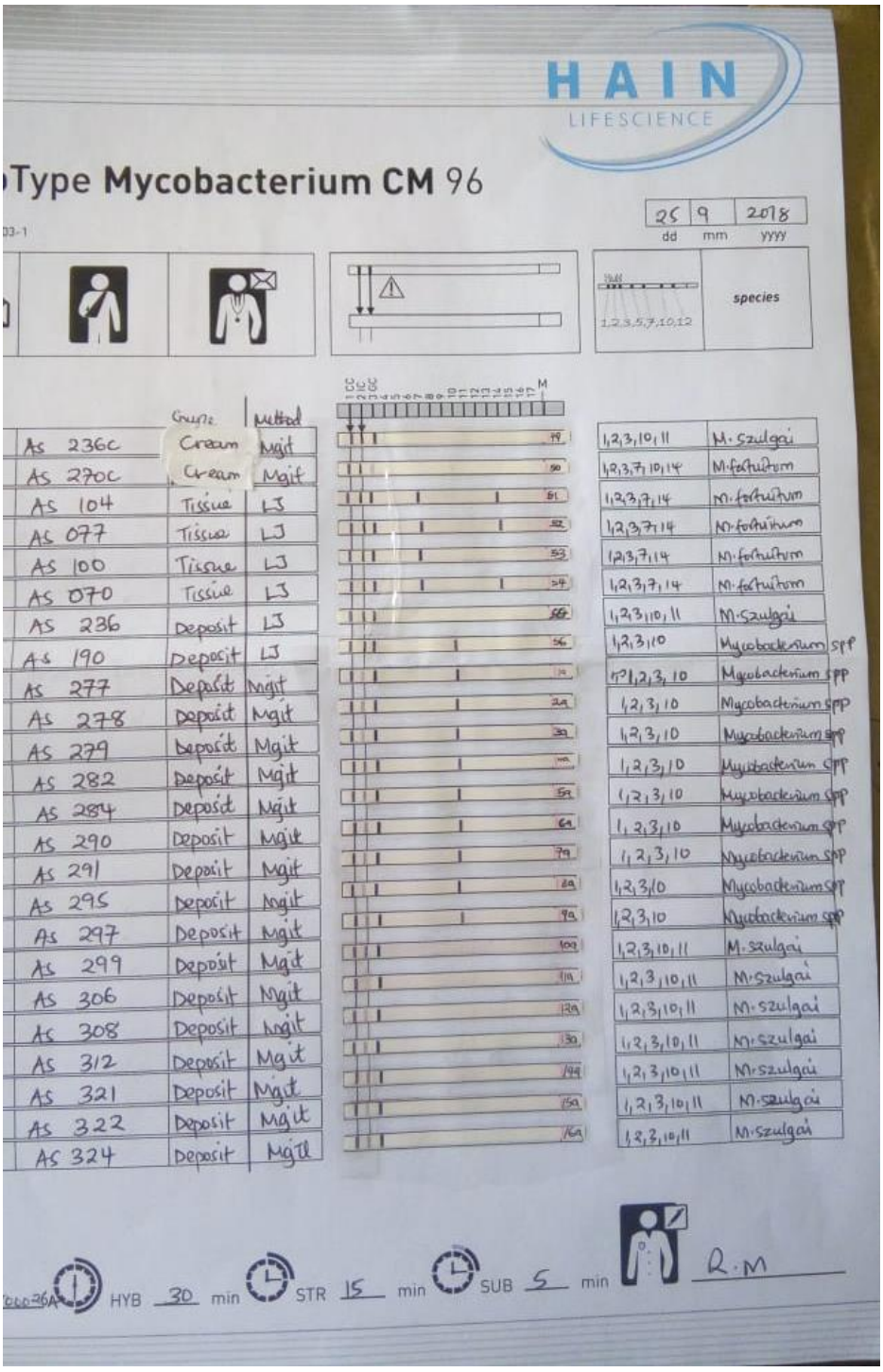
