

**EPIDEMIOLOGICAL STUDIES OF *MYCOBACTERIUM* SPECIES IN
SLAUGHTER CATTLE AND CONTAMINATIONS OF THE SLAUGHTER
FACILITIES ENVIRONMENT IN IRINGA MUNICIPALITY, TANZANIA**

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**A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
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ABSTRACT

This cross sectional study was carried out between August and December 2015 in order to determine the presence of Mycobacterial infections in slaughter cattle and assess the occurrence of Mycobacteria in the slaughter facility environment in Iringa Municipality, Tanzania. A total of 3817 slaughter cattle were examined tuberculous like lesions in lymph nodes, lungs and other visceral organs. Tissues with suspect lesions were collected for laboratory analysis. Effluent samples in the slaughter facility environment were collected early and late in the slaughter process in order to determine environmental contamination with Mycobacteria. Tissue samples were cultured using standard laboratory procedures and Mycobacterial species were identified using a multiplex PCR. Of the carcasses examined, 35 (0.9%) had lesions suggestive of tuberculosis. Ten of the 35(28.6%) samples culture positive and 1/10 (10%) was confirmed as *M. bovis*. The remaining 9 (90%) of isolates were non-tuberculous Mycobacteria (NTMs) which were identified as *M. lentiflavum* (3), *M. intracellulare/M. indicuspranii* (2), *M. lentiflavum/palustre/simiae*, (2) *M. goodii* (1) and *M. smegmatis* (1). Mycobacteria were detected in 25/172 (14.5%) of effluents samples. Five of the positive (20%) effluent samples were confirmed to be *M. bovis* and 20 (80%) were *M. kansasii*. This demonstrates that *Mycobacteria* are prevalent in slaughter cattle in Iringa Municipality, which is a public health concern. Notably, the majority of lesions were caused by NTM's. This study is the first to detect Mycobacteria in slaughter facility effluents in Tanzania. Routine disinfection of slaughter facility environment will minimize chances for persistent contamination. Further epidemiologic studies to establish the role of NTM's in clinical livestock disease are recommended. Studies on the magnitude of environmental contamination due to discharges of untreated slaughter facility effluents are also important.

DECLARATION

I, **Goodluck Charles Paul**, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

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LIST OF ABBREVIATIONS

BTB	Bovine Tuberculosis
DNA	Deoxyribose Nucleic Acid
HALI	Health for Animals and Livelihood Improvement
HIV/AIDS	Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome
L-J	Lowenstein- Jensen
<i>M.tb</i>	<i>Mycobacterium tuberculosis</i>
MAC	<i>Mycobacterium Avium Complex</i>
<i>MTBC</i>	<i>Mycobacteria Tuberculosis Complex</i>
NBS	National Bureau of Statistics
<i>NTM</i>	<i>Non Tuberculous Mycobacteria</i>
OIE	Office International des Epizootics (World Organisation for Animal Health)
PCR	Polymerase Chain Reaction
PHCT	Population and Housing Census of Tanzania
qPCR	Quantitative Polymerase Chain Reaction
RD	Region of Deletion
SCITT -	Single Comparative Intradermal Tuberculin Test
UCD	University of California Davis
UWAR	University of Warwick
ZN	Ziehl-Neelsen

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Tuberculosis (TB) is a contagious disease of both animals and humans. It is caused by genetically related bacteria in the *Mycobacterium tuberculosis* complex (MTBC) including *Mycobacterium bovis* and *M. tuberculosis*. *Mycobacterium tuberculosis* is the primary cause of human tuberculosis, whereas *M. bovis* causes bovine tuberculosis. Bovine tuberculosis (BTB) is an important disease of livestock and an emerging disease of humans in developing countries (Cleaveland *et al.*, 2007; Berg *et al.*, 2009). Bovine tuberculosis infection in humans mostly occurs in rural and peri-urban areas where animals and humans share the same environment (Cosivi *et al.*, 1998; Shiteye, 2007). Common practices of pastoral and agro-pastoral communities living in such areas include consumption of raw blood, milk and undercooked meat, increase the risk of exposure to *M. bovis* (Mfinanga *et al.*, 2003; Cleaveland *et al.*, 2007).

In Tanzanian livestock, the occurrence of BTB varies with location and production system. Prevalence ranges between 0 and 13.2% has been reported in cattle (Kazwala *et al.*, 2001; Shirima *et al.*, 2004; Durnez *et al.*, 2009; Swai *et al.*, 2012; Mwakapuja *et al.*, 2013). A study conducted by the Health for Animals and Livelihood Improvements (HALI) project reported 2% individual cattle prevalence and 18% herd level prevalence in cattle in Iringa rural district (HALI project, unpublished data) and also identified BTB in several wildlife species from the same ecosystem (Clifford *et al.*, 2013). In humans, TB is a common opportunistic infection particularly in immune compromised individuals such as HIV/AIDS patients (Raviglione *et al.*, 1995). Although human and animal TB is

frequently caused by bacteria in the *Mycobacterium tuberculosis* complex (MTBC), non tuberculosis Mycobacterial (NTM) species have been isolated in TB patients and from TB suspect lesions from cattle (Mbugi *et al.*, 2014). Therefore, the public health importance of NTM in humans is increasingly being recognized (Griffith *et al.*, 2007). NTM that have been isolated from TB patients and TB suspect lesions in slaughtered cattle include *Mycobacterium intracellulare*, *M. lentiflavum* and *M. fortuitum* (Durnez *et al.*, 2011; Palmer *et al.*, 2011; Shojaei *et al.*, 2011; Katale *et al.*, 2014).

Bovine tuberculosis is endemic in Tanzania, including in Iringa Region (Mwakapuja *et al.*, 2013). Currently there is no official screening and control program for the disease in cattle owned by traditional and commercial livestock keepers. In commercial or dairy farm herds, BTB screening is carried out to obtain BTB-free certification status at the expense of the dairy cattle owners, a cost that is prohibitive for most traditional cattle keepers. Without a BTB screening program, the cattle owner may unknowingly sell and buy infected cattle, and the only method for TB surveillance is identification of gross TB lesions at slaughter facilities during meat inspection.

Infection with zoonotic diseases at slaughter facilities, including BTB, is considered an occupational risk for slaughter facility workers and veterinarians (Swai *et al.*, 2012). The slaughter and meat inspection process can directly expose humans to BTB (Swai *et al.*, 2012). Infected cattle may shed *M. bovis* in feces and milk (Srivastava *et al.*, 2008) and contaminate the environment, which could pose an important risk for further transmission of the infection to humans and animals. In addition, most slaughter facilities lack effective sterilization protocols. For instance, contaminated knives may potentially carry zoonotic pathogens including *M. bovis* from an infected to an uninfected carcass during routine

meat inspection. This study used culture and molecular methods to determine the occurrences of MTBC and NTM's in TB suspect lesions collected during meat inspection from cattle as well as in the slaughter facilities environment in Iringa, Tanzania. The use of slaughter facility environmental sampling approach to assess the risk for human infection and carcass contamination has not been evaluated to date. Sequencing was used to establish genotypic profiles of the *Mycobacteria* isolates from the cattle tissue and environmental samples within and across slaughter facilities.

