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AGRICULTURE. MOROGORO, TANZANIA.

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ABSTRACT

A study on epidemiology bovine tuberculosis (BTB) in wildlife-livestock interface was undertaken in Katavi-Rukwa Ecosystem (KRE). The objective of the study was to generate epidemiology data of BTB in KRE interface. This study aimed to isolate members of the mycobacteria tuberculosis complex (MTC), determine the prevalence of the disease as well as evaluating Bovigam® and Stat Pak® serological diagnostic test.

In year 2010 to 2012, 789 cattle were examined by single comparative intradermal tuberculin test (SCITT) and 328 livestock tissues were cultured. One hundred and seventy eight wild animals were also sampled; among these, 119 tissue samples from buffalo, lion, leopard and hartebeest were sampled opportunistically during professional hunting and game cropping operations in the protected areas. Out of 789 cattle tested, SCITT results indicated prevalence of 4.6% [(95% confidence interval CI, 3.4-5.9)] and 10.7% [(95% CI, 7.8-11.9)] at ≥4 mm and >2 mm cut off, respectively. The prevalence was significantly affected by sex (female 5%, χ²= 56.4, p=0.03) and age (>2 years 5.8%, p=0.017). Typical tuberculous lesions were detected in 80% of tuberculosis reactor cattle tested from slaughter houses; among these, four isolates were MTC and were further genetically classified. Three isolates were identified as M. bovis strains and one isolate was M. tuberculosis. M. bovis isolates were grouped into three clusters of strains, and M. tuberculosis isolate was typified as East African Indian sub lineage (SIT 48, EAI1-SOM). Three clusters of M. bovis strains were identified as SB0133 and SB1467 reported elsewhere in the world while a new spoligotype pattern was identified and reported for the first time referenced as SB2191 in M. bovis spoligotype database. Non tuberculous mycobacteria were detected in 25.9% and 12.6% of livestock and wildlife tissue cultures, respectively suggesting transmission in area. Bovigam was in good agreement with SCITT (p=0.001) in diagnosis of disease. It is recommended to government authority to
use multisectoral, one health disease control strategy, in order to prevent the infected livestock to serve as a source of infection to wild animals or the other way around in the interface. Planning for BTB sensitization during disease control implementation is equally important.
DECLARATION

I, ZACHARIAH EPHRAIM MAKONDO, do hereby declare to the Senate of Sokoine University of Agriculture that this thesis 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.

____________________________  ________________________
ZACHARIAH EPHRAIM MAKONDO                  Date
(PhD. Candidate)

The above declaration is confirmed

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DEDICATION

This work is dedicated to GOD. To my parent Mr. and Mrs. Ephraim Makondo
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<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>BTB</td>
<td>Bovine tuberculosis</td>
</tr>
<tr>
<td>DALDO</td>
<td>District Agricultural and Livestock Development Officer</td>
</tr>
<tr>
<td>DR</td>
<td>Direct Repeats</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>GIS</td>
<td>Geographical Information System</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>KRE</td>
<td>Katavi Rukwa Ecosystem</td>
</tr>
<tr>
<td>LJM</td>
<td>Löwenstein–Jensen Media</td>
</tr>
<tr>
<td>MLDF</td>
<td>Ministry of Livestock Development and fisheries</td>
</tr>
<tr>
<td>MTC</td>
<td>Mycobacteria tuberculosis complex</td>
</tr>
<tr>
<td>NTM</td>
<td>Non tuberculous mycobacteria</td>
</tr>
<tr>
<td>OIE</td>
<td>World Organisation for Animal Health</td>
</tr>
<tr>
<td>PPD</td>
<td>Pure Protein Derivatives</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RD</td>
<td>Region of difference</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restricted Fragment length polymorphism</td>
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<tr>
<td>Rpm</td>
<td>Revolution per minute</td>
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<tr>
<td>SIT</td>
<td>Spoligo International Typing</td>
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<tr>
<td>SCITT</td>
<td>Single Comparative Intradermal Tuberculin Test</td>
</tr>
<tr>
<td>TANAPA</td>
<td>Tanzania National Parks</td>
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<tr>
<td>TAWIRI</td>
<td>Tanzania Wildlife Research Institute</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>ZN</td>
<td>Ziehl Nielsen stain</td>
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<tr>
<td>κ</td>
<td>Kappa</td>
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<td>FLI</td>
<td>Friedrick Loeffler Institute</td>
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CHAPTER ONE

1.0 INTRODUCTION

*Mycobacteria* are a family of small, rod-shaped bacilli that can be classified into three main groups for the purpose of diagnosis and treatment. These groups are: *Mycobacterium tuberculosis* complex (MTC) which can cause tuberculosis, *M. leprae* causes Hansen's disease or leprosy and Non tuberculous mycobacteria (NTM) which are all other *Mycobacteria* which can cause pulmonary disease resembling tuberculosis, lymphadenitis, skin disease, or disseminated disease. Infection with NTM may be asymptomatic or sub acute or chronic illness resembling pulmonary tuberculosis (Katoch, 2004). Generally tuberculosis is caused by members of the MTC. This group of MTC include *M. tuberculosis*, *M. bovis*, *M. caprae*, *M. microti*, *M. africanum*, *M. canetti* and *M. pinnipedii* (Brosch et al., 2002; Mishra et al., 2005).

Bovine tuberculosis (BTB) is a contagious chronic disease of cattle, caused by non motile, acid fast bacteria known as *M. bovis* indistinguishable from *M. tuberculosis* clinically, radiologically and pathologically (Awah-Ndukum et al., 2012; Ibrahim et al., 2012). It is associated with progressive emaciation (Aiello and Mays, 1998; OIE, 2009) and tubercle (granuloma) formation involving mostly the respiratory system but also in advanced cases, other organs such as lymph nodes, the alimentary tract, blood vessels, liver, spleen and the surfaces of body cavities are commonly affected (OIE, 2009). Bovine tuberculosis is of great economic importance to the livestock industry (Zinsstag et al., 2006) and also an important public health issue (Jiwa et al., 1997; Mwakapuja et al., 2013) transmissible to humans and wild animals. Suitable methods for tuberculosis molecular identification are mycogenus PCR, deletion typing and spoligotyping targeting the special direct repeat region (Kamerbeek et al., 1997) as well as the mycobacterial
interspersed repetitive units/variable number of tandem repeats (MIRU/VNTR) targeting short repetitive sequences scattered all over the chromosome (Supply et al., 2006).

1.1 Research Gap

The apparent variability of the prevalence and pathogenicity of BTB infection in wildlife and livestock in Tanzania leaves a gap to explore. The relationship between disease infection in livestock, wildlife and human populations and their role in disease maintenance is still a gap to fill. This study looked at BTB prevalence and explored the possibility of its transmission from livestock to wildlife in Katavi Rukwa Ecosystem.

1.2 Justification of the Study

In southern Tanzania there is limited information on the BTB disease as compared to northern part of the country (Mfinanga et al., 2004; Cleaveland et al., 2005) or elsewhere in Africa (Michel et al., 2007; Tschopp et al., 2009; Munyeme et al., 2010). Katavi Rukwa Ecosystem is one of the areas less studied and researched south of the Tanzania central railway line. This has drawn interest in exploring different topics for studies in KRE and particularly in wildlife of which animals like buffalo seem to play a role in maintaining the disease. Bovine tuberculosis is known to be persistent in wildlife and livestock population (Cleaveland et al., 2005) and a neglected zoonosis of major economic importance (Zinsstag et al., 2006; Hassanain et al., 2009; WHO, 2009, 2012;). The annual global TB incidence was predicted to increase to 10.2 million (Cosivi et al., 1998) by the year 2000 and consequently higher in recent years. Previous studies carried in Tanzania indicate M. bovis contributes 10.8% of human cervical adenitis (Mfinanga et al., 2004).
More studies show that Northern Tanzania had 0.7-1.06% BTB reactor rate (Shirima et al., 2003; Katale et al., 2012) and Southern Tanzania had 13.2% cattle reactor rate in selected areas (Kazwala et al., 2001) as compared to previously 0.2% prevalence of bovine tuberculosis in the study carried out in Tanzania Lake Zone cattle (Jiwa et al., 1997). Based on the skin test, another study done which involved smallholder and traditional system in Tanga reported individual animal prevalence to be 2% and 0% respectively, with the corresponding overall herd prevalence of 5.7% and 0%, respectively (Swai and Schoonman, 2012). In Tanzania, regional data indicate these up to 30% of total human tuberculosis cases are attributed to the extra pulmonary form (Kazwala et al., 2001). Substantial percentage of M. bovis is likely to be missed in human sputum samples through microscopic diagnosis. This is due to the extra pulmonary nature of the disease. This fact pose a great danger in human population especially in developing countries like Tanzania where animal control strategies such as test-and-slaughter or surveillance-segregation is almost impossible to implement. The scope of this study was to contribute existing knowledge on BTB prevalence and generate epidemiological data required to support prevention and control of the disease in wildlife-livestock-human interface areas in Tanzania.

1.3 Objectives

1.3.1 Overall objective

To study epidemiology of bovine tuberculosis in livestock-wildlife interface in Tanzania.

1.3.2 Specific objectives

i. To assess awareness and perceptions of Katavi Rukwa livestock keepers on BTB transmission
ii. To establish prevalence of *M. bovis* and other members of the MTC in wildlife and domestic ruminants in the vicinity of wildlife interface in Katavi-Rukwa ecosystem.

iii. To determine presence of NTM, molecular characterization and determination of genetic spatial-temporal variation in disease prevalence

iv. To compare the performance of Gamma interferon assay (Bovigam®) and Stat Pak® serological tests in diagnosis of the disease

The null hypothesis is that the transmission of BTB in the study area is the result of intermingling between livestock and wildlife.
CHAPTER TWO

2.0 LITERATURE REVIEW

Tuberculosis is a global threat widely distributed. The disease is caused by *Mycobacteria* species. The species are usually grown into culture to confirm the disease. *Mycobacteria* culture takes longer to grow. Most mycobacterial culture may take 4-8 weeks to grow; however, they can live and remain viable for a year. These cultures are fairly resistant to chemical disinfectants, and phenols (Aiello and Mays, 1998). Culture growth pH is mainly 4.5-8.0 at 37°C but it may be improved by adding glycerine, or pyruvate (Wilson and Miles, 1975). Several *Mycobacterium* species are pathogenic to man and animals. Tuberculosis (TB) caused by *M. bovis* is among the most widespread infectious zoonoses in the world. It is a major opportunistic infection in HIV-infected persons (Ravigilione *et al.*, 1988; Kazwala *et al.*, 2006; Alli *et al.*, 2010). At present, the vast majority of people carrying dual HIV and *Mycobacterium bovis* infections live in developing countries; although, few similar situations are also reported in developed countries (Dankner *et al.*, 1993; Kazwala *et al.*, 2006). Bovine tuberculosis (BTB), a disease caused by *M. bovis* is widespread throughout the world (Shitaye *et al.*, 2007). Joint global effort to fight the infection is needed for the control of the disease throughout the world.

On the other hand, situation with human tuberculosis is devastating. In 2007, an estimated 13.7 million people had active TB disease, with 9.3 million new cases and 1.8 million deaths (WHO, 2009; 2012). More so in year 2011 estimates remained higher up to 8.7 million new cases of TB.
2.1 **Species Affected**

Cattle are the primary hosts for *M. bovis*, but other domesticated and wild mammals can also be infected. Known maintenance hosts include brush–tailed opossums (and possibly ferrets) in New Zealand, badgers in the United Kingdom and Ireland, bison and elk in Canada, and kudu and African buffalo in southern Africa (Michel *et al.*, 2006; 2007). White-tailed deer in the United States have been classified as maintenance hosts (Brentensen *et al.*, 2007). Spillover hosts maintains the organism only when its population density is high. Species reported to be spillover are ferrets camels, llamas, many species of wild ruminants including deer and elk (Caley *et al.*, 2001); others in this list are elephants, rhinoceroses, foxes, coyotes, mink, primates, otters, seals, sea lions, hares, raccoons, bears, warthogs, cat, large cats (including lions, tigers, leopards, cheetahs etc.) and several species of rodents (Zumárraga, *et al.*, 2009). Some avian species, including mallard ducks appear to be resistant to experimental infection (Palmer *et al.*, 2011). Most mammals may be susceptible.

2.2 **Geographic Distribution**

Bovine tuberculosis is still widespread in Africa, parts of Asia and some Middle Eastern countries (Raviglione *et al.*, 1995; Pavlik *et al.*, 2002; Neill *et al.*, 2005). A considerable variation in the prevalence of tuberculosis has been reported throughout Latin America and the Caribbean (de Kantor and Ritacco, 2006). In some European Union member States or regions the eradication programmes were successful and several of these reached tuberculosis free status between 1980-2004 (Reviriego Gordejo and Vermeersch, 2006). Out of 55 African countries, 25 reported sporadic/low occurrence of bovine TB and out of 36 Asian nations, 16 reported a sporadic/low occurrence of bovine TB, and one (Bahrain) described the disease as enzootic (Cosivi *et al.*, 1998). The overall animal prevalence of BTB was found to be 6.8% in the livestock/wildlife interface areas in
Zambia (Munyeme et al., 2009). Prevalence of bovine tuberculosis increased in South Africa Kruger National Park buffalo sub-population from 13% in 2001 to 25% in 2003 (Michel et al., 2006). The prevalence rate of BTB has been reported to range from 3.4% (in smallholder production system) to 50% (in intensive dairy productions) and a range of 3.5% to 5.2% in slaughterhouses for various places of Ethiopia (Shitaye et al., 2007; Tigre et al., 2012).

Tanzania, like other developing countries has had number of constraints to fight BTB; such includes the lack of rapid and sensitive diagnostic systems, wildlife maintenance and spill over reservoir hosts, as well as transmission of the disease through unpasteurised milk, in insufficient or no control measure towards detection and eradication of the disease. The proportion of the disease in the country ranges from 0.2% to 13.2% according to the study carried in past years (Jiwa et al., 1997; Cleaveland et al., 2005; Kazwala et al., 2006; Mwakapuja et al., 2013).

Globally, it is estimated that by the year 2020, nearly one billion people will be newly infected with TB, 200 million people will get sick, and 35 million will die if TB control measures are not strengthened (WHO, 2009; 2012). The share of BTB in the global TB epidemic is little known although sporadic reports of cases are received from many African and Asian countries that indicate a substantial fraction (Mfinanga et al., 2004).

Tuberculosis studies in Tanzania have reported varied prevalence of BTB in several parts of the country. A survey in Lake Victoria zone identified an average of 0.2% of tuberculosis skin test reactors in a total of 8 190 cattle from 42 herds (Jiwa et al., 1997). Eastern Tanzania recorded 1% of the animals reacted positively to bovine tuberculin and the herd prevalence was at 10% (Katale et al., 2012).
In the Lugoba area, 1% of tuberculin reactors were found in cattle and herd prevalence was at 21% (Weinhaupl et al., 2000). Moreover, in the southern Highlands 13.2% of the cattle reacted positively to skin test and the herd prevalence was at 51% (Kazwala et al., 2001). Prevalence also have shown to vary with extensive and intensive production systems in the eastern zone of Tanzania whereby in extensive pastoral production systems reactor prevalence was 1% and in the intensive production systems was 2% (Shirima et al., 2003). Another cross sectional study of 10 549 cattle from 622 herds in northern Tanzania revealed a low SICCT reactor prevalence of 1% and a herd prevalence of 12% (Cleaveland et al., 2005). Swai and Schoonman, (2012) reported individual animal prevalences of BTB in the smallholder dairy and traditionally managed cattle at 2% and 0%, respectively in Tanga. Isolation and zoonotic transmission of $M. bovis$ was shown through molecular typing techniques (Kazwala et al., 2006) just like other studies isolation of strains of $M. bovis$ from wildlife (Cleaveland et al., 2005).

2.3 Transmission and Ecology

$M. bovis$ can be transmitted by the inhalation of aerosols, by ingestion, or through breaks in the skin. It is recognized that $M. bovis$ can be isolated from nasal mucus (Neill et al., 2005) this probably contributes to spreading infection. Populations of spillover hosts do not maintain $M. bovis$ indefinitely in the absence of maintenance hosts (Michel et al., 2006); some spillover hosts can become maintenance hosts if their population density is high. $M. bovis$ shed in respiratory secretions, faeces, milk, urine, vaginal or semen is considered rare but significant in nasal mucus (de Kantor and Ritacco, 2006). Nonhuman primates are usually infected by inhalation (Aiello and Mays, 1998) and can attain severe disease of lung and organs. Aerosol transmission also seems to be the main route of spread in badgers. $M. bovis$ can infect humans, primarily by the ingestion of unpasteurized dairy products but also in aerosols and through breaks in the skin. Raw or
undercooked meat can also be a source of the organism and contaminated environment might play a bigger role in the epidemiology of BTB (Tschopp et al., 2009). Transmission from bovine to cat has been reported in Argentina (Zumárraga, et al., 2011). Person-to-person transmission may be rare in immunocompetent individuals, but M. bovis has occasionally been demonstrated to be transmitted within small clusters of people such as alcoholics or HIV-infected individuals (http://www.cfsph.iastate.edu/Factsheets/pdfs/bovine_tuberculosis.pdf).

Wildlife animals have been implicated as reservoirs of M. bovis infection for cattle in many parts of the world. The pattern of infection in the population and potential risks to domestic livestock can be profoundly influenced by wild animals’ social organization and behaviour like that of badgers (Delahay et al., 2000) and probably other animals like buffalo. Movements and mixing of infected animals have long been considered a critical factor in the spread of livestock diseases as it has been suggested elsewhere (Kazwala et al., 2001; Gilbert et al., 2005). Likewise in this study, spatio-temporal distribution of M. bovis infection is essentially investigated to determine epidemiological and ecological study of the dynamics of bovine TB as it has been done in a wildlife population (Delahay et al., 2001) and livestock population (Porphyre et al., 2007; Okafor et al., 2011).

Disease problem at the wildlife-livestock interface are frequently bi-directional (Bengis et al., 2002) and normally a source of conflict between livestock owners and animal health officers on one side and wildlife conservationist on the other hand. Several studies on bovine tuberculosis transmission at wildlife interface have been done elsewhere in the world (Renwick et al., 2007; Munyeme et al., 2010). In Africa much studies have been carried out in Kruger ecosystem (Michel et al., 2006, 2010), Serengeti ecosystem in Tanzania (Cleaveland et al., 2005) and more recently at Mikumi ecosystem in Tanzania
(Mwakapuja *et al.*, 2013). Low levels of awareness among cattle owners on BTB need a special attention when planning for BTB sensitization and effective control implementation (Munyeme *et al.*, 2010). The present study contributes more to this aspect on a remotely and less researched interface area of Katavi-Rukwa ecosystem.

### 2.4 Incubation Period

The symptoms of bovine tuberculosis usually take three weeks or months to develop in cattle (Palmer *et al.*, 2011) or seldomly encountered (OIE, 2009) as the case in most animal species. In kittens experimentally infected by the parenteral route, the incubation period is approximately three weeks; it is probably longer under natural conditions (Aiello and Mays, 1998).