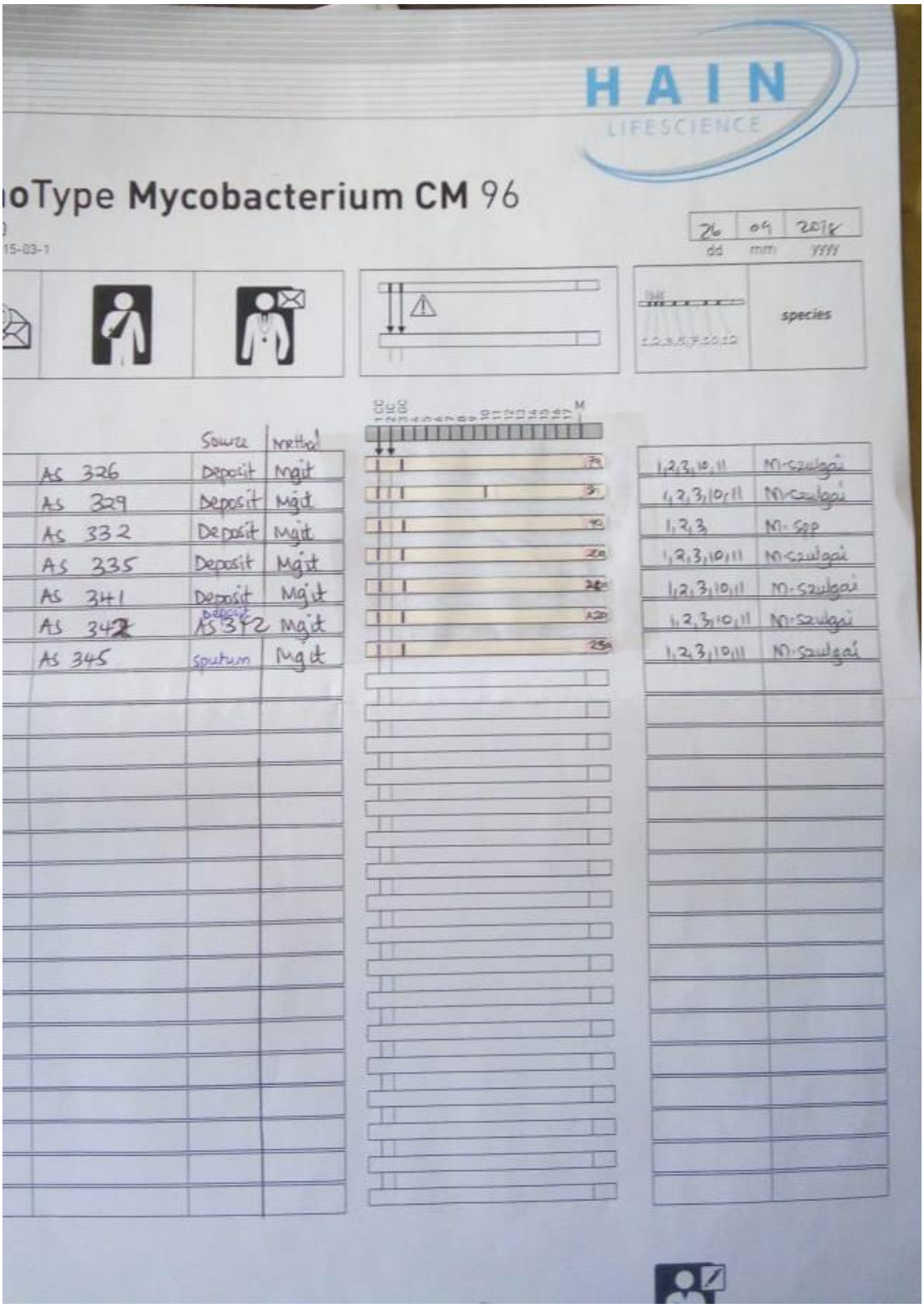
STR 15 min
SUB 5 min
R.M


