USE OF MOLECULAR METHODS TO DETECT SHEDDING OF *MYCOBACTERIUM BOVIS* IN CATTLE FAECES AND MILK

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ABSTRACT

Bovine tuberculosis (BTB) is a chronic bacterial disease caused by *Mycobacterium bovis*. It affects cattle and occasionally other animals and human. Several techniques exist for detection of BTB in animals. In Tanzania, Single Intradermal Comparative Tuberculin Test (SCITT) and abattoir meat inspection are the commonly used methods for screening of BTB. This study has used molecular biology method namely; RD4 Real time PCR (RD4 Rt PCR) to detect shedding *M. bovis* in milk and faecal samples from cattle tested by SCITT and Bovigam test. The study has also determined the detection limit of Immunomagnetic capture (IMC) and Centrifugation methods of *M. bovis* cells in spiked milk. A total of 63 cattle dairy form LITA farm Morogoro were tested for BTB with SCITT and Bovigam test. Faecal and milk samples were collected and RD4 Rt PCR was used to detect *M. bovis* DNA. BTB prevalence detected by SCITT and Bovigam was 3.1% and 3.1% respectively and BTB prevalence detected by RD4 Rt PCR in milk and faeces was 36.1% and 9.5% respectively. The kappa value between the SCITT and Bovigam was 1 and between shedding of *M. bovis* in milk and faeces detected using RD4 Rt PCR was 0.07. The detection limit of IMC and centrifugation coupled with Real time PCR ranges from 100 to 1 000 *M. bovis* cells per 500 µl milk and 10 to 100 *M. bovis* cells per 500 µl milk sample. High number of cattle was detected by Real time PCR to have BTB compared to SCITT or Bovigam. The confirmation of *M. bovis* in milk indicates potential root of transmission of *M. bovis* from cow to calf also to human who consume raw milk. Moreover, confirmation of *M. bovis* in feces indicates possible contamination of cattle carcasses during slaughter process. The study proposes that in order to improve BTB diagnosis for controlling and management of BTB, both conventional, SCITT or Bovigam and molecular methods in feces and milk need to be deployed in parallel but considering their own merits like cost and repeat access to animals.

DECLARATION

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

________________________________ __________________

Joseph Malakalinga Date

(MSc. Candidate)

The above declaration is confirmed by:

________________________________ __________________

Prof. Rudovick R. Kazwala Date

(Supervisor)

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LIST OF ABBREVIATIONS

16S rRNA	Subunit ribosomal Ribonucleic Acid gene
ABI	Applied Biosystems
BCG	Bacillus Calmette Guérin
Bovigam	Bovine interferon gamma
BSA	Bovine serum albumin
BTB	Bovine tuberculosis
Cat	Catalogue
CL	Confidence level
CMI	Cellular Mediated Immunity
C_T	Cycle at Threshold Value
DNA	Deoxyribonucleic Acid
EDTA	Ethyl Dimethyl Triacetate
ELISA	Enzyme linked immunosorbent Assay
IMC	Immunomagnetic capture
LITA	Livestock training Agency
M. bovis	*Mycobacterium bovis*
MPB	*Mycobacterium tuberculosis* Complex Protein
MTC	Mycobacterium tuberculosis complex
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PPD	Purified protein derivatives
Pr (a)	Agreement between Compared Techniques
Pr (e)	Likelihood of Random Agreement
RD4	Region of Difference four

rpm	Revolution per minute
Rt	Real Time
SACIDS	Southern African Centre for Infectious Surveillance
SCITT	Single Comparative Intradermal Tuberculin Test
SUA	Sokoine University of Agriculture
TB	Tuberculosis
TM	TaqMan
UK	United Kingdom
WHO	World Health Organization
K	Kappa value
AFB	Acid fast bacilli

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Bovine tuberculosis (BTB) is a chronic bacterial disease of animals caused by *Mycobacterium bovis* (*M.bovis*) BTB is also a zoonotic disease, and human beings can be infected by the consumption of non-pasteurised milk and its derivatives (Michel *et al*., 2010). Hence, BTB is still endemic worldwide (Humblet *et al.,* 2009). *M. bovis* pose a potential health hazard to both animals and humans (AL-Meshhadani *et al.,* 2000). Studies conducted from different countries revealed BTB prevalence ranging from 1.65% to 24.3% (Byarugaba *et al*., 2009; Thakur *et al.,* 2010; Boukary *et al*., 2011). Studies in Tanzania showed that the individual prevalence for pastoral cattle ranged from 1 to 13.2% while herd prevalence ranged from 11.6% to 51.0% (Katale *et al*., 2012). In human, extrapulmonary tuberculosis accounted over 20% of tuberculosis cases, some of the cases are caused by *M. bovis* (Mbugi *et al.,* 2012). The reports on BTB in Morogoro, the individual prevalence were 3.7% (Mwakapuja *et al*., 2013) and in small-scale dairy sector prevalence was 3.0% (Shirima *et al*., 2003). Other studies in Morogoro reported a prevalence of 3.0% (Mariki *et al*., 2013) and 2.5% (Durnez *et al*., 2009).