### 2.5 Clinical Signs and Postmortem Lesions

Tuberculosis is usually a chronic debilitating disease in cattle presenting with several clinical sins. Clinical signs include weakness, loss of appetite, loss of weight, fluctuating fever, intermittent cough, diarrhoea and swollen lymphnode. But mostly presented clinical signs are not specific to the disease because they may be observed in other diseases too. Bovine tuberculosis is characterized by the formation of granulomas (tubercles) where bacteria have localized. These granulomas are usually yellowish and either caseous, caseo-calcareous or calcified (Aiello and Mays, 1998; OIE, 2009) and often encapsulated. The presumptive diagnosis of tuberculosis can be made if the tissue has been examined to have characteristic gross and a histological lesion (such are caseous necrosis, mineralisation, epithelioid cells, multinucleated giant cells and macrophages). Since the typical tubercle lesions are often paucibacillary, the presence of acid-fast organisms in histological sections may not be detected (OIE, 2009), although *M. bovis*
can be isolated in culture. According to OIE recommendation, a traditional mycobacterial culture remains the gold standard method for routine confirmation of infection.

2.6 Diagnosis in animals

Tuberculosis can be difficult to diagnose basing only on the clinical signs; most are diagnosed by routine testing or found at the slaughterhouse. The differential diagnosis includes contagious bovine pleuropneumonia, Pasteurella or Corynebacterium pyogenes pneumonia, aspiration pneumonia (which is often secondary to chronic wasting disease in cervids), traumatic pericarditis, caseous lymphadenitis or melioidosis in small ruminants, and chronic aberrant liver fluke infestation. In live cattle, tuberculosis is usually diagnosed in the field using tuberculin skin test (OIE, 2009). The tuberculin test can be performed using bovine tuberculin purified protein derivatives (PPD) alone, or as SCITT that distinguishes reactions to M. bovis from those due to environmental Mycobacteria. In this test newly infected animals cannot be detected, positive results are primarily seen in animals infected for one to nine weeks (De la rua-Domenech et al., 2006). Other assays are typically used as ancillary tests to the tuberculin test. The lymphocyte proliferation and gamma-interferon (IFN-γ) assays are blood tests that measure cellular immunity (Gormley et al., 2006). The IFN-γ assay or Bovigam is particularly useful in animals that are difficult to capture or handle as they must be captured only once (Grobler et al., 2002; Munyeme et al., 2009), rather than twice for the tuberculin test. Following a slight modification, the IFN-γ assay has proved to be a valuable alternative to the tuberculin test (Gormley et al., 2006; Grobler et al., 2002; Munyeme et al., 2009). Field trials showed that Bovigam is more sensitive than the single intradermal tuberculin test for the diagnosis of tuberculosis (Grobler et al., 2002; Gormley et al., 2006). The gamma-interferon (IFN-γ) release or Bovigam® test was performed according to standard procedure (Kathryn, 2008 and Gormley et al., 2006).
Serological and blood based TB assays have drawn attention not only due to improving sensitivity and specificity in non-traditional livestock but also they require only a single animal capture (Lyashenko et al., 2006). Stat Pak test where coloured latex beads impregnated with antigen have also proved to be of value in BTB diagnosis. In this diagnostic test, the material flows along a membrane by capillary action across a line where antigen has been impregnated onto the membrane. Where coated latex beads have bound to antibody in the serum, these then binds to the membrane making a coloured line to detect the positive samples (Lyashchenko et al., 2006). The diagnostic sensitivity of Stat Pak varies but can be boosted to over 90% if used with intradermal tuberculin skin test especially in zoo animals (Lyashchenko et al., 2006).

Enzyme-linked immunosorbent assays (ELISAs) measure antibody titers for *M. bovis*. ELISA may complement tests of cellular immunity in anergic cattle. A presumptive diagnosis can also be made by histopathology and/or the microscopic demonstration of acid-fast bacilli. Direct smears from clinical samples or tissues may be stained with the Ziehl Neelsen stain. The diagnosis is confirmed by the isolation of *M. bovis* on selective culture media (OIE, 2009). *Mycobacteria* grow slowly, and cultures (in Lowenstein Jensen media) are incubated for eight weeks; growth usually becomes visible in 3 to 6 weeks. Lesioned organs compatible with tuberculosis lesions in slaughtered animals have been collected and cultured to isolate *Mycobacteria* (Oloya et al., 2007). The identity of the organism can be confirmed with biochemical tests and culture characteristics, or polymerase chain reaction (PCR) assays. The PCR test can also detect *M. bovis* directly in clinical samples (Hosek et al., 2006). Genomics has identified distinct deletions from their genomes that act as unique markers of each related *Mycobacteria* bacilli strain that were historically distinguished basing on host preference and biochemical traits (Hewinson et al., 2006).
2.6.1 Genotyping and deletion typing (RD)

Generally the diagnosis of *M. bovis* by PCR is fast, highly sensitive and of great value in epidemiological studies (Cedeno *et al.*, 2005; Sahraoui *et al.*, 2009). Molecular diagnosis of MTC involves *Mycobacteria* genus typing protocol that identifies species from the *Mycobacterium* genus, but also differentiate species of the *M. tuberculosis* complex from *M. avium* complex species. Most of the species that define the *M. tuberculosis* complex are included in the phylogenetic tree. The evolutionary relationship is based on specific “Region of Difference” (RD), genetic elements that are deleted along this phylogeny. The assumption of clonality among these species suggests that a lost RD cannot be restored. The fact that these RD regions are either present or deleted in a species of the *M. tuberculosis* complex helps to characterize unknown isolates (Brosch *et al.*, 2002). Deletion typing protocols includes three different regions: RD4, RD9, and RD10. Each of these protocol aims to find out if a specific strain has the RD-region present or deleted. A strain that has RD4 deleted is typed as *M. bovis* while a strain with RD9 present is most likely *M. tuberculosis*. Similarly, an isolate that has the RD9 deleted but the RD10 present can be classified as *M. africanum* (Brosch *et al.*, 2002; Berg, 2007, 2008a, 2008b; Berg *et al.*, 2011). The flow diagram for diagnosis is shown in Appendix 1a.

2.6.2 Spacer oligonucleotide typing (Spoligotyping)

Genetic fingerprinting techniques specifically known as spoligotyping can distinguish different and distinct groups of *M. bovis* strains. Spoligotyping as described by Kamerbeek *et al.* (1997) detects the polymorphism of the Direct Repeat (DR) chromosomal region that consists of unique spacer sequences (34-41 bp) interspersed with identical 36 bp direct repeats. These DR region are amplified by PCR with a biotin labelled primer and the presence or absence of 1 up to 43 spacer sequences is evaluated by hybridization to film captured oligonucleotides from a membrane (Oloya *et al.*, 2007).
Recently, studies have identified a clonal complex of *M. bovis* present at high frequency in cattle in population samples from several sub-saharan east and west-central African countries. This closely related group of bacteria is defined by a specific chromosomal deletion called region of difference African 1 (RDAf1) and can be identified by the absence of spacer 30 in the standard spoligotype typing scheme. This group of strains has been named as the African 1 (Af1) clonal complex and has defined the spoligotype signature of this clonal complex as being the same as the *M. bovis* BCG vaccine strain but with the deletion of spacer 30 (Müller *et al*., 2009). Another specific chromosomal deletion studied is region of difference African 2 (RDAf2) very distinctive to absence of spacers 3 to 7 in their spoligotype patterns. This second clonal complex of *M. bovis* in Africa was found at high frequency in east African cattle and with a distribution that does not overlap with the previously identified west-central African clonal complex (Berg *et al*., 2011). RDAf2 was identified in isolates from Uganda, Burundi, Ethiopia and Tanzania, reciprocally its deletion analysis showed that strains of RDAf2 are not deleted for RDAf1 and Berg *et al*., 2011 concluded that RDAf1 and RDAf2 were phylogenetically distinct. In this study spoligotyping patterns we used to compare the isolates obtained from Katavi Rukwa ecosystem.

### 2.6.3 Other Molecular methods

Molecular typing techniques such as RFLP analysis have been used to further characterize the isolates and determine the genetic profile of the mycobacteria strains (Kamerbeek *et al*., 1997; van Soolingen *et al*., 2001; Haddad *et al*., 2001, 2004; Kazwala *et al*., 2006; Oloya *et al*., 2007; Alli *et al*., 2010). Purified genomic DNA from mycobacterial strains is used. RFLP typing have been used in studying insertion particularly IS6110 RFLP analysis according to standardized protocol as studied in *M. tuberculosis* (van Embden *et al*., 2000) and *M. bovis* (van Soolingen *et al*., 2001).
Deoxyribonucleic acid (DNA) is digested with the restriction endonuclease enzyme, separated by agarose gel electrophoresis, and transferred to a nylon membrane by Southern blotting. The membrane is then hybridized with a probe targeting the right-hand site of the IS6110 element. The probes are labeled using the enhanced chemiluminescence detection system (ECL). The IS6110 RFLP patterns can be analyzed by using the BioNumerics software programs, and the dendrogram prepared respectively. However *M. bovis* from cattle, due to relatively fewer copies of the transposable element *IS6110* (Cousin *et al.*, 1998, Michel *et al.*, 2010) have been objectionably typed by RFLP (Mclernon, *et al.*, 2010). Recent studies discovered four or more copies of the insertion sequence IS6110 in East Africa *M. bovis* isolates that could be a rationale to authenticate typing by RFLP.

Strains of *M. bovis* or tuberculosis can often be further differentiated by VNTR typing due to its higher discriminatory power (Sahraoui *et al.*, 2009). Skuce *et al.*, 2002 has used the typing methods that have been developed, targeting the variable-number tandem repeats (VNTRs) of minisatellite-like mycobacterial interspersed repetitive units (MIRUs), or ETRs. The discriminatory power of VNTR analysis has been shown to be slightly lower than that of IS6110 RFLP but analysis showed VNTR typing to have many practical advantages over RFLP typing qualifying it to be used for epidemiological studies of *M. tuberculosis* such as Beijing strains (Kremer *et al.*, 2005). VNTR fingerprinting also has been used in general studies of clustering within and between ethnic groups (Sails *et al.*, 2011). Extensive worldwide databases of spoligo- and VNTR typing patterns facilitate the comparison of results from different countries and help to elucidate the distribution and spread of strains (http://www.Mbovis.org; http://www.miru-vntrplus.org/).
2.7 Non Tuberculous Mycobacteria

Recently, there has been an increase in disease caused by organisms broadly categorized as non tuberculous mycobacteria (NTM), a generic term for mycobacteria not included in the MTC and other than *M. leprae*. Of these NTM, *Mycobacterium avium* complex (MAC) species are the most common cause of human and animal disease globally (Shojaei *et al.*, 2011; Palmer *et al.*, 2011). Other NTM of importance are *M. intracellurare* and *M. fortuitum* (Durnez *et al.*, 2011). NTM are naturally resistant to most anti-tuberculosis drugs (Zhang *et al.*, 2013) and recently the infections due to NTM are growing steadily around the world. The clinical relevance of the MAC in humans has been amplified in recent decades with the increasing population of immunocompromised individuals and the AIDS pandemic (Griffith *et al.*, 2007). Other studies detected a wide range of potentially pathogenic NTM from the environment that suggests drinking untreated water and living in close contact with cattle or other domestic animals may be a risk factor associated with the possibility of humans and animals acquiring NTM infections from these ecosystems (Kankya *et al.*, 2011; Müller, *et al.*, 2011). A microbial danger to human and animal health due to the NTM is on a rise (Mohamed *et al.*, 2009a) because most of them are inherently resistant or partially susceptible to the standard anti-tubercular drugs (Gopinath and Singh, 2010). Several procedures are used alone or in various combinations for identification of NTM cultures. Such procedures include High Performance Liquid Chromatography (HPLC), biochemical Tests, 16S rDNA sequencing, and GenProbe technology.

2.8 Control

Bovine tuberculosis can be controlled by test-and-segregation or test-and-slaughter methods. These methods are guaranteed to eradicate tuberculosis from domesticated animals (Aiello and Mays, 1998; Reviriego Gordejo and Vermeersch, 2006). Affected
herds are re-tested periodically to eliminate cattle that may shed the organism. Unidirectional control strategy may not be feasible when the disease has established itself in wild animal maintenance host (Michel et al., 2006; Michel et al., 2010). It is prudent to contain or make effort in controlling and eventually eradicating the disease in livestock set up so to prevent wild life infection.

For the spatially distributed disease like bovine tuberculosis, it is extremely important to study and identify exceptionally high prevalence areas. This will help to tackle basic surveillance issues as to why the elevated prevalence in some geographical areas. Elevated prevalence and hotspots are tangled to area of high response or an elevated cluster for an event. Spatial temporal and space-time scan statistics are increasingly used for disease cluster detection and evaluation (Kelly, 2012) in many diseases including Foot and mouth disease (Perez et al., 2010; Dukpa et al., 2011), Rift valley fever (Diallo et al., 2011; Métras et al., 2012; Niu et al., 2012), tick borne disease (Chaput et al., 2002) and human tuberculosis (Tiwari et al., 2006). Limited use of the method is observed in bovine tuberculosis (Martinez et al., 2007; Porphyre et al., 2007; Kelly and More, 2011).
CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Ethics and Consent for Livestock Sampling

This study was approved by Sokoine University of Agriculture (SUA/ADM/R.1/8/NKASI/MPANDA/2009) and accepted by the respective district councils in the study area. The animal owners were requested to participate in the study and upon consent the sampling was carried out. TAWIRI and TANAPA approved research on wild animals at Katavi Rukwa Ecosystem (TANAPA/HQ/C.10/13).

3.2 Study Area

Study area extends from Lake Tanganyika towards Lake Rukwa encompassing the Katavi-Rukwa ecosystem and villages adjacent. This area lies south-east of Mahali Mountain National Park, east of Lake Tanganyika at Karema extending eastwards around the northern shoulder of the Ufipa plateau towards Lake Rukwa. The fieldwork was carried out in two districts Mpanda and Nkasi within Rukwa Region between August 2010 and August 2012 (Mpanda has now been transformed into Katavi region). Nkasi District is located South West of Tanzania between latitudes 6° 58´ and 8° 17´ South of Equator and Longitude 30° 20´ and 31°30´ east of Greenwich. The District is bordered by Mpanda District to the North, Sumbawanga District and Zambia to the South, Sumbawanga Municipal to the East and to the West by the Democratic Republic of Congo through Lake Tanganyika. Nkasi District with an area of 13 124 sq kms has a human population of about 228 885. Katavi Region was created in March 2012 replacing the district formerly known as Mpanda that lies between latitudes 5° 15´ and 7° 03´ South and Longitude 30° 02´ and 33°31´ East. This region of 564 604 population and land square kilometre of 45 843 bordered by Tabora region on East and Kigoma on the west. South of Mpanda there is Nkasi district (Fig. 1).
Figure 1: A study area where samples from livestock and wild animal were collected (A map of KRE and the adjacent places where samples from livestock and wild animals were collected. Insert is a map of Tanzania that shows the relative location of the study area)
In the field, skin tuberculin and gamma interferon (Bovigam) test was performed. These tests were run individually and then parallel for comparison. A clinical investigation was carried out. Sera, plasma and tissue samples were collected and submitted to the laboratory for a thorough culture and identification. Methods are well explained in each section. Rukwa area as whole is suitable for grazing if well-developed can hold 727 093 cattle equivalent to 508 965 Tropical Livestock Units (TLUs). According to Tanzania National Sample Census of Agriculture 2002/2003, Rukwa region had 504 727 cattle, Until 2006 the cattle population in Rukwa Region by district was 131 113 in Mpanda, 150 000 in Nkasi, 203 827 in Sumbawanga rural and 27 782 in Sumbawanga urban. The study was implemented in two Mpanda (recently Katavi region) and Nkasi Districts surrounding reserved area of the Katavi-Rukwa Ecosystem. However, until 2010 the estimate of cattle in these two study districts studied was about 350 000.

3.3 Study Population
Livestock were in different groups; first those from large, medium and smallholder agropastoral keepers, secondly those destined for slaughter at the slaughter houses but they can be retained for more than three days for tuberculin testing (those belonged to major butchermen in Rukwa) and thirdly those routinely slaughtered at the slaughter houses. On average the Mpanda slaughter house was slaughtering approximately 10 cattle per day as compared to three cattle per day at Nkasi slaughter house. Wildlife animals in the KRE were also studied, this included predators like lion, leopard and hyena as well their prey (buffaloes, eland, antelopes and others).

3.4 Study Design
Observational study was employed in detection of the disease, source, and route of infection during dry and rainfall season in 3 years of study. The assumption was made
that *Mycobacteria* strains can be transmitted at the interface between livestock, the maintenance host (e.g. Buffalo) and spillage hosts (mostly predators) all playing major role. A preliminary survey was carried out to study livestock and wildlife interaction within KRE, herd structure, drinking and grazing areas as well as movement of wild animals away from the national park. A band of two groups were considered whereby within each group it was assumed that there was interaction without clear demarcation in relation to animal movements. The groups are 1: livestock group (which have maximum and minimum contact to the Katavi Rukwa reserves), 2: wildlife group (sampled through targeted capture and opportunistic sampling strategy).

### 3.4.1 Wild Animal Sample Size Determination

This study was carried out in Katavi National Park and the surrounding game reserves in the whole of KRE (Fig. 1). Sampling was a cross sectional purposive approach. Criteria were firstly selecting buffalo groups that graze and drink water in shared points with livestock in proximity of the controlled areas. Second criterion was to choose animals that are relatively older and sick. Another reason for wild animal selection was due to availability and accessibility of animals, budgetary constraints especially procurement of anaesthetics such as etorphine hydrochloride; these drugs are costly (very expensive), not readily available and difficulty terrain of the ecosystem was an additional stumbling block to wildlife sampling due to forest, continuous cliff and seasonal streams.

### 3.4.2 Wild animal capture

For the wildlife group an opportunistic capture, and sampling of animals that are killed by hunters, naturally died and snare caught was performed. However, a targeted capture was also carried out by immobilization of older/suspected and known or possibly infected animals during routine veterinary work in the Katavi national park. The blood samples
were obtained from wild animals after immobilization by chemical restraint through remotely injected anesthetic drugs (darting). Targeted capture of free ranging buffalo ruminants was performed using a long barrel Dan inject No 1589 MOD JM dart gun US. This is a robust 25 bar pressure manometer fitted trigger dart gun with adapter that allows attachment of both 72g and 16g CO₂ cartridge cylinder. Etorphine hydrochloride (9mg M99 SA) combined with a tranquilizer xylazine (80mg Xylaject SA) were used in capture along with antidote diprenophine hydrochloride (25mg M5050 SA) in the 3 ml dart projectile syringes. Every buffalo captured was blind folded, and the blood drawn from the jugular vein (Kock and Burroughs, 2006). General sampling of wildlife animals included African buffaloes, lion leopard and others showed in results. Every hunter-killed animal during tourism hunting season in the ecosystem was sampled to establish disease status in the area by culture and isolation of the mycobacteria.