HAIN
LIFESCIENCE
Type Mycobacterium CM 96
25 | 9 | 2018
dd mm yyyy
species
1,2,3,5,7,10,12
HYB 30 min
STR 15 min
SUB 5 min
R.M


HAIN
LIFESCIENCE
oType Mycobacterium CM 96
15-03-1
26 09 2018
dd mm yyyy
species
Source
Method
AS 326 Deposit Mgit
AS 329 Deposit Mgit
AS 332 Deposit Mgit
AS 335 Deposit Mgit
AS 341 Deposit Mgit
AS 342 Deposit AS 342 Mgit
AS 345 sputum Mgit
1,2,3,10,11 M. szulgai
1,2,3,10,11 M. szulgai
1,2,3 M. spp
1,2,3,10,11 M. szulgai
1,2,3,10,11 M. szulgai
1,2,3,10,11 M. szulgai
1,2,3,10,11 M. szulgai

Appendix XV: Raw sequences of 16S rDNA gene of selected isolates

GTACGTAGTAGATTTRRTCKAACSKCAGGGTGCKTCRGGGGGCTCCMGWGG
CGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGCACTTTGGGATAAGCC
TGGGAAACTGGGTCTAATACCGAATATACCCTGCTGGCCGCATGGTCTGGK
GGGGGAAAGCTTTTGCGGTGTGGGATGGGCCCGCGGCCTATCAGCTTGTTG
GTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGT
GACCGGCCACACTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGC
AGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTG
GGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGACGAAGCGCA
AGTGACGGTACCTACAGAAGAAGGACCGGCCAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGGTCCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCT
CGTAGGTGGTTTGTCGCGTTGTTCGTGAAAACCGGGGGCTTAACCCTCGGCG
TGCGGGCGATACGGGCAGACTGGAGTACTGCAGGGGAGACTGGAATTCCTG
GTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGC
GGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGTACTAGGTGT
GGGTTTCCTTCCTTGGGATCCGTGCCGTAGCTAACGCATTAAGTACCCCGCC
TGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTAC
CTGGGTTTGACATGCACAGGACGCCGGTAGAGATATCGGTTCCCTTGTGGCC
TGTGTGCAGTGGTGCATGGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGTT
AAGTCCCGCAACGAGCGCAACCCTTGTCTCATGTGGCAGCACGTGATGKTG
CGACTCGKGAGAGACTGGCTGGGGATCCACTCGGACGCAACGGTGGCA

Sequence 1: Isolate 190_FPL*M. monecense*

ARGGTAMCCGCAWAMMATGCAAGTCGAACGGAAAGGCCCCTTCGGGGGT
GCTCGAGTGGCGAACGGSTGWGTAACACGTGGGTGATCTGCCCTGCACTTT
GGGATAAGCCTGGGAAACTGGGTCTAATACCGAATACACCCTGCTGGTCGC
ATGGCCTGGTGGGGGAAAGCTTTTGCGGTGTGGGATGGGCCCGCGGCCTAT
CAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGCC
TGAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTCCTACG
GGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCG
ACGCCGCGTGGGGGATGACGGCCTTCGGGTTGTAAACCCCTTTCAGTATCG
ACGAAGCCGTAAGGTGACGGTAGGTACAGAAGAAGCACCGGCCAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTG
GGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTCCGTGAAAACTCACAG
CTTAACTGTGGGCGTGCGGGCGATACGGGCAGACTGGAGTACTGCAGGGGA
GACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACAC
CGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAG
CGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGT
GGGTACTAGGTGTGGGTTTCCTTCCTTGGGATCCGTGCCGTAGCTAACGCAT
TAAGTACCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATT
GACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGC
GAAGAACCTTTACCTGGGTTGACATGCACAGGACGCGTCTAGAGATAGGCG
TTCCCTTGTGGCCTGTGTGCAGGTGGTGCATGCTGTCGTCAGCTCGTGTCGT
GGAGATKGTGGTTTAAGTCCCCGCAACGAAGCCGCAACYCTTGTCTCATGA
GGCCAGCMCGTTGATGATGAGGACTCGTGARARACTGGCCGGGTCAMCCTC
CGGGGAGGGAAAG

