EVALUATION OF IMMUNOMAGNETIC BEADS AND LOOP MEDIATED ISOTHERMAL AMPLIFICATION IN DETECTING *MYCOBACTERIUM TUBERCULOSIS* COMPLEX IN BOVINE MILK

BY

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Declaration

I **Rosaline N. Mwape** declare that the work presented in this dissertation was done by myself and has not been presented to this or any other university for the award of any degree.

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Date…….

Certificate of Approval

This thesis of Rosaline N. Mwape has been approved as fulfilment for the award of the degree of MASTER OF SCIENCE IN ONE HEALTH ANALYTICAL EPIDEMIOLOGY of the University of Zambia.

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Abstract

The study evaluated the use of immunomagnetic beads (IMB) in detecting *Mycobacterium tuberculosis* complex (MTC) in bovine milk. A total of 161 animals from traditional small scale farmers of Mphango and Itope areas of Chisamba and Rufunsa districts of Zambia were tested for tuberculosis using the comparative intradermal tuberculin skin test. Of the tested animals, 4 (2.5%, 95% CI=0.1%-4.9%) out of the 161 animals tested positive on tuberculin skin test and 12 (7.5%) showed inconclusive results.

Furthermore, milk samples from the 161 animals were tested for tuberculosis (TB) using the immunomagnetic beads technique and, DNA from the captured cells was used as samples for the loop mediated isothermal amplification (LAMP) test. A total of 24 (14.9%, 95% CI= 9.4%-20.4%) animals tested positive on IMB-LAMP. This technique was also able to detect 12 (8.3%, 95% CI=3.8%-12.8%) animals that initially tested negative with the tuberculin skin test as positives. The twenty four (24) milk samples that tested positive on IMB-LAMP were further cultured on Lowenstein-Jensen media supplemented with Tween 80. Out of the 24 samples, 4 (16.7%, 95% CI=1.8%-31.6%) showed signs of *Mycobacterium* growth and were considered positive. The rest 20 (83.3%) did not grow and were considered negative. The suspected culture positives were confirmed by multiplex PCR as *Mycobacterium bovis*.

The study has demonstrated that immunomagnetic beads can be used to capture MTC from milk of naturally infected animals and that the *Mycobacterium* can be isolated from milk samples of animals certified negative by the comparative intradermal tuberculin skin test. The study also demonstrated that the culturing technique, though regarded as a ‘Gold standard method’ need to be conducted alongside molecular tests.

The study recommends the use of IMB-LAMP as a complementary diagnostic test for detecting *Mycobacterium bovis* besides the intradermal tuberculin skin test and the culture method. It further suggests that milk be considered as a diagnostic specimen for tuberculosis in animals. Further studies may be carried out to establish the minimum number of mycobacterial cells that can be captured by the beads in milk samples.

Copyright

Dedications

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List of abbreviations and Acronyms

AFB	Acid-fast bacilli
AIDS	Acquired immune deficiency syndrome
bp	Base pairs
BTB	Bovine tuberculosis
CDC	Centre for disease control and prevention
CFSPH	Centre for Food Security and Public Health
CFU	Colony forming units
CI	Confidence interval
DDW	Distilled water
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and agriculture organisation
HIV	Human immunodeficiency virus
HPLC	High-Performance Liquid Chromatography
IL-6	Interleukin-6
IMB	Immunomagnetic beads
IMC	Immuno magnetic capture
IMS	Immuno magnetic separation
LAMP	Loop mediated isothermal amplification
LJ	Lowenstein Jensen
M. bovis	*Mycobacterium bovis*
MAbs	Monoclonal antibodies
mM	Micro molar

MOTT	*Mycobacterium* other than the *Mycobacterium* tuberculosis complex
MTC	*Mycobacterium tuberculosis* complex
OIE	World organisation for animal health
PCR	Polymerase chain reaction
m-PCR	Multiplex Polymerase chain reaction
PPD-A	Purified protein derivatives of avian tuberculin
PPD-B	Purified protein derivatives of bovine tuberculin
RD	Region of difference
TB	Tuberculosis
USA	United States of America
UV	Ultra violet
WHO	World health organisation
µl	Microliters

CHAPTER ONE

INTRODUCTION

Tuberculosis, a chronic bacterial disease of both man and animals, is caused by members of the *Mycobacterium tuberculosis* complex (MTC). Members of the MTC include *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium caprae*, *Mycobacterium microti*, *Mycobacterium pinnipedii* and *Mycobacterium canettii* (Jenkins *et al*., 2011). MTC causes ill-health among millions of people each year and ranks as the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (WHO, 2013). Bovine tuberculosis (BTB), mainly a disease for animals caused by *M. bovis*, continues to threaten livestock worldwide particularly in developing countries. It also has a very serious implication on the health of humans as it is a zoonotic disease (MacHugh *et al*., 2009). It has been classified as a risk 3 pathogen of public health importance (OIE, 2012), and the World Health Organisation (WHO) further classified it among the 7 neglected zoonotic diseases with a potential to infect man (Ereqat *et al*., 2013). Its negative effects are however difficult to ascertain because infected animals disseminate the bacteria for a non-determined period of time without showing clinical signs.

Previously, other members of the MTC apart from *M. tuberculosis* were considered to be *M. bovis*. Recently however, all members of the MTC are considered as independent species despite 99.9 percent of their genome being identical (Smith *et al*., 2006; OIE, 2009). Unlike other members of the MTC, *M. bovis* displays a wide range of hosts and its transmission to humans constitutes a public health risk (O'Reilly and Daborn, 1995). Rapid detection of MTC

strains is one of the most important factors in minimizing the maintenance and spread of tuberculosis (Hang'ombe *et al*., 2011; Wang *et al*., 2011). In most countries, especially those in the developed world, the reactive animals to the tuberculin test are usually slaughtered and owners are compensated. In some developing countries like Zambia however, the policy of compensating animal owners is not widely practiced due to overstrained public resources (Malama *et al*., 2013). The common practice, however, is that when the animal tests positive to the tuberculin skin test, the farmer takes the animal to the abattoir for slaughter. If the animal is found with generalized tuberculosis at the abattoir, it is condemned. However, if the disease is not generalized, the affected organs are condemned and the rest of the meat is passed as fit for human consumption.

Pasteurization of milk has largely contributed to the control of BTB in industrialized countries (Cosivi *et al*., 1998; Romero *et al*., 2006). In developing countries, this method is not widely practiced because most of the cattle are traditionally owned and people believe that drinking milk directly from cows is more nutritious and healthy. In Zambia for example, herdsmen usually depend on milk as their main source of food during many hours of cattle herding in the plains. This usually happens during the dry season when grass is very scarce and animals have to move long distances in search of food. The practice of drinking milk directly from the udder of cows by the Zambian herdsmen have been reported previously by Pandey *et al*., (2013).

The presence of the disease in wildlife coupled with lack of proper diagnostic tools and handling of animals at field level have further contributed to the difficulties in its eradication worldwide, and more so in resource constrained Sub-Saharan Africa. Some wild animals act as reservoirs and transmit the disease to domestic animals and humans at the human,

domestic and wild animal's interface where man and animals share common pool resources (water, land and food). Many wild animals have been implicated as reservoirs of the zoonotic *M. bovis* in different parts of the world (CFSPH, 2009). In Zambia, the Kafue lechwe antelope (*Kobus leche Kafuensis*) is a known wild animal reservoir of BTB (Malama *et al*., 2013). These animal species have contributed to the spread and maintenance of the disease in areas around the Kafue basin due to the constant interaction with domestic animals.

Severe economic losses due to death of animals (domestic and wild) and people in their productive ages have been attributed to tuberculosis (TB). Loss due to trade restrictions and chronic disease has severely affected developing countries. According to Figueiredo *et al*., (2012), worldwide annual losses to agriculture were estimated at $3 billion. The human burden of TB caused by *M. bovis* is still largely unknown in many countries because the signs are very similar to those caused by *M. tuberculosis* which is sorely a human pathogen. In some situations, the disease may also be a serious threat to endangered animal species such as the Iberian Lynx (*Lynx pardina*) (Aranaz *et al*., 2004; CFSPH, 2009).

Most of the people worldwide and approximately one third of the world's population are infected with bacteria belonging to the MTC (Chihota *et al*., 2007). Sub-Saharan Africa has reported the highest prevalence of TB since the advent of HIV and AIDS (Chihota *et al*., 2007). According to the 2006 World Health Organization data, mortality and morbidity statistics attributed to the disease included 14.6 million chronic active cases, 8.9 million new cases, and 1.6 million deaths (WHO, 2006). These cases were recorded mostly in developing countries with an expected 1% increase annually. For Zambia, the WHO estimates that the incidence of all forms of TB stands at 444/100,000 of the population (WHO, 2013).

Data on the incidence of TB in Zambia according to WHO (2013), which was estimated at 444 cases per hundred thousand of the population is quite alarming. One of the approaches that can be used to effectively manage the disease is the use of reliable diagnostic techniques. Currently, the commonly used technique to detect positive animals to TB in Zambia is the tuberculin test. This test is not very reliable because it gives false negatives depending on the animal's condition. The measurement of the hypersensitivity reaction site is subjective as it depends on the person doing the test and their experience. The procedure is very tedious as it involves follow ups and restraining animals which are semi wild. In this study, the use of the immunomagnetic beads technique for the detection of MTC in bovine milk was explored. The study is important as it may provide a complementary technique to the tuberculin test in milking animals.

1.1. Statement of the problem

In Zambia, the diagnosis of *M. bovis* in live cattle is usually done using the tuberculin skin test. This method involves injecting animals with purified protein derivatives of *M. bovis* intradermally. A positive test is indicated by a delayed hypersensitivity reaction (swelling). The tuberculin test can be performed using bovine tuberculin alone, or as a comparative test that distinguishes reactions to *M. bovis* from reactions to purified protein derivatives of *M. avium* (Schiller *et al*., 2010). However, false negative responses are sometimes seen in cows within 6 weeks of calving down, those in the early stages of infection and those in the late stages of the disease where the immune response is poor (Verma *et al*., 2014). It also has low specificity and sensitivity (Neill *et al*., 1994; Praud *et al*., 2014). Due to the strenuous work involved in restraining animals, false negatives and follow up measurements of the swelling makes this test very tedious. Furthermore, tuberculin skin test is not highly reliable especially when performed alone. Given that it is now mandatory in Zambia for all farmers who wish to

sell their milk to milk processing plants to have their animals certified as TB free using the tuberculin test, the authenticity of this certification is questionable considering the inconsistencies of the test. This study therefore explored the use of immunomagnetic technique as a diagnostic tool for detecting *M. bovis* in milk samples.

1.2. Study justification

Exploring the use of immunomagnetic technique for detecting *M. bovis* in milk samples is essential as it may be useful in certifying female animals as tuberculosis free alongside the tuberculin test, which sometimes gives inconclusive results. This technique may also be used by public health workers to test imported milk products for *M. tuberculosis* complex organisms. Further, this technique most likely may be useful to farmers in assessing the microbiological quality of milk. To the author's knowledge, this is the first time that the use of immunomagnetic beads was employed in milk to assess the recovery rate of organisms belonging to the MTC in Zambia. The study targeted traditional small scale farmers because they tend to keep their cattle under conditions that may favour TB transmission and maintenance. Furthermore, the traditional cattle produces minimal amounts of milk that may favour the applicability of the test. Specifically, the study was done in Chisamba and Rufunsa districts of Zambia because of previous total condemnation of animals at the abattoir due to TB (Farmer's group personal communication).

1.3. Study objectives

Main objective

To evaluate the possible application of IMB-LAMP technique in the diagnosis of tuberculosis directly from cattle milk.

Specific objectives

a. Direct detection of *M. tuberculosis* complex in milk using IMB-LAMP.

b. Isolation of *M. tuberculosis* complex from milk detected positive with IMB-LAMP.

CHAPTER TWO

LITERATURE REVIEW

2.1. Description of the organisms causing tuberculosis

Recent strict molecular genetic criteria suggest that all members of the MTC should be regarded as subspecies of *M. tuberculosis* (Brosch *et al*., 2002). This however is still a subject of debate and has not yet been accepted scientifically. These TB bacilli are described as, non-motile, non-sporulating, weakly Gram-positive, acid-fast bacilli (AFB) that appear microscopically as straight or slightly curved rods (Sakamoto, 2012). They are aerobic, acid alcohol fast and they are said to contain arabinose, galactose and meso-diaminopimelic acid in their cell wall (Goodfellow and Wayne, 1982). The mycobacteria have the ability to synthesize mycolic acid and this differentiates them from other bacteria within the order actinomycetes where they belong. They have the capacity to adapt to changes in their host's environment throughout infection. These changes include hypoxia, nutrient deprivation and exogenous stress (Rastogi, 1993). The adaptation to changes in a host and intracellular survival is attributed to the unique thick cell wall which contains glycolipids, mycolic acids, lipids and proteins.

2.2. Classification of *Mycobacterium*

Taxonomically, mycobacteria belongs to the genus *Mycobacterium* which is a single genus within the family *Mycobacteriaceae,* in the order Actinomycetales (Stackebrandt *et al*., 1997). The genus *Mycobacterium* contains opportunistic and strict pathogens that infect both man and animals. Mycobacteria species can be classified into two groups practically. According to Pfyffer *et al*., (1998), these groups include MTC and *Mycobacterium* other than

the *M. tuberculosis* complex (MOTT). The MOTT are also referred to as the 'non-tuberculous' or 'atypical mycobacteria' (Johnson and Odell, 2014).

This practical classification of mycobacteria species is however different from the classification of species based on their clinical importance. Rastogi *et al*., (2001) classified *Mycobacterium* into three groups based on the clinical importance. These groups include;

1. Strict pathogens, including the human pathogens *M. tuberculosis* and *M. leprae*, and the animal pathogen *M. bovis*.
2. Opportunistic (or potential) pathogens, including *M. simiae*, *M. avium* and *M. xenopi*.
3. Rare pathogens, including saprophytes such as *M. smegmatis* and *M. phlei*.

2.3. Routes of transmission and risk factors among animals

TB is contagious and can spread directly or indirectly from infected animals to susceptible ones in both wild and domestic animals. This transmission can be among animals of the same or different species. Wild animals and scavengers for example can acquire *M. bovis* under natural conditions through consumption of infected carcasses (Thoen *et al*., 2009). Domestic cattle are considered the true natural hosts of the bacterium and the principal reservoir of infection for other animals and man (De la Rua-Domenech, 2006a).

Factors such as overcrowding, close housing and improper management predisposes animals to TB (Verma *et al*., 2014). Tuberculosis is mostly a respiratory disease and the likely route of transmission is airborne though it can also be transmitted through other routes such as ingestion. De la Rua-Domenech (2006a) reported that the majority of natural infection in cattle were through aerosol infection.