3.4.3 Sampling for Bovigam® and Stat Pak®

The IFN-γ assay is useful in animals that are difficult to capture or handle as they must be captured only once. This is why all buffalos were tested for IFN-γ to maximise use of resource and time. Following a slight modification, the Bovigam® assay has proved to be a valuable tuberculosis test for cattle (Globler et al., 2002; Gormley et al., 2006; Kathryn, 2008). Animals were tested for Bovigam and Stat Pak to compare the performance of both tests. Samples were tested from 78 animals derived from buffalos, 59 captured during immobilization and 18 obtained from hunters. Only 115 cattle from slaughter house were selected for bovigam testing. This was possible at Mpanda slaughter house because they can keep animals in kraal for four days before slaughter. A total of 20 farmed cattle were tested as comparison for parallel test with SCITT diagnostic test.
3.4.4 Livestock sampling proportional to the size of clusters

Cattle herds were selected by a stratified cluster sampling proportional to the size of the cattle population in which the study unit was a village that was considered as a cluster or herd. By levels of district, division, ward, and village, the number of herds and individual animals were established. Sample size was calculated using the formula described by Bennett et al. (1991) of which from the two study districts 632 cattle were required. 32 clusters (villages) were selected (Table 1) and designed as herds. Each herd having 20 cattle samples. The sampling interval (K) is = Total number of cattle in all 32 selected villages / Number of herds (32) to sample. Then a random number below K is chosen to get the point of departure (e.g. 11) of the sampling. Next add the sampling interval (11+K = 280,000/32 + 11) until the required number of herds to sample is reached. The approach ensures that the size of the different clusters is considered appropriately.

The homogeneity between villages can be quantified by the intraclass correlation coefficient \( \rho(\text{roh}) = \frac{(\text{WithinVillageVariation})}{(\text{TotalVariation})} \). To consider this additional variability the standard error of the sampling distribution was enlarged by a factor called the design effect (D). D= Cluster inflation coefficient which is composed of the number of sampled animals (b) from each cluster (c) as mentioned above.

\[
D = 1 + (b-1)\rho
\]  

For \( \rho \) we use known estimates. It is assumed that \( \rho \) is 0.2 basing on expected prevalence of 13 \% to estimate interaction. Therefore for 20 animals per herd chosen D was calculated = 1 + (20–1) 0.2 = 1 + 3.8 = 4.8. This increases the total sample size considering a standard error \( (s.e. \) \) that measures the precision of our estimate of the same magnitude. The number of clusters can then be calculated as;

\[
c = \frac{p(1-p)D}{(s.e.)^2b}
\]  

.................................(2)
Table 1: Village selected and cumulative number of cattle used in the study

<table>
<thead>
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<th>Division</th>
<th>Ward</th>
<th>Village</th>
<th>Cattle number</th>
<th>Exactness (%)</th>
<th>Expected Prevalence</th>
<th>Cattle Tested</th>
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<td>13.5</td>
<td>42</td>
</tr>
<tr>
<td>Mwamapuli</td>
<td>Mwamapuli</td>
<td>Chala</td>
<td>5500</td>
<td>5</td>
<td>13.5</td>
<td>40</td>
</tr>
<tr>
<td>Mwamapuli</td>
<td>Mwamapuli</td>
<td>Chala</td>
<td>3376</td>
<td>5</td>
<td>13.5</td>
<td>32</td>
</tr>
<tr>
<td>Mwamapuli</td>
<td>Mwamapuli</td>
<td>Chala</td>
<td>2833</td>
<td>5</td>
<td>13.5</td>
<td>40</td>
</tr>
<tr>
<td>Mwamapuli</td>
<td>Mwamapuli</td>
<td>Chala</td>
<td>4294</td>
<td>5</td>
<td>13.5</td>
<td>25</td>
</tr>
<tr>
<td>Mwamapuli</td>
<td>Mwamapuli</td>
<td>Chala</td>
<td>2000</td>
<td>7</td>
<td>13.5</td>
<td>10</td>
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<tr>
<td>Mwamapuli</td>
<td>Mwamapuli</td>
<td>Chala</td>
<td>1200</td>
<td>5</td>
<td>13.5</td>
<td>20</td>
</tr>
<tr>
<td>Mwamapuli</td>
<td>Mwamapuli</td>
<td>Chala</td>
<td>2501</td>
<td>5</td>
<td>13.5</td>
<td>20</td>
</tr>
<tr>
<td>Mwamapuli</td>
<td>Mwamapuli</td>
<td>Chala</td>
<td>1500</td>
<td>5.4</td>
<td>13.5</td>
<td>17</td>
</tr>
</tbody>
</table>

It becomes possible to optimise the number of clusters (c) and the number of individuals within clusters (b) with regard to the aimed precision, the product (b*c) of which gives the desired sample size final (n) =632 livestock for the whole study at obtainable (s.e. )= 0.019 precision. The total sample size calculated for 32 herds was n=632 cattle. Looking at data of cattle provided in each of the village (herd) tested by tuberculin in terms of
statistical basis, the expected prevalence when planned to test the number of cattle and the value of exactness with the number finally tested is calculated and shown in a column (Table 1).

### 3.5 Tuberculin Skin Testing

All livestock, from which the specimens were collected, were ear tagged and shaved mark for identification and reading of skin swelling post tuberculin injection. The cattle population were categorized into males/female and young/old cattle; exotic or local breeds. The origin of the cattle was traced using documentation and tag.

Cattle were tested using tuberculin PPD induction. Cattle were restrained and shaved on one side of the neck in two sites (12 cm apart). After shaving one side of the cattle neck and recording the initial skin thickness, 0.1 ml of avian (A) Purified protein derivate PPD (A₀) and bovine (B) PPD as (B₀) tuberculin were injected intradermally. The blue and red McLintoch 2 ml automatic syringes (McLintoch-Glasgow, UK) were used to inject bovine and avian tuberculin respectively. Seventy two hours post-injection, the skin-fold thickness was measured again and recorded as A₇₂ and B₇₂ for avian and bovine PPD sites respectively (Lesslie *et al.*, 1976; OIE, 2009). The animals were considered negative if the increase of skin thickness represented by TT = (B₇₂ - B₀) - (A₇₂ - A₀) was <1. The animals were tuberculosis positive (reactors) if the increase in skin thickness (TT) was ≥4 mm and the rest considered inconclusive therefore retested to indicate their status (Appendix 2). Ameni *et al.* (2008, 2010) has described how to calculate true prevalence of bovine tuberculosis when using skin test in the above setup.
3.6 Gamma Interferon Immunoassay (Bovigam®)

Gamma-interferon (IFN-γ) assay (Appendix 3) can provide a means for the early identification of *Mycobacterium bovis* in infected animals. In this study the assay was used to test livestock and wild animals against the disease. Using the avian and bovine PPD tuberculin the samples (collected in heparin vacutainers), the blood was sensitized within 24 hours of collection and the supernatant recovered thereafter stored into liquid nitrogen or freezer. Blood samples were collected in heparinized vacutainer tubes and sensitized with both avian and bovine purified protein derivative tuberculin within 24 hours, incubated at 37°C and plasma harvested after 16 hours of incubation.

Blood sample was collected by jugular venepuncture into heparinised vacutainers. At room temperature blood was stored for atleast 30 hours of collection, aliquoted into three (1, 2 and 3) vials into which three substances were added; 100 μl of Phosphate buffered saline (nil or negative antigen), 100 μl of Bovine PPD antigen and 100 μl of Avian PPD antigen respectively. Samples were incubated at 37°C for 16 hours thereafter centrifuged and plasma harvested. Plasma was either tested immediately or stored at minus 20°C for several months. A summary of the assay procedure is given in appendix 3, each sample was assayed in duplicate and controls in triplicate. Microtitre plates coated with monoclonal antibody to gamma interferon are used as supplied for the assay. Plates are read using a microtitre plate reader with 450nm filter.

If the result of bovine purified protein derivatives optical density (Bov.PPD.OD) minus Nil antigen optical density (only added with phosphate buffered saline -nil.Ag.OD) AND Bov.PPD.OD – Av.PPD.OD ≥ 0.1 were considered positive if the positive bovine IFNγ OD > 0.700 (positive results must not deviate by more than 30% from mean OD*) Bov.PPD.OD – nil.Ag.OD < 0.1 AND Bov.PPD.OD – Av.PPD.OD < 0.1 were
considered negative if the negative bovine IFNγ OD < 0.130 (maximum variation of ± 0.04*) *If these conditions were not met then the test results were invalid thus repeated.

3.7 Stat Pak® Assay

Blood samples for Stat Pak test were collected in plain vacutainer tubes. The blood was left overnight and thereafter plasma decanted and stored in eppendorf tubes until test day. Serum samples were tested according to the rapid test Chembio Vet TB Stat Pak® manufacturer's instructions. A 20 µl of serum was added to the test strip sample area, then 3 drops (100 µl) of diluents added and the results were read visually after 20 minutes. The test results were only considered valid if a blue band was visible in the control area of the test strip. If a blue band also developed in the test area, the sample was considered antibody positive; if the test area remained clear, the sample was considered antibody negative. Any tests which failed to develop a line in the control area were repeated with a new strip.

3.8 Post-mortem and Tissues Sample and Data Collection

Tissue samples from animals with gross visible tubercle and nodular lesions were collected from slaughter houses/slabs through post-mortem. Similar tissue samples from dead animals especially in wildlife were collected. These samples were transported on ice or liquid nitrogen to the SUA-FVM for further processing. Ice pack and liquid nitrogen were used to maintain cold chain and -80°C temperature freezer for longer storage. Samples were collected between August 2010 and August 2012 from livestock and wildlife animals within KRE.

Main tissue samples were lymph nodes of the respiratory and alimentary system; they were collected from slaughtered cattle during routine meat inspection. The lymph nodes
were mainly retropharyngeal, sub mandibular, bronchial, and mediastinal. Tissue samples were collected from the two study districts’ slaughter house. The pooled lymph nodes from each animal were placed in a 25 ml plastic bag or screw capped plastic universal container. The containers were labelled properly with the tag number of the respective animal, and then stored in the freezers at −80°C prior to processing and inoculation.

The samples were cultured, sub cultured and subjected to different bacteriological, biochemical and molecular tests. Livestock (cattle, goat and sheep) antemortem and postmortem examination were performed at slab or slaughter house in the villages that surround the game area, gamma interferon and a skin test for animals was carried out. Most information on disease risk and other secondary data were captured through prepared structured questionnaires.

A total of 328 livestock tissue samples were collected from several slaughter houses in the study area from 2010 to 2012. It was convenient for Mpanda slaughter house to perform antemortem examination a day before slaughter because the businesspersons had built a boma nearby the slaughter house where they kept cattle awaiting slaughter. All of the cattle brought for slaughter to the slaughter houses or slabs were examined during antemortem for body condition, status, age, sex and breed. All ante-mortem examinations were followed up by postmortem inspections to look for suspect nodular and tuberculous lesions in the lungs, liver and in particular a range of lymph nodes (bronchial, mediastinal, parotid, and retropharyngeal LNs) and descriptions of the lesions were recorded. All sampled specimens from each animal were pooled together and kept in a liquid nitrogen container or cool boxes at 4°C and transported to the university laboratory in Morogoro. Tissue samples collected from the study area came from different areas.
To compare the diagnostic tests, Bovigam® Statpak® and Skin test were evaluated without considering the gold standard although some of the skin test results were confirmed positive in culture isolation. The gold standard for the *M. bovis* is isolation by culture (OIE, 2009; Ameni *et al*., 2010). As a rule of thumb the agreement between the tests was evaluated considering one of the tests (tuberculin) being a generally acceptable method. Kappa was calculated basing on estimation of the observed proportion of agreement and the expected proportion assuming chance agreement (Cohen, 1960; Gwet, 2008).

\[
\kappa = \frac{Pr(a) - Pr(e)}{1 - Pr(e)}
\]

Where Pr (a) is the relative observed agreement among methods, and Pr (e) is the hypothetical probability of chance agreement. If the methods are in complete agreement then \( \kappa = 1 \) in the equation. If there is no agreement among the methods other than what would be expected by chance, \( \kappa = 0 \). The statistic ranging from zero to one with a kappa value of about 0.4 to 0.6 indicates moderate agreement.

### 3.9 Bacteriological Examination

In the SUA-FVM Laboratory, bacteriological test was carried out to detect *M. bovis* and other members of MTC through culture and isolation. Specimens from all animals, which exhibited gross visible nodular and tuberculous lesions, were dissected and homogenised by using a stomacher blender. Two Löwenstein-Jensen (LJ) slants, supplemented with either sodium pyruvate or glycerol, were inoculated with 3 ml of the suspension and the cultures were incubated at 37° C for at least ten weeks, with weekly observation for any signs of growth. For cultures suspected to be contaminated, stored sediments were retreated for a longer duration and streaked onto a blood agar plate. Blood agar plates were
incubated overnight, and if any signs of contamination persisted, the sample was treated further by increasing the decontamination time. The positive cultures were those that provided colony morphology similar to that described by Kubica et al. (2003). Positive cultures were subsequently 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. *M. tuberculosis* produces eugonic growth on both media, while *M. bovis* favourably grows well on L-J pyruvate medium. Presence of Acid-Fast Bacilli were tested by Ziehl-Neelsen (ZN) staining and microscopy. The detailed description of each stage is described under each section.

All the processing of specimen from cattle and wildlife was carried in the certified bio safety cabinet. After one hour of thawing a pool of lymph nodes were taken from the containers aseptically using sterilized forceps and placed in sterile containers. Using sterile scalpel blades, samples were further macerated to obtain fine pieces, which were divided into two portions. One portion was stored at −80°C for future reference, while the other portion of chopped lymph nodes was placed in stomacher bag containing about 5 ml of distilled water and homogenized for two minutes using a stomacher 80 Lab blender (Seward Laboratory-London, U.K). The lymph nodes homogenate was then put into a universal container and 3% oxalic acid was added at equal volume to the universal bottle for decontamination. Decontamination was carried out for 45 minutes with intermittent shaking. The duration of the decontamination step was increased depending to the level of contamination. After the allotted decontamination time, the lymph node homogenate was centrifuged at 3000rpm for 15 minutes and the supernatant discarded while to the sediment a 2% NaOH mixed with an indicator-phenol red was added to neutralise the pH of the suspension. Neutralization was achieved when the suspension colour changed from purple to pink. Suspensions were then centrifuged and the supernatant discarded to leave
the sediment, 2ml of sediment were used as inoculum for the cultivation of mycobacteria into L-J Media.

For primary isolation of *Mycobacteria*, the egg media namely; Lowenstein Jensen with added pyruvate (LJ pyruvate) and glycerol (LJ glycerol) were used. The media compositions were as shown in Appendix 1b. Suspected colonies were stained with Ziehl Neelsen (ZN) according to Grange (1984). The presence of AFB and cording of bacilli were indicative of presence of *Mycobacteria* species. Briefly, a loopful of pure mycobacterial colony was spread to make an impression smear on a glass slide and heat-fixed using hotplate within the safety cabinet. Glass slides were then placed on a rack over a sink for ZN staining. The slide was flooded with carbolfuchsin and then heat fixed underneath burning methanol-cotton until steam rises without boiling. Then left to cool for 5 minutes and washed with running water. Decolorisation was done by acid alcohol (3ml of HCL into 97% ethanol) in 30 seconds and then rinse with running water. Counterstain with 1% Malachite green for 3 minutes and then washed with water. Slide was dried in air and observed under (at X100 magnification) oil immersion microscope. Pinkish-red rods were recorded as positive Acid Fast Bacteria (AFB).

Confirmed mycobacteria strains as AFB+ were sub-cultured in L-J media to obtain enough cells for molecular analysis. When enough colonies had grown on the surface of the solid culture media a single colon was harvested by scraping using sterile disposable plastic loop. The harvested cells were placed in a sterile 1.5 ml eppendorf containing 200 ul of nuclease free water and mixed thoroughly. The sealed eppendorf were placed in water bath and boiled at 80°C for 60 minutes in order to kill the cell, and also enable release of bacteria genome as extraction method for the DNA. After heat treatment the
eppendorf were transferred directly in the tray containing ice ready for PCR, and the remaining part was then stored at 4°C or -80°C for longer storage.

3.10 Molecular Diagnostic Tests

All strains of mycobacteria identified as AFB positive were subjected to molecular biology analysis using polymerase chain reaction (PCR). The first step towards carrying out the molecular biology tests was successful extraction of mycobacterial cells from culture.

3.10.1 Mycobacterial cells extraction from culture

The reagents and equipment used for extraction of mycobacterial cell were autoclaved to make them sterile before carrying out any molecular process, as reagents and equipment that are not sterile may contain DNA inhibitors that can interfere with results of PCR products as well. The heat killed extracted mycobacterial cells were centrifuged (at 1500rpm for 5 minutes) and then 2 µl of a sample supernatant was drawn and used for PCR procedures.

3.10.2 Determination of quality and quantity of the DNA

The quality and quantity of the extracted DNA was determined by subjecting the DNA to electrophoresis on 1.5% agarose gel followed by visual comparison of the intensity and conformation of bands of template DNA with those of molecular weight marker. A single clean band of the DNA with high intensity indicated that the DNA extracted was good in quality, and smearing signified poor quality.
3.10.3 Preparation of agarose gel

Agarose gel was made by mixing 1.65g of Agarose powder LE, Analytical Grade (Promega Madison, U.S.A) with 1 x TBE buffer filling to 150ml to obtain 1.5% concentration of the gel. Agarose was dissolved by heating the solution on a hot plate. A volume of 3.0 µl of ethidium bromide (0.06%v/v) solution was added to every 150 ml at 60°C of molten agarose to obtain the final concentration to 1.5 µg/ml. Molten agarose was then poured into the electrophoresis gel casting equipment and left for half an hour to set.

3.10.4 Loading of DNA into agarose wells

Before loading DNA samples, a 2 µl of loading blue dye 6X (Promega MADISON, WI USA) was added and mixed to every 8 µl of the DNA to be analyzed. For each analysis, the first well of the gel was loaded with DNA molecular marker 100 bp DNA ladder (Promega MADISON, WI USA) of 1 or 1.5 kb size. The molecular weight marker was run parallel with the DNA of sample, positive (*M. tuberculosis* was H37Rv and AF2122/97 for *M. bovis*) and negative control (Nuclease free water) in 1 x TBE buffer in a horizontal gel electrophoresis apparatus at a constant voltage of 100V for 90 minutes. Samples were electrophoresed in 1.5% agarose gel stained with Ethidium Bromide (3 µl into 110 ml-1 x TBE buffer trough) and observed under UV light.

3.11 Visualization of DNA

Visualization of bands was done by placing the electrophoresed agarose gel to a medium wavelength ultra-violet (UV) light transluminator (STX-20, Jencons Ltd., USA). A digital camera was used to document digital image of both DNA ladder and samples viewed within agarose gel and then the bands were evaluated.
3.12 Molecular identification of mycobacteria by PCR

This was carried out according to PCR protocol described by Berg, (2007); (2008a) and (2008b) that includes mycogenus specific multiplex PCR (Appendix 4) for identification of species from the *Mycobacterium* genus and to differentiate species of *M. bovis* from other members of the MTC.