Sequence 2: 321c_FPL*M. elephantis*

GTACAGTMGCTASAKTTTRATCATGSSKCARGGMSYYKYRGGRKRCTAMCM
GWGGCGAACGGGTGASTAACACGTGGGTGATCTGCCCTGCACTTTGGGATA
AGCCTGGGAAACTGGGTCTAATACCGAATATGACCACGCGCTTCATGGTGK
GTGGTGGAAAGCTTTTGCGGTGTGGGATGGGCCCGCGGCCTATCAKCTTGTT
GGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGG
TGACCGGCCACACTGGGACTGAGATACGGCCCASACTCCTACGGGAGGCAG
CAGTGGGGAATATTGCACAATGGGSGCAAGCCTGATGCAGCGACGCCGCGT
GAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAATAGGGACGAAGCGC
AAGTGACGGTACCTATAGAAGAAGGACCGGCCAACTACKTGCCAGCAGCCG
CGGTAATACGTAGGGTCCGAGCGTTGTCCGGAATTAYTGGGCGTAAAGAGC
TCGTAGGTGGTTTGTCGCGTTGTTCGTGAAAACTCACAGCTTAACTGTGGGC
GTGCGGGCGATACGGGCAGACTAGAGTACTGCAGGGGAGACTGGAATTCCT
GGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGG
CGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGA
ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGTACTAGGTG
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CTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCC
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CCTGGGTTTGACATGCACAGGACGACTGCAGAGATGTGGTTTCYCTTTGTGG
GCCTGTGTGCAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGWT
GGGGTTAAGTCCCGCAACGAGCAGCAACCCTYGTCTTCATGATGCCAGCAC
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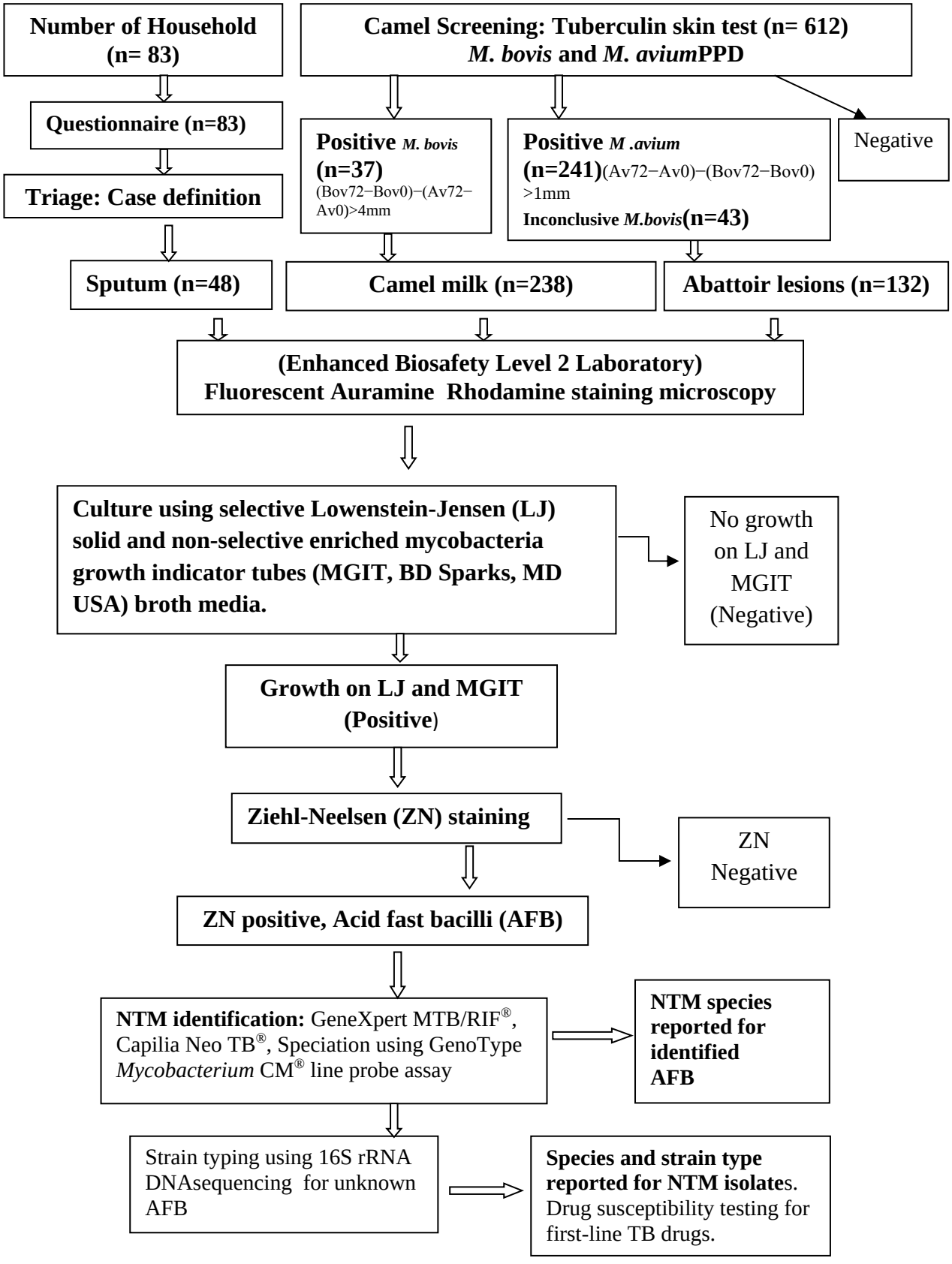
Sequence 3: 448_FPL*M. fortuitum*