1.2 Problem Statement and Justification

In Tanzania, studies conducted in slaughtered and live cattle have revealed that BTB is widespread and prevalence varies by region and production system (Kazwala *et al.*, 2001; Durnez *et al.*, 2009; Mellau *et al.*, 2010; Swai *et al.*, 2012; Mwakapuja *et al.*, 2013). Disease may be subclinical or clinical and the sizes of the lesions vary with stage of infection (O'Reilly *et al.*, 1995). Further, *M. bovis* can be shed in feces and milk, which contributes to environmental contamination and zoonotic transmission (Srivastava *et al.*, 2008).

Limited veterinary services and, possibly, lack of extension services results in sending infected animals to slaughter facilities. It is recommended that all animals sent for slaughter should be screened for diseases such as BTB at the farm level and reactors culled. This would minimize the risk of infected carcasses entering the human food chain as well as the risk of environmental contamination of the slaughter facility environment. Further, it would reduce the risk of zoonotic infection of slaughter facility workers. Unfortunately, resources to implement such screening programs are not available in most areas of Tanzania. Furthermore, many slaughter facilities have open drainage systems and

no means of waste treatment and therefore, drainage systems serves as potential sources of human exposure. There is also a tendency of local communities around the slaughter facilities to collect condemned materials, clotted blood, and gastrointestinal contents, which they use for different purposes like animal feed and manure. This study determined the prevalence of MTBC and NTM in TB suspect lesions of slaughtered cattle and whether or not MTBC and NTM were present in slaughter facility environmental samples. The results of this study can be used for formulating recommendations for carcasses handling and disinfection protocols, slaughter facility effluent drainage management and restructuring of the layout of slaughter facilities, with the ultimate goal of protecting slaughter facility workers and local communities from zoonotic diseases like tuberculosis and improving food safety.

1.3 Research Objectives

1.3.1 General objective

1.3.1.1 Epidemiological studies of *Mycobacterium* infections in cattle and assess environmental contamination of slaughter facilities in Iringa Municipality, Tanzania.

1.3.2 Specific objectives

- 1.3.2.1 To determine the presence of MTBC and NTM from cattle tissue samples from selected slaughter facilities.
- 1.3.2.3 To determine the occurrence of MTBC and NTM in slaughter facility effluents from floor drains.
- 1.3.2.4 To establish a genotyping profile of *Mycobacterium* species isolated from slaughter cattle and the slaughter facility environmental effluents.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Definition and Aetiology

Bovine tuberculosis is an infectious disease caused by *Mycobacterium bovis* and characterized by the formation of granulomatous lesions (tubercles) classically seen in the lungs and lymph nodes (Berg *et al.*, 2009). *M. bovis*, which is a member of the MTBC, is the primary cause of BTB in animals (Phillips *et al.*, 2001; Delahay *et al.*, 2002). Other members of the MTBC include *M. tuberculosis* (the causative agent of human TB), *M. africanum*, *M. canettii*, *M. microti*, *M. caprae*, *M. pinnipedii*, *M. suricattae* and *M. mungi* (Brosch *et al.*, 2002). Bovine tuberculosis can be transmitted from cattle to humans, mainly through consumption of contaminated milk and meat products (Kazwala *et al.*, 2001). Because of the routes of infection, the disease often manifests as extra pulmonary TB in people (WHO, 2006; Berg *et al.*, 2009).

2.2 Epidemiology

2.2.1 Prevalence of bovine tuberculosis in cattle in Tanzania

Several studies in Tanzania have reported the prevalence of BTB in cattle. In pastoral livestock systems, studies have reported individual cattle prevalence of 3.6 - 3.7% in the Morogoro region (Durnez *et al.*, 2009; Mwakapuja *et al.*, 2013) and 0-1% in the Tanga region (Shirima *et al.*, 2003; Swai *et al.*, 2012).

In smallholder dairy farms, studies have reported prevalence of up to 2% in Tanga (Swai *et al.*, 2012), 0.9% in Dar es Salaam, and 0.4 - 1.7% in Kibaha and Morogoro (Durnez *et al.*, 2009). In the southern highland zones of Tanzania including Iringa, Kazwala *et al.* (2001) reported a 13.2% prevalence of BTB in indigenous cattle. Studies in slaughterhouses in Morogoro, Tanga and Dar es Salaam detected tuberculous lung lesions in 1.9%, 0.3% and 0.7% of slaughtered cattle respectively (Mellau *et al.*, 2010; Swai *et al.*, 2012).

2.2.2 Disease prevalence in humans

Tuberculosis is a global problem with an estimated 9.6 million new human cases and 1.4 million deaths in 2014 (WHO, 2015). The estimated incidence rate for all forms of TB cases in Tanzanian residences was 155-561 cases per 100 000 populations in 2014 (WHO, 2015). The majority of human TB cases are caused by *Mycobacterium tuberculosis*, but BTB is increasingly being recognized as a public health problem (WHO, 2015). A concern regarding *M. bovis* infection in humans is that the bacterium is naturally resistant to pyrazinamide, one of the four first line antibiotics used to treat human tuberculosis (de la Rúa-Domenech, 2006).

Several studies in Tanzania have isolated *M. bovis* from patients with cervical adenitis. For example Kazwala *et al.* (2001) identified *M. bovis* in 7/44 (15.9%) of mycobacterial isolates from patients with tuberculosis in the Arusha region, and the majority of these patients had extra pulmonary lesions (Kazwala *et al.*, 2001a). Another study from the same region detected *M. bovis* in 7/457 (1.5%) patients with clinical mycobacterial adenitis (Mfinanga *et al.*, 2004). Similarly, a study in the Manyara region of Tanzania isolated *M. bovis* from 7/60 (11.7%) cervical adenitis cases (Cleaveland *et al.*, 2007).

Drinking raw milk, or eating undercooked meat from an infected animal, poor household ventilation, HIV infection and close contact with livestock have been identified as the most common risk factors for human infection with BTB in human (Mfinanga *et al.*, 2004; de la Rúa-Domenech, 2006; Fetene *et al.*, 2011). Limited awareness about these risks may contribute to the disease spread. For example, Mfinanga *et al.* (2003b) interviewed 426 family members from more than 5 tribal groups in the Arusha area and found that 40% of the people practiced behaviors that increased the risk of contracting BTB, such as drinking raw milk or eating undercooked meat and 75% had poor knowledge about the disease.

2.2.3 Disease transmission

Bovine tuberculosis is primarily transmitted among cattle through respiratory infection (O'Reilly *et al.*, 1995; Menzies *et al.*, 2000). Invisible droplets containing *M. bovis* may be exhaled or coughed by infected animals and then inhaled by other animals (Menzies *et al.*, 2000). Infected cattle may also shed the *M. bovis* in feces (Srivastava *et al.*, 2008) and contamination of pastures and water sources may provide an additional route of disease transmission (Cleaveland *et al.*, 2007). Human infection with bovine tuberculosis mainly occurs through consumption of contaminated or raw milk and eating undercooked meat from infected animals (Kazwala *et al.*, 2001). The disease in humans often manifests itself as extra pulmonary infections in the cervical lymph nodes leading to TB adenitis (Kazwala *et al.*, 2006; Cleaveland *et al.*, 2007; Berg *et al.*, 2009).