3.12.1 Genus Typing using Multiplex PCR

Mycogenus typing or multiplex PCR protocol identifyies species from the *Mycobacterium* genus, but also differentiate species of the *M. tuberculosis* complex from *M. avium*, *M. intracellulare* and other *Mycobacterium* species. The method can be applied to heat-killed bacterial suspensions or extracted genomic DNA. This multiplex PCR protocol uses six different primers. Firstly, it targets a sequence region within the 16S rRNA gene specific for the *Mycobacterium* genus that will exhibit a band at 1030 bp. The two primers MYCGEN-F and MYCGEN-R are designed to amplify a specific PCR product from genomic DNA of all known mycobacteria. Secondly, the PCR mix also includes primers that are specific for a hyper variable region of the 16S rRNA gene of *M. intracellulare* (MYCINT-F) and *M. avium* (MYCAV-R), respectively, giving one additional PCR product if the DNA template is any of these two species. Thirdly, species from the *M. tuberculosis* complex can also be identified due to the two primers (TB-1F, TB-1R) that target the MPB70 gene, specific for mycobacteria from the complex that shows band at 372 bp (Berg, 2008b; Appendix 4). All other Oligonucleotide primers used for molecular typing of mycobacteria isolates and sizes of the expected PCR products have been summarized in Table 2.
Table 2: Oligonucleotide primers used for molecular typing of mycobacteria isolates and sizes of the expected PCR products

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Present</th>
<th>Deleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>100μM MYCGEN-F</td>
<td>5'-AGAGTTTGATCCTGGGCTCAG-3'</td>
<td>1030 bp</td>
<td>NA</td>
</tr>
<tr>
<td>100μM MYCGEN-R</td>
<td>5'-TGCACACAGGCCACAAGGGA-3'</td>
<td>1030 bp</td>
<td>NA</td>
</tr>
<tr>
<td>100μM MYCAV-R</td>
<td>5'-ACCAGAAGACATGCTCTT-3'</td>
<td>1030 and 150 bp</td>
<td>NA</td>
</tr>
<tr>
<td>100μM MYCINT-F</td>
<td>5'-CCTTTAGGCCCATGTCTTTA-3'</td>
<td>1030 and 850 bp</td>
<td>NA</td>
</tr>
<tr>
<td>100μM TB1-F</td>
<td>5'-GAACAAATCCGGAGTTGACAA-3'</td>
<td>1030 and 372 bp</td>
<td>NA</td>
</tr>
<tr>
<td>100μM TB1-R</td>
<td>5'-AGCACGCTGCTCAATCATGTA-3'</td>
<td>1030 and 372 bp</td>
<td>NA</td>
</tr>
<tr>
<td>100μM RD4-FlankFW</td>
<td>5'-CTCGTCGAAGGCCACTAAAG-3'</td>
<td>335 bp</td>
<td>446 bp</td>
</tr>
<tr>
<td>100μM RD4-FlankRev</td>
<td>5'-AAGGCGAACAGATTCAGCAT-3'</td>
<td>Locus 2: Deletion typing RD 4</td>
<td></td>
</tr>
<tr>
<td>100μM RD4-InternalFW</td>
<td>5'-ACACGCTGGCGAAGTATAGC-3'</td>
<td>Locus 3: Deletion typing RD 9</td>
<td></td>
</tr>
<tr>
<td>100μM RD9-FlankFW</td>
<td>5'-AACACGGTCACGTTGCTG-3'</td>
<td>396 bp</td>
<td>575 bp</td>
</tr>
<tr>
<td>100μM RD9-FlankRev</td>
<td>5'-CAAACCAGACAGCTGCTTG-3'</td>
<td>Locus 4: Spoligotyping PCR</td>
<td></td>
</tr>
<tr>
<td>100μM RD9-InternalRev</td>
<td>5'-TTGCTTCCCCGCTCGTCTG-3'</td>
<td>Locus 4: Spoligotyping PCR</td>
<td></td>
</tr>
</tbody>
</table>

*DRa is biotinylated in the 5'-end. The table is adapted from Berg et al., 2009

3.12.1.1 Optimization of PCR conditions

The molecular laboratory work was done in four different laboratory rooms to prevent contamination of reagents with other DNA, as this would give wrong results. Extraction of DNA was done in the general tuberculosis designated laboratory followed by preparation of the DNA for PCR amplification in the molecular biology laboratory “A”. PCR reagents master mixing was carried out in the specific master mix molecular biology laboratory “B” and the PCR was set in the multipurpose molecular biology laboratory “C”. Amplification of the DNA by PCR was carried out after adjusting steps and setting the right cycles, temperature and time of the PCR thermocycler machine.
3.12.1.2 Preparation of DNA templates

DNA template from heat killed cell immediately quenched on ice before adding 2 μl of it in the 18 μl of the master mix in 0.2 ml flat cap PCR tube to make a final volume of 20 μl of the reaction mixture.

3.12.1.3 Preparation of the master-mix for Mycogenus PCR

The PCR amplification was performed in a final volume of 20 μl containing 26 samples reaction of a Qiagen product protocol as shown in Table 3. The reaction contains mixture for 22 samples plus two controls and the rest excess in case of pipetting error.

### Table 3: Mycogenus PCR 20 μl reaction mixture for 22 samples

<table>
<thead>
<tr>
<th>Reagent</th>
<th>X 1 reaction</th>
<th>X 26 reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase free water</td>
<td>6.2 μl</td>
<td>161.2 μl</td>
</tr>
<tr>
<td>100 μM Mycogen – Forward</td>
<td>0.3 μl</td>
<td>7.8 μl</td>
</tr>
<tr>
<td>100 μM Mycogen – Reverse</td>
<td>0.3 μl</td>
<td>7.8 μl</td>
</tr>
<tr>
<td>100 μM TB 1 – Forward</td>
<td>0.3 μl</td>
<td>7.8 μl</td>
</tr>
<tr>
<td>100 μM TB 1 – Reverse</td>
<td>0.3 μl</td>
<td>7.8 μl</td>
</tr>
<tr>
<td>100 μM MYCOCAVR – Reverse</td>
<td>0.3 μl</td>
<td>7.8 μl</td>
</tr>
<tr>
<td>100 μM MYCINTR – Forward</td>
<td>0.3 μl</td>
<td>7.8 μl</td>
</tr>
<tr>
<td>Qiagen Master mix (MgCl₂, DNTPs)</td>
<td>10 μl</td>
<td>260 μl</td>
</tr>
<tr>
<td>Total</td>
<td>18 μl</td>
<td>468 μl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>2 μl</td>
<td>Specific</td>
</tr>
<tr>
<td>Total reaction mixture</td>
<td>20 μl</td>
<td></td>
</tr>
</tbody>
</table>

3.12.1.4 Setting of the mycogenus PCR

The total reaction mixture of the DNA above was put into PCR reaction tubes and ran into the eppendorf thermocycler using the multiplex protocol that comprised of denaturation, annealing, and extension PCR cycle steps. The reactions were subjected to 35 cycles as shown in Table 4. The oligonucleotide primers used in the multiplex PCR for the amplification of DNA and their targets sites were;

1: MYCGEN-F (5’-AGA GTT TGA TCC TGG CTC GA-3’) forward primer for the detection of *Mycobacterium spp*
2: MYCGEN-R (5’-TGC ACA CAG GCC ACA AGG GA-3’) reverse primer for the detection of *Mycobacterium spp*
3: MYCAV-R (5’-ACC AGA AGA CAT GCG TCT TG-3’) primer for the detection of *Mycobacterium avium* complex strains
4: MYCINT-F (5’-CCT TTA GGC GCA TGT CTT TA-3’) primer for the detection of *Mycobacterium intracellulare* strains
3 and 4 is for detection of *Mycobacterium avium* complex
5: TB1-F (5’-GAA CAA TCC GGA GTT GAC AA-3’) and
6: TB1-R (5’-AGC ACG CTG TCA ATC ATG TA-3’) primers for the detection of MTC strains

**Table 4: The actual program sequence for the multiplex PCR**

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>2 minutes</td>
</tr>
<tr>
<td>Actual denaturation</td>
<td>94°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>62°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Extension/elongation</td>
<td>72°C</td>
<td>60 seconds</td>
</tr>
<tr>
<td>Final elongation</td>
<td>72°C</td>
<td>60 seconds</td>
</tr>
<tr>
<td>Cooling/hold</td>
<td>4°C</td>
<td>4 minutes</td>
</tr>
</tbody>
</table>

### 3.12.1.5 Analysis of PCR products

The PCR products were analyzed by using 1.5 % Agarose gel electrophoresis. A volume of 8μl PCR product (amplicon) was mixed with 2μl of loading dye. After mixing the PCR products were loaded in the wells according to number of amplicon and one of the well 1.5 kb molecular weight marker was added and run in a parallel track at 100 volts for 90 minutes.

### 3.12.2 Species Identification Using Region of Difference (RD4) based PCR

The PCR amplification was performed in a final volume of 20 ul containing X reaction of a Qiagen product protocol. The detail is shown in Table 5.
Table 5: RD 4 Deletion PCR 20 μl reaction mixture for 26 samples

<table>
<thead>
<tr>
<th>Reagent</th>
<th>X 1 reaction</th>
<th>X 26 reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O Qiagen</td>
<td>7.1 μl</td>
<td>184.6 μl</td>
</tr>
<tr>
<td>RD 4_Flank Rev 100 μM</td>
<td>0.3 μl</td>
<td>7.8 μl</td>
</tr>
<tr>
<td>RD 4_FlankFW 100 μM</td>
<td>0.3 μl</td>
<td>7.8 μl</td>
</tr>
<tr>
<td>RD 4_Internal 100 μM</td>
<td>0.3 μl</td>
<td>7.8 μl</td>
</tr>
<tr>
<td>Qiagen Master mix (MgCl₂, DNTPs)</td>
<td>10 μl</td>
<td>260 μl</td>
</tr>
<tr>
<td>Total</td>
<td>18 μl</td>
<td>468 μl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>2 μl</td>
<td>Specific</td>
</tr>
<tr>
<td>Total reaction mixture</td>
<td>20 μl</td>
<td></td>
</tr>
</tbody>
</table>

3.12.2.2 Setting of the RD 4 PCR

A PCR cycle comprised of denaturation, annealing, and extension steps. The reactions were subjected to 35 cycles as indicated in Table 6. The primers used in the PCR for the amplification of DNA were: RD4-FlankFW (5’- CTC GTC GAA GGC CAC TAA AG -3’) RD4- Flank Rev (5’- AAG GCG AAC AGA TTC AGC AT -3’) and RD4-InternalFW (5’- ACA CGC TGG CGA AGT ATA GC -3). Positive control primers were *M. tuberculosis* (H37Rv) and *M. bovis* (AF2122/97). Sequential program is presented in Table 6.

Table 6: The actual program sequence for the RD 4 PCR

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>15 minutes</td>
</tr>
<tr>
<td>Actual denaturation</td>
<td>95°C</td>
<td>60 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Extension/elongation</td>
<td>72°C</td>
<td>60 seconds</td>
</tr>
<tr>
<td>Final elongation</td>
<td>72°C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Cooling/hold</td>
<td>4°C</td>
<td>4 minutes</td>
</tr>
</tbody>
</table>

If the PCR product corresponds to 446bp (RD4-FlankFW + RD4- Flank Rev) then RD4 is deleted (i.e. *M. bovis*), whereas presence of RD 4 is shown by band size of 335 bp indicating *M. tuberculosis* or *M. africanum*. 
3.12.3.1 Species Identification Using Region of Difference (RD 9) based PCR

The PCR amplification was performed in a final volume of 20 μl containing X reaction of a Qiagen product protocol. Table 7 shows the mixture details.

Table 7: RD 9 Deletion PCR 20 μl reaction mixture for 22 samples

<table>
<thead>
<tr>
<th>Reagent</th>
<th>X 1 reaction</th>
<th>X 26 reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O Qiagen</td>
<td>7.1 μl</td>
<td>184.6 μl</td>
</tr>
<tr>
<td>RD 9_ Flank Rev 100 μM</td>
<td>0.3 μl</td>
<td>7.8 μl</td>
</tr>
<tr>
<td>RD 9_FlankFW 100 μM</td>
<td>0.3 μl</td>
<td>7.8 μl</td>
</tr>
<tr>
<td>RD 9_Internal 100 μM</td>
<td>0.3 μl</td>
<td>7.8 μl</td>
</tr>
<tr>
<td>Qiagen Master mix (MgCl₂, DNTPs)</td>
<td>10 μl</td>
<td>260 μl</td>
</tr>
<tr>
<td>Total</td>
<td>18 μl</td>
<td>468 μl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>2 μl</td>
<td>Specific</td>
</tr>
<tr>
<td>Total reaction mixture</td>
<td>20 μl</td>
<td></td>
</tr>
</tbody>
</table>

3.12.3.2 Setting of the RD 9 PCR

A PCR cycle comprised of denaturation, annealing, and extension steps. The reactions were subjected to 35 cycles as indicated in Table 8. The primers used in the PCR for the amplification of DNA were:

100 μM RD9-FlankFW 5’- AAC ACG GTC ACG TTG TCG TG -3’
100 μM RD9-FlankRev 5’- CAA ACC AGC AGC TGT CGT TG -3’
100 μM RD9-InternalRev 5’- TTG CTT CCC CGG TTC GTC TG -3’

Table 8: The actual program sequence for the RD 9 Deletion PCR

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>15 minutes</td>
</tr>
<tr>
<td>Actual denaturation</td>
<td>95°C</td>
<td>60 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Extension/elongation</td>
<td>72°C</td>
<td>60 seconds</td>
</tr>
<tr>
<td>Final elongation</td>
<td>72°C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Cooling/hold</td>
<td>4°C</td>
<td>4 minutes</td>
</tr>
</tbody>
</table>

The PCR results were judged for each sample by comparing with the DNA ladder. If the PCR product had a size of 396 bp (RD9-InternalFW + RD9- Flank Rev), this was interpreted as that RD9 is present (i.e. *M. tuberculosis*). However, if RD9 is deleted
(i.e. *M. africanum* and *M. bovis*), then it yields a PCR product of 575 bp (RD9-FlankFW + RD9-Flank Rev).

### 3.12.4 Strain Identification Using Spoligotyping

#### 3.12.4.1 Introduction

Spoligotyping procedure describes a genetic typing method, which is used to detect and type bacteria of the *Mycobacterium tuberculosis* complex, including *M. tuberculosis*, *M. africanum*, *M. microti*, *M. caprae*, and *M. bovis*. Spoligotyping was performed according to Berg, 2007 procedure that includes three main steps: 1) PCR amplification of specific spacer sequences of a strain, 2) hybridization to a spoligomembrane, and 3) detection. The PCR amplification of the spacers is accomplished by using the primers Direct Repeats DRa and DRb (i.e. DRa: 5'–GGT TTT GGG TCT GAC GAC–3' and DRb: 5'–CCG AGA GGG GAC GGA AAC–3’), which can anneal to all repeat sequences and thereby enables for amplification of all spacers that occur in the DR region of a specific strain. This method is based on the enzymatic amplification of the DR locus of *M. bovis* and detects the presence or absence of spacers within the DR locus. Culture positive clinical samples as well as directly collected specimens can identify the clonal nature of the isolates. Mixing of water, primers and mastermix was done as shown in Table 9. A 20 μl of a spoligotyping PCR mix is dispensed into PCR tubes and the tubes are sealed with a foil lid. Using clean filter tip add 5 μl of heat killed unknown cell samples and control samples (*M. tuberculosis* H37Rv, dH2O, *M. bovis* AF2122/97) to make a total of 25μl in each PCR tube. Seal all PCR tubes with a foil lid again and label them accordingly before running the thermocycler.
Table 9: Spoligotyping PCR reaction mixture for 10 samples

<table>
<thead>
<tr>
<th>Reagent</th>
<th>x 1 reaction</th>
<th>x 10 reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile HPLC water</td>
<td>3.5μl</td>
<td>35 μl</td>
</tr>
<tr>
<td>Primer 1 DRa</td>
<td>2 μl</td>
<td>20 μl</td>
</tr>
<tr>
<td>Primer 2 DRb</td>
<td>2 μl</td>
<td>20 μl</td>
</tr>
<tr>
<td>Qiagen mastermix</td>
<td>12.5 μl</td>
<td>125 μl</td>
</tr>
</tbody>
</table>

3.12.4.2 Spoligotyping PCR setting

Spoligotyping is the critical stage of the molecular epidemiology of this study that generated information on genetic variation and similarities of the *M. bovis* circulating at Katavi Rukwa wildlife – livestock interface. Strains were spoligo-typed according to the method by Kamerbeek *et al.* (1997) with minor modifications from Berg (2007), and the others as adapted from Berg (2008a, 2008b). All PCR and typing was performed at the FVM-SUA, but quality control and comparison of results was done at FLI, Germany.

A spoligotyping PCR amplification cycle comprises of denaturation, annealing, and extension steps. The reactions were subjected to 30 cycles in a thermocycler (Mastercycler personal-eppendorf AG). The detailed steps, their respective temperature and time are presented in Table 10.

Table 10: The actual program sequence for the Spoligotyping PCR

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>96°C</td>
<td>15 minutes</td>
</tr>
<tr>
<td>Actual denaturation</td>
<td>96°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>Extension/Elongation</td>
<td>72°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Final elongation</td>
<td>72°C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Soak/hold</td>
<td>4°C</td>
<td>4 minutes</td>
</tr>
</tbody>
</table>

3.12.4.3 Hybridization

Before hybridization, primary buffer 187.5 μl was add into the tubes containing the PCR products using clean pipette (20-200 μl) and put on the thermocycler for 10 minutes at 96°C by a denature program. Hybridisation oven and water bath were set both to 60°C.
Required buffers: Primary buffer (60°C), Secondary buffer (60°C), Secondary buffer (42°C), 2xSSPE (RT) at room temperature, these buffer were bought as a ready mix from supplier. The membrane was washed into 200 ml primary buffer at 60°C for 10 min. Followed by washing the miniblotter with detergent and rinsed thoroughly with distilled water. The support cushion and the membrane were placed, assemble and tightened to the miniblotter to prevent leakage between lanes, and any residual fluid removed by aspiration using the suction pump. 140 μl of primary buffer was added to the first and last lanes of the miniblotter, followed by loading 140 μl of the denatured PCR products. The lane after the last sample was also loaded with primary buffer. Hybridization was performed for 60 min at (60°C) in the hybridization oven; thereafter the samples removed completely from the miniblotter using the suction pump and disassembling the miniblotter. The membrane was washed twice in 200 ml secondary buffer at (60°C) for 10 min. The temperature of the hybridisation oven was changed to 42°C and the membrane transferred into the rolling bottle destined for Streptavidin-POD hybridization. Then incubation of the membrane with 5 μl Streptavidin peroxidase (Strep-POD) in 20 ml secondary buffer at 42°C for 1 hour. Also, the secondary buffer (42°C) placed into the hybridisation oven for equilibration at 42°C. Thereafter the membrane was washed twice in the destined rolling bottle in 200 ml secondary buffer for 10 min at 42°C. The membrane was washed twice in 200 ml 2xSSPE buffer for 5 min at room temperature. 10 ml enhanced chemiluminescent (ECL) reagent 1 was mixed with 10 ml of ECL reagent 2 and incubated with the membrane with for 2 minutes in the destined plastic container.