An infected animal can excrete the bacteria from its excretions and secretions for a long time and these materials may continue to pose a risk to other animals. As a result of this, the bacteria can also be transmitted through contaminated water, feed and pasture among domestic animals as well as between wild ones. Wild animal hosts have also been associated with the transmission of the bacteria to cattle through contact with excreted material onto pasture and feedstuffs via faeces, urine and/or aerosols (Sweeney *et al*., 2006). At the interface where domestic and wild animals share common pool resources such as water, food and land, the disease may be transmitted and maintained (Cleaveland *et al*., 2007; Munyeme *et al*., 2008). Some breeds of cattle may show resistance to the disease especially if they are kept in environments which allow the maintenance of the disease. Resistance to TB in some breeds has been reported previously (Dean *et al*., 2005; Vordermeier *et al*., 2012). Verma *et al*., (2014) described some resistance to BTB in the Brahman breed compared to the European breeds. Generally however, indigenous cattle in developing countries are assumed to be more resistant to the disease compared to exotic ones. Vordermeier *et al*., (2012) in their experimental study associated the high presence of Interleukin-6 (IL-6), a pleiotropic pro-inflammatory cytokine produced by innate immune cells as well as T cells in indigenous cattle compared to exotic breeds in Ethiopia to be responsible for resistance to BTB. The presence of IL-6 in tuberculous animals however has not been widely accepted and this, has been a subject of debate by many researchers (Vordermeier *et al*., 2012).

An infected dam poses a risk of infection to its young ones both unborn and the ones in their early life if it has tuberculous mastitis. O'Reilly and Daborn, (1995) reported the congenital transmission of the bacteria, though not a very common route and, Vitale *et al*., (1998) further isolated the bacteria in milk, making this a very important route of transmission to calves through ingestion.

2.4. Public health importance

The impact of TB on human health has been devastating worldwide. More than 3.5 million people die annually from TB, with BTB being responsible for 3% of these cases (Cosivi *et al*., 1998). Further, Oloya *et al*., (2008) estimated the prevalence of *M. bovis* to be at 7 % among pastoral communities in Karamoja region of Uganda. According to the WHO report of 2013, 8.6 million new TB cases and 1.3 million TB deaths were recorded (just under 1.0 million among HIV-negative people and 0.3 million HIV-associated TB deaths). There is uncertainty in all estimates of the burden of disease caused by TB (WHO, 2013). This uncertainty could probably be attributed to lack of proper research and reporting systems in most countries. Some cases of the disease also go undiagnosed in most developing countries where there are no proper health facilities in remote areas coupled with lack of proper laboratory facilities and equipment for diagnosis (Munyeme *et al*., 2010).

In Zambia, the extent of *M. bovis* involvement in the national tuberculosis burden is unknown and the situation is further compounded by poor or non-existence institutional system, and lack of control measures with limited research facilities (Munyene *et al*., 2010).

The public health importance of BTB cannot be over emphasised. This is because *M. bovis* causes a disease to man which cannot be differentiated clinically and pathologically from that caused by *M. tuberculosis* (Cosivi *et al*., 1998). Isolation of *M.bovis* from patients with clinical TB in developing countries makes this disease an important zoonosis. Malama *et al*., (2014) isolated *M. bovis* from sputum of two TB patients from Namwala district of Zambia and the strain was similar to the one isolated from cattle in the same area.

M. bovis, a pathogen which qualifies to be classified as having a higher risk of re-emerging due to its capability of infecting more than one host including wild animals and man has recently been highlighted as a public health problem due to the HIV/AIDS pandemic (Cosivi *et al*., 1998). HIV weakens the immune system of infected individuals making them susceptible to most opportunistic microorganisms including *M. bovis* which is usually lethal. Historically, it was assumed that human TB had its origin as a zoonosis, with *M. tuberculosis* evolving from *M. bovis* by specific adaptation of an animal pathogen to the human host at the time of cattle domestication some 10,000 to 15,000 years ago (De la Rua-Domenech, 2006a). This assumption has however been discarded after many molecular analysis of the MTC which now show that, the genome of *M. bovis* is smaller than that of *M. tuberculosis* (Brosch *et al*.,2002)

Transmission of *M. bovis* to man from other animals besides cattle happens occasionally. The transmission of the disease from other animals to man is sometimes considered as an occupational disease especially to people who work with animals and most of the time come in close contact with animals that are either alive or slaughtered. Such kind of workers who are at risk of infection due to their occupations include veterinarians, meat inspectors, farmers, butchery and abattoir workers (De la Rua-Domenech, 2006a).

The most likely routes of transmission to man from animals are through aerosol which is the principal route of transmission (De la Rua-Domenech, 2006a). Close physical contact between humans and potentially infected animals is present in some communities, especially in developing regions (Cosivi *et al*., 1998). This is so because, cattle are at a core centre in most social events that take place in those countries such as funerals, dowry payments and weddings. Having cattle is also a sign of prestige and wealth in most cattle keeping African

tribes so, most of the animals are allowed to live for a long time making the old ones with chronic infections continue to pose a risk to owners. In a review by Humblet *et al*., (2009), age was highlighted to be a risk factor for BTB with old animals being at a higher risk compared to young ones.

Ingestion of infected milk and meat is a very important mode of transmission of the disease from infected animals to man. Pandey *et al*., (2013) cultured and isolated *M. bovis* from milk of 3 animals that tested positive on tuberculin. In a similar study, Jia *et al*., (2012) were able to culture and isolate the bacteria's DNA from milk. These findings clearly indicated that the viable bacteria from milk could easily be transmitted to man and establish disease. The milk transmission route is very important especially in countries where the disease is uncontrolled, some milk is consumed unpasteurized and most of the meat found in informal markets is uninspected. Unpasteurized milk and milk products continue to be regarded as the main vehicle for transmission in countries where BTB is prevalent and eradication programmes are patchy or non-existent (Ashford *et al*., 2001). Milk borne infection has been found to be a principal cause of cervical lymphadenopathy (scrofula), abdominal and other forms of extra pulmonary TB (Cosivi *et al*., 1998). In countries like Zambia where milk is not usually boiled by most people in rural areas before use, TB due to *M. bovis* may possibly be the major cause of extra pulmonary tuberculosis in humans (Pandey *et al*., 2013). This mode of transmission however has been reported to be of little significance in urban areas of industrialised countries despite a high incidence of TB in national cattle herd due to a wide spread and compulsory pasteurization of milk (De la Rua-Domenech, 2006a).

Many dairy products such as cheese and sour milk have been reported as potential risks of BTB to man due to the resistance of the bacteria to chemicals and because it is unaffected by

the acidic pH of such products. The use of raw milk in the production of cheese and other dairy products is another potential public health risk associated with tuberculous cattle. Viable Mycobacteria (including *M. bovis*) have been found to survive in mature unpasteurized cheeses (Spahr and Schafroth, 2001). The presence of viable *M. bovis* in sour milk which was traditionally processed from indigenous cattle in Namwala district of Zambia was reported by Sitima, (1997).

Food hygiene mostly of food originating from traditional cattle maybe a good preventive measure although it is difficult to implement in developing countries. In most countries of sub-Saharan Africa, there is active competition between large-scale milk and meat processing companies and the informal sector. The informal sector can ignore standards of hygiene and quality and products are often sold directly to the final consumers (Cosivi *et al*., 1998).

There have also been reports of the bacteria being transmitted directly through mucous membrane or open skin. This mode of transmission was mainly of historical interest, but is extremely rare these days, especially in industrialized countries (De la Rua-Domenech, 2006a). In the past, cutaneous or mucosal transmission was an occasional source of localized skin, tendon and lymph node lesions, otitis and conjunctivitis in milkers, those regularly dressing carcases of tuberculous animals and veterinarians exposed during surgical interventions or necropsies (Ashford *et al*., 2001). Whatever the route of infection, the precise infectious dose of *M. bovis* for humans remains unknown (O'Reilly and Daborn, 1995).

The presence of the disease in wild animals continue to pose a risk to humans especially those who live in or near game management areas. Most of the times, the transmission of the disease to man happens if they share common pool resources such as water at the

human/domestic animal/wildlife interface areas (Cleaveland *et al*., 2007; Munyeme *et al*., 2008). The consumption of uninspected game meat in interface areas is also of public health concern especially in developing countries where it serves as a main source of protein for the local people.

2.5. Clinical manifestation of tuberculosis in animals

TB presents itself in a chronic form with a progressive formation of tubercles in affected organs especially the lungs, with undulating fever and a cough. According to OIE, (2009) and Muma *et al*., (2013), the infection is often subclinical. When present, clinical signs are not specifically distinctive and can include weakness, anorexia, emaciation, dyspnoea, enlargement of lymph nodes, and cough, particularly with advanced tuberculosis. There is also loss of condition, a low grade fever and a roughened hair coat.

After infection, nonvascular nodular granulomas known as tubercles may develop. Characteristic tuberculous lesions occur most frequently in the lungs and the retropharyngeal, bronchial and mediastinal lymph nodes (FAO, 2000). Lesions can also be found in the mesenteric lymph nodes, liver, spleen, on serous membranes and in other visceral organ (FAO, 2000). There is also bronchopneumonia, nodules on the pleura and peritoneum. Some active lesions may be surrounded by hyperemic periphery while chronic ones may contain caseous material in the centre or calcification (FAO, 2000).

2.6. Hosts and Reservoirs of infection

MTC include a wide range of bacteria which affect different animal species. In this grouping, *M. bovis* which is zoonotic has a wide range of host animal species, which include cattle, goats, bisons, antelopes, humans and non-human primates, and can cause disease in

susceptible hosts (O'Reilly and Daborn, 1995). Cattle are however considered to be the primary host for the bacteria (CFSPH, 2009). Among the hosts, some animal species may be termed as maintenance, spillover and reservoir hosts. Maintenance hosts are sometimes referred to as reservoirs of the disease. These are mainly wild animals that are capable of transmitting the disease to domestic animals and humans (CFSPH, 2009). Known maintenance hosts in different regions 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 (CFSPH, 2009). In Zambia, the kafue lechwe antelope is also a known reservoir of the disease (Munyeme *et al*., 2009).

Humblet *et al*., (2009) distinguished two categories of hosts, namely: maintenance hosts (those capable of maintaining and spreading the infection) and spill over hosts. Spill-over hosts (usually 'dead-end' hosts, in which the incidence and pathology of the disease indicates that they play no significant role in its onward transmission). Spill-over hosts may also be 'amplifier' hosts if they act as incidental sources of TB for livestock or other species (Morris *et al*., 1994; De Lisle *et al*., 2001). Infected humans can also act as amplifier hosts of BTB, presenting a potential source of *M. bovis* for animal and human contacts (De la Rua-Domenech, 2006a). White-tailed deer in the Michigan area of the United States of America have been classified as maintenance hosts (CFSPH, 2009). However, some workers classify White-tailed deer as a spill over host that maintains the organism only when its population density is high (Bruning-Fann *et al*., 2001; Kaneene *et al*., 2002). Species reported to be spill over hosts include sheep, goats, horses, pigs, dogs, cats, ferrets, camels, llamas, many species of wild ruminants including deers and elk, elephants, rhinoceroses, foxes, coyotes, mink, primates, opossums, otters, seals, sea lions, hares, raccoons, bears, warthogs, large cats

(including lions, tigers, leopards, cheetahs and lynx) and several species of rodents (CFSPH, 2009).

Little is known about the susceptibility of birds to *M. bovis*, although they are generally thought to be resistant (CFSPH, 2009). Experimental infections have recently been reported in pigeons after oral or intra tracheal inoculation and in crows after intra peritoneal inoculation. Some avian species, including mallard ducks, appear to be resistant to experimental infection (CFSPH, 2009).

2.7. Diagnostic methods

Existing strategies for long-term BTB control/eradication campaigns are being reconsidered in many countries because of the development of new testing technologies, increased global trade and continued struggle with wildlife reservoirs (Schiller *et al*., 2010). Rapid detection and diagnosis of MTC is very important as this can help in minimizing TB spread (Hang'ombe *et al*., 2011; Wang *et al*., 2011). The discovery of a diagnostic test with a high sensitivity and specificity will be a breakthrough in its eradication. There are different methods that are used in the diagnosis of TB in both the dead and live animals at the moment.

In live animals, diagnosis mainly depends on clinical manifestations of the disease achieved during antemortem inspection, skin testing, and subsequent identification of the pathogen by biochemical tests (Vitale *et al*., 1998). In dead animals, this can be achieved using postmortem inspection techniques and histopathology of tissues from pathological lesions. The TB diagnostic methods/approaches used currently are summarised below:

2.7.1. Antemortem examination

In cattle, clinical evidence of TB is usually lacking until very extensive lesions have developed (OIE, 2009). BTB is normally a chronic and subclinical disease. Antemortem examination of animals may not give a definitive diagnosis of the disease. This is so because an infected animal may live for a long time without showing clinical signs and there are many diseases besides TB that may show signs such as emaciation, coughing and enlargement of superficial lymph nodes. These reasons make ante mortem examination not to be a very reliable diagnostic tool.

2.7.2. Delayed hypersensitivity reaction tests

In many countries, BTB in live animals is diagnosed based on delayed hypersensitivity reactions using different types of tuberculin tests. Tuberculin is a concentrated sterile culture filtrate of tubercle bacilli grown on glycerated beef broth (OIE, 2009). This is normally achieved by injection of purified protein derivatives of bovine (PPD-B) tuberculin alone or as a combination of bovine tuberculin and avian tuberculin on the caudal fold of the tail or the mid neck (OIE, 2009). The skin of the mid neck is said to be more sensitive to tuberculin hypersensitive reactions than that of the caudal fold (Schiller *et al*., 2010; Verma *et al*., 2014). Due to this difference in the sensitivity of the skin, more dosage of tuberculin is needed when injecting on the caudal fold compared to the mid neck region. Cell mediated hypersensitivity acquired through infection can be demonstrated systemically by fever, conjunctivitis or dermally by a local swelling (Fentahun and Luke, 2012).

The different types of tuberculin tests are; Short thermal, Stormont, Single intradermal and comparative intradermal (Radostits *et al*., 2007).

a. The short thermal test involves the subcutaneous injection of 4ml of PPD-B into the mid neck of an animal whose rectal temperature is not more than 39ºC at the time of injection. Four, six and eight hours after injection, the animal's rectal temperature is monitored. If the rectal temperature rises to 40ºC, the animal is considered positive for tuberculosis (Radostits *et al*., 2007; Verma *et al*., 2014).

b. The Stormont test which is considered to be more sensitive than the short thermal test is done by injecting PPD-B into the mid neck intra dermally. After seven days of initial injection, another injection is given on the same site as the first one. Twenty four hours later if the skin thickness increases to 5 mm or more, the animal is considered positive. The whole test requires 3 visits to the farm making it very tedious especially on the part of restraining animals (Whelan *et al*., 2003; Radostits *et al*., 2007). In rural parts of Zambia with poor animal handling/ restraining facilities (Figure1), the test is very difficult to implement.