2.3 Diagnosis of tuberculosis in live animals

2.3.1 Clinical signs

Bovine tuberculosis is very difficult to diagnose, because clinical signs are non-specific and thus difficult to differentiate from other disease conditions such as trypanosomosis,

paratuberculosis, starvation, contagious bovine pleuropneumonia (CBPP), chronic metritis and the effect of age. The clinical signs in severely affected animals may include low grade and intermittent fever, loss of body condition, coughing, and swelling of peripheral superficial lymph nodes (Radostits *et al.*, 1994). Tuberculosis is commonly diagnosed through tuberculin testing and serological methods in live animals. Lately, new diagnostic techniques such as serology and molecular biology have been developed such as Multi-Antigen Print Immuno Assay (MAPIA), the fluorescence polarization assay, a rapid immunochromatographic test, a 96-well multiplex system, a dual path platform assay, a chemiluminescent platform, an improved ELISA, an interferon gamma test and qPCR (Schiller *et al.*, 2010; OIE, 2016).

2.3.2 Tuberculin skin test (TST) method

The tuberculin skin test is regarded as the standard method for detection of bovine tuberculosis in live animals (OIE, 2016). The test involves measuring the thickness of a shaved area of the skin on the neck or tail fold with a vernier caliper (Hauptner Herberholz, Germany), followed by intradermal injection of bovine tuberculin purified protein derivative (PPD, Veterinary Laboratories Agency, Weybridge, UK) into the measured area, remeasuring the skin fold is performed after 72 hours to detect swelling due to a delayed hypersensitivity reaction (Monaghan *et al.*, 1994). TST test has been used for ante-mortem diagnosis of latent and active TB infection in animals, for test and slaughter programs as well as TB eradication campaigns (OIE, 2009), however, false negative results have been reported. Several factors can lead to false negative results, including testing newly infected (3-6 weeks) or immune suppressed animals; using low potency, expired, or inappropriately stored PPDs; using incorrect PPD doses or injection sites, or if different persons take the first and second skin measurement (Rua-Demenech

et al., 2006; Humblet *et al.*, 2011). The test has a sensitivity and specificity that ranges between 96-98.8% and 68-95%, respectively (Monaghan *et al.*, 1994).

2.3.3 Interferon gamma test (IFN- γ)

The interferon gamma test is an in vitro cellular assay for bovine tuberculosis (Wood *et al.*, 2001). The test can be used in parallel with the intradermal tuberculin test to allow for detection of a greater number of infected animals before they become a source of infection for other animals as well as a source of contamination of the environment (Gormley *et al.*, 2006). The test method measures the concentration of interferon-gamma (IFN- γ), which is released by specific T-cells due to TB infection (Rose *et al.*, 2012). There are little cross-reactions with other diseases (Rose *et al.*, 2012). Many countries are using the IFN- γ assay as a serial test to the skin test in order to increase test specificity (Schiller *et al.*, 2009).

2.4 Diagnosis of Tuberculosis in slaughtered animals

2.4.1 Post-mortem examination/inspection

Post-mortem examination of slaughtered animals involves visual inspection under light, hand palpation and incision of the organs and tissues to look for the presence of tuberculous lesions or granulomas. The lesions are characterized as thick greenish yellow exudates, cheesy masses and/or tubercles of various sizes and numbers (Corner, 1994). During the examination, particular emphasis is given to tissues/organs such as lymph nodes, lungs, liver and kidneys. Tissues and/or organs with suspect pathological changes are then submitted for culture in order to confirm the disease. Visual inspection as a detection method requires trained personnel who know how to identify the typical TB lesions.

2.5 Laboratory Tests

2.5.1 Bacteriology

Suspect TB lesions collected from slaughtered animals are cultured on Lowenstein-Jensen (L-J) media with glycerol or pyruvate for the purpose of isolation of *Mycobacterial* species as described by Wood *et al.* (1992). Growth of *Mycobacteria* is confirmed through demonstrations of Acid-fast bacteria by Ziehl Neelsen (ZN) staining and microscopic examination as described by Neill *et al.* (1994). The method is regarded as a definitive diagnosis for TB in animals (OIE, 2016). However, when using culture due to slow growth nature of *Mycobacteria* it takes about 6-8 weeks to confirm the disease.

2.5.2 Molecular methods

2.5.2.1 Diagnostic PCR

Recently, new, rapid, sensitive molecular diagnostic methods have been developed, that can detect bacterial DNA in a variety of matrices such as water, milk, urine, feces, and soil (Sweeney *et al.*, 2006; King *et al.*, 2015). Detection of TB using Polymerase chain reactions (PCR) can provide fast and reliable results and significantly reduces the time to confirmation (i.e. from several weeks to one day), thus allowing the possibility of taking timely control measures to prevent further spread of the disease in herds as well as in the community (Antognoli *et al.*, 1997; Miller *et al.*, 2001). The method amplifies DNA fragments of *Mycobacteria* to identify *Mycobacteria* species, regardless of their cell viability (De la Rúa-Domenech *et al.*, 2006; Medeiros *et al.*, 2010). However, the method can be applied in parallel with bacteriological culture for biological specimen such as milk as well as the Single Comparative Intradermal skin test (SCIT) method in live animals. For example, *M. bovis* DNA has been detected in milk samples that were collected from

SCIT positive cows (Zumárraga *et al.*, 2012; Figueiredo *et al.*, 2012). In addition, a study in Brazil detected *M. bovis* in milk samples in 5/8 (62.5%) milk samples from SCIT negative cows (Zarden *et al.*, 2013) indicating that the higher sensitivity of the molecular method may be useful in detecting animals that are shedding bacteria in the milk while still testing negative on traditional BTB tests.

2.5.2.2 Real time PCR

Real time PCR assays can be used both qualitatively and quantitatively to determine the occurrence of *Mycobacterium* DNA in a sample. The method utilizes specific primers, targeting the MPB70 antigen, a gene specific to MTBC (Young *et al.*, 2005; Courtenay *et al.*, 2006). There are several advantages over conventional PCR as it allows an absolute quantification by using a standard curve comparison approach of known target sequence numbers. For example, a complete genome sequence of *M. bovis* has been used to design primers flanking a region of difference (RD4) between the sequences of *M. bovis* DNA from other members of the complex (Brosch *et al.*, 2002). Therefore, the presence of *M. bovis* is confirmed by using a fluorescent (TaqMan) probe which discriminates *M. bovis* from other members of the complex as it hybridizes both the 5' and 3' RD4 deletion flanking sequences, which only occur directly adjacent to each other in *M. bovis* (Brosch *et al.*, 2002).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area and Population

This study was carried out from August to December 2015 in four selected slaughter facilities, Mlandege, Ipogolo, TRM and Kihesa, which serve as sources of meat supplies in Iringa Municipality. Iringa Municipal Council has a human population of 151 345 (PHCT, 2013). In 2012, Iringa region had a cattle population of 664 272 heads, 96.7% of which were indigenous cattle kept by agro pastoralists and pastoralists under traditional farming systems (NBS, 2014). The slaughtered animals mostly were adult cattle and predominantly of local breeds. The bulk of the slaughter cattle came from livestock markets within the Iringa region and adjoining areas of Dodoma region. Most of these livestock markets receive livestock from the villages bordering the wildlife-protected areas including the Ruaha National Park. However, as there is no mandatory livestock identification system in Tanzania, it is difficult to trace the exact household of origin of the slaughter animals. Further, animals may be sold multiple times at different markets before arriving at the slaughter facility. Consequently, only the most recent movement of the animal could be traced in this study by recording the information on the movement permit or verbally asking the seller. Mapping was conducted using QGIS (version 2.14.3) (Fig. 1).