Appendix XVI: Diagnostic algorithm



Appendix XVII: Culture media composition

Löwenstein-Jensen (LJ) media

1. Coagulated eggs
2. Glycerol or sodium pyruvate as source of energy
3. Malachite green
4. Asparagine
5. potato starch
6. Mineral salt solution consisting of;- potassium dihydrogen phosphate, sodium citrate and Magnesium sulfate
7. Antibiotics (nalidixic acid and penicillin).

Mycobacteria growth indicator tube (MGIT) media (modified middlebrook 7H9 broth)

1. MGIT growth supplement (containing: Oleic acid; Albumin; Dextrose; and Catalase)
2. MGIT antibiotic mixture (containing: Polymixin B; Amphotericin B; Nalidixic acid; trimethoprim; and azlocillin)
3. A fuorescence quenching-based oxygen sensor to detect mycobacterial growth

Appendix XVIII: Publications from the research

Tropical Animal Health and Production
https://doi.org/10.1007/s11250-019-02054-2

REGULAR ARTICLES

A cross-sectional epidemiological investigation of nontuberculous mycobacteria of public health importance in slaughter camels in Samburu County, Kenya

Lucas Luvai A. Asaava[1] · Michael M. Gicheru[1] · Moses Mwangi[2] · Edwin Mwangi[3] · Ernest Juma[3] · Ruth Moraa[3] · Adan Halakhe[1] · Willie Abela Githui[3]