Single comparative intradermal tuberculin test (SCITT) is used for detecting live animals with *M. bovis* infection (Boukary *et al*., 2011). BTB diagnosis in Tanzania is done by SCITT, abattoir meat inspection and rarely by culture or molecular techniques (Katale *et al.,* 2012). Regardless of SCITT to lack of sensitivity and specificity (de la Rua-Domenech *et al.,* 2006), it is still being used alone for BTB diagnosis in Tanzania. In Tanzania, the main screening scheme for BTB in cattle is SCITT, although other studies have reported *M. bovis* in milk using molecular methods of SCITT negative cattle (Zumárraga *et al*.,

2012; Zarden *et al*., 2013). Still there are number of factors affecting the performance of SCITT including poor nutrition, chronic diseases, early infection and operator error (de la Rua-Domenech *et al.,* 2006). The implementation of other test to confirm the SCITT negative animal will assist in the control and management of BTB in Tanzania. Other test that is used for BTB diagnosis is Bovine interferon gamma assay (Bovigam). Bovigam is an *in vitro* laboratory assay of a cell mediated immune response, its sensitivity and specificity is between 81.8% and 100% respectively (Wood and Jones 2001). In New Zealand and other countries, Bovigam has been implemented in routine screening to confirm SCITT negative animals (Wood and Jones. 2001). In Tanzania, Bovigam is used to establish BTB prevalence in Gairo, Morogoro (Mariki *et al*., 2013).

Shedding of *M. bovis* in milk is reported by culture (Kazwala *et al.,* 1998; Zarden *et al.,* 2013) and also by direct detection of *M. bovis* DNA in milk sample using molecular methods from SCITT negative animals (Zumárraga *et al*., 2012; Zarden *et al*., 2013). In United Kingdom, Sweeney *et al*., (2006) reported shedding of *M. bovis* in badger feces using Real time PCR. Molecular techniques such as PCR have shown to have promising results on SCITT negative animals (Zarden *et al*., 2013).

In Tanzania, molecular methods are used mainly after culture from tissue and milk samples (Kazwala *et al*., 1998; Mwakapuja *et al.,* 2013). The purpose of those techniques was to differentiate *Mycobacterium tuberculosis* complex from non tuberculous Mycobacteria and classify them to species level (Kazwala *et al*., 1998; Mwakapuja *et al.,* 2013). No study has used molecular techniques directly on clinical samples. Milk and faeces are easily obtained from live animal compared to tissue samples for which animals have to be slaughtered to obtain the tissue. This study is aimed at using molecular methods notably Real time PCR in milk and faeces to diagnose BTB in cattle tested by SCITT and

Bovigam test. The screening was done at LITA farm known to have a history of BTB as detected by SCITT method.

1.2 Problem Statement and Study Justification

BTB diagnosis in Tanzania is done by SCITT, abattoir meat inspection and rarely by culture or molecular techniques (Katale *et al.,* 2012). The SCITT is a worldwide diagnosis test, which lacks both sensitivity and specificity (de la Rua-Domenech *et al.,* 2006). In a state of anergy, chronic infection, early stage of BTB disease also operator error are the causes of false negative results of SCITT (de la Rua-Domenech *et al.,* 2006), therefore leaving BTB positive cattle in the herd which might remain as a source of infection in the herd. Culture is the best for diagnosis but involves the use of harsh decontamination procedures which kill some *M. bovis*. The procedure is time consuming; it requires up to eight weeks showing positive culture. This is due to slow growing nature of *M. bovis*. PCR is sensitive, fast, rapid, diagnosing even samples with non-viable *Mycobacteria* (De la Rua-Domenech *et al.,* 2006; Medeiros *et al.,* 2010). These molecular methods especially the real time PCR are rapid, reliable, and highly sensitive, therefore can be used as alternative tools for the detection of many infectious agents (Malhotra-Kumar *et al*., 2008). Molecular methods are used to detect *M. bovis* cells in milk from SCITT negative animals (Zarden *et al.,* 2013). Also, a molecular method such as RD4 real time PCR is used to detect *M. bovis* DNA in faeces, urine and soil (Sweeney *et al.,* 2006). Since 2008, Bovine tuberculosis research team at SUA has observed several BTB outbreaks in the study farm at LITA farm. Although cattle were repeatedly screened using the SCITT and positive reactors culled by slaughtering, the disease has reoccurred in multiple times. This implies that the tuberculin skin test does not accurately diagnose the disease in some infected cattle and these animals act as the source of infection in the study farm.

In order to improve the BTB detection, the present study has used Real time PCR to assess the shedding of *M. bovis* in Milk and faeces from positive and negative SCITT and Bovigam tested cattle. The study will generate information which will help control and management of BTB in livestock farms.