The Mlandege, Ipogolo and TRM slaughter facilities are owned by Iringa Municipal Council and the maximum slaughter capacity for each is 50 cattle per day. The Kihesa slaughterhouse is privately owned and slaughters up to 5 cattle per day. All the cattle presented for slaughter at the four slaughter facilities during the study period formed the

study population.

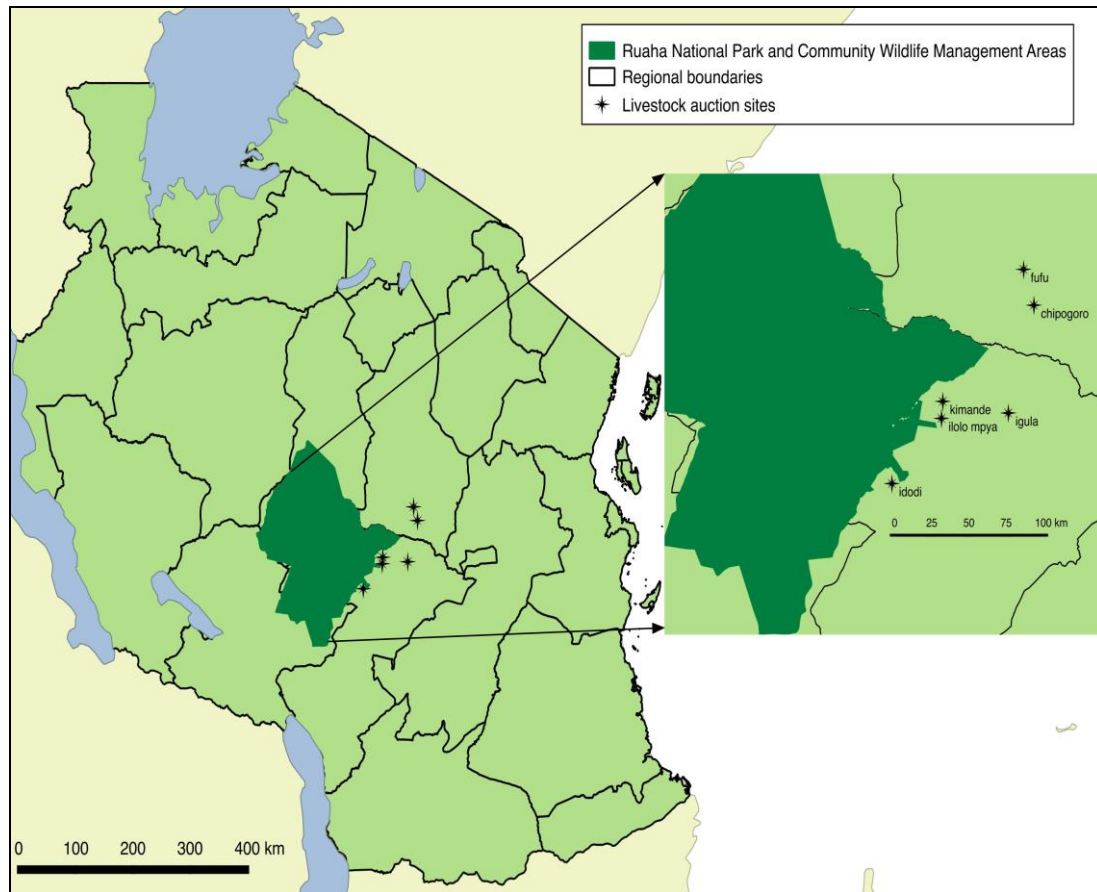


Figure 1: A map of study area showing livestock markets in Iringa and nearby areas of Dodoma region which are the major sources of slaughter cattle in different slaughter facilities in Iringa Municipality

3.2 Study Design and Sample Size

A cross-sectional study design was adopted for this study. The four selected study slaughter facilities were visited daily. The meat inspectors performed a thorough postmortem examination of all the cattle slaughtered with focus on tuberculous and granulomatous lesions. Only tissue lesions consistent with TB were purposively collected for laboratory analysis.

3.2.1 Postmortem examination of cattle carcasses for TB lesion detection

The postmortem inspection was carried out specified in meat hygiene regulation of animal disease Act no 17 of 2003, briefly meat inspection involve visual inspection, palpation and incision of the organs and tissues. Particular emphasis was given to the examination of high risk tissues/organs such as lymph nodes, lungs, liver and kidneys. The cut surfaces of tissues and/or organs were examined under light for the presence of granulomas with thick white, greenish, or yellow exudate, cheesy masses and/or gritty tubercles of various sizes and numbers. Such lesions are characteristic of TB infection (Corner, 1994) and were therefore sampled for laboratory diagnosis.

3.2.2 Sample collection and handling

3.2.2.1 Tuberculous tissues and/or organs

Portions of tissues and/or organs consistent with TB lesions as described in 3.2.1 were collected during regular meat inspection procedures and placed into plastic bags (Whirl pak, Nasco, WI), labeled and immediately stored in a cool box with ice during the field work. The samples were subsequently transported to the HALI project field office in Iringa within two hours of collection and frozen temporarily in a -20°C freezer before transportation on ice to Sokoine University of Agriculture (SUA) in Morogoro for laboratory analysis.

3.2.2.2 Sampling of slaughter facility environments

Environmental samples (effluents) were collected twice during the slaughter process. Timing of sampling was based on the type of slaughter activities, although a strict distinction was not always possible as activities occasionally overlapped. The first sampling was conducted early in the slaughter process, during flaying and evisceration.

The second sampling was conducted after meat inspection, during carcass dissection and floor washing. The samples were taken at the outlet of the open floor drainage. A total of 200 ml of effluents was collected into a new plastic bottle, labeled, and stored in a cool box with ice. One hundred milliliters of the collected effluent was then filtered through a sterile Microfil filter (0.22 μm x 47 mm, Microfil V, Millipore, Billerica, MA, USA). The filter paper was temporarily frozen at -20°C before transport in a liquid nitrogen-based dry shipper to University of Warwick, UK for laboratory analysis.