Received: 28 May 2019 / Accepted: 19 August 2019

Abstract
The diagnosis of tuberculosis (TB) in camels at slaughter houses heavily relies on post mortem (PM) meat inspection to detect granulomatous lesions; however, the sensitivity of this technique is not perfect. The objective of this study was to isolate and characterize mycobacteria associated with suspect TB pathological lesions at PM. At PM, 1600 camels were examined in two county slaughterhouses. One hundred and thirty two, 8.25% (132/1600) (Binomial CI 95% 6.95–9.71%), suspect granulomatous lesions were found. Twenty seven, 1.69% (27/1600) (Binomial CI 95% 1.11–2.45%), were confirmed as acid-fast bacilli (AFB) using Ziehl-Neelsen (ZN) staining after culture. Speciation using the GenoType® Mycobacterium assay (Hain Lifesciences, Nehren, Germany) found a majority isolates to be *Mycobacterium fortuitum* (17), the other species identified included *M. szulgai* (2), *M. scrofulaceum* (3), *M. marinum* (1), *M. intracellulare* (1), *M. gordonae* (1), and 2 unidentified mycobacteria species. The types of lesions observed were nodular, caseous masses involving whole organs or cavities, and purulent masses. The highest proportion of suspect lesions were observed in the right, left bronchial lymph nodes, and the mediastinal lymph nodes (59.54%), followed by the retropharyngeal lymph nodes (12.21%), the medial lobe (10.67%), and the left lateral and quadrate lobes of the lungs (17.58%). The 6–7 age category had higher odds (OR = 2.5) of culture positivity. It was concluded that a variety of NTM species of medical importance were associated with TB lesions in the thoracic lymph nodes and lungs. There is need to unravel the public health significance of these mycobacteria.

Keywords Zoonotic mycobacteria · Meat inspection · Camels · Culture

Introduction

There are approximately 25 million dromedary camels (*camellus dromedaries*) worldwide, 44% of which are found in East Africa, with a potential of global market of camel products of 10 billion USD. In Kenya, as at 2017 the camel population was estimated at approximately three million heads mostly important for milk, meat, and hides (FAOSTAT 2017). The breeds of Kenyan dromedaries based on geographical locations and socio-ethnological classification include the Somali, Gabbra, Rendile, and Turkana breeds (Mburu et al. 2002). In pastoral settings similar to the study area, TB is ranked as one of the most common diseases in humans and camels (Lamuka et al. 2017). Movement restriction of camels and camel products due to TB has economic implications such as denying incomes to the camel-rearing communities and foreign

This article belongs to the Topical Collection: Camelids
Guest Editor: Bernard Faye

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[2] Kenya Medical Research Institute (KEMRI) Center for Public Health Research (CPHR), P.O.Box 54840-00200, Kenyatta National Hospital Grounds, Nairobi, Kenya

[3] Kenya Medical Research Institute (KEMRI) Center for Respiratory Diseases Research (CRDR), P.O. Box 54840-00200, Kenyatta National Hospital Grounds, Nairobi, Kenya

Published online: 26 August 2019

Received: 14 October 2019 | Revised: 29 May 2020 | Accepted: 16 June 2020

DOI: 10.1111/zph.12754

ORIGINAL ARTICLE

WILEY

Isolation, identification and associated risk factors of non-tuberculous mycobacteria infection in humans and dromedary camels in Samburu County, Kenya

Lucas L. A. Asaava[1] | Willie A. Githui[2] | Moses Mwangi[3] | Edwin Mwangi[2] | Ernest Juma[2] | Ruth Moraa[2] | Adan Halakhe[1] | Michael M. Gicheru[1]

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Emails: asaava.lucas@ku.ac.ke; azaale@gmail.com

Funding information
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Abstract