Figure 1: Animal restraining/handling facility in rural Chisamba

The most common tuberculin tests practiced in many countries are the single and comparative intradermal tests. These tests are widely used because of availability, low running costs, long historical use and a lack of alternative tests (Schiller *et al*., 2010).

c. The single intra dermal test involves the injection of PPD-B on the mid neck or caudal fold of the tail and subsequent detection of an increase in skin thickness is checked 72 hours after injection (OIE, 2009; Verma *et al*., 2014). Because animals are frequently exposed to or infected with various non-tuberculous mycobacteria, cross-reactive responses to PPD-B may occur as many antigens contained within PPD-B are shared between non-tuberculous and tuberculous mycobacteria (Schiller *et al*., 2010).
d. A comparative intradermal test is done to differentiate reactions of MTC and those of *Mycobacterium avium* complex or non-tuberculous *Mycobacterium* (OIE, 2009). This is normally done by measuring the initial skin thickness and injecting PPD-B and purified protein derivatives of avian (PPD-A) tuberculin on the mid neck with the distance between the two injections sites of 12 cm. The results are read 72 hours after injection by measuring the final skin thickness. According to the OIE report (2009), the difference between initial and final skin thickness of less than 2mm, is be considered negative; between 2mm and 4mm the results are said to be inconclusive, and above 4mm, it is considered positive for BTB.

The world organization for animal health (OIE, 2009) recommends that a comparative intra dermal tuberculin test on live animals be done to test for TB. Though this kind of testing is recommended, it is known that the skin test lacks sufficient sensitivity and specificity in many cases (Francis *et al*., 1978; Seiler, 1979; Neill *et al*., 1994; De la Rua-Domenech *et al*., 2006b; Praud *et al*., 2014). It is very tedious as it involves restraining of animals that maybe semi wild. The measurement of the skin swelling is subjective as it highly depends on the

experience of the one doing the test. It also has low degree of standardization, and imperfect test accuracy (De la Rua-Domenech *et al*., 2006b). It is a good test for identification of the presence of TB in animals at herd level but, not a very good one when it comes to diagnosis of TB at individual animal level (Dawson and Trapp, 2004). According to the report by Neil *et al*., (1992), *M. bovis* may be isolated from the secretions of skin-test-negative cattle. Zarden *et al*., (2013) isolated *M.bovis* from tuberculin skin test negative animals and Verma *et al*., (2014) in their review highlighted that *M. bovis* may be isolated from tuberculin skin test negative animals. Muma *et al*., (2013) also reported of the underestimation of the prevalence of BTB in Zambia since most of the tests were based on the tuberculin test which is not a very reliable.

2.7.3. Polymerase Chain Reaction (PCR)/ DNA finger printing

Considering the drawbacks revealed by many approaches in the diagnosis of TB in live animals, several alternatives are being sort for rapid and specific diagnosis of the disease. Molecular based approaches, especially polymerase chain reaction (PCR) assays are most promising for diagnosis in live animals (Figueiredo *et al*., 2010).

The use of PCR in the detection of MTC has been reported previously by many researchers (Vitale *et al*., 1998; Cardoso *et al*., 2009; Figueiredo *et al*., 2010; Pandey *et al*., 2013). The technique has been widely evaluated for the detection of *M. tuberculosis* complex in clinical samples (mainly sputum) in human patients and has recently been used for the diagnosis of TB in animals (OIE, 2009). The introduction of PCR and nucleic acid hybridization has greatly reduced identification time and improved the level of detection in clinical specimens (Clarridge *et al*., 1993). Vitale *et al*., (1998) reported excellent sensitivity, specificity, and positive and negative predictive values for the PCR-dot blot test performed using milk

samples and lymph node aspirates, and recommended that these type of samples could be considered useful in the screening for TB in cattle, as it has higher accuracy than the skin test. PCR techniques offer high sensitivity, and have been successfully used for diagnosing BTB in several types of naturally infected organic materials such as tissue, blood and nasal exudates (Romero *et al*., 1999; Cardoso *et al*., 2009; Figueiredo *et al*., 2010). In Zambia, Pandey *et al*., (2013) reported for the first time, isolating DNA of *Mycobacterium bovis* from freshly drawn milk from three tuberculin reactor cows using PCR.

Genotyping of bacterial isolates or PCR products is increasingly becoming a standard tool for epidemiological disease control and eradication (Schiller *et al*., 2010). Methods of DNA finger printing have been developed which enable the differentiation of strains of *M. bovis* and also show the transmission and spread patterns together with the origin of the bacteria. The most commonly used method is spoligotyping (from 'spacer oligotyping'), which allows the differentiation of strains inside each species belonging to the *M. tuberculosis* complex, including *M. bovis*, and can also distinguish *M. bovis* from *M. tuberculosis* (Heifets and Jenkins, 1998). Multiplex PCR (m-PCR) is also one of the laboratory method that can be used in the differentiation of species belonging to the MTC. The identification of different species belonging to the MTC is also important for appropriate patient management (Nakajima *et al*., 2010). Many researchers have demonstrated successfully the use of m-PCR which provide valuable information on the region of difference (RD) in the chromosome of MTC (Warren *et al*., 2006; Nakajima *et al*., 2010 and Hang'ombe *et al*., 2011). Warren *et al*., (2006) for example have used m-PCR (deletion analysis) using the primers indicated in table 1.

Table 1: PCR primer sequences and corresponding amplification product sizes indicating the presence or absence of genomic regions of difference in different MTC members

Primer sequence	RD	*M. tuberculosis*	*M. bovis*	*M. bovis* BCG
5'AAGCGGTTGCCGCCGACCGACC3'* 5'CTGGCTATATTCCTGGGCCCGG3'* 5'GAGGCGATCTGGCGGTTTGGGG3'*	1	RD 1 present (146 bp)	RD1 present (146 bp)	RD1 present (146 bp)
5'ATGTGCGAGCTGAGCGATG3' 5'TGTACTATGCTGACCCATGCG3' 5'AAAGGAGCACCATCGTCCAC3'	4	RD4 present (172 bp)	RD4 present (268 bp)	RD4 present (268 bp)
5'CAAGTTGCCGTTTCGAGCC3' 5'CAATGTTTGTTGCGCTGC3'* 5'GCTACCCTCGACCAAGTGTT3'*	9	RD9 present (235 bp)	RD9 absent (108 bp)	RD9 absent (108 bp)
5'GGGAGCCCAGCATTTACCTC3' 5'GTGTTGCGGGAATTACTCGG3' 5'AGCAGGAGCGGTTGGATATTC3'	12	RD 12 present (369 bp)	RD12 absent (306 bp)	RD12 absent (306 bp)

* Primer sequences according to Parsons *et al*., 2002

Though the molecular based assays have been widely accepted, they are expensive and not every laboratory can afford them, more especially those in economically strained countries. Most times, they are used as last resort tests or for research purposes. False-positive and false negative results, particularly in specimens containing low numbers of bacilli, have reduced the reliability of these test (OIE, 2009). In addition to the high running costs and false results, reduced sensitivity of PCR assays in the detection of MTB complex bacteria compared to culture techniques has previously been reported (Schiller *et al*., 2010).

2.7.4. Traditional culture / microscopy methods

Identification of the *Mycobacterium* is based on the traditional method with the Ziehl-Neelsen acid-fast stain and on the pigmentation, growth rate, gross and microscopic colony morphologies of cultures of the isolated causative organism (Vitale *et al*., 1998). The bacteria from pathological lesions or fluids of suspected animals are normally grown on Lowenstein-Jensen media which should be tightly closed to avoid drying. *M. bovis* normally shows dysgenic colonies whereas colonies for *M. tuberculosis* are more eugenic in shape and are aerophilic (Radostits *et al*., 2007). Glycerol inhibits the growth of *M. bovis* because, it lacks a functioning pyruvate kinase meaning it cannot use the carbohydrate to generate energy (Keating *et al*., 2005). The growth of *M. bovis* is enhanced by lipids particularly sodium pyruvate which act as a sole source of energy (Keating *et al*., 2005).

Traditional mycobacterial culture is considered the gold standard method for routine confirmation of infection and diagnosis (OIE, 2009). The Ziehl-Neelsen stain is very rapid but lacks specificity and cannot be used to distinguish between the various members of the family *Mycobacteriaceae* (Vitale *et al*., 1998). The slow-growing mycobacteria require highly selective media containing antibiotics and antifungals, and harsh decontaminating procedures such as acid washes, to reduce growth of competing organisms (Donoghue *et al*., 1997; Palomino and Portaels, 1998). It can take about 4 to 8 weeks for the bacteria to grow fully on selective media and obtain a good growth. The dormant and dead microorganisms cannot grow on media. These attributes make it not to be a preferred method of diagnosis and in informing immediate treatment decisions.

Histopathology can also be used as a diagnostic tool from tissues prepared from suspected animals. The presumptive diagnosis of mycobacteriosis can be made if the tissue has

characteristic histological lesions such as, caseous necrosis, mineralisation, epithelioid cells, multinucleated giant cells and macrophages (OIE, 2009). This type of test require specific equipment for tissue preparation that may not be readily available in most laboratories of developing countries such as Zambia.

2.7.5. Postmortem examination

Postmortem examination of animals can also be used as a form of diagnosis and this is done during meat inspection in slaughter houses. The diagnosis is normally presumed when organs especially the lungs and associated lymph nodes, appear with gross pathological lesions such as caseous material, abscesses and mineralisation. The finding of a tuberculous animal at slaughter should initiate an investigation through the skin test of the herd of origin and any other potentially exposed animals (Whipple *et al*., 1996; Olea-Popelka *et al*., 2008). The success of such practices however cannot be ascertained. In countries like Zambia, this is not normally done because of poor animal identification at routine postmortem inspection in different slaughter facilities country wide. In the USA, only 50–70% of such investigations result in identification of the herd of origin and in finding all exposed animals because of the lack of uniform animal identification regulations combined with inconsistent record-keeping (Kaneene *et al*., 2006).

2.7.6. Loop-mediated isothermal amplification (LAMP)

M. bovis, a very slow growing bacteria requires a rapid diagnostic tests in order to make fast clinical decisions. Several diagnostic methods have been developed but most of them have a low detection limit. Recently, the LAMP system has been developed and has been found to have a low detection dose for most organisms including members of the MTC (Saharan *et al*., 2014). This method has been found to amplify very few copies of the target DNA with high

specificity, efficiency and rapidity under isothermal conditions by using four specifically designed primers (Saharan *et al*., 2014). The repetitive insertion sequence IS6110 is used as a target gene for members of the MTC (Aryan *et al*., 2010). According to previous reports (Mori *et al.*, 2001; Aryan *et al*., 2010; Hang'ombe *et al*., 2011; Saharan *et al*., 2014), this test fast, does not require a lot of complicated equipment and it is cheap.

The method has been used in many studies to detect the presence of microorganisms. Hang'ombe *et al*., (2011) demonstrated that loop-mediated isothermal amplification (LAMP) can be used to directly detect MTC from processed clinical and abattoir specimens in lechwe and cattle. Further, Kaewphinit *et al*., (2013) also detected the DNA of *M. tuberculosis* from clinical sputum samples using LAMP.

2.7.7. Blood based tests

Diagnostic blood tests are also available and include gamma interferon assay, which uses an enzyme-linked immunosorbent assay (ELISA) as the detection method for interferon, the lymphocyte proliferation assay, which detects cell-mediated immune responses and the indirect ELISA which detects antibody responses (OIE, 2009). Due to the cost and complex nature of these laboratory-based assays, they are usually used as ancillary tests to maximize the detection of infected animals (parallel testing), or to confirm or negate the results of an intra-dermal skin test (OIE, 2009).

The gamma interferon assay listed by OIE as an alternative test to international trade in 2009 is used alongside the sandwich ELISA that uses two monoclonal antibodies to bovine gamma interferon. This *in vitro* assay is a laboratory-based test that detects specific cell-mediated immune responses by circulating lymphocytes (Schiller *et al*., 2010). This test does not

require second or subsequent visits to tested animals and may be helpful in anergic animals. It also has a high sensitivity (Schiller *et al*., 2010). It however has low specificity (Schiller *et al*., 2010) and high running costs as well as difficulties faced in the standardization of the tuberculins (De la Rua-Domenech *et al*., 2006b). Further, poor humoral immune responses exhibited by animals infected with TB may affect the results.

2.7.8. Biochemical tests

Several biochemical tests have been developed to aid in the diagnosis of TB in animals. These tests are based on the ability of *Mycobacterium* to reduce or deaminate certain compounds. These tests include:

a. The nitrate reduction test where nitrate is reduced to nitrite and, this is indicated by a color change to red (Carter, 1984).
b. Another test is the deamination of pyrazinamide broth containing pyrazinamide by young culture of *Mycobacterium*. A positive reaction is given by a pink band in the agar (Fentahun and Luke, 2012).
c. Other biochemical tests include, niacin production and urease tests (OIE, 2009).

2.7.9. Immunomagnetic separation

Immunomagnetic separation (IMS) using small super- paramagnetic particles or beads coated with antibodies against surface antigens of cells, has been shown to be efficient for the isolation of certain eukaryotic cells, proteins and nucleic acids from biological fluids (Safarık and Safarıkova´, 1999). The technique has found its way in several medical applications (Olsvik *et al*., 1994). It can also be used in separating cells from stool, water, bone marrow and food (Li *et al*., 1996; Safarık and Safarıkova´, 1999). Recently, the principle has also been found useful in separating prokaryotic cells (Olsvik *et al*., 1994). Immunomagnetic

enrichment is assisted by the fact that bacteria immunologically bound to magnetic beads usually remain viable and can continue to multiply if nutritional requirements are provided (Torensma *et al*., 1993).

This technique has an advantage of being able to separate a large quantity of target cells from crude samples using magnetism in combination with conventional separation or identification methods to recover a pure sample of target cells. Compared to other methods, IMS has been found to be simple, fast and in a way, may be considered a sample enrichment stage prior to carrying out other methods (Safarık and Safarıkova´, 1999). Luk and Lindberg, (1991) described IMS to be a simple and powerful tool for quick and effective extraction of bacteria from clinical samples, including *M. tuberculosis* organisms. Further, IMS could potentially circumvent the need for chemical decontamination, by selectively capturing *M. bovis* cells and separating them from other bacteria in the samples, thereby retaining viability of *M. bovis* cells (Sterwart *et al*., 2013). In general, the magnetic separation procedure is gentle, facilitating the rapid handling of delicate cells from an unfriendly environment (Safarik and Safarıkova´, 1999). For the separation of prokaryotic cells, initial decontamination is sometimes not required. The method does not require expensive consumables or equipment.