1.3 Objectives

1.3.1 Overall objective

To demonstrate presence of *M. bovis* in milk and faeces of cattle

1.3.2 Specific objectives

i. To detect and determine prevalence of BTB infection in cattle using SCITT and Bovine gamma interferon test (Bovigam)

ii. To compare the shedding of *M. bovis* in milk and faeces in cattle using RD4 real Time PCR

iii. To assess the performance of Immunomagnetic capture and centrifugation methods in improving detection levels of mycobacterium in clinical specimens

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Bovine Tuberculosis

BTB is a chronic bacterial disease of animals and humans caused by *M. bovis*. BTB remains a major public health issue. According to the WHO, in 2004, mortality and morbidity statistics included 14.6 million chronic active cases, 8.9 million new cases, and 1.6 million deaths, mostly in developing countries with an expected 1% increase annually (WHO, 2006). BTB is re-emerging in a number of developed countries due to environmental changes, the movement of people and animals, closer inter-species contacts, and changes in animal management (Proano *et al*., 2009). *M. bovis* has a wide host range making eradication difficult and resulting in some animals becoming wildlife reservoirs of the disease. In dairy cattle, the disease causes weight loss, decreased milk production, and lowered reproductive rate (Proano *et al*., 2009). The costs of diagnosis and treatment of cattle and humans and the costs of correct disposal of infected animal carcasses have an additional impact.

2.2 Aetiology

M. bovis is a member of the Mycobacterium *tuberculosis complex* belonging to family *Mycobacteriaceae* which includes *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium canettii*, *Mycobacterium microti* and *M. bovis BCG* (Brosch *et al.,* 2002). *M. bovis* is a Gram positive, acid fast, rod shaped, and aerobic bacterium.

2.3 Genome Structure

The sequence results revealed *M. bovis* genome to have a sequence of 4 345 492 base pairs in length in a single circular chromosome (Garnier *et al*., 2003). There are 3 952 genes

encoding proteins in the genome (Garnier *et al.,* 2003). *M. bovis* genome is >99.52% similar at the nucleotide level to *Mycobacterium tuberculosis* (Garnier *et al*., 2003). Comparative sequencing with *M. tuberculosis* showed 11 deletions from the genome of *M. bovis*, which are 1-12.7 kb in size, and that has been confirmed by the genome sequence data (Garnier *et al*., 2003). *M. bovis* have one unique locus termed TbD1, which is not present in the *M. tuberculosis* strain; this gives the clue that deletion has been the mechanism in shaping the *M. bovis* genome (Garnier *et al*., 2003).

2.4 Transmission

Transmission of *M. bovis* can occur between animals, from animals to humans and vice versa and rarely, between humans (Fig. 1). *M. bovis* infection is transmissible from cattle to humans directly by aerogenous route and through direct contact with materials contaminated with nose and mouth secretions from an infected animal (Thomas *et al*., 2010). Research findings revealed that at risk individuals are persons in contact with potentially infected animals such as veterinarians, abattoir workers, meat inspectors, autopsy personnel, farmers, milkers, animal keepers (as well as those in the zoo), animal dealers, laboratory personnel, owners of potential tuberculous pets (e.g. monkeys) (Ofukwu *et al.,* 2006; Yumi *et al*., 2007). Indirectly, human acquires the disease from animal sources by ingestion of meat and meat products from slaughtered infected cattle and consumption of unpasteurised infected milk (Thoen *et al*., 2006).

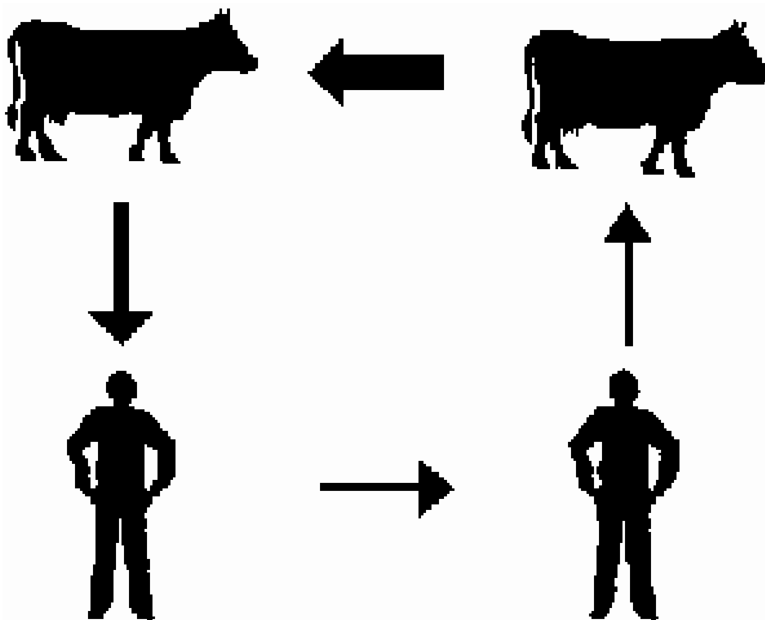

Figure 1: Cycle of *M. bovis* transmission between cattle and humans. The thickness of the arrows suggests level of probability (Source: Grange and Collins, 1987)

2.5 Host Range

M. bovis affects most mammalian hosts, including humans, cattle, pigs, domestic cats, wild carnivores and omnivores , it rarely affects equids or sheep (Delahay *et al.,* 2002; Phillips *et al.,* 2003). There is no enough information on the susceptibility of birds to *M. bovis*, although they are generally thought to be resistant.