Finally set the hybridisation oven to 80°C for the stripping later on.

3.12.4.4 Exposure and detection of the membrane

The membrane is wrapped in a clean, transparent plastic foil. Into the dark room place the wrapped membrane facing up in a film cassette and position a new X-ray film on top.
The membrane was exposed for 15 - 20 min and then the film obtained was incubated for 1-2 min in the developer, rinsed with water and incubated for 4 min in the fixer and at least for 2 minutes in the water bath. The film was washed under running water and then allowed to air-dry ready for interpretation. The whole hybridization and detection sequential steps can be summarized as shown in (Fig. 3).

![Diagram](image)

**Figure 2:** Schematic view of hybridization and detection of spacers on a spoligomembrane, symbolized by positive binding of spacer 19 (Adapted from Berg, 2007)

### 3.12.4.5 Spoligotyping results

All sequence of the results were recorded in a “Spoligotyping excel workbook” and in a digitized manner of spacers from the autoradiograph results. For each isolate, the presence or absence of each of the 43 oligonucleotides was recorded as 1 (presence) or 0 (absence). The digitized obtained patterns (a series of 43 digit binary numbers) were transferred and identified to species strain by comparison with the sequences of spoligotyping pattern in the internet open source database accessible at [www.Mbovis.org](http://www.Mbovis.org).

Spoligotyping showing the deletion of Spacers 3, 9, 16 and the last 5 spacers (39-45) indicates that *M. bovis* is present.
3.13 Identification of non tuberculous mycobacteria (NTM) species

Sequencing the bacterial 16S ribosomal DNA gene (16S rDNA) has emerged as one of the best method to identify bacteria rapidly. Isolates that could not belong to *Mycobacterium tuberculosis* complex in the present study were submitted to 16S rDNA sequencing targeting the hypervariable region A and B for identification to their species level. Bacterial suspension for the NTM was submitted to FLI Jena, Germany for sequencing. The PCR for sequencing was performed according to methods by Kirschner and Bottger, (1998) using primer 271. The PCR product was eluted from agarose gel using QIAquik gel extraction kit (Qiagen, Hilden, Germany) and the sequencing done at GATC Biotech Konstanz Germany thereafter edited and analysed.

To advance the identification of NTM species, a partial sequencing of the 65 Kilodalton Heat Shock Protein (hsp65) gene was used and then identified by comparing unknown sequences to reference databases by a FASTA BLAST search. This reaction amplified a 439-bp fragment within the *hsp65* gene using the primers Tb11 (5′-ACCAACGATGGTGTGTCATC-3′) and Tb12 (5′-CTTGTGAACCGCATACCCT-3′) (Senna et al., 2008; Franco et al., 2013)

3.14 Spatial-temporal variation

3.14.1 Introduction

The use of Spatial-temporal variation detection of disease clusters is highly useful in surveillance of this disease. This helps in uncovering the reason behind the spread of the disease, and making suitable policies to control risk factors and eventual disease eradication. In this studies, statistics was demonstrated in the spatial scan developed by Martin Kulldorff (2012) in SaTScan® software that detects and provide inference for spatial and space-time disease clusters.
3.14.2 GIS reading and spatial temporal variations
Places of sample collection in this study were geo-referenced by use of hand held Global Positioning System (GPS) and the data were stored in a Geographical Information System (GIS) enabled database to allow incorporation of attribute data that can be used in epidemiological analysis, disease surveillance and risk analysis.

3.14.3 Questionnaire survey
Individual farmers were subjected to a questionnaire survey across the study districts of Mpanda and Nkasi. The questionnaire survey focused on assessing awareness on definition of tuberculosis, causes, and symptoms, modes of transmission and prevention or control measures employed in handling tuberculosis infections. The ability of the respondent to clearly mention in local Kiswahili or English, any of the aspects above would qualify the respondent as more knowledgeable. Possible source of bias were handled by engaging trained interviewer who administered a designed standardized questionnaire to each study participant as a tool for data collection.

3.15 Data Analysis
Quantitative data collected from the field and sampling were recorded and coded in the Microsoft Excel 5.0, and exported to EpiInfo® Version 3.1 (CDC, 2009) for Windows. The analysis in the EpiInfo® involved frequency, cross tabulation, mean, standard deviation and related statistics that were obtained and used to describe the characteristics and trends of variables. General linear models GLN) was used to assess the association between the different risk factors (age, sex, herd size, location, and animal tested) and results of SCITT of individual animals using STATA for Windows (version 12.0; StataCorp, 4905 Lakeway Drive College Station, Texas 77845 USA, 800-STATA-PC) with a p-value of <0.05 considered statistically significant. The kappa test was used
statistically for assessing the agreement between serological diagnostic methods measured on a dichotomous scale. It was used to measure the proportion of agreement beyond that to be expected by chance.

Arc GIS 9.3® in couple with SaTScan™ softwares was used to analyse spatial and temporal patterns of the disease both in the wildlife and livestock. By using the maximum spatial cluster size of 50% of the total population at risk, the spatial temporal as well as purely spatial cluster analysis was run to scan for area with high rate disease using a poisson (probability model) discrete statistic.

Characterization involving spoligotyping were analysed in the Bionumerics software. Obtained sequences of NTM were edited and analyzed in Bioedit http://www.mbio.ncsu.edu/BioEdit/bioedit.html and sequences were compared to available sequences in GenBank by the (National Centre for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/) NCBI Blast sequence alignment tool. The isolates were determined to species based on the maximum score and maximum identity values on NCBI Blast alignment, a maximum score and maximum identity of ≥ 99% were accepted.
CHAPTER FOUR

4.0 RESULTS

4.1 Farmers’ Knowledge and Perception on BTB Transmission

A questionnaire was used to collect the information and knowledge of farmers on bovine tuberculosis disease and its transmission. Respondents were from 26 villages of Mpanda (Katavi region) and Nkasi district. Generally, respondents were agropastoralists rearing mostly indigenous Tanzania short horn zebu with few exotic breeds. Few exotic breeds were Ayrshire and Friesian kept under zero grazing in Mpanda town. The knowledge on the role of various risk factors responsible for the occurrence and spread of BTB between cattle and people were assessed by a questionnaire on a total of 67 farm owners (households). Such disease risk factors include poor knowledge of the disease, drinking raw milk, eating raw meat, sharing pasture or water with wildlife, inhaling air from infected human and eating dead animals. The result is shown in the text and also depicted in Table 11. To compliment their knowledge about the disease, 97% of respondents mentioned the clinical manifestation of the disease in livestock as coughing and all of them mentioned emaciation. However, these clinical signs are not specific to only BTB. Proportion of respondent’s consuming raw milk was 77.6 %, while 3% boil and all do sour milk at some points. All respondents said they graze livestock communally and share watering points. During dry season, respondents 49/67 (73.1 %) move their livestock closer to protected areas in search for pastures and water.
Table 11: Questionnaire result on cattle owners’ awareness and behaviour related to bovine tuberculosis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Awareness of Bovine TB n (%)</th>
<th>Drinking raw milk n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mpanda (Katavi) region</td>
<td>51 (86.1)</td>
<td>51 (91)</td>
</tr>
<tr>
<td>Nkasi district</td>
<td>16 (56.2)</td>
<td>16 (67.1)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>61 (91)</td>
<td>61 (83.6)</td>
</tr>
<tr>
<td>Female</td>
<td>6 (97.3)</td>
<td>6 (73.4)</td>
</tr>
<tr>
<td>Age(years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;40</td>
<td>55 (76.4)</td>
<td>55 (98.1)</td>
</tr>
<tr>
<td>&lt;40</td>
<td>12 (82.1)</td>
<td>12 (17.9)</td>
</tr>
<tr>
<td>History of human TB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11 (98.5)</td>
<td>11 (85.4)</td>
</tr>
<tr>
<td>No</td>
<td>56 (61.5)</td>
<td>56 (55)</td>
</tr>
<tr>
<td>History of BTB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>42 (86.7)</td>
<td>42 (78.6)</td>
</tr>
<tr>
<td>No</td>
<td>25 (62.7)</td>
<td>25 (89.2)</td>
</tr>
<tr>
<td>Cattle owned</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>9 (67.4)</td>
<td>9 (13.4)</td>
</tr>
<tr>
<td>11-100</td>
<td>32 (79.8)</td>
<td>32 (47.8)</td>
</tr>
<tr>
<td>&gt;100</td>
<td>26 (88.3)</td>
<td>26 (38.8)</td>
</tr>
<tr>
<td>Indigenous</td>
<td>65 (97)</td>
<td>65 (99.3)</td>
</tr>
<tr>
<td>Exotic</td>
<td>2 (100)</td>
<td>2 (0)</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STD 7</td>
<td>61 (95.6)</td>
<td>61 (78.7)</td>
</tr>
<tr>
<td>None</td>
<td>6 (56.1)</td>
<td>6 (100)</td>
</tr>
<tr>
<td>Sharing water and pasture with wild animals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>57 (65.1)</td>
<td>57 (73.7)</td>
</tr>
<tr>
<td>No</td>
<td>10 (66.3)</td>
<td>10 (65.4)</td>
</tr>
</tbody>
</table>

n = number of respondents, (%) = proportions of response

Respondents were asked if they have heard about BTB, 80.6% have heard of bovine tuberculosis, or tuberculosis in animals. Of the cattle owners who had heard of tuberculosis in animals, only 5.9% (4/67) had an idea that the disease can be spread through air. However, 80.6% of the cattle owners believed that the disease is transmitted from wildlife to their animals.
Table 12: Overall farmers’ perception on bovine tuberculosis disease

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Level</th>
<th>Respondents (n=67)</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human TB case – coughing evidence</td>
<td>Yes</td>
<td>37</td>
<td>55.2</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>30</td>
<td>44.8</td>
</tr>
<tr>
<td>Human TB case – emaciation evidence</td>
<td>Yes</td>
<td>39</td>
<td>58.2</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>28</td>
<td>41.8</td>
</tr>
<tr>
<td>Dead animal disposal-Bury</td>
<td>Yes</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>66</td>
<td>98.5</td>
</tr>
<tr>
<td>Dead animal eating</td>
<td>Yes</td>
<td>56</td>
<td>83.6</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>11</td>
<td>16.4</td>
</tr>
<tr>
<td>Dead animal selling</td>
<td>Yes</td>
<td>64</td>
<td>95.5</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>3</td>
<td>4.5</td>
</tr>
<tr>
<td>Sell milk to neighbour</td>
<td>Yes</td>
<td>5</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>62</td>
<td>92.5</td>
</tr>
<tr>
<td>Sell meat to neighbour</td>
<td>Yes</td>
<td>67</td>
<td>100</td>
</tr>
<tr>
<td>Interaction with wild animal</td>
<td>Yes</td>
<td>67</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>54</td>
<td>80.6</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>13</td>
<td>19.4</td>
</tr>
<tr>
<td>TB from wildlife to livestock</td>
<td>Don’t know</td>
<td>40</td>
<td>59.7</td>
</tr>
</tbody>
</table>

4.1.1 Evidence for clustering of BTB based on purely spatial analysis

Twenty five locations in the study area with a population of 59 720 cattle were analysed. Statistic identified the most likely significant cluster for high occurrence of BTB at Mpanda outskirts. The overall relative risk (RR) within the cluster was 10.58 with an observed number of 9 cases detected during the year 2010–2012, compared with 1.08 expected cases. A statistically significant secondary cluster for high occurrence of BTB was also detected at Mirumba and Mamba with RR = 6.08 and 2.16, respectively. Twenty two percent were the annual cases per 100 000 cattle tested. Table 13 shows the detailed statistics. Table 14 also shows statistic scan in relation to high or low rate of disease using retrospective purely spatial statistic.
Table 13: High rate spatial BTB clusters detected using maximum spatial cluster size of 50% of population at risk

<table>
<thead>
<tr>
<th>Cluster with location ID</th>
<th>No. of cases</th>
<th>Expected cases</th>
<th>Relative Risk</th>
<th>Log likelihood Ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Most likely cluster*</td>
<td>9</td>
<td>1.08</td>
<td>10.57</td>
<td>12.0824</td>
<td>0.053</td>
</tr>
<tr>
<td>Secondary cluster**</td>
<td>5</td>
<td>0.92</td>
<td>6.08</td>
<td>4.60880</td>
<td>0.084</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.97</td>
<td>2.14</td>
<td>0.856636</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*the most likely cluster detected was located around Mpanda town outskirts.
** Secondary clusters detected at Mirumba and Mamba

Table 14: High or low rate spatial BTB clusters detected using maximum spatial cluster size of 50% of population at risk

<table>
<thead>
<tr>
<th>Cluster with location ID</th>
<th>No. of cases</th>
<th>Expected cases</th>
<th>Relative Risk</th>
<th>Log likelihood Ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Most likely cluster*</td>
<td>9</td>
<td>1.32</td>
<td>17.63</td>
<td>27.7899</td>
<td>0.013</td>
</tr>
<tr>
<td>Secondary cluster 1**</td>
<td>3</td>
<td>2.13</td>
<td>3.71</td>
<td>3.22</td>
<td>0.08</td>
</tr>
<tr>
<td>Secondary cluster 2**</td>
<td>5</td>
<td>1.13</td>
<td>4.81</td>
<td>3.72</td>
<td>0.35</td>
</tr>
<tr>
<td>Secondary cluster 3**</td>
<td>1</td>
<td>7.86</td>
<td>0.11</td>
<td>5.35</td>
<td>0.08</td>
</tr>
</tbody>
</table>

*Most likely primary significant cluster was at Mpanda outskirts, **Secondary cluster 1 was at Urwira, **Secondary cluster 2 at Mirumba (not significant), and **Secondary cluster 3 were Chala and Mashete

4.1.2 BTB clustering basing on space-time analysis

Retrospective Space-Time analysis scanning for clusters with high rates using the Discrete Poisson model results were somewhat far distinct from the purely spatial analysis in the areas of high incidence of BTB particularly in the most likely primary clusters detected. Using the same spatial window that could include up to 50% of population at risk and a maximum temporal window of 50% without including purely spatial clusters, the most likely statistically significant cluster for high occurrence of BTB was again found to exist at Mtisi, Ibindi, Itenka, Kakese, Mpanda, Urwira, Kiwajense, Kabungu, Mirumba, Katuma, Kashishi, Usevya, Paramawe, Tupindo. Figure 4 shows how hotspots were overlapping at Nkasi districts where farmers move between Mirumba and Paramawe in search of water and pasture close to the Katavi national park. Thirty number of BTB cases were detected in these 14 villages of the most likely primary cluster. The BTB relative risk for this cluster was 17.34, highly significant (p = 0.036) observing 103.8 annual cases of BTB per 100 000 cattle. Retrospective Space-Time
analysis scanning for clusters with high or low rates using the same model returned the same villages statistic at p = 0.04.

Figure 3: A study area location with radius (0.69) showing spatial temporal BTB hotspots (A map of KRE showing spatial temporal BTB hotspots for samples from livestock and wild animals collected. Insert is a map of Tanzania that shows the relative location of the study area)
4.1.3 Prevalence of Bovine Tuberculosis

A single comparative intradermal tuberculin test was performed in 789 adult cattle during the study period including 146 cattle that were tested prior to slaughter in the slaughter houses. Eleven (7.5 %) out of cattle tested in slaughter house were also positive. Thirty two clusters were studied, whereby only one cluster from Nkasi district was skin test positive reactor. Out of these 32 herds there were 15 positive clusters which make up to 46.9 % skin reactor cattle herd prevalence.

In the present study, by using 92.3% specificity and 72.7% sensitivity for the 4 mm cut-off, the positive predictive value was 88.9% while the negative predictive value was 80%. The apparent prevalence is 3.7% therefore the true prevalence was 4.6 % (95% CI, 3.4–5.9%) for the 4-mm cut-off. For the 2 mm cut off the true prevalence was 10.7% (95% CI [7.8-11.9]). Prevalence of 12-18% of disease was observed at Mpanda outskirts and Mirumba relatively higher as compared to other place like Paramawe in Nkasi districts. Nkasi place had generally 3% of disease proportion in villages that had positive reactors. General presentations of tuberculosis positive reactors are given in the Fig. 4.
4.1.4 Factors Associated with TB Positive Reactors

In performing univariate and multivariate analysis, a random effect logistic regression analysis was performed with herd (village) treated as a random effect to account for the difference in herd sizes because animals within herd are considered to form a cluster. Odds ratio OR=1 was used as a reference level to the risk factors that contribute to BTB positivity reactors. The risk factors were sex, location and number of animal as shown in Table 15.
Table 15: Univariate analysis of risk factors for cattle tuberculin reactors using the general linear model (GLM) with herd (village) as random effect

<table>
<thead>
<tr>
<th>Factors</th>
<th>Proportional % (n/N)</th>
<th>χ2 value</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>5.1 (30/584)</td>
<td>56.40</td>
<td>11(1.449-85.062)</td>
<td>0.03*</td>
</tr>
<tr>
<td>Male</td>
<td>4.4 (9/205)</td>
<td>0.34</td>
<td>9(0.508-2.464)</td>
<td></td>
</tr>
<tr>
<td>Breed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ayrshire cross</td>
<td>0.0 (0/6)</td>
<td></td>
<td></td>
<td>0.32</td>
</tr>
<tr>
<td>Friesian cross</td>
<td>0.0 (0/8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSH Zebu</td>
<td>5.0 (39/775)</td>
<td>0.1</td>
<td>1.3(0.831-3.244)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young &lt; 2 yrs</td>
<td>5.8 (3/52)</td>
<td>0.017*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adults &gt; 2 yrs</td>
<td>4.9 (38/772)</td>
<td>5.67</td>
<td>8.9(1.817-42.317)</td>
<td>0.017*</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mpanda</td>
<td>8.4 (36/426)</td>
<td>11.06</td>
<td>13 (1.443-84.785)</td>
<td>0.27</td>
</tr>
<tr>
<td>Nkasi</td>
<td>0.8 (3/363)</td>
<td>1.53</td>
<td>0.8(0.714-3.20)</td>
<td></td>
</tr>
<tr>
<td>1-8</td>
<td>6.3 (2/32)</td>
<td>1.00</td>
<td>0.76(0.227-2.312)</td>
<td>0.84</td>
</tr>
<tr>
<td>Animal Tested</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-14</td>
<td>4.7 (11/234)</td>
<td>1.78</td>
<td>0.92(0.369-3.413)</td>
<td></td>
</tr>
<tr>
<td>&gt;15</td>
<td>5.4 (28/523)</td>
<td>1.53</td>
<td>0.793(0.198-1.603)</td>
<td></td>
</tr>
</tbody>
</table>