The method works on two principles. The first one is the inherent magnetic properties contained by some cells which allows them to be attracted by a magnetic field of a magnetic separator. There are only two types of such cells in nature, namely red blood cells (erythrocytes) containing high concentrations of paramagnetic haemoglobin, and magnetotactic bacteria containing small magnetic particles within their cells (Safarik and Safarıkova´, 1999). Because it depends on inherent magnetic properties, all the cells either active, dormant or dead can be separated by this method. The second type involves tagging of

non-magnetic cells with a magnetic label in order to make them be attracted to a magnetic field when introduced in a crude nonmagnetic mixture. The cells which have acquired magnetic properties can be separated using a magnetic separator (Safarik and Safarıkova´, 1999). The isolation of bacteria from clinical specimens with specific monoclonal antibodies (MAbs) coupled to magnetic beads can generate samples with minimal nonspecific DNA contamination and reduced levels of factors inhibitory to PCR (Li *et al*., 1996). After magnetic separation, the pure harvested cells can then be identified using other laboratory tests such as culture, PCR, LAMP, ELISA and Chromatography (Safarik and Safarıkova´, 1999).

Many studies have been conducted successfully using IMS to separate different cells from crude samples. The Immunomagnetic capture technique was successfully adapted to extract cells of *Mycobacterium bovis* from naturally infected soil and badger faeces by Sweeney *et al*., (2006). Grant *et al*., (1998) also demonstrated the successful isolation of *M. paratuberculosis* from milk using immunomagnetic separation. In other studies, Apaire-Marchais *et al*., (2008) demonstrated the use of IMS to separate cells of Candida yeast species from artificially contaminated human blood using magnetic beads coated with monoclonal antibodies. IMS has also been described as an improved detection method for *M. bovis* from lymph node tissues (Stewart *et al*., 2013).

Data on the use of Immunomagnetic beads to detect MTC cells from biological samples such as milk from naturally infected animals is limited. Hence this study attempted to explore the use of Immunomagnetic beads technique in the detection of MTC cells in bovine milk.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Back ground of study areas

The study was conducted in Chisamba and Rufunsa districts of Central and Lusaka provinces of Zambia (Figure 2). Specifically in Mphango area under Chief Bundabunda of Chisamba and Itope area under Chief Chamuka of Rufunsa. Chisamba and Rufunsa were recently gazetted as districts in 2014 and data regarding population of animals and humans is limited. Cattle rearing is one of the most important livelihood activity of the local people as they depend on milk and meat as sources of protein and other macro and micronutrients. Most times, the milk is consumed directly from cattle without any form of treatment and at times directly from the udders of cows. They also earn a sizable income from selling milk and meat products in the nearby markets of Chongwe and Lusaka.

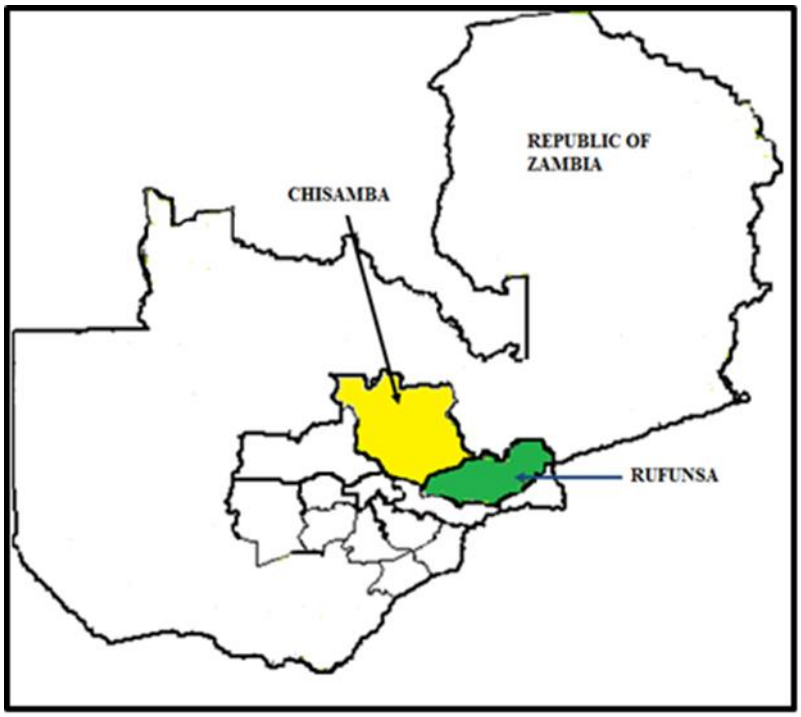

Figure 2: Map showing study areas of Chisamba and Rufunsa districts

Most of their animals are reared in a free range manner and it is a common practice for one farmer to own animals in more than one herd. Every after two weeks animals in the respective herds are allowed to gather at one central place for dipping. Such practices allow the spread and maintenance of TB among domestic animals.

The two areas were chosen because of the past history of total condemnation of the animals which were taken for slaughter to abattoirs due to bovine tuberculosis (farmer's group personal communication in 2013).

3.2. Study design

The study was done in two phases. The first phase was a field experiment in which the comparative intradermal tuberculin skin test was done on female lactating animals in the chosen project areas (Itope of Rufunsa and Mphango of Chisamba districts). The tuberculin test was conducted to provide a comparative basis with the assay under investigation (IMB-LAMP). The second phase was laboratory based in which milk samples from all animals sampled were assayed using IMB-LAMP, culture and m-PCR.

3.3. Sampling

The two stage cluster sampling method was used. The first stage involved purposively identifying villages with the history of TB in animals in both districts. The second stage involved randomly selecting animal herds to be included in the study within the identified villages.

The sample size (n) was calculated using the following formula as described by Lwanga and Lameshow, (1991).

$$n = \left(\frac{Z_{\alpha/2}}{E} \right)^2 * P(1-P)$$

Where Z is a standard normal critical value at 95% confidence level = 1.96, E the standard error and P is the anticipated proportion.

An assumption was made that P was not to exceed 20% based on the previous published data on the prevalence of TB in Zambia. A prevalence of 6.3% was reported by Muma *et al*., (2013) whereas Pandey *et al*., (2013) reported a much lower value of 2.6%. The anticipated precision was estimated between 15%-25% (5% within true value).

Based on this formula, the calculated sample size (n) was 246 animals but only 195 animals were sampled. This was due to the limitation on the number of animals available for sampling as most of them were dried due to scarcity of food, considering it was a dry season (September to November). Further, other herds had gone far to look for pasture in the bush where they could not be reached for weeks.

Out of the 195 animals that were tested with the comparative tuberculin test, milk was only collected from 161 animals because some farmers did not bring back their animals 72 hours later for the second tuberculin skin measurement.

3.4. Inclusion criteria

Only female lactating animals from traditional small scale farmers were included in the study.

3.5. Comparative intradermal tuberculin skin test

The comparative tuberculin skin test was done according to the OIE (2009) procedure. This involved shaving the left mid neck on two areas with the distance of 12 cm between them. After that, the skin fold thickness of the two shaved areas was measured with a venier calipers and recorded. A 0.2 ml volume of PPD-B was injected on the dorsal and 0.2ml of PPD-A was injected on the ventral shaved areas respectively.

The skin-fold thicknesses of the two shaved areas were re-measured 72 hours after injection. The tuberculin test was interpreted according to the guidelines provided by OIE (2009). Where the difference between the initial and final skin thickness of less than 2 mm was recorded, an animal was considered negative. Between 2 and 4 mm, the results were considered inconclusive or suspicious and above 4 mm positive. Milk was thereafter collected aseptically from each animal in falcon tubes and labelled correctly. The volume of milk collected was according to the production of each animal considering that indigenous cattle are not high producing animals. The collected milk samples were packed in a cooler box with ice and transported to the University of Zambia, school of Veterinary Medicine, Biosafety Laboratory level 3 (BSL-3) where they were stored in the refrigerator at -10ºC until analysis.

3.6. Immunomagnetic separation

One hundred and sixty one (161) milk samples from the animals that tested positive and negative on tuberculin test were subjected to IMC procedure. One millilitre (1 ml) of milk from each animal was mixed with 1ml of sodium hydroxide (NaOH) and incubated at room temperature for 2 minutes. Thereafter, an equal volume of TB beads solution (Microsens

Medtech ltd London, UK) was added to the mixture and incubated at room temperature (Figure 3).

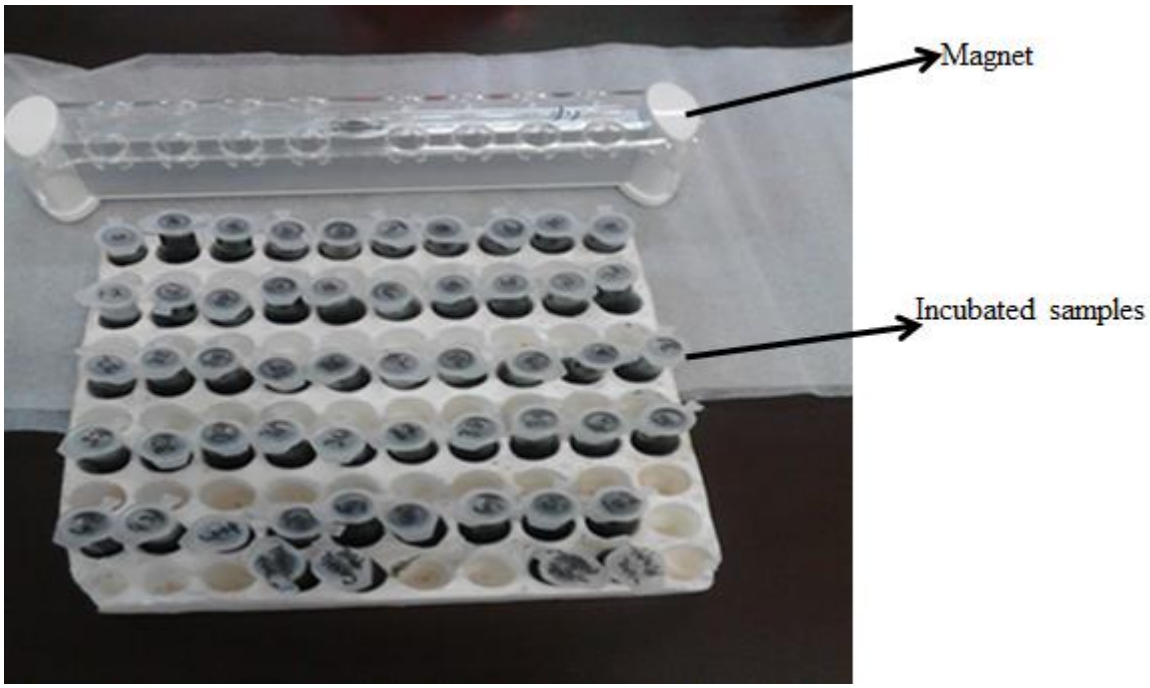

Figure 3: A magnet and incubated samples at room temperature

After 2 minutes of incubation, the beads were removed from the mixture using a specialized magnet (Figure 4), and the supernatant was decanted.

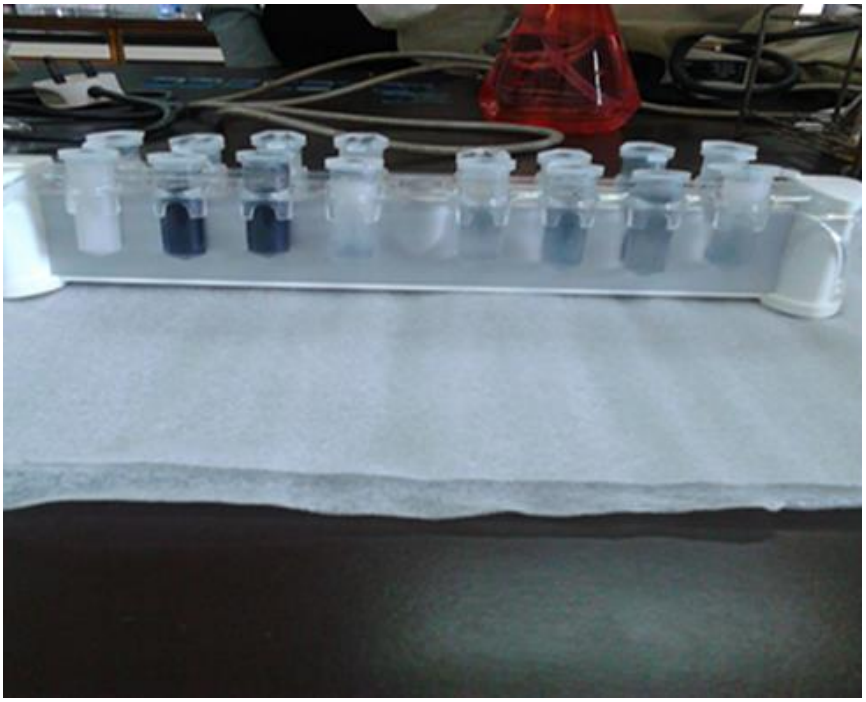

Figure 4: Immunomagnetic capture of MTC on beads by the magnet

After that, the beads containing MTC were washed using the TB beads wash solution and later centrifuged at 20,000 ×*g* for 10minutes. The supernatant was decanted and 150 µl elution buffer (Microsens Medtech ltd London, UK) was added to remove MTC cells from the beads. The mixture was centrifuged to obtain the supernatant containing MTC cells.

3.7. DNA extraction

The supernatant containing MTC cells was put into the Eppendorf tubes and subjected to DNA extraction using a heat block at 95 ºC for 60 minutes.

3.8. Loop mediated isothermal amplification (LAMP) method

The LAMP procedure that was used was the one described by Hang'ombe *et al*., (2011) with slight modifications. The LAMP reagents in each tube were 10 × LAMP buffer, 200mM Tris-HCl (pH 8.8), 100mM KCl, 100 mM $(NH_4)_2$ SO_4, 1% Tween 20, 10 mM dNTPs (2.5mM each), 5M betane, 100mM $MgSO_4$, DDW (distilled water), Bst DNA polymerase (8 unit/ µl,

New England Biolabs), Primer mix (Six primer mixture) (Table 2) and Fluorescent reagent (Eiken Co.)