3.3 Laboratory Sample Analysis

3.3.1 Culture of Mycobacterium from tissue and/or organ samples

Tuberculous tissue samples were processed and cultured at the Sokoine University of Agriculture TB Laboratory as described by OIE (2009). Briefly, each TB suspect tissue and/or organ sample was chopped into small pieces using a sterile scalpel blade and homogenized in a stomacher 80-laboratory blender (Seward Laboratory, London, UK) for 2 minutes under biosafety cabinet level 2 conditions. Tissue and/or organ homogenates were transferred to a sterile 50 ml universal container and centrifuged for 10 minutes at 30 revolutions per minute. An equal volume of 3% Oxalic acid was added for decontamination purposes in a safety cabinet, followed by intermittently shaking of the mixture for at least 30 minutes.

After decontamination, the supernatant was discarded and a 2% solution of Sodium Hydroxide was added to the remaining sediment in order to neutralize the oxalic acid. Phenol Red solution (1%) was added to the final mixture of the acid and base to check if the neutral point of pH 7 had been attained. Aliquots of the remaining sediments were inoculated onto Lowenstein- Jensen (L-J) media with Pyruvate and Glycerol (BDH

Chemicals Ltd, Poole, UK). Samples were incubated at 37 °C and observed for 2-3 consecutive days to assess the quality of decontamination. In absence of contamination signs, the cultures were observed weekly for growth of *Mycobacterium* colonies for 6-8 weeks. Positive cultures with colony morphological features as described by Vestal and Kubica (1966) were sub cultured onto another set of media and incubated for another two to four weeks. The first step towards species identification was the visual observation of growth on L-J glycerol and L-J pyruvate media. *Mycobacterium tuberculosis* produces eugonic growth on both media, while *M. bovis* grows well on L-J pyruvate medium. The suspect colonies were subjected to Acid Fast Bacteria (AFB) analysis as a preliminary confirmatory test for Mycobacteria as described by Ziehl-Neelsen. Briefly, the bacterial smears were prepared and allowed to air dry. Then the smears were heat fixed and covered with carbol fuchsin stain. Heat was applied to the mixture until vapour began to rise at about 60°C. The slide was allowed to stain for another 5 minutes and then washed with clean water. The smears were covered with 3% v/v acid alcohol for 5 minutes to allow for sufficient decolourization and then washed with clean water. Lastly, the smears were covered by malachite green stain for 1-2 minutes and then washed with clean water. The slides were left on a draining rack for air-drying and examined microscopically, using a 100X oil immersion objective. The results were interpreted as positive when bright red, straight or slightly curved rods, occurring singly or in small groups were seen.

3.3.2 DNA extraction and Mycobacterium identification

All culture positive samples were subjected to the Mycogenus typing heat-killed method. Briefly, a mixture of colony material and 100 µl of distilled water was added to 1.5ml screw capped eppendorf tube and incubated at 80 °C for 1 hour to allow for inactivation of the Mycobacterial cells. The heat-killed cells were stored at 4 °C until further laboratory

analysis. Identification of *Mycobacteria* was done by using a multiplex PCR protocol. This method amplifies species-specific DNA fragments that differentiates the MTBC) from *M. intracellulare*, *M. avium* and other *Mycobacterial* species as described by Wilton and Cousins (1992) and Gordon *et al.* (1999). Six oligonucleotide primers for *Mycobacteria* were used. Firstly, two primers, MYCGEN-F and MYCGEN-R, were designed to target and amplify a sequence of highly conserved region within the 16S rRNA gene that is specific for the *Mycobacterium* genus. Secondly, the PCR mix also included two primers, MYCINT-F and MYCAV-R, that are specific for a hyper variable region of the 16S rRNA gene of *M. intracellulare* and *M. avium*, respectively. The additional PCR products were used to identify any DNA template of the two species. Control strains, *M. tuberculosis* H37Rv (ATCC 25618) and *M. bovis* AF2122/97 (ATCC BAA-935) were obtained from the Tuberculosis laboratory at the SUA Faculty of Veterinary Medicine.

The PCR amplification master mixture had a total volume of 20 µl which included 10 µl HotStar Taqmaster Mix (Qiagen, UK), 6.2 µl nuclease free water (Qiagen, City, UK), 0.3 µl of each primer, and 2 µl heat-killed DNA template. The samples were run in a PCR thermocycler machine (Bio-Rad, Hercules, USA). The denaturation process was performed for 10 minutes at 95°C, and the annealing process involved 35 cycles for 1 minute, 0.5 minute and 1 minute at 95°C, 61°C and 72°C respectively. Gel electrophoresis was then performed at 100V, with a one hundred base pair (100 bp) DNA ladder and blue loading dye (Promega Corporation, Madison, USA). Ethidium bromide was used as the staining reagent. The mycogenus typing results were interpreted based on the size of the PCR fragments.

3.3.3 Deletion analysis

The analysis involved PCR amplification of species-specific DNA fragments as described by Gordon *et al.* (1999) and Brosch *et al.* (2002). The analysis was performed to identify the presence or deletion of a “Region of Difference” (RD) in the species of MTBC. The RD4 was targeted and amplified using primers RD4 Flank-Fw (5'-CTC GTC GAA GGC CAG TAA AG-3'), RD4 Flank-Rev (5'-TTG CTT CCC CGG TTC GTC TG-3'), and RD9-InternalRev (5'-TTG CTT CCC CGG TTC GTC TG-3'). The primers were mixed with 7.1 µl of nuclease free water (Qiagen). The other reactions were the same as for the Mycobacterium typing, except that the denaturation step was performed at 95 °C for 15 minutes.

3.4 Analysis of Slaughter facility Effluent Samples by Quantitative PCR

The DNA from the effluent samples was extracted using a Fast DNA spin kit for soil (MP Biomedicals, Santa Ana, CA, USA) and analyzed using quantitative Polymerase Chain Reaction (qPCR) as described by King *et al.* (2015). This qPCR assay can quantify the genome of *M. bovis* and *M. tuberculosis* in environmental samples and differentiate *M. bovis* and *M. tuberculosis* from other members of MTBC.

3.5 Data Analysis

All data were entered into a Microsoft Excel spread sheet. For each slaughter facility, the number of animals slaughtered each day, the number of animals with gross lesions and the number of *M. bovis* and NTM detections in tissues and effluents (environmental samples) were summarized using descriptive statistics. The prevalence of *M. bovis* and NTM's during the study period were calculated for each of the four slaughter facilities and compared among slaughter facilities using Fisher exact tests. Mann-Whitney and Kruskal

Wallis tests were used to compare pathogens genome equivalents in environmental samples for the first and second sampling per day and between different sampling days within a slaughter facility and among the four slaughter facilities.

CHAPTER FOUR

4.0 RESULTS

4.1 Sources of slaughter cattle in Iringa Municipality

A total of 3817 cattle carcasses were examined during the five-month study period. During this period a total of 3817 cattle were slaughtered as follows: 1941 at Mlandege, 1149 Ipogolo, 655 at TRM and 72 at Kihesa. Generally, the highest number of slaughter cattle was recorded to be sourced from Kimande livestock market 1899/3817 (50%) in the Iringa region followed by Chipogoro livestock market which is in Dodoma region (Table 1).