Non-tuberculous mycobacteria are of public health significance, and zoonotic infection is attributed to the sociocultural practice of consumption of raw milk and the close human–livestock contact in pastoral communities. This study aimed at isolation, identification of mycobacteria from human sputum and camel milk and risk factors assessment in Samburu East, Kenya. Six hundred and twelve camels and 48 people presumed to have tuberculosis (TB) from 86 households in Wamba and Waso regions were screened. Camels were categorized into Somali, Turkana and Rendile breeds. Single intradermal comparative tuberculin test (SICTT) was used as a herd-screening test on lactating camels and a milk sample collected from reactive camels. Sputum samples were collected from eligible members of participating households. A standard questionnaire on possible risk factors for both humans and camels was administered to respective household heads or their representatives. Total camel skin test reactors were 238/612 (38.9%). Milk and sputum samples were analysed at KEMRI/TB research laboratory for microscopy, GeneXpert®, culture and identification. Isolates were identified using 16S rRNA gene sequencing at Inqaba biotec in South Africa. Sixty-four isolates were acid-fast bacilli (AFB) positive of which *M. fortuitum* (3), *M. szulgai* (20), *M. monacense* (5), *M. lehmanni* (4), *M. litorale* (4), *M. elephantis* (3), *M. duvalii* (3), *M. brasiliensis* (1), *M. arcueilense* (1) and *M. lentiflavum* (1) were from milk; *M. fortuitum* (1), *M. szulgai* (2) and *M. litorale* (1) were from humans. Risk factors included the following: Turkana breed (OR = 3.4; 95% CI: 1.2–9.3), replacements from outside the County (OR = 2.1; 95% CI: 0.3–12.3), presence of other domestic species (small stock; OR = 4.6) and replacement from within the herd (OR = 3.2; 95% CI: 0.7–14.7). Zoonotic risk practices included raw milk consumption, shared housing and handling camels. Monitoring of zoonotic NTM through surveillance and notification systems is required.

KEYWORDS
dromedary camel, epidemiology, Kenya, milk, non-tuberculous mycobacteria, zoonosis

Appendix XIX: Conferences

International food safety conference, May, 2019

ST2-5-005S: Zoonotic Non-tuberculous Mycobacteria isolated in dromedary camel milk and sputum of associated household members in Samburu East, Kenya.

Lucas Luvai Azaale Asaava[a,*] Willie Abela Githui[b], Moses Mwangi[c], Edwin Mwangi[d], Ernest Juma[e], Ruth Moraa[f], Adan Halakhe[g] and Michael M. Gicheru[h]
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Background: Nontuberculous Mycobacteria (NTM) are recognized as important human pathogens, in addition, they confound diagnosis and therapy of tuberculosis due to *Mycobacterium tuberculosis* (MTB). NTM infection may occur through ingestion, inhalation or dermal contact. Among the species of zoonotic importance from camelids are *M. chelonae*, *M. fortuitum*, *M. marinum*, *M. scrofulaceum*, *M. xenopi*, *M. simiae*, *M. szulgai*, *M. kansasii*, *M. leprae* and *M. avium*. The aim of this study was to identify the Mycobacteria from camel milk and sputum of associated household members and assess risk factors for infection in Samburu East, Kenya. **Methodology:** A 30-40ml composite milk sample was collected in sterile 50ml falcon tubes from all camels classified as tuberculin skin test positive or inconclusive according the *Office International des Epizooties* (OIE) criteria. Sputum was obtained from members of participating households with TB case definition for tuberculosis. All samples were submitted to KEMRI/CRDR for mycobacteriology, confirmatory culture, speciation and strain typing using HAIN® line probe assay. A standard questionnaire was administered to participating households. **Results:** A total number of 226 camel milk samples were collected, 36/226 (15.9%) showed the presence of acid fast bacilli (AFB) on culture after 8 weeks. Of the human sputum samples, 3/48 (6.25%) showed presence of AFB on culture after 8 weeks. The species identified using HAIN® CM assay from milk culture isolates were *M. fortuitum* (1), *M. szulgai* (19), and 17 unknown *Mycobacteria* species. *Mycobacterium fortuitum* (1), *M. szulgai* (1) and one unknown *Mycobacteria* species were identified from sputum. Turkana breed of camel (OR=3.4; 95% CI: 1.2 – 9.3), source of new introductions; Outside the county (OR=2.1; 95% CI: 0.3-12.3) and; No introductions (OR=3.2; 95% CI: 0.7 – 14.7) were found to be strongly associated with camel milk culture positivity. **Conclusion:** Preliminary data shows that NTM exist in both camel milk and sputum of household members suggesting cross transmission due to habitual consumption of raw unpasteurized camel milk among Samburu community.