The LAMP mixture for the positive control was 2 µl of known DNA, 1 µl of Bst DNA polymerase and 22 µl of TB-LAMP premix. For the negative control, an equal volume of distilled water was used in place of the known DNA. Two microliter (2 µl) of extracted DNA was added to 22 µl of TB-LAMP premix and 1 µl of Bst DNA polymerase. Two microliter (2 µl) of the extracted DNA (sample) was mixed with 1 µl of Bst DNA polymerase and 22 µl of TB-LAMP premix. The mixtures for the samples, positive and negative controls were put in tubes containing LAMP reagents. The tubes were then incubated in the water bath at 64ºC for 60 minutes. The results were obtained by observing the reactions under UV lamp. The positive reaction showed a stronger intensity compared to the negative reaction and these were compared to the positive and negative controls respectively.

Table 2: Primers used for LAMP reaction

Name of primer	Sequence
FIP	CACCCACGTGTTACTCATGCAAGTCGAACGGAAAGGTCT
BIP	TCGGGATAAGCCTGGACCACAAGACATGCATCCCGT
F3	CTGGCTCAGGACGAACG
B3	GCTCATCCCACACCGC
FLP	GTTCGCCACTCGAGTATCTCCG
BLP	GAAACTGGGTCTAATACCGG

3.9. Culture

Twenty four samples of milk that tested positive on LAMP were cultured on Lowenstein-Jensen (L-J) medium as described by Pandey *et al*., (2013) with slight modifications. One millilitre (1ml) aliquots of the sediments from each milk sample were spread on the surface of each of the LJ medium slants with glycerol and pyruvate. Cultures were incubated aerobically at 37ºC for 8 weeks and observed weekly for signs of growth. The bacteria that showed small, moist, creamy to yellowish smooth flat colonies were identified to be *Mycobacterium*.

3.10. DNA extraction from cultured samples

The suspected isolates were then subjected to DNA extraction using DNAZol (Invitrogen, Carlsbad, CA, USA) and the extracted DNA was subjected to multiplex PCR for species identification. About 500 µl of DNAZol was put into the Eppendorf tubes followed by the addition of the colonies removed from the L-J media. The mixture was allowed to stand for about 5-10 minutes and then subjected to active DNA microsmash at 4000 $\times g$ for 1 minute. The resulting mixture was removed from the microsmash and centrifuged at 15000 $\times g$ for 10 minutes. The supernatant was transferred to clean Eppendorf tubes to which 1 ml of absolute alcohol was added. The mixture was then centrifuged at 15000 $\times g$ for 10 minutes and the supernatant decanted. Further, 70% alcohol was added to the mixture and centrifuged at 3000 $\times g$ for 10 minutes and supernatant discarded. The dry tubes were put up side down for three minutes and later eluted with TE buffer.

3.11. Multiplex PCR

The extracted DNA was subjected to m-PCR. The PCR procedure was done according to the procedure described by Hang'ombe *et al*., (2011). Extracted DNA from *Mycobacterium*

bacterial cultures was used as a template. Primer pairs for cfp32 (a specific gene for MTC), RD9 (region of difference 9 seen only in *M. tuberculosis* and *M. canettii*), and RD12 (region of difference 12 deleted in *M. bovis*, *M. caprae*, and *M. canettii*) were used by Nakajima *et al*., (2010) as shown in table 3.

Table 3: Primers used for multiplex PCR

Target locus	Primer name	Primer sequence	Location	Size (bp)
cfp32	Rv0577F	5' ATGCCCAAGAGAAGCGAATACAGGCAA 3'	671166-192	786
	Rv0577R	5' CTATTGCTGCGGTGCGGGCTTCAA 3'	671951-928	
RD9	Rv2073cF	5' TCGCCGCTGCCAGATGAGTC 3'	2330579-598	600
	Rv2073cR	5' TTTGGGAGCCGCCGGTGGTGATGA 3'	2331173-150	
RD12	Rv3120F	5'GTCGGCGATAGACCATGAGTCCGTCTCCAT3'	3485558-587	404
	Rv3120R	5' GCGAAAAGTGGGCGGATGCCAG 3'	3485961-940	

The general PCR recipe contained 7.4 µl H2O, 2 µl 10 x Taq buffer, 2 µl dNTPs (2.5 mM each), 0.2 µl Taq (Takara, Japan), 1 µl target DNA, 2.2 µl of 10 µM cfp32 primers, 0.7 µl of 5 µM RD9 primers, and 0.8 µl of 5 µM RD12 primers. Appropriate negative controls consisting of PCR mix without target DNA were included. The PCR was performed using the following program: denaturation for 1 min at 98ºC followed by 35 cycles of 5 sec at 98ºC, 20 sec at 58ºC, and 1 min at 68ºC with final extension for 5 min at 72ºC in a thermalcycler (iCycler, Bio-Rad Laboratories Inc., CA, USA). All PCR products were identified by gel electrophoresis on a 2.0% agarose gel and were visualized by ethidium bromide staining.

3.12. Data analysis

Descriptive statistical data was analysed using S-PLUS Windows professional 2001. The other non-numerical data was analysed based on the generated laboratory results.

CHAPTER FOUR

RESULTS

4.1. Comparative intradermal tuberculin skin test

A total of 161 cattle were tested using the comparative intradermal tuberculin skin test. Out of the 161 cattle, 114 (70.8%) were from Chisamba district in Mphango area and 47 (29.2%) were from Rufunsa district in Itope area. The specific details on the comparative intradermal tuberculin results are indicated in Appendices 1 and 2. From the two districts, 4 (2.5%, 95% CI=0.1%-4.9%) tested positive on the comparative intradermal tuberculin skin test, 12 (7.5%) were provisionally considered suspects or inconclusive and 145 (90.1%) tested negative.

Figure 5 summarises the results of the comparative intradermal tuberculin skin test for the animals investigated in the Mphango area of Chisamba district. Out of the 114 animals tested, (2.60%, 95% CI=0%-5.5%) were positive, 7.0% showed inconclusive results or were provisionally considered as suspects and 90.40% were negative.

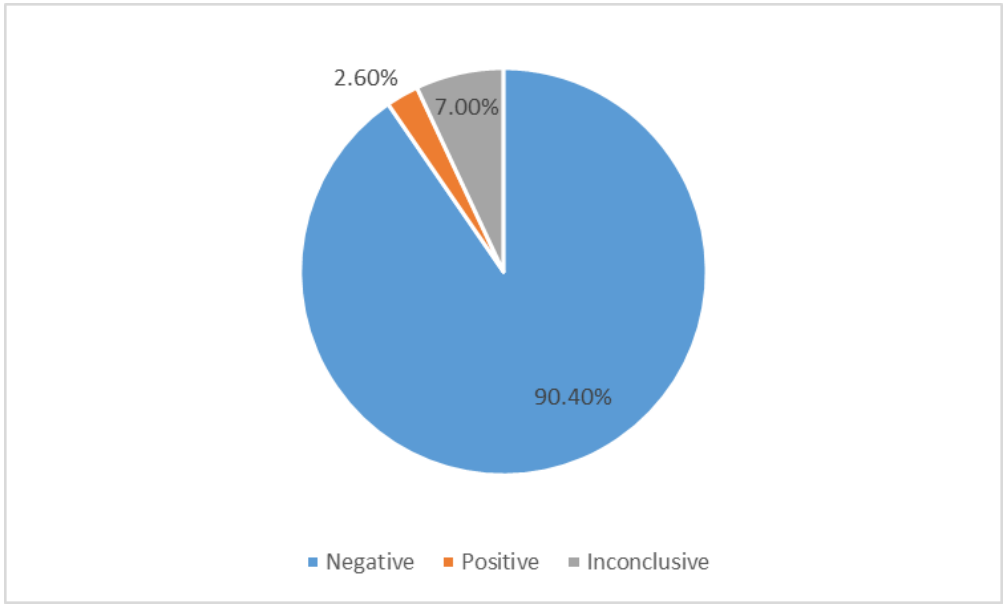

Figure 5: Comparative intradermal tuberculin skin test results for Chisamba district

The results of the comparative intradermal tuberculin skin test for the animals of Itope area of Rufunsa district are summarized in Figure 6. Out of the 47 animals that were investigated, (2.1%, 95% CI=0%-6.2%) tested positive, 8.5% were inconclusive and 89.4% tested negative.

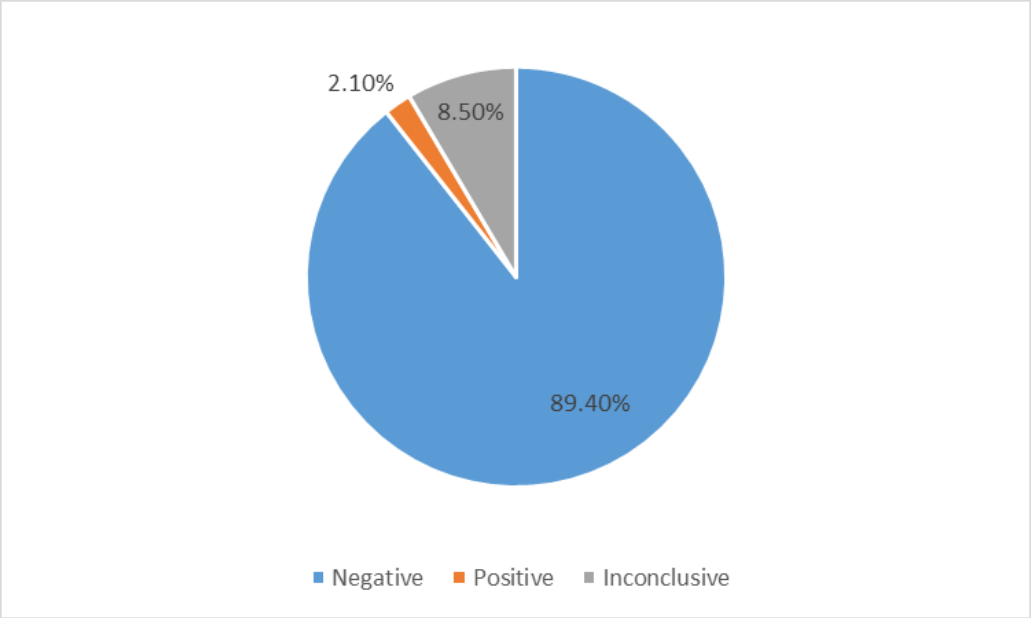

Figure 6: Comparative intradermal tuberculin skin test results for Rufunsa district

4.2. Immunomagnetic beads and LAMP

Milk samples from the 161 animals that were tested with the comparative intradermal tuberculin skin test were subjected to Immunomagnetic capture technique and later LAMP. From the 161 milk samples collected from the two districts, 24 (14.9%, 95% CI =9.4%-20.4%) tested positive and 137 (85.1%) tested negative.

In Chisamba district, (16.7%, 95% CI=9.9%-23.6%) of animals were found TB positive and 83.3% negative (Figure 7).

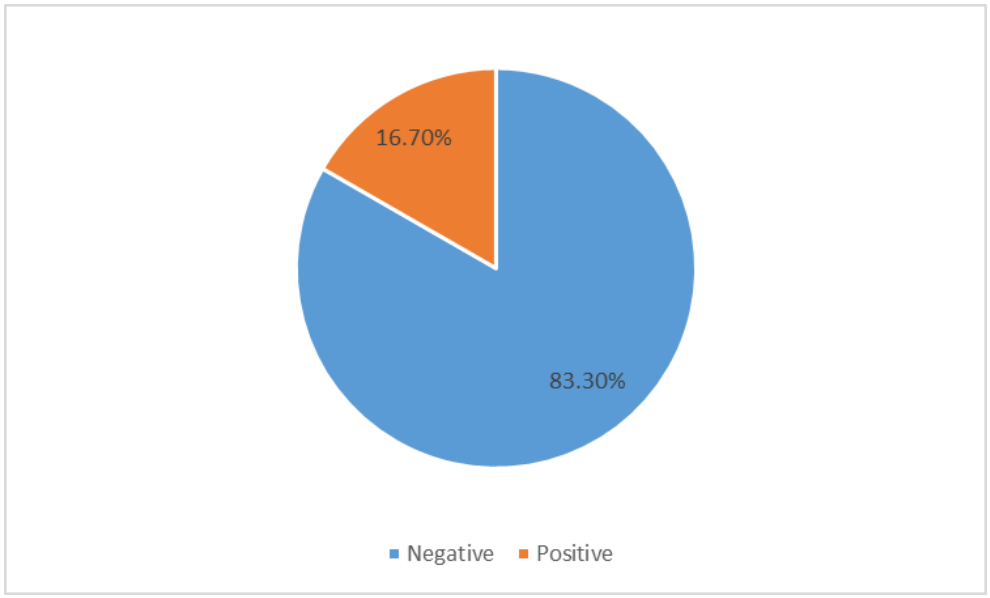

Figure 7: IMB-LAMP results for milk samples from Chisamba district

The IMB – LAMP results for milk samples from Rufunsa district are summarized in Figure 8. From the milk samples collected, (10.6%, 95 % CI=1.8%-19.4%) were positive and 89.4% negative (figure 8).

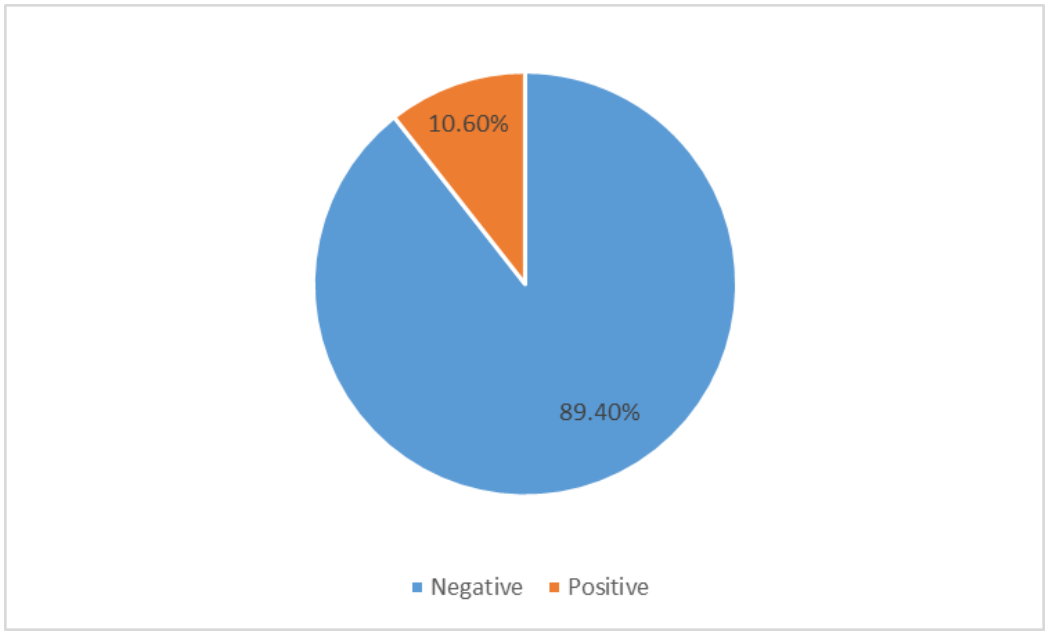

Figure 8: IMB-LAMP results for milk samples from Rufunsa district

The IMB – LAMP results were interpreted using the UV illuminator. A positive result showed stronger intensity than a negative one (see examples in Figure 9).