Table 1: Cattle source results from four different slaughterhouse facilities in Iringa Municipality, Tanzania (n=3817)

Slaughter facility	Animal markets				Total
	Chipogoro (%)	Kimande (%)	Mlowa (%)	Nyang'olo (%)	
Mlandege	750 (19.6)	675 (17.7)	363 (9.4)	153 (4.0)	1941 (50.7)
Ipogolo	0 (0.0)	1037 (27.2)	1 (0.03)	111 (2.9)	1149 (30.2)
Kihesa	1 (0.03)	24 (0.7)	6 (0.2)	41 (1.0)	72 (1.9)
TRM	0 (0.0)	163 (4.4)	269 (7.0)	223 (5.8)	655 (17.2)
Total	751 (19.7)	1899 (50.0)	639 (16.6)	528 (13.7)	3817 (100.0)

Note that the information on the sources of slaughter cattle was obtained from the cattle sellers or the butcherman

4.2 Prevalence of gross tuberculous lesions in cattle carcasses in Iringa Municipality

The overall prevalence of carcasses with TB lesions was 35/3817 (0.9%). The prevalence of gross tuberculous lesions in carcasses examined in different slaughter facilities were 8/1941 (0.4%), 22/1149 (2%), 3/72 (4.2%) and 2/655 (0.3%) at Mlandege, Ipogolo, Kihesa and TRM, respectively (Table 2). Most of the lesions encountered involved the lungs and the associated respiratory lymphnodes.

Table 2: Total number of cattle slaughtered and gross tuberculous lesions in cattle carcasses in Iringa Municipality, Tanzania

Slaughter facility	Number of cattle slaughtered	Number (%) of cattle carcasses with gross lesions	Number (%) of organs with TB lesions		
			Lungs	Lymph nodes ^a	Liver
Mlandege	1941	8 (0.4)	5 (63.0)	2 (25.0)	1 (12.5)
Ipogolo	1149	22 (2.0)	3 (14.0)	17 (77.0)	2 (9.0)
TRM	655	2 (0.3)	0 (0.0)	2 (100.0)	0 (0.0)
Kihesa	72	3 (4.2)	0 (0.0)	3 (100.0)	0 (0.0)
Total	3817	35 (0.9)	8 (23.0)	24 (68.0)	3 (8.0)

^aLymph nodes include retropharyngeal and mediastinal lymph nodes

4.3 Mycobacterial Tissue Culture Results

The Mycobacteria tissue culture results are summarized in Table 3. Of the 35 carcasses with gross tuberculous lesions, 10 (28.6%) had Mycobacterial growth on cultures (Fig. 2a). The Z-N staining of the 10 Mycobacteria isolates showed presence of bright red, straight or slightly curved rod cells occurring either singly or in small groups and were all confirmed as AFB positive (Fig. 2b). Of the 10 Mycobacteria isolates, majority (17.2%) were from Ipogolo slaughter facility. *Mycobacterium bovis* constituted only 10% of all 10 isolates (Table 3).

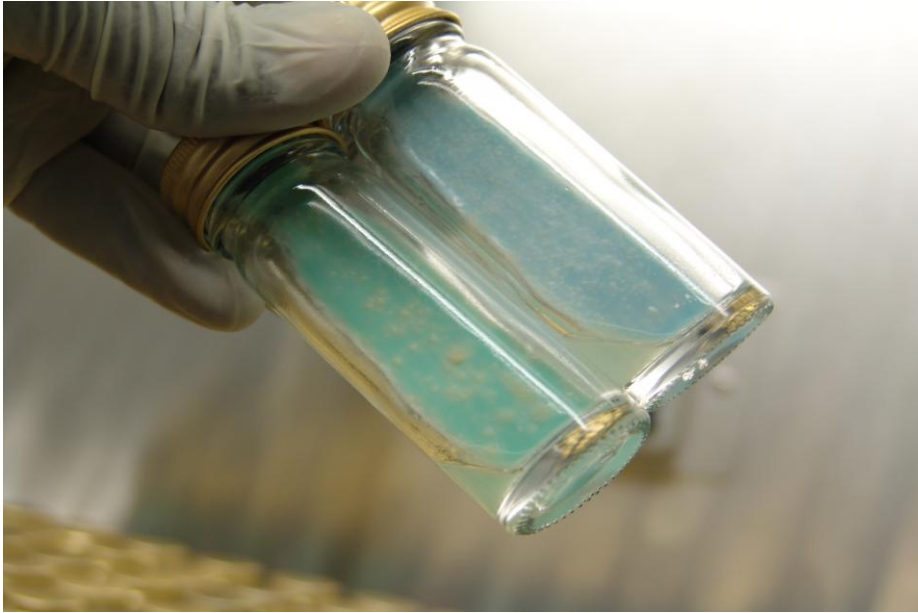


Figure 2a: Culture positive on L-J media. Morphologically, the colonies appear rough, brown and granular

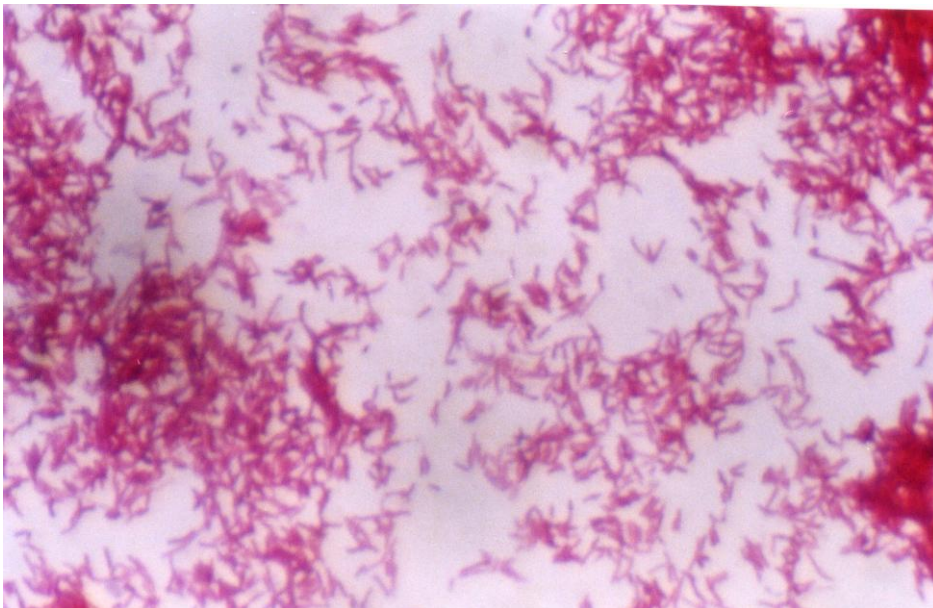


Figure 2b: AFB positive smear. Note the cells that appear bright red, straight or slightly curved rods are Mycobacteria

Table 3: Mycobacterial culture results from four slaughter facilities in

Iringa Municipality, Tanzania

Slaughter facilities	Number of tissue lesions cultured	Number with Mycobacterial growth colony seen (%)	Confirmed <i>Mycobacterium</i> species <i>M. bovis</i> (%)	NTM (%)
Mlandege	8	3 (8.6)	0 (0.0)	3 (30.0)
Ipogolo	22	6 (17.2)	0 (0.0)	6 (60.0)
Kihesa	3	1 (2.8)	1 (10.0)	0 (0.0)
TRM	2	0 (0.0)	0 (0.0)	0 (0.0)
Total	35	10 (28.6)	1 (10.0)	9 (90.0)

4.4 Typing of the Isolated *Mycobacterium* species

Molecular typing of *Mycobacterium* isolates showed that nine exhibited a band size of 1030 bp which is consistent with MTBC (Fig. 3). One *Mycobacterium bovis* isolate was confirmed via PCR in a tuberculous lung lesion from cattle slaughtered at Kihesa slaughter facility and sourced from Kimande livestock market. The other 9 culture positive samples were identified as NTMs (Fig. 3). The NTMs were further sequenced and identified as *M. lentiflavum* (3), *M. intracellulare* / *M. indicuspranii* (2), *M. lentiflavum/palustre/simiae* (2), *Mycobacterium goodii* (1) and *M. smegmatis* (1).