2.6 Immunology of the Disease

BTB is featured by the formation of granulomas (tubercles) that are usually yellowish and either caseous, caseo-calcareous or calcified and are sometimes encapsulated (De Lesle *et al*., 2002). *M. bovis* is an intracellular pathogen; macrophages are responsible for dissemination and destruction of *M. bovis*. Also invade other monocytic cells. Various disease diagnostic methods rely on the detection of humoral (antibody) responses to the

disease agent. T lymphocytes are responsible for the immunological response of *M. bovis* in infected animal. This process called cell mediated immune (CMI) response which is both way of defense and the causative of chronic inflammation characteristic of *M. bovis* infections (de la Rua-Domenech *et al.,* 2006).

2.7 Clinical Signs

BTB usually has a prolonged course, and symptoms take months or years to appear. The usual clinical signs includes; weakness, loss of appetite, weight-loss, fluctuating fever, intermittent hacking cough, diarrhea, large prominent lymph nodes. However, the bacteria can also lie dormant in the host without causing disease (OIE, 2009). In many cases, the course of the infection is chronic and signs may be lacking, even in advanced cases when many organs may be involved. When present, clinical signs vary; lung involvement may be manifested by a cough, which can be induced by changes in temperature or manual pressure on the trachea (OIE, 2009). Dyspnoea and other signs of low-grade pneumonia are also evidence of lung involvement. In advanced cases, lymph nodes are often greatly enlarged and may obstruct air passages, the alimentary tract, or blood vessels (OIE, 2009). Lesions are usually non-odoriferous. Other anatomical sites can be infected and should be examined. Macroscopically, tuberculous granulomas usually have a yellowish appearance and are caseous, caseo-calcareous, or calcified in consistency (OIE, 2009) occasionally; its appearance may be purulent. The appearance may be more purulent in cervids and camelids. Some non tuberculous granulomas may be indistinguishable macroscopically from tuberculous Granulomas (OIE, 2009). The caseous centre is usually dry, firm, and covered with a fibrous connective capsule of varying thickness. Lesion size ranges from small enough to be missed by the unaided eye, to involvement of the greater part of an organ. Serial sectioning of organs and tissues may be required to detect the small lesions contained within the tissue (OIE, 2009).

The human form of *M. bovis* infection has similar clinical forms as that caused by *M. tuberculosis* (Kirk *et al*., 2003; Ofukwu *et al.,* 2006). Cervical lymphadenopathy (which primarily affects the tonsilar and pre-auricular lymph nodes), intestinal lesions, chronic skin tuberculosis (lupus vulgaris), and other non pulmonary forms are particularly common (Cosivi *et al*., 1998). In young children infected with *M. bovis* typically have abdominal infections and older patients suffer from swollen and sometimes ulcerated lymph glands in the neck (Nwanta *et al*., 2010). The symptoms may include fever, cough, chest pain, cavitation and hemoptysis (Shitaye *et al*., 2006).

2.8 Detection

There are several techniques for BTB diagnosis which include tuberculin skin test, microscope, culture, Bovine gamma interferon assay (Bovigam), Molecular techniques such as PCR, real time PCR and other.

2.8.1 Single comparative intradermal tuberculin test (SCITT)

Single comparative intradermal tuberculin test (SCITT) has been applied for identifying live animals with *M. bovis* infection (Boukary *et al*., 2011). The skin tests are the international standard for diagnosis of bovine TB in cattle herds and individual animals (OIE, 2009). It is a delayed-type hypersensitivity response to the intradermal injection of purified protein derivative (PPD); this is the complex water-soluble fraction of the heat-treated products of growth and lysis of *M. bovis*. Upon injection of bovine PPD into the skin of an animal not sensitized to tuberculin antigens, there is no significant local inflammatory response. However, if tuberculin is injected into an animal whose immune system has been sensitized by infection with *M. bovis* or by exposure to cross-reacting antigens, it triggers an inflammatory response and swelling at the injection site that reaches its greatest intensity after 48–72 hours of incubation (Pollock *et al*., 2003).

The interpretation of the SCITT test is based on the observation that *M. bovis* infected cattle tend to show a greater response to bovine tuberculin than to avian tuberculin, whereas infections with other Mycobacteria promote the reverse relationship (Pollock *et al*., 2003). This test, therefore, allows better discrimination than the SCITT between animals infected with *M. bovis* and those sensitized to tuberculin after exposure to organisms of the *M. avium* complex or to environmental non pathogenic *Mycobacteria*.

In cattle subjected to the skin test false negative tuberculin reactions can occur for a variety of reasons, newly, infected cattle generally do not react to the intradermal injection of tuberculin (de la Rua-Domenech *et al.,* 2006). Hypersensitivity to tuberculin usually develops in cattle between 1 and 9 weeks after infection with *M. bovis*, depending on animal and test factors, but for most animals a full response is likely to develop between 3 and 6 weeks post-infection (de la Rua-Domenech *et al.,* 2006).