Keywords: zoonotic, Nontuberculous *Mycobacteria*, dromedary camel, camel milk, human sputum, risk factors, Samburu, Kenya

ST2-5-006S: Isolation Of Listeria Species In Milk And Meat Products In Nairobi And Its Environs And The Implication In Food Safety

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Background: Listeriosis, caused by *Listeria monocytogenes*, is among the most important foodborne diseases worldwide. The disease is said to be the leading cause of death in reported cases of food poisoning with mortality rates of between 30%-50%. **Objective:** The aim of the study was to determine the occurrence of Listeria species, specifically *Listeria monocytogenes* in milk and meat products sold in retail markets in the study area. **Methodology:** The study was conducted in the Nairobi Metropolitan region where 350 milk and 220 meat products were collected from selected retail markets. Isolation and identification was done as per the FDA/BAM method. DNA from identified colonies was extracted and multiplex PCR done to identify the genus and species of Listeria. **Results:** Out of the 570 samples, 8.59% tested positive for Listeria species. Twenty one (42.8%) isolates were from milk products namely; milk powder (5.8%), short life milk (1.5%), long life milk (4.83%) and milk from dispensing machines (80%). The rest, (57.2%) were obtained from meat products namely; ham (5.4%), brawn (17.8%), polony (29.6%), salami (16.7%) and ready to eat meat (5.19%). Speciation of the isolates confirmed 22 as *L. monocytogenes* (3.86%), a majority of which (77.27%) were from milk products while the rest (22.72%) were from meat products. The highest prevalence, 68.18 %, was from dispensed milk while the lowest, 4.54%, was from short life milk, long life milk and ham. Of the other 27 Listeria isolates, two were identified as *L. welshimeri* while three were identified as *L. innocua*. The rest were unidentified Listeria. **Conclusion:** These results have far reaching

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ISOLATION OF ZOONOTIC NON TUBERCULOUS MYCOBACTERIA AMONG DROMEDARY CAMELS AND HOUSEHOLD MEMBERS IN SAMBURU COUNTY, KENYA

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Background: Zoonotic nontuberculous Mycobacteria (NTM) infect a wide range of domestic animals wildlife and man causing various diseases. The aim of this study was to isolate and identify the Mycobacteria from camels and associated household members and to assess zoonotic risk in Samburu East, Kenya.
Materials and methods: This was a cross sectional, one-health approach study involving lactating camels (n=611) from 83 households and slaughtered camels (n=1600). A semi-structured questionnaire was administered to each of the household and symptomatic household members (n=48) were identified. All samples were analysed at KEMRI/CRDR enhanced BSL2 laboratory.

Results: Fifty five, 23.1% (55/238) camel milk samples were AFB positive after culture. One hundred and thirty two, 8.25% (132/1600) suspect granulomatous lesions were found. Twenty seven, 1.69% (27/1600) were AFB positive after culture. Seven, 14.58% (7/48) human sputum samples were AFB positive and included M. fortuitum (1), M. szulgai (2) and four unknown Mycobacteria species. A majority of the NTM isolates were

found to be M. fortuitum (62.9%) in post mortem lesions and M. szulgai (52.7%) in milk, the other species identified include M. scrofulaceum (11%), M. marinum (3.7%), M. intracellulare (3.7%), M. gordonae (3.7%). The MIC (µg/mL) for ethambutol, rifampicin and isoniazid was 1->8, 1->64 and >1 respectively. Higher odds were found for culture positivity among camel breed (OR=3.4) and source of replacements of camels (OR=2.1, 3.2).

Conclusions: A variety of NTM were isolated from lymph nodes of the thoracic cavity. The interaction between camels and humans and consumption of raw camel milk explains the similarity of isolates and drug susceptibility. It is important to note that isolates were highly resistant to first-line TB drugs.

Keywords: Nontuberculous Mycobacteria, dromedary camel, epidemiology, Kenya