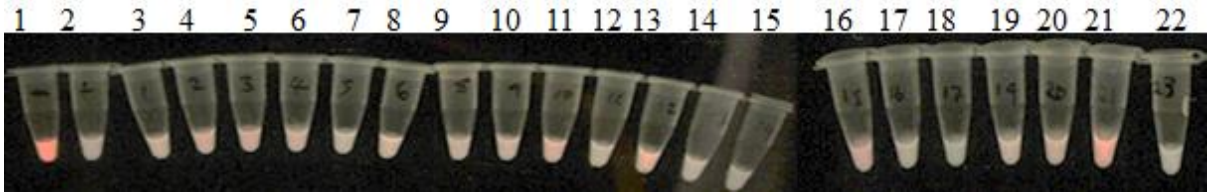

Figure 9: LAMP results of tested milk samples. 1: Negative and 2 positive controls, with the other tubes indicating sample results.

4.3. Comparison of positive and negative results of comparative tuberculin skin test and IMB-LAMP

A summary of positive, negative and inconclusive results for the two tests namely Comparative intradermal tuberculin skin test and IMB-LAMP are shown in table 3. Out of the 12 animals that showed inconclusive on the comparative intradermal tuberculin test, 4 (33.3%) were negative on IMB-LAMP, and, 8 (66.7%, 95% CI=40.0%-93.4%) were positive. All the 4 animals that were positive on the tuberculin test were also positive on IMB-LAMP. From the 145 animals that were tuberculin skin test negative, 133 (91.7%) were also IMB-LAMP negative while 12 (8.3%, 95% CI=3.8%-12.8%) came out positive for tuberculosis on IMB-LAMP.

Table 4: Summary of results on tuberculin skin test and Immunomagnetic beads-LAMP

Type of test	Number of animals	Negatives	Positives	Inconclusive	Proportion of positives	CI 95%
Comparative intradermal tuberculin skin test	161	145	4	12	2.5%	0.1%-4.9%
IMB-LAMP	161	137	24	0	14.9%	9.4%-20.4%

4.4. Culture

Twenty four milk samples that tested positive on IMB-LAMP were cultured on L-J media (Figure 10). Out of the 24 milk samples, growth of *Mycobacterium* was only observed in 4 (16.7%, 95% CI=1.8%-31.6%). The rest 20 (83.3%) did not grow and were considered negative.

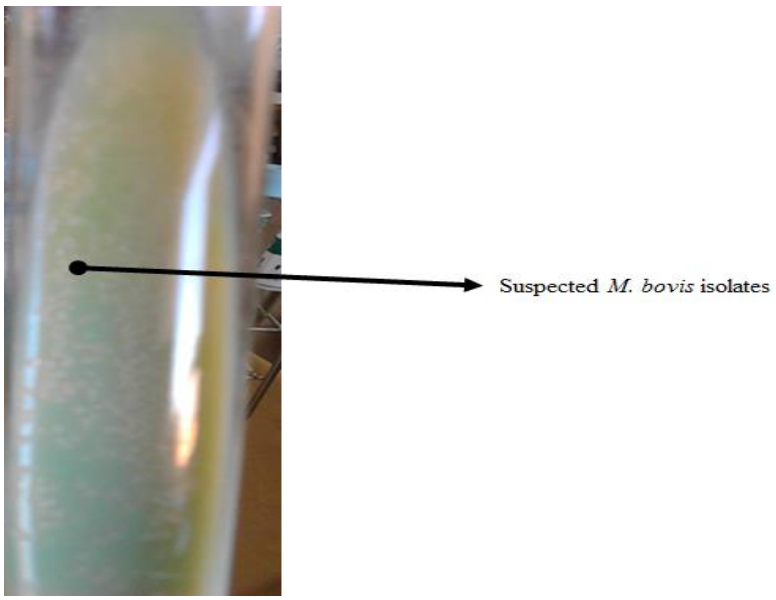

Figure 10: Suspected Mycobacterial cultures on L-J media

Multiplex PCR

The milk samples that showed mycobacterial growth on L-J media were subjected to m-PCR for species identification. The selected samples analysed by m-PCR showed amplifications of region of difference (cfp 32) typical of *M. bovis* (Figure 11).

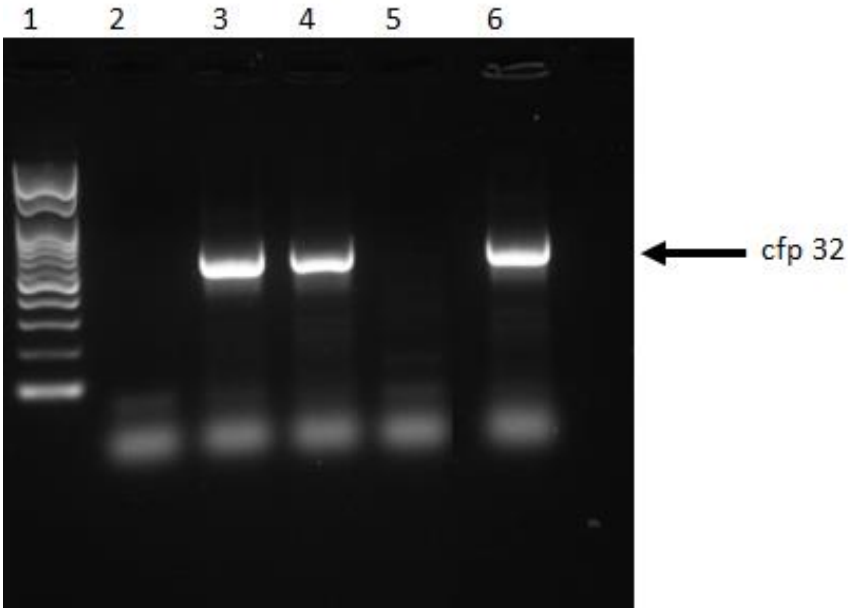

Figure 11: Typical Multiplex PCR banding Patterns of mycobacterial strains. Lanes: 1,100bp ladder; 2, Negative control; 3, Positive control with the other bands indicating sample results.

CHAPTER FIVE

DISCUSSION

5.1. Significance of the results obtained

From the experiments that were carried out (IMB-LAMP) and culture, *Mycobacterium* DNA and cells respectively were isolated from milk. IMB was successfully adapted to extract cells of MTC in milk from naturally infected animals. This is the first study to demonstrate the use of this technique to isolate the bacteria directly from milk in naturally infected animals in Zambia.

In other studies elsewhere, Sweeney *et al*., (2006) demonstrated its use in capturing *Mycobacterium* from environmental samples where the badgers live. Stewart *et al*., (2013) demonstrated the applicability of the technique in the isolation of *Mycobacterium* from lymph node tissues. In the study by Grant *et al*., (1998), isolation of non-tuberculous *Mycobacterium* was achieved using the same test. A review by Strain *et al*., (2011b), described IMS as a promising technique that might be a viable alternative to tissue culture.

This study has demonstrated that the IMB technique is a very fast and efficient way of recovering both viable and nonviable *Mycobacterium* from the crude samples. The test utilises the equipment that is not complicated. Further, the test does not require harsh chemical decontamination which might be harmful to the bacterial cells. Corner *et al*., (1995) reported that, while the chemical agents are effective in controlling contamination, they are to some extent toxic to *M. bovis* and a sizable proportion of this bacteria may be lost in the process.

From the results obtained, it is clearly evident that the sensitivity of IMB in combination with LAMP is considerably higher than for the other two techniques that were used in this study. IMB-LAMP was able to detect more positives than the tuberculin and culture techniques. Evidently, 24/161 (14.9%, 95% CI=9.4%-20.4%) tested positive on IMB-LAMP compared to 4/161 (2.5%, 95% CI=0.1%-4.9%) that tested positive on tuberculin and 4 (16.7%, 95% CI=1.8%-31.6%) on culture respectively.

The direct detection of *M. bovis* using culture is generally regarded as the most sensitive and therefore the 'gold standard' for determining TB infection status (Murphy *et al*., 2010). However, the sensitivity of the tissue culture method is greatly influenced by a number of factors. Strain *et al*., (2011a) described TB as a disease where the 'Gold standard' test is deficient. According to Strain *et al*., (2011b), factors that may influence the culture method include, the number of tissues examined, the aseptic methods used to take tissues, the processing of tissues prior to culture and the methods and materials used for culture including the number and type of media used and the length of incubation time. *M. bovis* is a slow growing organism which might be out grown by faster growing non mycobacterial species making the identification of *M. bovis* in samples impossible (Strain *et al*., 2011b).

From the 24 samples that were initially detected as positives on IMB-LAMP, only 4 (16.7%, 95% CI=1.8%-31.6%) were able to grow on the media after culturing. From this finding, it is therefore very reasonable to assume that some milk samples most likely, contained *Mycobacterium* which was not sufficient enough to grow on media and thus could not compete with other opportunistic microorganisms. This could also be an indication that some *Mycobacterium* were not viable enough to grow on media. These data therefore justifies the suitability of the IMB-LAMP technique considering it was able to detect the nonviable or

dormant *Mycobacterium* cells that were undetected by the culture method probably due to low concentration of *Mycobacterium* present in the milk samples. In other studies, Gallagher *et al*., (1998) noted that if the number of *M. bovis* present in tissues are in very small numbers, (1 to 10^5 cfu per gram of tissue), the growth on media may be affected. In a review by Strain *et al*., (2011b) it is documented that this lack of growth or the number of viable bacteria may further be compounded by chemical decontamination which is normally done prior to culture to prevent overgrowth of contaminating microorganisms. Figueiredo *et al*., (2012) also reported about lack of growth of some *M.bovis* on media due to the chemical decontamination that they used. Many researchers have clearly indicated that the culture method may not be completely relied upon when the knowledge of the presence of TB in the animals is in question. It is important that bacteriology is supported by a recognised confirmatory step (Strain *et al*., 2011b). The use of a molecular confirmation step such as spoligotyping may improve the specificity of the culture to as high as (>99.99%) (Skuce *et al*., 2005). Figueiredo *et al*., (2012) also described the use of High-Performance Liquid Chromatography (HPLC) and m-PCR to improve the diagnosis and control of BTB considering that some samples did not grow on culture due to the decontamination procedure. In this study, *M. bovis* was identified and confirmed by the results of the m-PCR, which targets three genetic regions that are cfp32, RD9 and RD12.

It is very intriguing to note that the IMB-LAMP findings provided a promising window of complementarity to the tuberculin skin test. The IMB-LAMP technique was clearly able to isolate the bacteria DNA from 12 (8.3%) (95% CI=3.8%-12.8%) out of the 145 animals that tested negative to the tuberculin skin test. This finding is in agreement with Zarden *et al*., (2013) who were able to isolate the bacteria from animals that tested negative on tuberculin. The findings from this study therefore indicate that the tuberculin test most likely gave false

negative results. This observation is in agreement with other previously published reports (Neill *et al*., 1994; Praud *et al*., 2014).

It is also very interesting to note that out of the 12 animals that gave inconclusive results or were provisionally considered as suspects on the tuberculin skin test, 8 (66.7%) (95% CI=40.0%-93.4%) of these animals came out positive on IMB-LAMP technique. This significant finding suggests that the IMB can be used to test animals with inconclusive results on tuberculin skin test immediately instead of waiting for three months to retest the animals. Considering that humans are in constant contact with their animals and, animals are usually housed together and mingle during feeding and drinking of water, the period of waiting can pose a risk to other animals and humans especially if the animal has the disease.

5.2. Public health significance of the results

Evidence from the positive culture results indicated that the bacteria remains viable in milk and, can therefore can be a vehicle of transmission to humans. The isolation of bacteria from milk further signifies its public health importance and this calls for serious control measures to be put in place in order to control BTB.

In Zambia and other developing countries, tuberculosis in humans is controlled using the BCG vaccination which is given to babies in their first two weeks of life. This preventive measure cannot be fully achieved if the disease in animals is neglected given that it is zoonotic and no known vaccination is available in animals. Malama *et al*., (2014) reported isolating a strain of *M. bovis* in animals that was similar to the one isolated from a TB human patient in Namwala district of Zambia.

The viability of the bacteria in milk is also a very important finding. Though some cells could not grow on media, probably due to low cultural detection levels, there is still need to take a precautionary approach. Gallagher *et al*., (1998) noted that if the number of organisms are below the limits of cultural detection, no bacteria can be seen on growth media. However, this does not indicate that the *Mycobacterium* cannot infect man if it is below the cultural detection limit since the culture method is affected by a number of factors for bacteria to grow. According to O'Reilly and Daborn (1995), the infectious dose of *M. bovis* to humans is unknown.

Most of the sampled animals showed subclinical cases of TB. It is therefore very difficult for a farmer to tell if the animals are sick or not. The practices of drinking milk directly from the udders of animals and that of unpasteurized one by the local people in the study areas put them at a higher risk of contracting TB from diseased lactating animals. The practice of drinking milk directly from the udders of animals has been reported before by Pandey *et al*., (2013). In a study by Sweeney *et al*., (2006), the bacteria were isolated from environmental samples, suggesting that, people living in close contact with tuberculous animals are at risk of contracting the disease. The use of unpasteurised milk in the manufacturing of dairy products also put people at a higher risk of contracting BTB. The presence of viable bacteria in sour milk has been reported previously (Sitima *et al*., 1997). In the trace back of 35 infection cases linked to *M. Bovis* in New York City, CDC, (2005) discovered that most of these cases were associated with the consumption of cheese made from non-pasteurized milk imported from an endemic area of BTB.

CHAPTER SIX

CONCLUSIONS

1. The study has revealed that immunomagnetic beads can be used to capture MTC from milk of naturally infected animals.

2. *Mycobacterium* can be isolated from milk samples of animals certified negative by the intradermal tuberculin skin test.

3. The study also demonstrated that in order to carry out proper and reliable diagnosis of bovine tuberculosis, a multi-technique approach is required.

4. The isolation of viable *Mycobacterium* from milk suggests that, milk can act as a source of infection of zoonotic TB to humans in Zambia.