A state of anergy may develop in cattle with advanced or generalized TB in animals subjected to stress (de la Rua-Domenech *et al.,* 2006). Topical or systemic administration of glucocorticoids can also lead to a significant reduction in the size of the bovine tuberculin reaction in infected cattle co-infected with *M. bovis* and viruses that depress the function of lymphocytes and macrophages (such as the bovine viral diarrhea virus) may have a transient effect on diagnostic assays for TB (de la Rua-Domenech *et al.,* 2006). Additionally, diminished cell mediated immune responses associated with malnutrition have been documented in both humans and laboratory animals (de la Rua-Domenech *et al.,* 2006). As with any diagnostic test, operator errors and faulty equipment may also lead to false negative skin test results, for instance, when tuberculin is not lodged into the skin, the dose injected is insufficient, or test results are read too early or too late after tuberculin injection (de la Rua-Domenech *et al.,* 2006). Lastly, when using the standard

interpretation of the SCITT, the recognition of some *M. bovis* infected cattle with tuberculous lesions can be temporarily masked by prior exposure to Mycobacteria of the *M. avium*–intracellulare complex, if the skin reaction to avian tuberculin in those animals exceeds that to bovine tuberculin (Hope *et al*., 2005). In comparative skin tests, animals infected with Mycobacteria other than the MTB complex generally develop reactions to avian tuberculin much greater than those to bovine tuberculin. (de la Rua-Domenech *et al.,* 2006).

2.8.2 Gamma interferon assay

The Gamma Interferon assay (Bovigam) is an in vitro blood test developed in Australia for the diagnosis of BTB in cattle (Wood and Jones, 2001). The test was approved as an official diagnostic method for bovine TB in Australia in 1991 (de la Rua-Domenech *et al.,* 2006). The efficiency of Bovigam test has been tested in several studies conducted in Australia, Brazil, Ethiopia, Great Britain, Republic of Ireland, Italy, New Zealand, Northern Ireland, Spain and the USA (Wood and Jones, 2001). In these trials, the sensitivity of the gamma-IFN test was varied between 73.0% and 100%, with a median value of 87.6%. Its median specificity was 96.6%, with a range of 85.0–99.6% (de la Rua-Domenech *et al.,* 2006). The test in New Zealand was introduced to screen the SCITT negative animals. It has advantages of not been affected by early BTB infection, but it is also affected by other chronic infection, parturition and dexamethasone treatment. Gamma interferon has an important role in the immune responses to tuberculous Mycobacteria as the major macrophage activation factor. As with the skin tests, the principle underpinning the interferon gamma test is the detection of the host's CMI response against *M. bovis* infection (Pollock *et al.,* 2005).

2.8.3 Microscopy

Microscopy is the simplest and most rapid procedure currently available to detect acid-fast bacilli (AFB) in clinical specimen by zienhl-Neelsen staining method or its modifications (Cheesbrough *et al*., 2000). Microscopy is cheap to perform, specific enough to indicate treatment in countries where TB is prevalent, and can be completed within hours if necessary (WHO, 2006). The limitation of microscopy for detection of TB is that it requires a large number of bacilli to be present in order for the result to be positive (5 000 –10 000 bacilli per ml) of sample (Khagi *et al.,* 2009). However, this renders the test unable to detect less advanced cases. The results of microscopy can be influenced by the type of specimens, thickness of the smear, extent of decolarization, type of counter stain used, training and experience of the person examining the smear (Medappa *et al.,* 2002). Still microscope cannot differentiate species of *Mycobacteria*.

2.8.4 Culture

Isolation of mycobacteria from sputum samples by culture still represents the corner stone on which definitive diagnosis of TB relies and is currently the gold standard for the primary isolation of mycobacteria (WHO, 2006). At present, mycobacterial culture is performed on Lowenstein Jensen medium (Pai *et al.,* 2009). The major constraint of culturing mycobacteria in conventional media is its slow growth which necessitates a mean incubation period of at least 4 weeks (Medappa *et al.,* 2002). Also, mycobacterial culture method is feasible only if > 100 *M. tuberculosis* bacilli are present in one ml of specimen. The specificity of the culture method is close to 100% but it is affected by the use of decontaminating agents which reduce the *M. bovis* cells during the culturing procedures. Also samples like faeces, soil and milk are highly contaminated with other microbes which are resistant to decontaminants, upon inoculation in media these

contaminants cover all media surfaces and therefore difficult to isolate individual colonies of *Mycobacteria*.

2.8.5 Polymerase chain reaction

The polymerase chain reaction (PCR) is a scientific tool in molecular biology used to identify a particular gene by amplifying a single or a few copies of a piece of DNA following its steps and generate thousands to millions of copies of a particular DNA sequence of that gene. PCR is molecular tool used in medical and biological research labs for a variety of applications. There are several types of PCR used in detection of disease agents in biological samples. Conventional PCR uses only primers that bind to a complimentary strand adjacent the targeted DNA sequence (Huard *et al.,* 2003). Real time PCR has added advantages of being more sensitive and specific by having extra reagent called DNA probe which bind the targeted DNA sequence **(**Malhotra-Kumar *et al*., 2008). A combination of primer and probe together has increase specificity and sensitivity of Real time PCR. There are several Conventional PCR and Real time PCR developed to detect the *M. bovis* in biological samples differing in their primers used to identify a particular DNA sequence, discriminatory power of *Mycobacteria* species.