OR=Odds Ratio and CI=Confidence Interval *statistically significant

In multivariate analysis all variables resulting from univariate analysis were carried on for further analysis, a forward selection approach was used to include variables from the model based on a likelihood ratio test. Results from multivariate analysis indicate that, some of the risk factors (age, location, sex, and animal tested; Table 16) which were considered into this study significantly contributed to positive reactivity to BTB. As indicated in Table 16, sex of animals was significantly associated with the result of Single Comparative Intradermal Tuberculin Test of individual cattle (OR = 11.06; CI; 1.449 – 84.875). This reflects that females were 11.06 times more likely to test positive than males. On the other hand, significant association depicted in result of skin test (SCITT) positivity with location indicate the highest prevalence of BTB was found in Mpanda district (8.4 %) as compared to Nkasi district (0.8 %).
Table 16: Multivariate analysis of risk factors for cattle tuberculin reactors using the general linear model (GLM) with herd (village) as random effect

<table>
<thead>
<tr>
<th>Factors</th>
<th>χ² value</th>
<th>OR 95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>41.06</td>
<td>11.1(1.489-84.875)</td>
<td>0.03*</td>
</tr>
<tr>
<td>Male</td>
<td>5.71</td>
<td>9.8(0.727-45.464)</td>
<td></td>
</tr>
<tr>
<td>Breed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ayrshire cross</td>
<td>1.1</td>
<td>0.2(0.831-1.233)</td>
<td>0.20</td>
</tr>
<tr>
<td>Friesian cross</td>
<td>1.1</td>
<td>0.2(0.831-1.233)</td>
<td>0.20</td>
</tr>
<tr>
<td>Age category</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adults &gt; 2 yrs</td>
<td>10.3</td>
<td>10.4(0.6-4.2)</td>
<td>0.014*</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mpanda</td>
<td>9.26</td>
<td>11 (1.443-84.785)</td>
<td>0.04*</td>
</tr>
<tr>
<td>Nkasi</td>
<td>7.13</td>
<td>6 (0.714-3.200)</td>
<td></td>
</tr>
<tr>
<td>1-8</td>
<td>0.2</td>
<td>0.37(0.034-0.10)</td>
<td></td>
</tr>
<tr>
<td>9-14</td>
<td>1.88</td>
<td>1.2 (0.359;0.671)</td>
<td>0.23</td>
</tr>
<tr>
<td>Animal Tested</td>
<td>&gt;15</td>
<td>1.71</td>
<td>0.7 (0.198;1.6030)</td>
</tr>
</tbody>
</table>

OR=Odds Ratio and CI=Confidence Interval *statistically significant

4.1.4 Evaluation of Bovigam® and Stat Pak® in livestock samples

In recent years, a quite number of novel diagnostics have been developed or improved that might be suitable for BTB diagnosis in wildlife and livestock. Among the most promising tests in this regard are Bovigam® (AG, Prionics). Stat Pak is a rapid lateral-flow test that detects antibodies by using a multi-antigen cocktail of recombinant proteins of M. bovis. The current study evaluated the performance of both Bovigam and Stat Pak methods of tuberculosis detection. Out of 77 samples evaluated in this test. Bovigam showed good performance by identifying 19 positive reactors as positive from 20 positive cattle tested.

4.1.5 Comparison of Laboratory tests methods using Kappa test

In this test, a total of 77 samples were used to compare the performance of the two diagnostic tests against the standard tuberculin skin test (SCITT). Bovigam was in good agreement with skin test in diagnostic results (p=0.001) when compared with skin test (Standard test). Statistically there was no significant difference (p=0.32) between Bovigam and Stat Pak diagnostic test. A Table 17 shows the summary and agreement status between all test methods performed.
Table 17: Assessment of agreement of BTB diagnostic tests

<table>
<thead>
<tr>
<th>SCITT and Bovigam Comparison</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCITT Positive</td>
<td>19</td>
<td>5</td>
<td>24</td>
</tr>
<tr>
<td>SCITT Negative</td>
<td>1</td>
<td>52</td>
<td>53</td>
</tr>
<tr>
<td>SCITT Total</td>
<td>20</td>
<td>57</td>
<td>77</td>
</tr>
</tbody>
</table>

\( p = 0.001 \quad \kappa = 0.80 \)

<table>
<thead>
<tr>
<th>SCITT and Stat Pak Comparison</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCITT Positive</td>
<td>18</td>
<td>14</td>
<td>32</td>
</tr>
<tr>
<td>SCITT Negative</td>
<td>19</td>
<td>26</td>
<td>45</td>
</tr>
<tr>
<td>SCITT Total</td>
<td>37</td>
<td>40</td>
<td>77</td>
</tr>
</tbody>
</table>

\( p = 0.112 \quad \kappa = 0.14 \)

<table>
<thead>
<tr>
<th>Bovigam and Stat Pak Comparison</th>
<th>Bovigam-Stat Pak Positive</th>
<th>Bovigam-Stat Pak Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCITT Positive</td>
<td>14</td>
<td>18</td>
<td>32</td>
</tr>
<tr>
<td>SCITT Negative</td>
<td>25</td>
<td>20</td>
<td>45</td>
</tr>
<tr>
<td>SCITT Total</td>
<td>39</td>
<td>38</td>
<td>77</td>
</tr>
</tbody>
</table>

\( p = 0.32 \quad \kappa = 0.20 \)

4.1.6 Wildlife Blood based Assay Test results

Bovigam assay identified five positive (6.4 %) out of 78 buffalo samples in the study area (Fig. 5), all these samples were also positive to Stat Pak. More buffalos sample tested negative by Bovigam but turned Stat Pak positive. Of the total 10 buffaloes’ herds in Katavi, only two had positive antibody test reactors. There was no significant difference between the two serological diagnostic tests used in this study \( \chi^2 = 72.5, \ p = 0.39 \), the results are shown in Table 18. Figure 5 shows diversity of the KRE that has identified location of five buffalo in Katavi Nationa Park that reacted positive to both Bovigam and Stat Pak.
**Figure 5: Wildlife tuberculosis positive samples in Katavi Rukwa ecosystem**

(A map of KRE showing wildlife places where samples from wild animals were collected. Insert is a map of Tanzania that shows the relative location of the study area)
Table 18: Distribution of BTB positive reactors in Katavi Rukwa Ecosystem buffalos based on Bovigam and Stat Pak tests

<table>
<thead>
<tr>
<th>Factors</th>
<th>Total Tested</th>
<th>Bovigam positive (%)</th>
<th>Stat Pak positive %</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>18</td>
<td>1(5.6)</td>
<td>2(11)</td>
<td>0.39</td>
</tr>
<tr>
<td>Male</td>
<td>60</td>
<td>4(6.7)</td>
<td>4(6.7)</td>
<td></td>
</tr>
<tr>
<td>Year</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2010</td>
<td>42</td>
<td>0(0)</td>
<td>2(4.8)</td>
<td>0.33</td>
</tr>
<tr>
<td>2011</td>
<td>13</td>
<td>1(7.7)</td>
<td>1(7.7)</td>
<td></td>
</tr>
<tr>
<td>2012</td>
<td>23</td>
<td>4(17.4)</td>
<td>6(26.1)</td>
<td></td>
</tr>
<tr>
<td>Sampling Procedure</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hunter killed</td>
<td>14</td>
<td>0(0)</td>
<td>0(0)</td>
<td>0.41</td>
</tr>
<tr>
<td>Routine activities</td>
<td>5</td>
<td>0(0)</td>
<td>0(0)</td>
<td></td>
</tr>
<tr>
<td>Immobilized</td>
<td>59</td>
<td>5(8.5)</td>
<td>6(10.2)</td>
<td></td>
</tr>
</tbody>
</table>

4.1.7 Bacteriological Identification of *M. bovis*

Sixty five percent of all tissue samples were collected from Mpanda slaughter house and town outskirts. Out of 328 tissue samples collected; all were inoculated and 103 cultures grown. Culture growths in the LJM-G were 78 and 82 in LJM-P, among these 23 cultures were found to grow in only one of either media. Fifteen isolates were thought to be cocci not related to mycobacteria, but when they were tested by PCR they all except one turned to be mycobacteria. From all 102 samples grown, only five were members of MTC that were later confirmed by molecular methods. The remaining mycobacteria were also confirmed as various types of NTM. A detail from each sample is presented in Table 19

Table 19: Tissue samples cultured and the microscopy identification

<table>
<thead>
<tr>
<th>Factors</th>
<th>Samples collected</th>
<th>LJM-G growth (%)</th>
<th>LJM-P growth (%)</th>
<th>Mycobacteria (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>63</td>
<td>11(17.5)</td>
<td>12(19.0)</td>
<td>18(28.6)</td>
</tr>
<tr>
<td>Male</td>
<td>264</td>
<td>67(25.4)</td>
<td>70(26.5)</td>
<td>85(32.2)</td>
</tr>
<tr>
<td>Species</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>294</td>
<td>78(26.5)</td>
<td>82(27.9)</td>
<td>100(34.0)</td>
</tr>
<tr>
<td>Caprine</td>
<td>33</td>
<td>3(9.1)</td>
<td>3(9.1)</td>
<td>3(9.1)</td>
</tr>
<tr>
<td>Age category</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>296</td>
<td>78(26.4)</td>
<td>92(31.1)</td>
<td>96(32.4)</td>
</tr>
<tr>
<td>Young</td>
<td>31</td>
<td>3(9.7)</td>
<td>5(16.1)</td>
<td>7(22.6)</td>
</tr>
<tr>
<td>Animal status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Health</td>
<td>315</td>
<td>72(22.9)</td>
<td>76(24.1)</td>
<td>100(31.7)</td>
</tr>
<tr>
<td>Sick</td>
<td>2</td>
<td>1(50)</td>
<td>1(50)</td>
<td>0</td>
</tr>
<tr>
<td>Weak</td>
<td>4</td>
<td>1(25)</td>
<td>1(25)</td>
<td>3(75)</td>
</tr>
<tr>
<td>Emaciated</td>
<td>5</td>
<td>4(80)</td>
<td>4(80)</td>
<td>0</td>
</tr>
<tr>
<td>Specimen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>2</td>
<td>0</td>
<td>1(50)</td>
<td>1(50)</td>
</tr>
<tr>
<td>Mediastinal</td>
<td>318</td>
<td>75(23.6)</td>
<td>8025.2</td>
<td>97(30.5)</td>
</tr>
<tr>
<td>Retropharyngeal</td>
<td>6</td>
<td>3(50)</td>
<td>2(33.3)</td>
<td>4(66.7)</td>
</tr>
<tr>
<td>Prescapular</td>
<td>1</td>
<td>0</td>
<td>1(100)</td>
<td>1(100)</td>
</tr>
</tbody>
</table>
4.1.8 Results of Mycobacterial Genus Typing

One hundred and two mycobacteria exhibited band 1030 bp except those which belongs to MTC which had two bands, the first 1030 bp and 372 bp in addition (Fig.7) that confirms a member of MTC. Ninety seven samples were tested for NTM. Five MTC strains detected were collected at slaughter house from cattle originating in different parts of the study districts. All four MTC were from Mpanda (Katavi region) and only one strain was collected from Nkasi District.

![PCR products by Mycogenus typing of mycobacteria isolates from cattle](image)

**Figure 6:** PCR products by Mycogenus typing of mycobacteria isolates from cattle. MAKER (M), Samples: 1 (MP189), 2 (MP183), 3 (MP112), 4 (MP31) 5 (MP224), 6 (MP43), 7 (MP93), 8 (MP58), 9 (MP110) 10 *M. tuberculosis*, 11 Water, 12 *M. bovis* MAKER (M). Sample 4 is negative, and sample 11 is nuclease free water as a negative control. 10 and 12 are control positive.

4.1.9 Deletion typing

Deletion typing was performed on all detected MTC. In this case only five members of MTC isolated were evaluated. PCR result for each sample was judged by comparing with the DNA ladder and the positive controls. Fig. 8 shows sample number 3 repeated as 11 which has a 335 bp indicating presence of either *M. tuberculosis* or *M. africanum* species.
In order to typify the species, a second deletion typing RD 9 was run. Fig. 9 shows Sample 3 (3) to have had 396 bp that typified it to be *M. tuberculosis*.

**Figure 7: PCR products by RD4 deletion typing of mycobacteria isolates from cattle**

MAKER(M), 1 (MP224), 2 (MP181R), 3 (MP62), 4 (MP10), 5 (MP190), 6 (MP523), 7 (MP93), 8 (MP10), 9 (MP110), 10 *M. bovis*, 11 *M. tuberculosis* (positive control), 12 Water (negative), S13 *M. bovis* (positive control), MAKER(M).

**Figure 8: PCR products by RD9 deletion typing of mycobacteria isolates from cattle**

MAKER 1 (MP224), 2 (MP181P), 3 (MP62), 4 (MP10), 5 (MP523), 6 (MP62), 7 *M. bovis*, 8 *M. tuberculosis* (positive control), 9 Water (negative), 10, *M. bovis* (positive control), MAKER. Sample 6 (MP62) is *M. tuberculosis*.
4.1.10 Spoligotyping results

Spoligotyping was performed on all five of MTC. Among these *M. bovis* were three and only one *M. tuberculosis* isolate were confirmed, all samples came from slaughter houses. All four *M. bovis* isolates obtained in this ecosystem exhibited three different spoligotype patterns (Fig. 9).

![Spoligotype autorad showing patterns of four strains of M. bovis and M. tuberculosis detected](image)

Figure 9: Spoligotype autorad showing patterns of four strains of *M. bovis* and *M. tuberculosis* detected

1; *M. tuberculosis* SIT 48, group 1 *M. bovis* 2-4; SB1467, group 2 5 and 7(SB0133); *M. bovis* group 3 6; SB2191. Control positive (*M. tuberculosis* was H37Rv, AF2122/97 for *M. bovis*) and negative control (Nuclease free water) are not shown in the autorad.

The strains identified during spoligotyping were digitized as 1s indicating presence of the spacer whilst 0s indicate the absence or lack of spacer. This was used to compare the strain in the www.Mbovis.org database. Each single pattern of the above digitized strain was typed into www.Mbovis.org to retrieve the associated name. Three of the strain isolate matched the SB names in the database. One strain did not match any, so it was assigned a new SB. All of these isolates lacked spacer number 3, 6, 9, 16 and 39 to 43; this is a usual characteristic that distinguishes all *M. bovis* from *M. tuberculosis*. However, in addition to these deleted spacers in samples 5 and 7 in Fig. 5 were *M. bovis* group common to Tanzania isolates (namely SB0133) that lacked in addition spacers 4, 5, and 7.
But differently from \textit{M. bovis} group 2-4 unusual to Tanzania isolates named SB1467. The SB1467 lacks usual spacers of \textit{M. bovis} and spacers 4, 5 and 7 similar to SB0133. Interestingly SB1467 has additional lacking spacer number 15 a character distinguishes from usual Tanzania strains. SB2191 is a newly identified. This strain exhibits lack of spacers 4, 5 and 7 just like SB1467 but also lacks specifically 11 and 12 spacers in addition to the usual \textit{M. bovis} pattern. This new strain (Sample 6 in Fig. 9) was a newly identified and was assigned SB2191 pattern in the Mbovis database. Sample number one (1) in the autorad has spacers full in the sequence except number 34 and 41 that was identified as \textit{M. tuberculosis} which according to its clonal specialty was identified to be EAI1-SOM, SIT 48 which has a pattern value 77777777413731 in Spoligo International Typing database http://www.pasteur-guadeloupe.fr:8081/ SITVIT_ ONLINE/. This is generally represented as East Africa Indian (EAI) lineage.

\textbf{4.1.11 Genetic relatedness of \textit{M. bovis} isolates}

Strains of \textit{M. tuberculosis} are 98 to 100% closely related and more so to \textit{M. bovis}. This fact makes it difficult to discriminate the stains by conversional methods (Brosch \textit{et al.}, 2002). A simplified method of calculating genetic relatedness between \textit{M. bovis} strain was modified and used by Kazwala \textit{et al.} (2006). It gives a percentage of genetic relatedness value (GRV) as shown in the formula

\[ GRV = \left( \frac{\# \text{ spacer positions shared by patterns} - \# \text{ spacer positions different between the patterns}}{\text{Total spacer positions common}} \right) \times 100 \]

Table 20 gives the complete list of strain and the GRV from known mycobacteria strain.
Table 20: Comparison of Mycobacteria strains by genetic relatedness value

<table>
<thead>
<tr>
<th>Strain comparisons</th>
<th>GRV</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB0133 to <em>M. bovis</em> (AF2122/97)</td>
<td>91.18</td>
</tr>
<tr>
<td>SB425 to <em>M. bovis</em> (AF2122/97)</td>
<td>67.65</td>
</tr>
<tr>
<td>SB1467 to <em>M. bovis</em> (AF2122/97)</td>
<td>88.24</td>
</tr>
<tr>
<td>SB2191 to <em>M. bovis</em> (AF2122/97)</td>
<td>82.35</td>
</tr>
<tr>
<td>SB1467 to SB0133</td>
<td>96.77</td>
</tr>
<tr>
<td>SB2191 to SB0133</td>
<td>90.32</td>
</tr>
<tr>
<td>SB2191 to SB1467</td>
<td>93.33</td>
</tr>
<tr>
<td>SB1467 to SB0425</td>
<td>76.67</td>
</tr>
<tr>
<td>SB1467 to SB1252</td>
<td>96.67</td>
</tr>
<tr>
<td>SB2191 to SB1252</td>
<td>96.55</td>
</tr>
<tr>
<td>SB0133 to SB0425</td>
<td>74.19</td>
</tr>
<tr>
<td>SB2191 to SB0425</td>
<td>79.31</td>
</tr>
</tbody>
</table>

Relatedness value analysed showed 96.6% genetic relatedness of SB2191 to the SB1252 (60-53-5F-7F-FF-60) strain isolated from cattle in United Kingdom. The GRV was lower when SB2191 is compared to SB1467 (93.3%) and SB0133 (90.3%). Not as much relatedness was observed for SB1467 towards SB0425 (76.7%) but very closely related to SB0133 (96.8%).