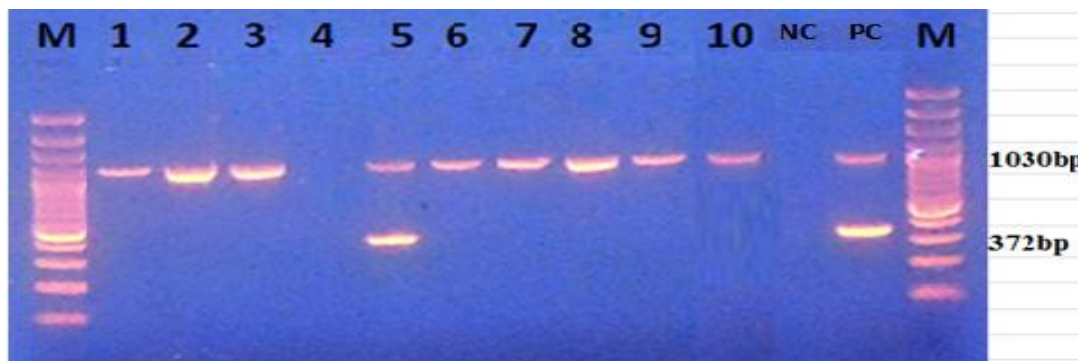


Figure 3: PCR products by Mycogenus typing of mycobacteria isolates from cattle tissue collected at four slaughter facilities in Iringa, Tanzania. Lanes marked M is a molecular weight marker (100 bp) while lanes marked 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 were Mycobacteria samples; lane NC is negative control (water); lane PC is positive control (*M. bovis*). Note that a sample in lane 4 is negative

4.5 Slaughter Facility Effluent Sample Results

Table 4 summarizes results of *Mycobacterial* species detected in

slaughter facility effluent samples in Iringa Municipality. A total of 172 effluent samples were collected during the study period. Eighty six samples were taken early in the slaughter process (first sampling) and the other 86 samples late in the slaughter process (second sampling). Mycobacteria were detected in 25 (14.5%) of effluent samples by qPCR. *Mycobacterium bovis* was detected in 5 (2.9%) of the effluent samples and *M. kansasii* in 20 (11.6%) samples (Table 4).

Table 4: *Mycobacterial* species detected in slaughter facility effluent samples in Iringa Municipality

Slaughter facility	No. of effluents (1 st sampling)	No. (%) of effluent with Mycobacterial	No. of effluents (2 nd sampling)	No. (%) of effluent with Mycobacterial	Total no. (%) of effluent with Mycobacterial	No. (%) of Mycobacterial species identified	
						<i>M. bovis</i>	<i>M. kansasii</i>
Mlandege	23	2 (2.3)	23	4 (4.7)	6 (3.5)	5 (2.9)	1 (0.6)
Ipogoro	23	4 (4.7)	23	3 (3.5)	7 (4.1)	0 (0)	7 (4.1)
TRM	20	4 (4.7)	20	4 (4.7)	8 (4.7)	0 (0)	8 (4.7)
Kihesa	20	2 (2.3)	20	2 (2.3)	4 (2.3)	0 (0)	4 (2.3)
Total	86	12 (14)	86	13 (15.1)	25 (14.5)	5 (2.9)	20 (11.6)

CHAPTER FIVE

5.0 DISCUSSION

The purpose of this study was to assess the occurrence of *Mycobacterial* infection in slaughter cattle and the slaughterhouse environment in Iringa Municipality, Tanzania. The overall prevalence of tuberculous lesions in cattle carcasses was 0.9% with the highest proportion of lesions (62.8%) recorded at Ipogolo slaughter facility. *M. bovis* constituted 10% of the isolated Mycobacteria while the five NTM species were isolated in the remaining 90% of culture positive samples. These findings indicate that slaughter cattle in the south-central Tanzania are infected with different *Mycobacteria* species that may pose a threat to personnel working with meat value chain and lastly the final meat consumers in Iringa Municipality, Tanzania. The TB prevalence (0.9%) in cattle carcasses from this study is consistent with previous slaughter facility surveys, which reported prevalence ranging between 0.32% at a Tanga slaughter facility in north-eastern Tanzania (Swai *et al.*, 2012) and 0.7% at Arusha slaughter facility in northern Tanzania (Mellau *et al.*, 2010). A higher prevalence of TB lesions ranging between 2% and 3.3% has been reported in carcasses in Morogoro (Kambarage *et al.*, 1995; Mariki *et al.*, 2013).

Similarly, Asseged *et al.* (2004) and Awah-Ndukum *et al.* (2007) reported 0.8% and 1.5% prevalence of tuberculosis in Cameroon and Ethiopia, respectively. Studies in other countries have reported high rates of TB lesions in slaughter animals (Cadmus *et al.*, 2008; Cadmus and Adesokan, 2009). Many reasons may account for the differences in prevalence which include the exposure status. Presence of high number of infected animals in the cattle population may enhance faster rates of spread of the diseases especially under the extensive management system which is common in Tanzania.

Nevertheless, diagnosis through postmortem examination during meat inspection based on gross pathological lesions has several drawbacks which some lesions may be mistakenly regarded as TB like lesions.

The low prevalence of bovine tuberculosis recorded in slaughtered cattle in this study is in line with the prevalence of 2% reported in live animals in the Iringa region (Clifford *et al.*, 2013). Generally, in Tanzania the occurrence of BTB in live cattle varies between 0 to 13.2% (Kazwala *et al.*, 2001; Shirima *et al.*, 2004; Durnez *et al.*, 2009; Swai *et al.*, 2012; Mwakapuja *et al.*, 2013). Poor animal husbandry systems have been reported to intensify the spread of the disease among live animals, as a large proportion of cattle in the country are kept by traditional pastoralists who migrate searching for grazing areas and may share water sources with other livestock herds as well as wild animals, such practices may increase the risk of tuberculosis transmission in animals.

Analysis of slaughter facility effluents indicated that 14.5% contained Mycobacteria of which *M. bovis* was detected in 2.9% and *M. kansasii* in 11.6% of the effluent samples. Mycobacteria were detected in a relatively high proportion of effluent samples from the slaughter facilities, which suggest that the slaughterhouse effluents are potential sources of environmental contamination with pathogens. The effluent may serve as a source of infection for any humans and animals that come in contact with the effluent. Chemical treatment of wastes originating from slaughter facilities is recommended in order to avoid potential spread of zoonotic diseases.