The *M. tuberculosis* complex (MTC) is comprised of bacterial organisms that genetically share identical 16S rRNA sequences (Boddinghaus *et al*., 1990). This has resulted to difficulties to in differentiation of one species to another and one strain to another. Some PCR developed for BTB diagnosis includes Mycogenus PCR, Deletion typing PCR.

The Mycogenus PCR is multiplex PCR which uses six different primers that helps to identify *Mycobacterium* genus, *M. tuberculosis* complex, *M. avium* and *M. intracellulare* by targeting 16S rRNA gene for *Mycobacterium* genus, hyper variable region of the 16S

rRNA gene of *M. intracellulare* and *M. avium* and MPB70 of MTC (Gordon *et al*., 1999; Brosch *et al*., 2002). Still, their challenges on the techniques like failure to differentiate the MTC members into their species and other *Mycobacterium* genus than *M. intracellulare* and *M. avium*

M. tuberculosis and *M. bovis* genomes are 99.95% related at the nucleotide level (Garnier *et al*., 2003), DNA microarrays revealed 16 regions of difference (RD1 to RD16) (Behr *et al*., 1999.) in their genome. These, Regions of difference (RD) are present or deleted in some members of MTC have been utilized for differentiation of members of MTC (Fig. 2). RD9 is specifically used for detection of *M. tuberculosis* and differentiate it from other MTC members (Pinsky and Banaei, 2008.); the challenge is RD also present in *M. canettii* (Brosch *et al*., 2000). Therefore another PCR is needed to differentiate *M. tuberculosis* from *M. canettii.* RD12 is absent in *M. canettii*, which has been targeted for the specific detection of *M. canettii* in a complex conventional PCR methodology (Huard *et al.,* 2003). RD4 is deleted in *M. bovis* by detecting its absence helps to discriminate *M. bovis* from other MTC. Deletion typing is limited to MTC members, Therefore this method cannot be used for *Mycobacteria* other than MTC which are now becoming of public health importance.

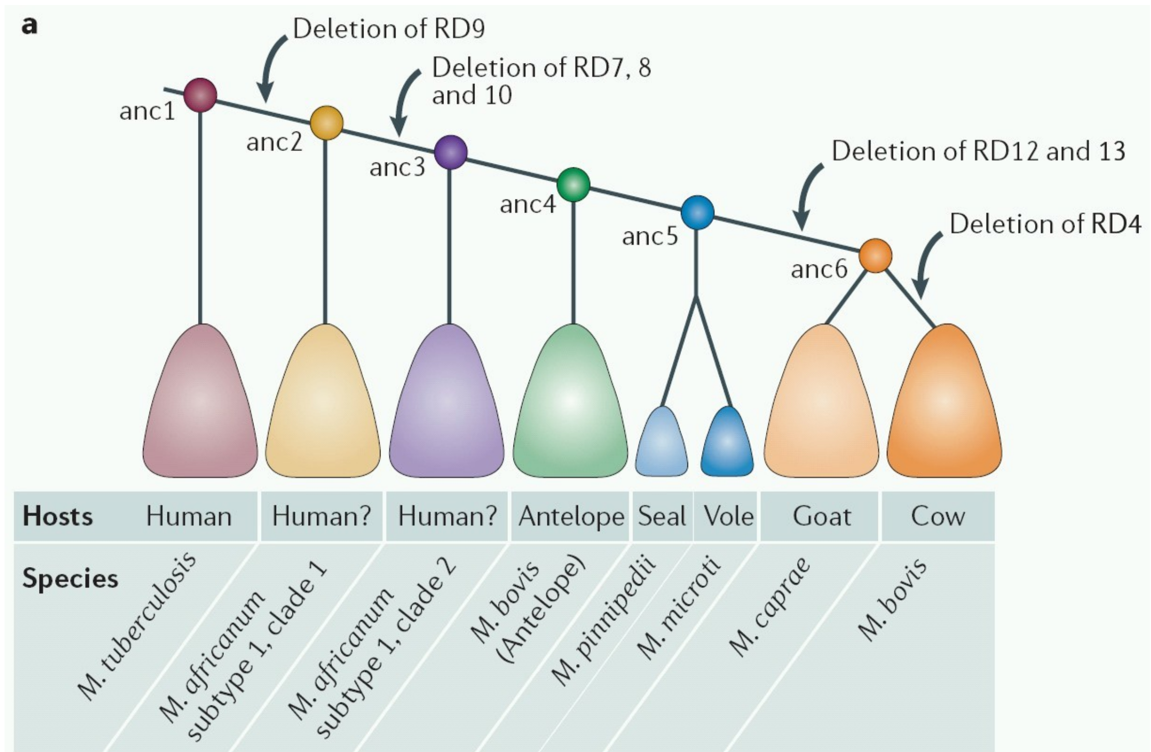

Figure 2: Phylogeny of the *M. tuberculosis* complex. Source: (Smith *et al.*, 2006)

Real time PCR has advantages over conventional PCR because it allows absolute quantification by comparison to a standard curve of known target sequence numbers. The complete genome sequence of *M. bovis* has been used to design primers flanking a region of difference (RD) between the sequence of *M. bovis* DNA and that of other *M. tuberculosis* complex members (Brosch *et al.,* 2002). The RD4 real time PCR has been used to detected *M. bovis* DNA in faeces, soil and urine (Sweeney *et al.,* 2006). This study has used the RD4 real time PCR to detect the *M. bovis* DNA in milk and faeces. The presence of *M. bovis* is confirmed by using a fluorescent (TaqMan) probe which discriminates *M. bovis* from other *M. tuberculosis* complex members since it hybridizes with both the $5^{/}$and $3^{/}$ RD4 deletion flanking sequences, which only occur directly adjacent to each other in *M. bovis* (Brosch *et al.,* 2002).