4.1.12 Isolation of Non Tuberculous Mycobacteria from livestock

All *Mycobacteria* identified (103) by microscopy as AFB positive were subjected to *Mycobacterium* genus PCR. Out of these tissue samples, 87 mycobacteria were sequenced for NTM. Overall, mycogenus PCR detected (87/328) 25.9% mycobacteria other than MTC of all tissue samples cultured. All these NTM were sequenced and identified to species level. Samples from cattle were 84 and 3 from goats; The NTM were confirmed 3/30(10%) in goats and 84/328(25.6%) in cattle. Table 21 shows the different NTM identified to species level.
Table 21: Selected NTM isolated from Katavi cattle and goats 2010 to 2012

<table>
<thead>
<tr>
<th>Ser No.</th>
<th>SUA ID</th>
<th>FLI ID</th>
<th>Species</th>
<th>NTM species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MP502</td>
<td>12MA1251</td>
<td>Cattle</td>
<td>M. peregrinum</td>
</tr>
<tr>
<td>2</td>
<td>MP26</td>
<td>12MA1252</td>
<td>Cattle</td>
<td>M. kansasii and gastri</td>
</tr>
<tr>
<td>3</td>
<td>MPZ35</td>
<td>12MA1259</td>
<td>Goat</td>
<td>M. nonchromogenicum</td>
</tr>
<tr>
<td>4</td>
<td>MP243</td>
<td>12MA1261</td>
<td>Cattle</td>
<td>M. palustre</td>
</tr>
<tr>
<td>5</td>
<td>MP35</td>
<td>12MA1263</td>
<td>Cattle</td>
<td>M. kansasii and gastri</td>
</tr>
<tr>
<td>6</td>
<td>MP121</td>
<td>12MA1269</td>
<td>Cattle</td>
<td>M. lentiflavum</td>
</tr>
<tr>
<td>7</td>
<td>MP83</td>
<td>12MA1293</td>
<td>Cattle</td>
<td>M. kansasii and gastri</td>
</tr>
<tr>
<td>8</td>
<td>MP107</td>
<td>12MA1288</td>
<td>Cattle</td>
<td>M. kansasii and gastri</td>
</tr>
<tr>
<td>9</td>
<td>MP91</td>
<td>12MA1294</td>
<td>Cattle</td>
<td>M. goodie, M. moriokaens, M. smegmatis</td>
</tr>
<tr>
<td>10</td>
<td>MP63</td>
<td>12MA1298</td>
<td>Cattle</td>
<td>M. kansasii and gastri</td>
</tr>
<tr>
<td>11</td>
<td>MP146</td>
<td>12MA1301</td>
<td>Cattle</td>
<td>M. kansasii and gastri</td>
</tr>
<tr>
<td>12</td>
<td>MP166</td>
<td>12MA1304</td>
<td>Cattle</td>
<td>M. kansasii and gastri</td>
</tr>
<tr>
<td>13</td>
<td>MP170</td>
<td>12MA1310</td>
<td>Cattle</td>
<td>M. intracellulare</td>
</tr>
<tr>
<td>14</td>
<td>MP53</td>
<td>12MA1313</td>
<td>Cattle</td>
<td>M. kansasii and gastri</td>
</tr>
<tr>
<td>15</td>
<td>MP30</td>
<td>12MA1315</td>
<td>Cattle</td>
<td>M. kansasii and gastri</td>
</tr>
<tr>
<td>16</td>
<td>MP167</td>
<td>12MA1319</td>
<td>Cattle</td>
<td>M. lentiflavum</td>
</tr>
<tr>
<td>17</td>
<td>MP231</td>
<td>12MA1255</td>
<td>Cattle</td>
<td>M. nonchromogenicum</td>
</tr>
<tr>
<td>18</td>
<td>MPZ35</td>
<td>12MA1260</td>
<td>Goat</td>
<td>M. indicus pranii and M. intracellulare</td>
</tr>
<tr>
<td>19</td>
<td>MP 227</td>
<td>12MA1292</td>
<td>Cattle</td>
<td>M. hibernae and M. engbaekii</td>
</tr>
<tr>
<td>20</td>
<td>MP 502</td>
<td>12MA1250</td>
<td>Goat</td>
<td>Mycobacterium fortuitum, M. porcinum</td>
</tr>
<tr>
<td>21</td>
<td>MP 26</td>
<td>12MA1251</td>
<td>Cattle</td>
<td>Mycobacterium peregrinum, M. septicum</td>
</tr>
<tr>
<td>22</td>
<td>MP 243</td>
<td>12MA1261</td>
<td>Cattle</td>
<td>Mycobacterium intracellulare, M. indicus pranii</td>
</tr>
<tr>
<td>23</td>
<td>MP 183</td>
<td>12MA1262</td>
<td>Cattle</td>
<td>Mycobacterium kansasii, M. gastri</td>
</tr>
<tr>
<td>24</td>
<td>MP 121</td>
<td>12MA1269</td>
<td>Cattle</td>
<td>Mycobacterium lentiflavum</td>
</tr>
<tr>
<td>25</td>
<td>MP 33</td>
<td>12MA1270</td>
<td>Cattle</td>
<td>Mycobacterium kansasii, M. gastri</td>
</tr>
<tr>
<td>26</td>
<td>MP 17</td>
<td>12MA1297</td>
<td>Cattle</td>
<td>Mycobacterium kansasii, M. gastri</td>
</tr>
</tbody>
</table>

Two samples MP 183 and MP 17 were shown to have mixed M. Kanssii and M. gastri species using 16S rRNA but further test using hsp65 gene confirmed all as M. kansasii sub-species VI. A summary of NTM isolated in higher percentage are shown in Fig. (11). Sixteen tissue samples were having one isolate species of NTM. These species were M. hibernae, M. engbaekii, M. septicum, M. arupense and M. godii. Although, Mycobacterium avium was not isolated in both livestock and wildlife but M.
intracellulare a member of the Mycobacterium avium complex was isolated in the livestock.

![Bar chart showing number of NTM isolated from livestock tissues in Katavi Rukwa Ecosystem](chart.png)

**Figure 20: Number of NTM isolated from livestock tissues in Katavi Rukwa Ecosystem**

### 4.1.13 Isolation of Non tuberculous mycobacteria from wild animals

One hundred and eleven out of 119 tissue samples from different animal species inoculated into culture media showed growth. Thirty two tissue samples were confirmed to be AFB positive by microscopy. DNA from these 32 isolates was tested for mycogenus PCR. Only 15 (46.9%) NTM out of 32 wild animals tested were isolated (Table 22). These 15 (12.6%) NTM were identified by 16S rDNA sequence analysis to species level. The species detected were grouped into the following: *Mycobacterium lentiflavum*, *Mycobacterium fortuitum*, and *Mycobacterium peregrinum*. Hsp65 sequencing returned the same species in group.
Table 22: Frequency of culture isolation of NTM from wildlife samples in Katavi Rukwa Ecosystem 2010-2012

<table>
<thead>
<tr>
<th>Species</th>
<th>Number collected</th>
<th>AFB Positive</th>
<th>NTM Isolated</th>
<th>NTM prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leopard (Panthera pardus)</td>
<td>5</td>
<td>4</td>
<td>M. lentiflavum</td>
<td>3 (60)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M. fortuitum,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M. peregrinum</td>
<td></td>
</tr>
<tr>
<td>Buffaloes (Syncerus caffer)</td>
<td>52</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kudu (Tragelaphus strepsiceros)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Impala (Aepyceros melampus)</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hartebeest (Alcelaphus buselaphus)</td>
<td>10</td>
<td>2</td>
<td>M. fortuitum</td>
<td>3 (25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M. fortuitum,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M. peregrinum</td>
<td></td>
</tr>
<tr>
<td>Lion (Panthera leo)</td>
<td>12</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zebra (Equus burchelli)</td>
<td>7</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Eland (Taurotragus oryx)</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Baboon (Papio anubis)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Warthog (Phacochoerus africanus)</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Bushpig (Potamochoerus larvatus)</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hippopotamus (Hippopotamus amphibius)</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Reedbuck (Redunca arundinum)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Waterbuck (Kobus ellipsiprymnus)</td>
<td>2</td>
<td>1</td>
<td>M. fortuitum</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Elephant (Loxodonta africana)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Crocodile (Crocodylus niloticus)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Oribi (Ourebia ourebi)</td>
<td>2</td>
<td>2</td>
<td>M. fortuitum</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>119</td>
<td>32</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER FIVE

4.0 DISCUSSION

The present study has demonstrated five isolates of the *Mycobacterium tuberculosis* complex species from cattle in the Katavi Rukwa ecosystem, four were *M. bovis* and one was *M. tuberculosis*. Using microarray spoligotyping (Anke *et al.*, 2012) the strain of *M. tuberculosis* was identified to be SIT 48 EA11-SOM commonly isolated in East Africa and India (Srivastava *et al.*, 2008) and recently in Brazil (Magdinier *et al.*, 2012). SIT database identifies this as East African Indian lineage. Other studies have reported the isolation of general *M. tuberculosis* from milk (Ameni and Erkihun, 2007; Regassa *et al.*, 2010) and meat inspection (Kazwala *et al.*, 2006; Berg *et al.*, 2009; Cadmus, 2009; Cadmus *et al.*, 2011; Mohamed *et al.*, 2009b). *M. tuberculosis* individual cattle prevalence in the slaughter house in the present study was 0.3% which is comparable to the findings of other studies in Sudan and Ethiopia (Sulieman and Hamid, 2002; Ameni *et al.*, 2011). The study did not attribute the infection to risk associated with people living with animals because farmers were not sharing houses with cattle. However, people sharing houses with small ruminants were commonly observed. The transmission could paradoxically occur if water or pasture is contaminated with human sputum (Munyeme *et al.*, 2009; Silaigwana *et al.*, 2012) and other sharing mechanisms such as mouth to mouth (Ameni and Erkihun, 2007; Gumi *et al.*, 2012). SB2191 was sampled from the Usevya slaughter slab, but records indicate to have originated from communities herding cattle around the Katavi Rukwa Ecosystem. The strain has never been isolated elsewhere globally, either in wildlife or livestock. The strain can be a result of evolution of the other strains in livestock, but the source could also be a spillover infection from wild animals as the wildlife-livestock interaction is higher in this area due to scarce in grazing areas and
water sharing causing the movement of cattle as far as possible towards the wild animals and vice versa.

The fact that all positive isolates were from the thoracic lymphnodes also suggests that the respiratory infection could be responsible for the animal to animal transmission. High rate spatial and space-time clusters identified three villages (Mpanda, Mirumba and Mamba) of the study area to be hotspots for Bovine tuberculosis. These new hotspots add to previously researched areas of Serengeti (Cleaveland et al., 2005; Katale et al., 2013) and Mikumi Selous (Mwakapuja et al., 2013) Ecosystems. Critically, these hotspots were so close to wildlife protected (richer in water and pastures) areas of Katavi National Park. These areas have good supply of water and pastures through-out the year hence they attract animals and influence congregation that could lead to disease transmission as shown in previous studies (Vander Wal et al., 2012; Mwakapuja et al., 2013). Although smaller numbers of goat were tested but neither tissue nor blood was found positive except for few non tuberculoues mycobacteria.

Results indicate that farmers move their animals closer to protected areas. More than 65% respondents indicated that they normally share grazing and water with wild animals. Some farmers at Mirumba were illegally grazing their cattle as far inside the park particularly during the dry season. This scenario led to intermingling of the livestock and wild animals. Few instances of buffalo herds coming out of the park were noticed in far north of the Katavi National Park. Uncontrolled animal movement in the Ecosystem indicates that there is no significant barrier between livestock and wild animals. The situation favours the hypothesis that livestock may be a source of BTB transmission to the wildlife, although the possibility of wild animals transmitting the disease to livestock was not demonstrated in this study due to lack of isolated strains.
It is important to mark down the hotspot special patterns of the disease. The present study using both space-time and spatial temporal analysis identified significant disease primary hotspot at Mpanda outskirts, Mirumba, Mamba. Urwira, Chala and Mashete were also returned as secondary hotspots. Since clustering of the disease was observed to occur within the hotspot, then these areas have to be targeted for control, to reduce the impact and eventually eradicate the disease on the livestock. Regular skin test of cattle and removal of reactors is imperative because the spatial and temporal dynamics of disease in host populations go hand in hand with the timing of the disease control strategy.

Molecular characterisation of the strains of *M. bovis* using spoligotyping revealed the presence of four isolates grouped into three clusters of spoligotype patterns. These four strains confirm bovine tuberculosis in the study area. Study on relatedness (Penlap et al., 2012; Kazwala et al., 2006) revealed presence of strain groups. The SB0133 (Hex code 60-5F-5F-7F-FF-60) which was frequently isolated in KRE has also been reported earlier in Tanzania (Kazwala et al., 2006) but also was reported to be the most dominant in free ranging cattle in Tanzania and other eastern African countries (Berg et al., 2009; Müller et al., 2009; Tsegaye et al, 2010). The SB0133 strain is also not uncommon elsewhere (Njanpop-Lafourcade et al., 2001), SB1467 (Hex code 60-5F-1F-7F-FF-60) was among the isolates in this study. This species has been isolated in Uganda (Oloya et al., 2007), and closely relates to SB0133. SB1467 could be a mutant from the normal SB0133, which is not uncommon in Tanzania. Nevertheless this strain as it is might have been originated from Uganda due to animal movement as the agropastoralists from the Sukuma tribe seem to migrate from Lake Zone neighboring Uganda and all the way to Katavi Rukwa Ecosystem.
The results obtained indicate a true prevalence (4.6 %) of the disease in the study area which is slightly high compared to recent studies in Northern Tanzania (Katale et al., 2013). In the same comparison, herd prevalence was recorded less (43.8%) in this study compared to previous record (46.9%). The prevalence is lower if compared to 13.2 % reported by Kazwala et al. (2001) in Southern Tanzania but higher than 0.70-1.06% that was reported by Shirima et al. (2003) in Northern Tanzania. The present study has demonstrated prevalence higher than 0.2 % recorded in Tanzania Lake Zone (Jiwa et al., 1997). The herd prevalence was relatively higher (43.8%) if compared to the previous studies reported at 8.44-13.17% in different studies (Kazwala et al., 2001).

The study also compared the performance of the blood based test assays. By using Kappa test, the Bovigam test showed 80% compliance to the tuberculin test at relatively higher detection of the positive reactor animals. Significantly, there was good agreement between Bovigam (80 %, p=0.001)) than Stat Pak (14 %) in comparison to skin test (SCITT). This means there was a poor agreement to skin test when compared to Stat Pak. The difference between the two diagnostic tests: Bovigam and Stat Pak was not significant (p=0.10). Bovigam or gamma interferon (blood test) is a sensitive live animal test that can identify animals in an earlier stage of infection compared to other tests. In this study, Bovigam was demonstrated to be in good agreement with skin test in most cases, which suggest that the test is good to be used in screening or parallel with skin testing.

The role of wildlife in the epidemiology of BTB has been greatly underestimated, both in developing countries as well as in the developed world (Michel et al., 2006). The present study has recorded a Bovigam test prevalence of 6.4% (n=78) in Katavi National Park buffalos which is slightly higher as compared to serological assay (enzyme immunoassay)
that detected antibodies to *M. bovis* in one of 17 (6%) buffalos from Tarangire national park in northern Tanzania (Cleaveland *et al.*, 2005), but lower than 13% for the study done in 2001 at Kruger National Park using the same modified gamma interferon assay (Michel *et al.*, 2006). The same author reported 25% prevalence in 2003 in the same national park. No reports have confirmed BTB in Tanzanian buffalos despite previous prevalence report by Cleaveland *et al.* (2005) in other wild animals like wildebeest (11 %), Topi (11 %) and Kudu. Buffaloes’ herds or groups in Katavi tend to migrate out of the park during the dry season in search of pasture and water, but they return in greater number when it rains. Buffalos herds prevalence was 20% that is enormously significant although slightly lower compared to 34.5% demonstrated in Kruger National Park (Michel *et al.*, 2006) using Bovigam test.

Small bachelor herds or groups of buffalos were accessed and captured in the national park during dry season. In wet season larger herds of buffalos were seen and the team had the scope of choosing capture of buffalos from both sex. This behaviour may have contributed to variation in the performance of the diagnostics between dry season and wet season (Michel *et al.*, 2006).

Due to the limited and variable sensitivity of the serologic assay, it is difficult to determine the true prevalence of infection in animals basing on these tests. They are conservatively considered too insensitive to detect tuberculosis in both cattle and wildlife. Michel *et al.* (2010) demonstrated a sensitivity of 10.4% in buffaloes and 25% in lion by enzyme immunosorbent assay. Also compared performance of the gamma interferon assay in African buffaloes under the recommended guidelines for interpretation of test results and found a high sensitivity (92.1%) at the cost of a greatly reduced specificity
Generally the Bovigam test is promoted for use particularly in screening of bovine tuberculosis in both livestock and wildlife. Although there was no isolation of \textit{M. bovis} (n=119) in Katavi Rukwa Ecosystem wild animal, still there is a possibility that wild animal may contract infection from livestock because this study confirmed the presence of \textit{M. bovis} in cattle. This situation of constantly intermingling with wild animals in the national park and controlled game areas has also been demonstrated by Cleaveland \textit{et al.} (2005). Evidently, now bovine tuberculosis is becoming endemic disease in many of agropastoral livestock keepers in almost all corners of the country. These facts pose a danger of the disease to establishing itself in the wild animals through livestock if not controlled.

NTM were detected in 25.9% and 12.6% of livestock and wildlife tissue cultures, respectively. Most of the NTM were detected from health animals as reported in other studies (Kankya \textit{et al.}, 2011). These NTM were isolated in single or as mixed infection in different samples. This study indicates relatively higher isolation of NTM in comparison to other studies (Kankya \textit{et al.}, 2011; Katale \textit{et al.}, 2013). Isolation is continuously on increase that poses a threat to both animals and human. Although most NTM species may not be a true respiratory pathogen, but may demonstrate clinical disease in ungulate species (Thorel \textit{et al.}, 2001) and extrapulmonary disease in immunocompromised humans (Tschopp \textit{et al.}, 2009; Kankya \textit{et al.}, 2011). This study, as reported elsewhere (Kankya \textit{et al.}, 2011) have shown the most non-tuberculous mycobacteria isolated in high numbers as \textit{M. kansasii} (30%) and \textit{M. gastri} (30%). Other NTM species in relatively low numbers were \textit{M. fortuitum}, \textit{M. peregrinum}, \textit{M. intracellulare} and many others. \textit{M. fortuitum} may cause post-surgical infections that pose threat to human health (Gopinath and Singh, 2010). Though neglected but NTM infections are very important (Jarzembowski \textit{et al.},...
2008). Several NTM species are now causing disease in human (Katoch, 2004). *M. peregrinum* in most studies was demonstrated as a group of *M. fortuitum* that frequently cause infection (Müller *et al.*, 2009; Kankya *et al.*, 2011).

Generally most of NTM are considered non-pathogenic in most species, but of recent years these mycobacterial species have been associated with granulomatous lesions in both cattle and humans (Kazwala *et al.*, 2006; Müller *et al.*, 2011). *M. kansasii* is most frequently (Kankya *et al.*, 2011) cause of lung infections and its prevalence in the world has increased. In this study, similarly the highest percentage of isolation was both *M. kansasii* and *M. gastri*.

The above fact show importance of these isolated NTM as it has been shown elsewhere (Franco *et al.*, 2013; Senna *et al.*, 2008). In livestock and wild animals, NTM may cause immune reactions and thus interfere with tuberculin skin test or Bovigam tests. Since this is supported (Palmer *et al.*, 2011), then cross reactivity and skin test interference may be expected in area where there is high prevalence of NTM.