Tuberculosis is also a zoonotic disease, with HIV/AIDS patients being at highest risk of infection (Raviglione *et al.*, 1995). Pastoral and agropastoral communities may drink fresh

blood and raw milk and eat raw or partially cooked meat, which predisposes them to infection with bovine tuberculosis. Daily contact with infected animals may also increase the risk of zoonotic disease transmission. It is suggested that all cattle sent for slaughter need to be screened for BTB at the farm level and reactors not be sent to slaughter facilities could reduce the infection risks of humans.

The current study found that the majority of TB lesions (24/35) involved the lungs and associated retropharyngeal and bronchial lymph nodes. Disease transmission from cattle to cattle is thought to occur primarily through inhalation of infectious aerosols. Previous studies also predominantly detected lesions in the lungs and the associated lymph nodes, with lesions in the mesenteric lymph nodes, liver, spleen and serous membranes being less common (Menzies and Neill, 2000; OIE, 2009). It is therefore recommended that proper identification of respiratory lymph nodes be emphasized during training of meat inspectors in order to minimize the chance of missing of TB suspect lesions during inspection.

Of the 35 carcasses with TB suspect lesions, only 10/35 (28.6%) had growth on cultures and was confirmed to be AFB positive. It is possible, as the duration between sample collection and submission for laboratory cultures may have negatively influenced the likelihood of mycobacteria isolation or the collected tuberculous lesions were not accurately identified by the meat inspectors (Biffa *et al.*, 2010). In addition, there are several other infectious agents such as fungi, *Staphylococcus*, *Actinomyces* and *Actinobacillus* species, which are also known to cause granulomatous lesions that resemble that of TB in animals (Biffa *et al.*, 2010). Such infectious agents would not be identified with the methods used.

The majority of *Mycobacteria* isolated were NTMs. Studies by Makondo *et al.* (2014) and Mbugi *et al.* (2014) reported similar isolates of NTMs in wildlife, humans and livestock in the Katavi ecosystems in the Sourthen and the Serengeti ecosystems in the northern highlands in Tanzania. Some of these NTMs, for example *M. intracellulare* are considered pathogenic, as they have been isolated from TB lesions both in animals and humans particularly in individuals with HIV/AIDS (Kankya *et al.*, 2011). Further studies are needed to understand the importance of NTM's in the development of TB like lesions in wildlife, livestock and humans.

Our study was the first to detect *M. bovis* in the slaughterhouse environment. Detection of *M. bovis* from the effluent samples on days where no TB lesions were collected in cattle tissues may indicate that some infected carcasses were missed during regular meat inspection process, or the slaughterhouse environment was persistently contaminated with *Mycobacteria* species including *M. bovis*. *Mycobacteria* species may survive in the environment for several weeks due to a number of factors including dormancy and a thick waxy cell wall that offers desiccation resistance (Maddock, 1933; Young *et al.*, 2005; Fine *et al.*, 2011; Gengenbacher and Kaufmann, 2012). In this study *Mycobacteria* species were more frequently detected in the environment during the later stages of the slaughter process when activities like meat inspection, carcass splitting, and cleaning of the rumen, abomasums and reticulum were performed. A study by Srivastava *et al.* (2008) reported that *Mycobacteria* may be shed through feces and milk and removal of the gastrointestinal tract and dissection of the udder may have additionally contributed to environmental contamination. As the risk of slaughter facility environmental contamination increases with the progress of the slaughter process, slaughter equipments such as knives could potentially be source of meat contamination.

It is common that effluents from slaughter facilities in many towns in Tanzania are not properly treated to control biohazards. This also applies in Iringa municipality whereby, septic tank system is used in effluent management of which the tanks are worn out and the effluents drains to an open land where animals and humans access it with no restriction. In addition, since the effluents are not chemically treated, they can find their way to water bodies where further transmission to other animals and humans can occur. In urban areas of Tanzania, effluents are used for irrigation of vegetable gardens, a practice that can further pose a threat to consumers of vegetables. Also the slaughter facility effluents should be well treated before being discharged into the environment so as to minimize spread of zoonotic pathogens including Mycobacteria.

Mycobacterium kansasii was not isolated from any of the suspected TB lesions collected, however, it was detected in the environment. The pathogenicity of this *Mycobacterial* species is unclear, but a study conducted in Great Britain reported that *M. kansasii* was isolated from internal organs of skin test reactive calves, suggesting that the calves possibly were skin test reactors due to exposure to *M. kansasii* (Vordermeier *et al.*, 2007; Williams *et al.*, 2009; Houlihan, 2010; Tschopp *et al.*, 2010). In humans, *M. kansasii* has been reported to cause lung infections that result into severe tuberculosis-like disease and its prevalence is increasing globally (Morris *et al.*, 2011).

In the current study, the sources of slaughtered cattle were assessed and it was observed that animals were from numerous villages within and across the study region. Although the individual household of origin could not be identified the findings showed that many of the slaughtered cattle were recorded from Kimande, Nyang'oro and Mlowa livestock

markets which receives cattle from the villages bordering protected areas such as the Wildlife Management Area and Ruaha National Park, where there is high interactions between wild animals and livestock. In addition, Chipogoro livestock market in Dodoma region receives cattle from different villages that border the Kizigo and Mhezi Game Reserves and; Ruaha National Park from the Dodoma region. Therefore, it is recommended that there have to be mandatory livestock identification system in Tanzania, which will be of use in tracing the households of origin of all the slaughter animals.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Based on the findings of this study, it is therefore concluded that:

- i. The source of slaughter cattle in Iringa Municipality mainly are villages within the Iringa rural district.
- ii. The prevalence of mycobacteria in cattle carcasses was 0.9%) and in the slaughter facility environment is 14.5%
- iii. The identified species of Mycobacteria in the carcasses were *M. bovis*, *M. lentiflavum*, *M. intracellulare*/*M. indicuspranii*, *M. lentiflavum/palustre/simiae*, *M. goodii* and *M. smegmatis* while *M. bovis* *M. kansasii* were detected in the environmental samples.
- iv. The viability of the Mycobacteria that were detected from the slaughterhouses environment were not assessed in this study, however the findings still indicate that contamination of the slaughter facility environment occurs and possibly could pose a risk for carcass contamination and human infections.

6.2 Recommendations

- i. Regular education and refresher courses for meat inspectors and other novel transmission routes in order to increase understanding for risk factors of potential zoonotic disease transmission routes between people, animals and their shared environment.
- ii. Use of a closed drainage systems to all slaughter facilities are recommended as people may access it for different reasons when drains in an open drainage systems to the surrounding communities.
- iii. Chemical treatment of slaughter facilities effluents is also important in order to safeguard the health of nearby communities by reducing survival of dangerous zoonotic pathogens of public and animal health importance.
- iv. The novelty of this study to be applied in various matrices such as milk and water for detecting infectious diseases including Mycobacteria and included when formulating disease testing and control strategies.
- v. Further investigations are warranted to better understand novel transmission routes and the role of NTM's in both animals and human tuberculosis epidemiology.

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