2.8.6 Immunomagnetic capture

Immunomagnetic capture (IMC) is a newly technique used to extract cells of *M. bovis* from mixed cell communities with a polyclonal antibody to *M. bovis* BCG and thus enabling cultivation of *M. bovis* from soil samples (Sweeney *et al.,* 2006). IMC was also used to detect *M. bovis* DNA in badger faeces and to monitor environment contamination to *M. bovis* (Sweeney *et al.,* 2006). A good specificity of IMC can be improved by using a monoclonal antibody which binds MBS43 (Wiker *et al.,* 1996), MPB83, is a glycosylated cell wall-associated protein in *M. bovis* (Hewinson *et al.,* 1996), this monoclonal antibody has been used to separate *M. bovis* in a biological sample from other members of the *M. tuberculosis* complex (Goodge *et al.,* 1994).

The IMC-PCR is reported to achieve a sensitivity of 100% for detecting *M. avium* subsp. *paratuberculosis* in faeces (Khare *et al*., 2004). The IMC improvement was done by coupling with bead beating and real-time PCR and found to be a very effective method for the isolation, separation, and detection of *M. avium* subspecies, *Paratuberculosis* from milk and feces samples from cattle and American bison (Khare *et al.,* 2004).

2.8.7 *Mycobacterium bovis* shedding in BTB infected cattle

It has been reported that, for cattle slurry/manure to act as a source of *M. bovis*, at least one animal in the herd must be infected and excreting bacteria in faeces, urine or milk that has been disposed of in the slurry lagoon (de la Rua-Domenech *et al.,* 2007). It is assumed that all infected animals excrete, sporadically, at some stage post-infection (Francis *et al.,* 1946). The chance of excretion and the number of mycobacteria excreted by an individual animal depends upon infectious dose, site and level of infection, and the amount of time the animal has been infected or severity of infection (Phillips *et al.,* 2003). Excretion of *M. bovis* in faeces, urine or milk is more likely to occur in cases of generalized or advanced

tuberculosis, normally characterized by disseminated infection and lesions in organs such as the liver, kidneys and udder, or in the meninges and serous cavities (Neill *et al.,* 2001).

Current information from meat inspection and post mortem examination indicates that few infected cattle exhibit lesions in the intestine or mesenteric lymph nodes (Liebana *et al.,* 2008). Feces deposits are rarely examined for the presence of *M. bovis*, and as a result, empirical data on shedding of *M. bovis* in feces is limited. A few studies have investigated the excretion of *M. bovis* in naturally infected cattle. Report on viability of tubercle bacilli was demonstrated in the feces of six apparently healthy cows (Williams and Hoy, 1927). In a subsequent study, 24% of feces samples from cows were found to be positive for *M. bovis* following inoculation of guinea pigs (cited by Williams and Hoy, 1930). The authors concluded that the chief source of infection of the feces originated from the lungs, supported by the fact that at the post mortem examinations only one cow showed any visible evidence of tuberculous infection of the mucous membrane of the intestine. Furthermore, cows were observed to swallow coughed sputum and *M. bovis* was demonstrated repeatedly in sputum. According to Makkaievskaya (cited by de la Rua-Domenech, 2007), 93% of cattle with clinical TB and 43% of reactors without clinical signs shed *M. bovis* bacilli in their feces.

Where there is infection in the herd, either detected or undetected, routes that could lead to contamination of milk with *M. bovis* include via feces and from the environment but the main risk are from direct contamination of the milk in the udder. Contamination of milk can occur before the animal tests positive on the skin test or before clinical signs of infection are apparent (ACMSF, 2010). A study conducted in Brazil by Zarden *et al.* (2013) collected milk samples from 8 SCITT negative animals for examination by culture and PCR and found that 5 milk samples were positive for *M. bovis* one by culture and 4 by

PCR. In other studies, Zumárraga *et al.* (2012) reported positive PCR results for milk samples from bulk tank from TB-suspected herds and also certified TB-free herds and Figueiredo *et al*. (2010) reported the identification of specific *M. bovis* DNA in 12% of milk samples from skin test negative cattle. Despite of several methods exist for detection of BTB disease still there are a lot of challenges on BTB detection in animals and animal products.

In Tanzania, Real time PCR is rarely used for BTB diagnosis from biological sample, Conventional PCR is mostly used to confirm positive culture results. Due to the evidence exist on shedding of *M. bovis* in biological samples such as milk and feces and the misdiagnosis of BTB cattle by SCITT, My study has used RD4 Real time PCR for BTB diagnosis in feces and milk from cattle of LITA farm SCITT and Bovigam tested.