In studying risk factors related to BTB, the questionnaire generated information from farmers’ response contributed many useful findings. Respondents from Mpanda (Katavi region) level of disease awareness were higher (86.1%) as compared to others in Nkasi district. This shows a strong association between having a BTB positive or tuberculosis history and level of awareness by the cattle owners. This finding is in agreement with results obtained in similar study in Zambia and Uganda (Munyeme *et al.*, 2010, Kankya *et al.*, 2010). Low levels of disease awareness among cattle owners need a special attention. Nkasi district may be given priority when planning for BTB sensitization during disease control implementation. Although female in this study have shown high level of
awareness of disease but unfortunately most of them still drink raw milk. Male have shown to drink raw milk too. Results indicate 91% of Mpanda farmers drink raw milk; most of these are those who have no formal education. Most of respondents preferred raw milk because they think boiled milk is tasteless. This could predispose respondents to infection because this is a major risk factor (Katale *et al.*, 2012) for humans to get infection with BTB. Amen *et al.* (2007) have shown the association of BTB potential risk to lack of understanding and bad behavior of drinking raw milk. If compared, respondents who had history of BTB or human TB were very much aware of the disease and they demonstrated shift from drink of raw milk. This is in agreement with study done in Zambia (Munyeme *et al.*, 2010).
CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

This is the first report on the prevalence and molecular characterisation of *M. bovis* in the Katavi Rukwa Ecosystem. Five isolates of the MTC species was observed. Three of these *M. bovis* were common Tanzanian strains but one strain SB 2190 was isolated for the first time. Not only that the isolate was new to the study area but it has never been isolated before worldwide. The finding is a new knowledge to the tuberculosis research in Tanzania. *M. tuberculosis* in cattle was also isolated. This is not common in livestock although fewer studies indicate such isolates in East Africa. Further study is recommended to explore on the possibility of the *M. tuberculosis* transmission in livestock.

Bovine tuberculosis has been confirmed in the study area. It is highly recommended to the government to initiate a deliberate control strategy to prevent the disease from spreading to other areas. Since the extent of the disease is low, a test and slaughter might be suitable. Although compensation for the lost animals (tuberculosis positive forced to be slaughtered) is not practiced in Tanzania; however, through private public partnership, it is possible to join effort to make sure that the reactors are slaughtered and if possible compensation is provided to the difficult cattle owners who decline to willingly dispose reactors.

Although *M. bovis* was not isolated in wildlife there are still possibilities of wild animals to acquire tuberculosis infection from livestock because the disease has been confirmed in cattle from the study area. Nevertheless, the blood based assay analysis of wild samples also returned positive cases. Livestock illegally enters the protected game areas in search
of pasture and water. In Tanzania, there is no management strategy policy for bovine tuberculosis in wild animals particularly for buffalos that are considered as maintenance host. Therefore, it is very important to constructively focus resources on surveillance in order to determine the distribution and rate of spread of the disease. Transmission between livestock and wildlife was not confirmed. However, isolation of identical species of NTM from livestock and wildlife could suggest the possibility of transmission of these species of mycobacteria between livestock and wild animals.

The prevalence of human tuberculosis caused by *M. bovis* and non tuberculous mycobacteria is not known in the area and therefore calls for further investigations. Coupled with the fact that there was a case of cattle diagnosed to have *M. tuberculosis*, it is highly recommended to extend further research on human tuberculosis in the study area. Since there may be a possibility of human tuberculosis infecting cattle or vice versa, a call is made for medical and veterinary professional to work together under one health initiative. Further demonstration of many NTM detected in slaughter houses call for necessary action. Especially *M. kansasii, M. fortuitum* and *M. intracellulare* that were isolated could be a problem when they infect human being. A detailed study is highly recommended to establish the prevalence in both animals and human especially now with the rise of HIV and immunocompromised patients.

It is recommended to governmental authority to have disease surveillance and control strategy especially in livestock, so that the infected livestock cannot serve as a source of infection to wild animals or the other way around in the interface. Planning for BTB sensitization during disease control implementation is equally recommended in order to raise the level of disease awareness.
REFERENCES


Appendix 1: Tests for TB diagnosis in the laboratory from samples collected both in wildlife and livestock animals
Appendix 1b: Media composition of the Löwenstein–Jensen

i) LJ glycerol medium (LJM-G) was made up of 61.7% whole egg, 36.9% (v/v) IUT buffer salt solution (50mM K$_2$PO$_4$; 25mM Na$_2$HPO$_4$.2H$_2$O; 1.6mM MgSO$_4$.7H$_2$O; 14mM citric acid; 67mM L-Asparagine; 0.2% glycerol) and 2.4% (v/v) of 1.2% (w/v) malachite green. Add egg to buffer salt and then the Malachite green to complete the solution.

ii) LJ pyruvate medium (LJM-P) was made of 61.6% whole egg, 36.9% (v/v) pyruvate medium buffer salt solution (50mM K$_2$PO$_4$; 25mM Na$_2$HPO$_4$.2H$_2$O; 114mM sodium pyruvate; 14mM citric acid), 1.2% v/v of 1% (w/v) malachite green and 0.25% (v/v) of 1% (w/v) trypan blue. Add Egg into buffer salt followed by Malachite green and lastly with Trypan blue solution. After mixing all the components, 4ml of the medium was dispensed into 30ml glass universals and then insipissated at 85°C for one hour to solidify the media. L-J glycerol medium had a pale green colour, while L-J pyruvate medium had pale blue. About 0.1ml of the sediments from each sample was spread on the surface of each media using a sterile disposable pipette and, in order to avoid sedimentation of inoculums at the bottom of the slope, at incubation chamber all the slopes were laid horizontally overnight before being placed vertical for continued incubation at 37°C until culture growth or at least ten weeks.
Appendix 2: Procedure for the Single Comparative Intradermal Tuberculin Test

(as Performed under EU regulation 64/432/EEC and OIE 2009)

1. Identify the animal by its ear tag number and record identification

2. Shave two sites in the middle third of the neck on one side (the right hand side is used in this test), one above the other, separated by about 12cm.

3. At each site, a fold of skin should be measured using a calliper and the measurement recorded.

4. PPD-bovine and PPD-avian (Central Veterinary Institute, Lelystad, The Netherlands) should be injected (0.1ml) intradermally. The upper site should be used for the avian PPD and the lower site for the bovine PPD.

5. Re-measure after 72 hours the same skin fold at each site using callipers and record the measurement. The same operator should make the measurements on both occasions.

7. Standard interpretation is then applied to the results

a. If the reaction to bovine PPD is > 4.0 mm greater than to avian PPD the test is considered positive, between 1.0 and 4.0 mm is inconclusive and < 1.0mm is considered negative.
Appendix 3: Procedure summary for Bovigam® immunoassay

Add 50 μl of green diluent to wells
↓
Add 50 μl of test and control samples (control last) and mix
↓
Incubate for 60 minutes
↓
Wash (repeat six times) and drain
↓
Add 100 μl of conjugate to wells (diluted to working strength in blue diluent)
↓
Incubate for 60 minutes
↓
Wash (repeat six times)
↓
Prepare enzyme solution
↓
Add 100 μl of enzyme to wells
↓
Incubate 30 minutes (protect from direct sunlight)
↓
Add 50 μl of enzyme stopping solution and mix
↓
Read absorbance in five minutes using 450 nm filter and again with a 620-650 nm filter
Appendix 4: Procedure summary for genus typing PCR of *Mycobacterium* species

1. Initial denaturation at 95°C for 10 mins

2. Followed by 35 cycles of: Denaturation at 95°C; 1 min → annealing primer at 61°C; 30 seconds
   → Extension at 72°C; 2 mins.

3. Final elongation at 72°C; 10 mins

4. Hold at 4°C

Electrophoresis performed at 100 volts on Agarose 1.5% gel of 1x TAE buffer and ethidium bromide at 0.3 µg/ml.

**RESULTS**

1. 1030 base pairs— all members of the genus of *Mycobacteria* (i.e. primers Mycgen-F/R)
2. 180 base pairs— M. Avium subspecies including M. Paratuberculosis (primers Mycgen – F/M YcAv – R) plus 1030 base pairs
3. 850 base pairs— M. Intracellulare (primers Myc Int-F/ Mycgen-R) in addition to 1030 base pairs
4. 372 base pairs— M. Tuberculosis complex (with TB-1-F/ TB-1-R) in addition to 1030 base pair genus product.

Two bands indicate a specific PCR product and respective species-specific PCR product
Appendix 5: Bovine tuberculosis research questionnaire for animal owners

**A. General**

Name of investigator: Cluster: ........................................

Farmer’s Name: ........................................

GPS

Date: d d m m y y y y

ID: ........................................................................

3000 Mozambique

**B. Herd**

**Breed**

a. Maasai Yes 1 No 2 Don’t know 3
b. Zebu Yes 1 No 2 Don’t know 3
c. Ankole Yes 1 No 2 Don’t know 3
d. Boran Yes 1 No 2 Don’t know 3
e. Other ......................................................................

**Number of animals tested**

**Age groups**

Years 0-1 male female 1-3 male female 4-10 male female >10 male female

<10 = 1, <20 = 2, <30 = 3, <30 = 4

**C. Tuberculosis general**

1. Do you often see emaciated cattle in your herd?

   Yes 1 No 2 Don’t know 3

2. When, during rainy season?

   Yes 1 No 2 Don’t know 3
3. When, during dry season?

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
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<td>3</td>
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</table>

4. What do you think is the reason of the emaciation?

<table>
<thead>
<tr>
<th></th>
<th>Starvation</th>
<th>Age</th>
<th>Disease</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Yes</td>
<td>No</td>
<td>Don’t know</td>
<td>Don’t know</td>
</tr>
<tr>
<td>b</td>
<td>Yes</td>
<td>No</td>
<td>Don’t know</td>
<td>Don’t know</td>
</tr>
<tr>
<td>c</td>
<td>Yes</td>
<td>No</td>
<td>Don’t know</td>
<td>Don’t know</td>
</tr>
<tr>
<td>d</td>
<td></td>
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</tbody>
</table>

5. Which disease?

<table>
<thead>
<tr>
<th></th>
<th>Nagana</th>
<th>Worms</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Yes</td>
<td>No</td>
<td>Don’t know</td>
</tr>
<tr>
<td>b</td>
<td>Yes</td>
<td>No</td>
<td>Don’t know</td>
</tr>
<tr>
<td>c</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

6. Have you heard of TB?

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
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<td></td>
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<td>3</td>
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</tbody>
</table>

7. Are you aware of any case of Tb in your family or village?

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>3</td>
<td></td>
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</tbody>
</table>

8. What signs do you observe?

<table>
<thead>
<tr>
<th></th>
<th>Coughing</th>
<th>Emaciation</th>
<th>Lnn. swelling</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Yes</td>
<td>No</td>
<td>Don’t know</td>
<td>Don’t know</td>
</tr>
<tr>
<td>b</td>
<td>Yes</td>
<td>No</td>
<td>Don’t know</td>
<td>Don’t know</td>
</tr>
<tr>
<td>c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d</td>
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</table>

9. Have you seen enlargement of Lnn. in the neck area of these people?

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
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<td>2</td>
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<td>3</td>
<td></td>
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</tbody>
</table>


10. For how long were infected people treated?
   a. 1-2 weeks  Yes 1  No 2  Don’t know 3  
   b. up to 6 month Yes 1  No 2  Don’t know 3  
   c. Other

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<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
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</table>

   **D. Tuberculosis livestock**

11. Have you heard of tuberculosis disease in cattle or any other livestock?
    Yes 1  No 2  Don’t know 3  

12. Do you remember a case of TB in your herd?
    Yes 1  No 2  Don’t know 3  

13. How was the case managed?
    a. Slaughtered  Yes 1  No 2  Don’t know 3  
    b. Sold  Yes 1  No 2  Don’t know 3  
    c. Other

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td></td>
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</tbody>
</table>

14. Do you know how the animals become infected?
    a. Contact  Yes 1  No 2  Don’t know 3  
    b. Milk  Yes 1  No 2  Don’t know 3  
    c. Other

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
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</table>

15. Is there any meat inspection done during home slaughter?
    Yes 1  No 2  Don’t know 3  

16. Is there any meat inspection done at the slaughter house/slabs?
    Yes 1  No 2  Don’t know 3  

17. Do you know whether TB can be transmitted from cattle to humans?

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
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<td>3</td>
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</table>

18. How does the transmission take place?

<table>
<thead>
<tr>
<th></th>
<th>Contact</th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.</td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>b.</td>
<td>Milk</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>c.</td>
<td>Other</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

19. Do you drink milk?

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
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</table>

20. Do you treat your milk before consumption?

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
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</tbody>
</table>

21. How do you treat your milk?

<table>
<thead>
<tr>
<th></th>
<th>Boiling</th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.</td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>b.</td>
<td>Souring</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>c.</td>
<td>Other</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

22. Do you sell your milk?

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

23. To whom do you sell?

<table>
<thead>
<tr>
<th></th>
<th>Neighbour</th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.</td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>b.</td>
<td>Market</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>c.</td>
<td>Other</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
24. Do you consume raw meat/blood?

Yes 1  No 2  Don’t know 3

E. Management livestock

25. What do you do with sick animals?

a. Treatment  Yes 1  No 2  Don’t know 3
b. Slaughter  Yes 1  No 2  Don’t know 3
c. Destroy  Yes 1  No 2  Don’t know 3
d. Sell  Yes 1  No 2  Don’t know 3
e. Other

26. What do you do with dead animals?

a. Burry  Yes 1  No 2  Don’t know 3
b. Burn  Yes 1  No 2  Don’t know 3
c. Eat  Yes 1  No 2  Don’t know 3
d. Sell  Yes 1  No 2  Don’t know 3
e. Other

27. Where do you sell your animals?

a. Village  Yes 1  No 2  Don’t know 3
b. Market  Yes 1  No 2  Don’t know 3
c. Middle man  Yes 1  No 2  Don’t know 3
d. Other

28. Where do you buy your animals?

a. Village  Yes 1  No 2  Don’t know 3
b. Market  Yes 1  No 2  Don’t know 3
c. Middle man  Yes 1  No 2  Don’t know 3
29. Do you exchange animals with neighbours or friends?
   Yes 1 ☐ No 2 ☐ Don’t know 3 ☐

30. How is grazing organized?
   a. Communal Yes 1 ☐ No 2 ☐ Don’t know 3 ☐
   b. Individual Yes 1 ☐ No 2 ☐ Don’t know 3 ☐
   c. Other ☐

31. Do you use seasonal grazing grounds in other areas?
   Yes 1 ☐ No 2 ☐ Don’t know 3 ☐

32. Do your animals share watering points with other herds?
   Yes 1 ☐ No 2 ☐ Don’t know 3 ☐

33. Do you keep livestock in your house?
   a. Cattle Yes 1 ☐ No 2 ☐ Don’t know 3 ☐
   b. Sheep/goats Yes 1 ☐ No 2 ☐ Don’t know 3 ☐
   c. Other ☐

34. Which livestock diseases do you experience in your herd/farm?
   a. Nagana Yes 1 ☐ No 2 ☐ Don’t know 3 ☐
   b. East coast Yes 1 ☐ No 2 ☐ Don’t know 3 ☐
   c. Anthrax Yes 1 ☐ No 2 ☐ Don’t know 3 ☐
   d. Brucellosis Yes 1 ☐ No 2 ☐ Don’t know 3 ☐
   e. Other ☐
35. Do you institute preventive measures to your animals?

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
<th>Don’t know</th>
</tr>
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<tbody>
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<td>1</td>
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<td></td>
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</table>

36. Which measures do you apply?

<table>
<thead>
<tr>
<th>Measure</th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Vaccination</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>b. Dipping</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>c. Deworming</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>d. Other</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

37. Where do you take your animals during droughts?

<table>
<thead>
<tr>
<th>Location</th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Stay</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>b. Elsewhere</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>c. Other</td>
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</tr>
</tbody>
</table>

38. Do you use cow dung to construct your house/grain storage facilities?

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
</tr>
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<tbody>
<tr>
<td>1</td>
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</table>

**F. Wildlife**

39. Do you know whether Tb infects wildlife?

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
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<tbody>
<tr>
<td>1</td>
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<td>2</td>
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</tr>
</tbody>
</table>

40. If yes, which species are more frequently affected?

<table>
<thead>
<tr>
<th>Species</th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Buffalo</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>b. Lion</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>c. Antelope</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>d. Baboon</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>e. Other</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

41. Does your livestock interact with wildlife?

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td></td>
<td>2</td>
<td>3</td>
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</tbody>
</table>
42. Do you know if the disease can be transmitted between wildlife and livestock?

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
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</thead>
<tbody>
<tr>
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</table>

G. Socioeconomic (Household) characteristics

43. Type of dwelling/housing

<table>
<thead>
<tr>
<th></th>
<th>Thatch house</th>
<th></th>
<th>Brick</th>
<th></th>
<th>Both</th>
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<tbody>
<tr>
<td>1</td>
<td>Yes</td>
<td>2</td>
<td>No</td>
<td>3</td>
<td>Don’t know</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

44. Main occupation of head of household

<table>
<thead>
<tr>
<th></th>
<th>Herder</th>
<th>Agr/past</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yes</td>
<td>2</td>
<td>Don’t know</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

45. Do have any other source of income?

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>Don’t know</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

46. If yes, which activities do you have?

<table>
<thead>
<tr>
<th></th>
<th>Shop</th>
<th>Bar/Cafe</th>
<th>Machine</th>
<th>Restaurant</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yes</td>
<td>2</td>
<td>Don’t know</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

47. Which household assets do you have?

<table>
<thead>
<tr>
<th></th>
<th>Phone</th>
<th>Car</th>
<th>Tractor</th>
<th>Bicycle</th>
<th>Motorcycle</th>
<th>Generator</th>
<th>Solarpanel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yes</td>
<td>2</td>
<td>Don’t know</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
48. Do you know how much you spend on medicine for treatment of your livestock?

Yes 1  No 2  Don’t know 3

48. If YES how much money do you spend on treatment and prophylaxis in a year

a. <100,000 Yes 1  No 2  Don’t know 3
b. <500,000 Yes 1  No 2  Don’t know 3
c. <1000000 Yes 1  No 2  Don’t know 3
d. <5,000,000 Yes 1  No 2  Don’t know 3

49. What benefits do you get from your livestock?

a. Food Yes 1  No 2  Don’t know 3
b. Draught Yes 1  No 2  Don’t know 3
c. Soc. value Yes 1  No 2  Don’t know 3
d. Other 1

50. What medicine do you use to treat your animals?

a) Antibiotics Yes 1  No 2  Don’t know 3
b) Antitryps Yes 1  No 2  Don’t know 3
c) Other 1

51. What is the market price of cattle?

Age groups

Oxen  Bull  Cow  Calf

<100,000 = 1, <200,000 = 2, <300,000 = 3, <400,000 = 4

52. What veterinary public services do you receive in your livestock?

a. Diagnosis Yes 1  No 2  Don’t know 3
b. Treatment Yes 1  No 2  Don’t know 3
c. Slaughter house Yes 1  No 2  Don’t know 3
d. Vaccine Yes 1  No 2  Don’t know 3
e. Others 1