CHAPTER SEVEN

RECOMMENDATIONS

The findings from this study have provided an intriguing perspective that might stimulate the interest of other researchers in exploring quick diagnostic techniques for *Mycobacterium*. Based on the present study, the following recommendations are made;

1. The use of IMB-LAMP as a complementary diagnostic test for detecting *Mycobacterium bovis* besides the intradermal tuberculin skin test and the culture method.

2. That milk be considered as a diagnostic specimen for tuberculosis in animals.

3. Further studies to establish the minimum number of mycobacterial cells that can be captured by the beads in milk samples.

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APPENDICES

Appendix 1: Results of the comparative intradermal tuberculin skin test of animals from Chisamba district

Animal I.D	Initial skin thickness (mm)		Final skin thickness (mm)		Difference in thickness (mm)	Results interpretation
8	*Bovine PPD*	7.04	*Bovine PPD*	7.28	0.24	Negative
	Avian PPD	9.25	*Avian PPD*	9.22	-0.03	
22	*Bovine PPD*	5.88	*Bovine PPD*	8.27	2.39	Suspect
	Avian PPD	5.77	*Avian PPD*	6.24	0.47	
75	*Bovine PPD*	5.99	*Bovine PPD*	8.39	2.4	Suspect
	Avian PPD	7.04	*Avian PPD*	8.24	1.2	
2	*Bovine PPD*	6.26	*Bovine PPD*	7.13	0.87	Negative
	Avian PPD	7.20	*Avian PPD*	7.24	0.04	
11	*Bovine PPD*	6.31	*Bovine PPD*	7.16	0.85	Negative
	Avian PPD	7.37	*Avian PPD*	8.71	1.34	
20	*Bovine PPD*	8.41	*Bovine PPD*	10.21	1.8	Negative
	Avian PPD	8.40	*Avian PPD*	8.70	0.3	
9	*Bovine PPD*	10.92	*Bovine PPD*	11.3	0.38	Negative
	Avian PPD	8.88	*Avian PPD*	9.8	0.92	
17	*Bovine PPD*	8.36	*Bovine PPD*	8.22	-0.14	Negative
	Avian PPD	8.94	*Avian PPD*	9.89	0.95	
14	*Bovine PPD*	5.19	*Bovine PPD*	6.34	1.15	Negative
	Avian PPD	6.27	*Avian PPD*	6.45	0.18	
18	*Bovine PPD*	6.69	*Bovine PPD*	11.63	4.94	Positive
	Avian PPD	9.81	*Avian PPD*	14.61	4.8	
3	*Bovine PPD*	6.72	*Bovine PPD*	7.85	1.13	Negative
	Avian PPD	5.43	*Avian PPD*	6.11	0.68	

Animal I.D	Initial skin thickness (mm)		Final skin thickness (mm)		Difference in thickness (mm)	Results interpretation
5	*Bovine PPD*	5.85	*Bovine PPD*	7.01	1.16	Negative
	Avian PPD	7.09	*Avian PPD*	8.19	1.10	
1	*Bovine PPD*	7.43	*Bovine PPD*	8.30	0.87	Negative
	Avian PPD	7.43	*Avian PPD*	8.36	0.93	
19	*Bovine PPD*	5.98	*Bovine PPD*	7.97	1.99	Negative
	Avian PPD	6.24	*Avian PPD*	9.28	3.04	
24	*Bovine PPD*	7.36	*Bovine PPD*	8.96	1.6	Negative
	Avian PPD	5.16	*Avian PPD*	9.10	3.94	
23	*Bovine PPD*	9.32	*Bovine PPD*	10.2	0.88	Negative
	Avian PPD	7.79	*Avian PPD*	9.84	2.05	
15	*Bovine PPD*	8.96	*Bovine PPD*	8.61	-0.35	Negative
	Avian PPD	7.61	*Avian PPD*	10.54	2.93	
12	*Bovine PPD*	7.67	*Bovine PPD*	9.57	1.90	Negative
	Avian PPD	8.96	*Avian PPD*	10.56	1.6	
6	*Bovine PPD*	6.89	*Bovine PPD*	7.12	0.23	Negative
	Avian PPD	8.55	*Avian PPD*	11.81	3.26	
16	*Bovine PPD*	9.87	*Bovine PPD*	11.03	1.16	Negative
	Avian PPD	9.98	*Avian PPD*	10.58	0.6	
7	*Bovine PPD*	7.25	*Bovine PPD*	9.55	2.3	Suspect
	Avian PPD	8.35	*Avian PPD*	8.24	-0.11	
64	*Bovine PPD*	4.23	*Bovine PPD*	9.29	5.06	Positive
	Avian PPD	9.75	*Avian PPD*	12.33	2.58	
10	*Bovine PPD*	8.05	*Bovine PPD*	9.61	1.56	Negative
	Avian PPD	7.24	*Avian PPD*	7.37	0.13	
26	*Bovine PPD*	6.16	*Bovine PPD*	6.91	0.75	Negative
	Avian PPD	5.48	*Avian PPD*	8.99	3.51	
4	*Bovine PPD*	6.59	*Bovine PPD*	7.88	1.29	Negative

Animal I.D	Initial skin thickness (mm)		Final skin thickness (mm)		Difference in thickness (mm)	Results interpretation
	Avian PPD	9.32	*Avian PPD*	9.39	0.07	
21	*Bovine PPD*	9.11	*Bovine PPD*	10.21	1.1	Negative
	Avian PPD	12.80	*Avian PPD*	13.88	1.08	
27	*Bovine PPD*	8.69	*Bovine PPD*	8.62	-0.07	Negative
	Avian PPD	9.84	*Avian PPD*	12.50	2.66	
13	*Bovine PPD*	6.00	*Bovine PPD*	7.07	1.07	Negative
	Avian PPD	8.01	*Avian PPD*	8.23	0.22	
112	*Bovine PPD*	6.44	*Bovine PPD*	7.33	0.89	Negative
	Avian PPD	7.04	*Avian PPD*	6.89	-0.15	
114	*Bovine PPD*	5.65	*Bovine PPD*	5.63	-0.02	Negative
	Avian PPD	5.85	*Avian PPD*	5.64	-0.21	
159	*Bovine PPD*	6.20	*Bovine PPD*	6.05	-0.15	Negative
	Avian PPD	5.95	*Avian PPD*	5.89	-0.06	
149	*Bovine PPD*	8.29	*Bovine PPD*	7.72	-0.57	Negative
	Avian PPD	9.54	*Avian PPD*	9.92	0.38	
158	*Bovine PPD*	5.52	*Bovine PPD*	6.02	0.5	Negative
	Avian PPD	6.78	*Avian PPD*	7.35	0.57	
154	*Bovine PPD*	6.81	*Bovine PPD*	5.80	-1.01	Negative
	Avian PPD	8.30	*Avian PPD*	7.56	-0.74	
161	*Bovine PPD*	7.64	*Bovine PPD*	7.79	0.15	Negative
	Avian PPD	7.56	*Avian PPD*	7.78	0.22	
152	*Bovine PPD*	6.80	*Bovine PPD*	7.64	0.84	Negative
	Avian PPD	7.60	*Avian PPD*	9.09	1.49	
148	*Bovine PPD*	5.12	*Bovine PPD*	4.61	-0.51	Negative
	Avian PPD	7.00	*Avian PPD*	6.78	-0.22	
160	*Bovine PPD*	7.48	*Bovine PPD*	6.98	-0.5	Negative
	Avian PPD	8.48	*Avian PPD*	6.59	-1.89	

Animal I.D	Initial skin thickness (mm)		Final skin thickness (mm)		Difference in thickness (mm)	Results interpretation
153	*Bovine PPD*	6.67	*Bovine PPD*	7.33	0.66	Negative
	Avian PPD	9.50	*Avian PPD*	11.53	2.03	
156	*Bovine PPD*	5.74	*Bovine PPD*	6.91	1.17	Negative
	Avian PPD	6.47	*Avian PPD*	6.51	0.04	
157	*Bovine PPD*	4.92	*Bovine PPD*	5.15	0.23	Negative
	Avian PPD	5.92	*Avian PPD*	6.97	1.05	
155	*Bovine PPD*	7.01	*Bovine PPD*	7.37	0.36	Negative
	Avian PPD	6.46	*Avian PPD*	7.17	0.71	
151	*Bovine PPD*	5.27	*Bovine PPD*	5.55	0.28	Negative
	Avian PPD	5.14	*Avian PPD*	5.19	0.05	
150	*Bovine PPD*	6.72	*Bovine PPD*	6.47	-0.25	Negative
	Avian PPD	8.31	*Avian PPD*	8.69	0.38	
54	*Bovine PPD*	7.45	*Bovine PPD*	8.07	0.62	Negative
	Avian PPD	8.70	*Avian PPD*	8.08	-0.62	
58	*Bovine PPD*	8.92	*Bovine PPD*	8.68	-0.24	Negative
	Avian PPD	10.60	*Avian PPD*	11.53	0.93	
73	*Bovine PPD*	8.62	*Bovine PPD*	10.33	1.71	Negative
	Avian PPD	8.30	*Avian PPD*	8.64	0.34	
70	*Bovine PPD*	7.66	*Bovine PPD*	7.92	0.26	Negative
	Avian PPD	9.99	*Avian PPD*	11.25	1.26	
47	*Bovine PPD*	6.00	*Bovine PPD*	8.38	2.38	Suspect
	Avian PPD	6.30	*Avian PPD*	6.75	0.45	
63	*Bovine PPD*	7.87	*Bovine PPD*	8.79	0.92	Negative
	Avian PPD	8.90	*Avian PPD*	8.74	-0.16	
57	*Bovine PPD*	5.90	*Bovine PPD*	8.34	2.44	Suspect
	Avian PPD	6.02	*Avian PPD*	6.64	0.62	
62	*Bovine PPD*	5.21	*Bovine PPD*	6.27	1.06	Negative

Animal I.D	Initial skin thickness (mm)		Final skin thickness (mm)		Difference in thickness (mm)	Results interpretation
45	*Avian PPD*	4.65	*Avian PPD*	5.37	0.72	
	Bovine PPD	7.33	*Bovine PPD*	7.72	0.39	Negative
	Avian PPD	8.66	*Avian PPD*	8.74	0.08	
61	*Bovine PPD*	5.31	*Bovine PPD*	7.30	1.99	Negative
	Avian PPD	8.49	*Avian PPD*	9.60	1.11	
65	*Bovine PPD*	6.44	*Bovine PPD*	10.57	4.13	Positive
	Avian PPD	10.03	*Avian PPD*	12.14	2.11	
46	*Bovine PPD*	8.85	*Bovine PPD*	8.92	0.07	Negative
	Avian PPD	11.69	*Avian PPD*	11.39	-0.3	
53	*Bovine PPD*	7.16	*Bovine PPD*	8.69	1.53	Negative
	Avian PPD	9.55	*Avian PPD*	9.95	0.4	
50	*Bovine PPD*	8.21	*Bovine PPD*	7.92	-0.29	Negative
	Avian PPD	10.78	*Avian PPD*	10.94	0.16	
72	*Bovine PPD*	4.76	*Bovine PPD*	6.05	1.29	Negative
	Avian PPD	5.02	*Avian PPD*	5.97	0.95	
48	*Bovine PPD*	7.24	*Bovine PPD*	7.53	0.29	Negative
	Avian PPD	6.82	*Avian PPD*	7.10	0.28	
59	*Bovine PPD*	6.06	*Bovine PPD*	8.87	2.81	Suspect
	Avian PPD	6.97	*Avian PPD*	7.01	0.04	
74	*Bovine PPD*	8.35	*Bovine PPD*	10.02	1.67	Negative
	Avian PPD	9.81	*Avian PPD*	9.89	0.08	
55	*Bovine PPD*	6.66	*Bovine PPD*	6.35	-0.31	Negative
	Avian PPD	7.12	*Avian PPD*	8.29	1.17	
60	*Bovine PPD*	7.56	*Bovine PPD*	8.25	0.69	Negative
	Avian PPD	10.41	*Avian PPD*	11.39	0.98	
67	*Bovine PPD*	5.37	*Bovine PPD*	6.96	1.59	Negative
	Avian PPD	8.70	*Avian PPD*	12.65	3.95	

Animal I.D	Initial skin thickness (mm)		Final skin thickness (mm)		Difference in thickness (mm)	Results interpretation
56	*Bovine PPD*	6.66	*Bovine PPD*	8.34	1.68	Negative
	Avian PPD	8.34	*Avian PPD*	7.96	-0.38	
66	*Bovine PPD*	6.22	*Bovine PPD*	6.35	0.13	Negative
	Avian PPD	6.95	*Avian PPD*	6.62	-0.33	
51	*Bovine PPD*	6.86	*Bovine PPD*	8.46	1.6	Negative
	Avian PPD	6.75	*Avian PPD*	7.66	0.91	
68	*Bovine PPD*	6.51	*Bovine PPD*	6.70	0.19	Negative
	Avian PPD	7.84	*Avian PPD*	8.30	0.46	
49	*Bovine PPD*	7.75	*Bovine PPD*	7.53	-0.22	Negative
	Avian PPD	8.27	*Avian PPD*	9.29	1.02	
137	*Bovine PPD*	6.71	*Bovine PPD*	7.85	1.14	Negative
	Avian PPD	5.68	*Avian PPD*	6.65	0.97	
139	*Bovine PPD*	5.90	*Bovine PPD*	6.17	0.27	Negative
	Avian PPD	5.55	*Avian PPD*	7.18	1.63	
143	*Bovine PPD*	7.83	*Bovine PPD*	7.27	-0.56	Negative
	Avian PPD	9.33	*Avian PPD*	10.33	1	
146	*Bovine PPD*	7.84	*Bovine PPD*	8.02	0.18	Negative
	Avian PPD	8.37	*Avian PPD*	7.89	-0.48	
145	*Bovine PPD*	8.59	*Bovine PPD*	9.14	0.55	Negative
	Avian PPD	10.05	*Avian PPD*	12.12	2.07	
130	*Bovine PPD*	5.35	*Bovine PPD*	5.57	0.22	Negative
	Avian PPD	5.20	*Avian PPD*	5.68	0.48	
132	*Bovine PPD*	6.30	*Bovine PPD*	7.01	0.71	Negative
	Avian PPD	7.55	*Avian PPD*	8.75	1.20	
140	*Bovine PPD*	5.79	*Bovine PPD*	5.74	-0.55	Negative
	Avian PPD	6.48	*Avian PPD*	4.93	-1.55	
129	*Bovine PPD*	6.83	*Bovine PPD*	8.18	1.35	Negative

Animal I.D	Initial skin thickness (mm)		Final skin thickness (mm)		Difference in thickness (mm)	Results interpretation
131	*Avian PPD*	6.16	*Avian PPD*	7.38	1.22	
	Bovine PPD	4.86	*Bovine PPD*	5.38	0.52	Negative
	Avian PPD	5.35	*Avian PPD*	6.06	0.71	
138	*Bovine PPD*	7.12	*Bovine PPD*	7.85	0.73	Negative
	Avian PPD	8.44	*Avian PPD*	9.50	1.06	
147	*Bovine PPD*	5.67	*Bovine PPD*	6.16	0.49	Negative
	Avian PPD	7.84	*Avian PPD*	7.93	0.09	
142	*Bovine PPD*	5.02	*Bovine PPD*	5.26	0.24	Negative
	Avian PPD	6.26	*Avian PPD*	6.31	0.05	
134	*Bovine PPD*	6.23	*Bovine PPD*	6.37	0.14	Negative
	Avian PPD	7.07	*Avian PPD*	7.34	0.27	
144	*Bovine PPD*	6.48	*Bovine PPD*	5.63	-0.85	Negative
	Avian PPD	6.42	*Avian PPD*	7.83	1.41	
136	*Bovine PPD*	6.85	*Bovine PPD*	6.69	-0.16	Negative
	Avian PPD	8.95	*Avian PPD*	9.22	0.27	
135	*Bovine PPD*	6.60	*Bovine PPD*	7.93	1.33	Negative
	Avian PPD	7.55	*Avian PPD*	8.06	0.51	
133	*Bovine PPD*	8.13	*Bovine PPD*	7.36	-0.77	Negative
	Avian PPD	7.84	*Avian PPD*	8.75	0.91	
141	*Bovine PPD*	5.91	*Bovine PPD*	7.08	1.17	Negative
	Avian PPD	6.69	*Avian PPD*	7.07	0.38	
116	*Bovine PPD*	4.87	*Bovine PPD*	4.99	0.12	Negative
	Avian PPD	5.67	*Avian PPD*	5.93	0.26	
111	*Bovine PPD*	6.97	*Bovine PPD*	7.90	0.93	Negative
	Avian PPD	8.74	*Avian PPD*	8.41	-0.33	
109	*Bovine PPD*	7.10	*Bovine PPD*	7.26	0.16	Negative
	Avian PPD	6.16	*Avian PPD*	7.03	0.87	

Animal I.D	Initial skin thickness (mm)		Final skin thickness (mm)		Difference in thickness (mm)	Results interpretation
106	*Bovine PPD*	5.66	*Bovine PPD*	7.40	1.74	Negative
	Avian PPD	6.54	*Avian PPD*	7.50	0.96	
113	*Bovine PPD*	8.72	*Bovine PPD*	10.73	2.01	Suspect
	Avian PPD	10.45	*Avian PPD*	10.51	0.06	
108	*Bovine PPD*	6.69	*Bovine PPD*	6.18	-0.51	Negative
	Avian PPD	7.79	*Avian PPD*	6.07	-1.72	
107	*Bovine PPD*	4.91	*Bovine PPD*	4.76	-0.15	Negative
	Avian PPD	4.55	*Avian PPD*	4.37	-0.18	
110	*Bovine PPD*	7.02	*Bovine PPD*	6.15	-0.87	Negative
	Avian PPD	7.61	*Avian PPD*	7.59	-0.02	
115	*Bovine PPD*	6.50	*Bovine PPD*	5.52	-0.98	Negative
	Avian PPD	5.80	*Avian PPD*	6.31	0.51	
82	*Bovine PPD*	6.52	*Bovine PPD*	7.54	1.02	Negative
	Avian PPD	7.67	*Avian PPD*	8.97	1.3	
84	*Bovine PPD*	5.29	*Bovine PPD*	4.60	-0.69	Negative
	Avian PPD	5.09	*Avian PPD*	6.53	1.44	
76	*Bovine PPD*	6.89	*Bovine PPD*	8.18	1.29	Negative
	Avian PPD	5.32	*Avian PPD*	5.84	0.52	
77	*Bovine PPD*	6.82	*Bovine PPD*	7.72	0.9	Negative
	Avian PPD	6.54	*Avian PPD*	7.32	0.78	
79	*Bovine PPD*	6.63	*Bovine PPD*	7.39	0.76	Negative
	Avian PPD	8.70	*Avian PPD*	7.93	-0.77	
85	*Bovine PPD*	7.24	*Bovine PPD*	7.60	0.36	Negative
	Avian PPD	6.13	*Avian PPD*	7.73	1.6	
83	*Bovine PPD*	6.00	*Bovine PPD*	6.95	0.95	Negative
	Avian PPD	6.75	*Avian PPD*	6.10	-0.65	
86	*Bovine PPD*	6.63	*Bovine PPD*	7.61	0.98	Negative

Animal I.D	Initial skin thickness (mm)		Final skin thickness (mm)		Difference in thickness (mm)	Results interpretation
78	*Avian PPD*	7.20	*Avian PPD*	8.25	1.05	Negative
	Bovine PPD	9.00	*Bovine PPD*	10.09	1.09	
	Avian PPD	9.82	*Avian PPD*	11.21	1.39	
80	*Bovine PPD*	8.48	*Bovine PPD*	7.83	-0.65	Negative
	Avian PPD	11.02	*Avian PPD*	11.81	0.79	
81	*Bovine PPD*	9.28	*Bovine PPD*	9.34	0.06	Negative
	Avian PPD	10.84	*Avian PPD*	10.77	-0.07	
117	*Bovine PPD*	6.58	*Bovine PPD*	6.01	-0.57	Negative
	Avian PPD	6.29	*Avian PPD*	6.39	0.1	
119	*Bovine PPD*	7.36	*Bovine PPD*	8.25	0.89	Negative
	Avian PPD	7.90	*Avian PPD*	9.10	1.2	
120	*Bovine PPD*	5.50	*Bovine PPD*	9.05	3.55	Suspect
	Avian PPD	7.65	*Avian PPD*	9.77	2.12	
121	*Bovine PPD*	5.35	*Bovine PPD*	5.42	0.07	Negative
	Avian PPD	6.82	*Avian PPD*	7.27	0.45	
118	*Bovine PPD*	9.05	*Bovine PPD*	9.10	0.05	Negative
	Avian PPD	7.99	*Avian PPD*	7.90	-0.09	

Appendix 2: Results of the comparative intradermal tuberculin skin test of animals from Rufunsa district

Animal I.D	Initial thickness (mm)		Initial thickness (mm)		Difference in thickness (mm)	Results interpretation
162	*Bovine PPD*	8.48	*Bovine PPD*	9.77	1.29	Negative
	Avian PPD	7.99	*Avian PPD*	9.26	1.27	

Animal I.D	Initial thickness (mm)		Initial thickness (mm)		Difference in thickness (mm)	Results interpretation
124	*Bovine PPD*	8.59	*Bovine PPD*	7.82	-0.77	Negative
	Avian PPD	8.58	*Avian PPD*	7.16	-1.42	
128	*Bovine PPD*	9.77	*Bovine PPD*	8.74	-1.03	Negative
	Avian PPD	9.84	*Avian PPD*	9.88	0.04	
122	*Bovine PPD*	8.16	*Bovine PPD*	7.72	-0.44	Negative
	Avian PPD	8.50	*Avian PPD*	8.45	-0.05	
123	*Bovine PPD*	7.76	*Bovine PPD*	9.19	1.43	Negative
	Avian PPD	10.70	*Avian PPD*	11.17	0.47	
125	*Bovine PPD*	8.12	*Bovine PPD*	7.84	-0.28	Negative
	Avian PPD	7.74	*Avian PPD*	8.88	1.14	
126	*Bovine PPD*	8.33	*Bovine PPD*	7.57	-0.76	Negative
	Avian PPD	7.96	*Avian PPD*	9.74	1.78	
127	*Bovine PPD*	6.90	*Bovine PPD*	8.53	1.63	Negative
	Avian PPD	7.85	*Avian PPD*	9.55	1.70	
98	*Bovine PPD*	7.47	*Bovine PPD*	13.84	6.37	Positive
	Avian PPD	7.01	*Avian PPD*	7.25	0.24	
93	*Bovine PPD*	7.23	*Bovine PPD*	10.58	3.35	Suspect
	Avian PPD	6.43	*Avian PPD*	10.49	4.06	
99	*Bovine PPD*	7.82	*Bovine PPD*	8.28	0.46	Negative
	Avian PPD	7.74	*Avian PPD*	9.37	1.63	
96	*Bovine PPD*	7.97	*Bovine PPD*	8.69	0.72	Negative
	Avian PPD	10.06	*Avian PPD*	10.76	0.70	
90	*Bovine PPD*	7.74	*Bovine PPD*	9.20	1.46	Negative
	Avian PPD	9.87	*Avian PPD*	10.48	0.61	
87	*Bovine PPD*	6.59	*Bovine PPD*	7.91	1.32	Negative
	Avian PPD	7.85	*Avian PPD*	8.41	0.56	
88	*Bovine PPD*	6.78	*Bovine PPD*	7.91	1.13	Negative

Animal I.D	Initial thickness (mm)		Initial thickness (mm)		Difference in thickness (mm)	Results interpretation
94	*Avian PPD*	7.68	*Avian PPD*	7.84	0.16	
	Bovine PPD	7.53	*Bovine PPD*	7.87	0.34	Negative
	Avian PPD	8.76	*Avian PPD*	10.00	1.24	
95	*Bovine PPD*	5.90	*Bovine PPD*	6.30	0.40	Negative
	Avian PPD	6.72	*Avian PPD*	7.49	0.77	
102	*Bovine PPD*	7.22	*Bovine PPD*	8.73	1.51	Negative
	Avian PPD	6.81	*Avian PPD*	8.54	1.73	
100	*Bovine PPD*	6.98	*Bovine PPD*	7.81	0.83	Negative
	Avian PPD	7.08	*Avian PPD*	9.17	2.09	
91	*Bovine PPD*	6.51	*Bovine PPD*	5.78	-0.73	Negative
	Avian PPD	5.68	*Avian PPD*	6.74	1.06	
103	*Bovine PPD*	9.80	*Bovine PPD*	8.35	-1.45	Negative
	Avian PPD	8.78	*Avian PPD*	8.33	-0.45	
105	*Bovine PPD*	11.06	*Bovine PPD*	11.04	-0.02	Negative
	Avian PPD	10.81	*Avian PPD*	12.54	1.73	
101	*Bovine PPD*	7.52	*Bovine PPD*	7.58	0.06	Negative
	Avian PPD	7.86	*Avian PPD*	8.85	0.99	
89	*Bovine PPD*	7.18	*Bovine PPD*	8.16	0.98	Negative
	Avian PPD	9.21	*Avian PPD*	9.98	0.77	
97	*Bovine PPD*	7.03	*Bovine PPD*	6.06	-0.97	Negative
	Avian PPD	9.41	*Avian PPD*	7.60	-1.81	
92	*Bovine PPD*	6.92	*Bovine PPD*	6.87	-0.05	Negative
	Avian PPD	8.69	*Avian PPD*	12.43	3.74	
104	*Bovine PPD*	7.31	*Bovine PPD*	7.27	-0.04	Negative
	Avian PPD	8.22	*Avian PPD*	8.85	0.63	
31	*Bovine PPD*	7.28	*Bovine PPD*	6.62	-0.66	Negative
	Avian PPD	6.94	*Avian PPD*	8.66	1.72	

Animal I.D	Initial thickness (mm)		Initial thickness (mm)		Difference in thickness (mm)	Results interpretation
43	*Bovine PPD*	7.73	*Bovine PPD*	7.74	0.01	Negative
	Avian PPD	7.15	*Avian PPD*	8.40	1.25	
38	*Bovine PPD*	7.73	*Bovine PPD*	7.63	-0.1	Negative
	Avian PPD	7.58	*Avian PPD*	6.70	-0.88	
44	*Bovine PPD*	7.13	*Bovine PPD*	7.63	0.5	Negative
	Avian PPD	8.16	*Avian PPD*	8.48	0.32	
30	*Bovine PPD*	5.37	*Bovine PPD*	5.77	0.4	Negative
	Avian PPD	6.12	*Avian PPD*	5.94	-0.18	
33	*Bovine PPD*	6.22	*Bovine PPD*	6.60	0.38	Negative
	Avian PPD	6.87	*Avian PPD*	7.36	0.49	
36	*Bovine PPD*	5.84	*Bovine PPD*	6.11	0.27	Negative
	Avian PPD	5.24	*Avian PPD*	6.53	1.29	
29	*Bovine PPD*	11.12	*Bovine PPD*	11.42	0.30	Negative
	Avian PPD	11.73	*Avian PPD*	11.71	-0.02	
39	*Bovine PPD*	6.26	*Bovine PPD*	9.00	2.74	Suspect
	Avian PPD	6.95	*Avian PPD*	7.53	0.58	
28	*Bovine PPD*	7.17	*Bovine PPD*	7.75	0.58	Negative
	Avian PPD	8.04	*Avian PPD*	8.53	0.49	
40	*Bovine PPD*	9.51	*Bovine PPD*	9.11	-0.4	Negative
	Avian PPD	7.58	*Avian PPD*	9.50	2.12	
35	*Bovine PPD*	6.50	*Bovine PPD*	6.69	0.19	Negative
	Avian PPD	6.33	*Avian PPD*	6.69	0.36	
34	*Bovine PPD*	6.92	*Bovine PPD*	6.78	-0.14	Negative
	Avian PPD	5.88	*Avian PPD*	7.93	2.05	
42	*Bovine PPD*	6.35	*Bovine PPD*	6.97	0.62	Negative
	Avian PPD	7.92	*Avian PPD*	6.55	-1.37	
37	*Bovine PPD*	7.07	*Bovine PPD*	8.26	1.19	Negative

Animal I.D	Initial thickness (mm)		Initial thickness (mm)		Difference in thickness (mm)	Results interpretation
32	*Avian PPD*	6.26	*Avian PPD*	7.02	0.76	
	Bovine PPD	6.67	*Bovine PPD*	7.20	0.53	Negative
	Avian PPD	6.75	*Avian PPD*	7.12	0.37	
41	*Bovine PPD*	6.25	*Bovine PPD*	5.65	-0.6	Negative
	Avian PPD	6.32	*Avian PPD*	7.27	0.95	
25	*Bovine PPD*	8.32	*Bovine PPD*	8.21	-0.11	Negative
	Avian PPD	8.98	*Avian PPD*	9.36	0.38	
69	*Bovine PPD*	8.06	*Bovine PPD*	8.26	0.20	Negative
	Avian PPD	10.00	*Avian PPD*	11.07	1.07	
71	*Bovine PPD*	5.31	*Bovine PPD*	7.31	2.00	Suspect
	Avian PPD	6.19	*Avian PPD*	10.84	4.65	