CHAPTER THREE

3.0 MATERIALS AND METHODOLOGY

3.1 Study Area

The study was conducted in Morogoro region at Livestock Training Agency (LITA) farm. Morogoro region is located between 6° 9′ 0″ S and 37° 40′ 0″ E. The topography of Morogoro is predominantly plain with rainfall ranging between 900 and 1 400 mm annually. The average temperature in Morogoro is 30° C due to its lowland nature. The individual BTB prevalence for pastoral cattle in Tanzania ranges from 1% to 13.2% while herd prevalence ranged from 11.6% to 51.0% (Katale *et al*., 2012). Studies conducted in Morogoro on BTB, showed that the individual prevalence ranges from 2.5% - 3.7% (Shirima *et al.,* 2003; Durnez *et al*., 2009; Mwakapuja *et al*., 2013: and Mariki *et al*., 2013). LITA farm is located at 6º 50′ 37.8″ and 37 º 39′ 59.8″. The farm is 1.5 km from Morogoro town centre, and western nearby SUA.

The Farm has mixed animals adult and young animals which include cattle, goat and sheep. This study used the dairy cattle as study animals. Milk from dairy cattle is sold to people living nearby the farm and other are sold in Morogoro town center. Since 2008, several BTB outbreaks were observed at LITA farm, although cattle repeatedly were screened using the SCITT and positive reactors removed by slaughtering, the disease has reoccurred many times. This implies that the tuberculin skin test may not accurately diagnose the disease in some infected cattle and act as the source of infection in the LITA farm. This study used milk and faecal samples to improve the detection of the BTB in LITA farm by using Real time PCR in parallel with SCITT and Bovigam test.

3.2 Study Design

The study design used is cross sectional study design carried out at LITA farm. SCITT is an *in vivo* test that was directed to 63 cattle in the LITA farm, blood and faecal samples were first collected before SCITT from all 63 animals. Additional milk sample were collected from 35 lactating cattle among a group of 63 cattle. The biological samples such as blood and milk were transported to the TB laboratory situated at the SUA in the Faculty of Veterinary Medicine. All laboratory procedures were performed at TB laboratory. Blood was used to detect interferon gamma upon *in vitro* Sensitization with Purified protein derivative of bovine and avium. DNA was extracted from collected blood and milk sample, then Real time PCR was used to detect *M. bovis* DNA. Field and laboratory work were performed for three months from January to March, 2014.

3.3 Sample Size Calculation

Sample size was estimated by the formula:

n= (1-(1-CL)/d) x (N-(d-1)/2)………………………………………...…………………. (1)

Where; n = required sample size, N =63, population size, *d* = 3, number of expected diseased animals in the herd, CL = 0.95, confidence level as a fraction (Flahault *et al*., 2005).

n= (1-(1-0.95)/3)(63-(3-1)/2)……………………..…………………………………….. (2)

n=61

3.4 Sample Collection

3.4.1 Milk

Milk samples were collected in the morning during milking time. The udder was cleaned with soap and left to dry. Then methylated spirit was applied on udder surface, all this was to reduce contaminants. The middle milk during milking from all four tits was pulled in

one 50 ml falcon tube. A total of 50 ml milk samples were collected from each of the 35 enrolled lactating cows. Then collected milk samples were labeled accordingly and transported to the TB laboratory at SUA and stored in a refrigerator waiting for laboratory analysis in the next day.

3.4.2 Faeces

Cattle were mobilized in the morning and restrained in a crush system. Sterile gloves were used for collection of faeces sample direct from the rectum. Gloves were worn on hand was introduced per rectum and feces placed into 50 ml falcon tube. Faecal samples were collected from all 63 SCITT tested cattle and transported in a cool box to TB laboratory for further laboratory analysis.

3.4.3 Blood

Cattle were restrained in a crush, the blood was collected from jugular vein, if missed was collected from the coccygeal artery in the tail. Blood collected into heparin, plain and EDTA vacutainer tube using vacutainer needle and vacutainer holder from each SCITT tested cattle.

3.5 Intradermal Tests

The Bovine Tuberculin Purified Protein derivatives 3 000 (PPD-B) and Avian Tuberculin Purified Protein derivatives (PPD-A) 2 500 (Prionics Lelystad BV, Lelystad, Netherlands) were used in SCITT. Animals were restrained in a crush, two different spots were created by shaving 12 cm apart using razor blade at the middle third of the neck of each animal. The thickness of the skin was measured prior injecting PPDs followed by intradermal injection of 0.1 ml of PPD-A and PPD-B by using HAUPTNER MUTO (Hauptner

Herberholz, Germany) syringe . The thickness of skin folds at each sites were measured and recorded again at 72 hours after PPD inoculation. PPD reaction of each animal was calculated using the formular: (PPD-B_{72} – PPD-B_0) – (PPD-A_{72} – PPD-A_0). Interpretation of the skin reactions based on the manufacturer's instructions and OIE applying a 4-mm cut-off, animals were categorised as positive reactors when a reaction to bovine tuberculin PPD was more than 4 mm, greater than the reaction to avian tuberculin PPD. A reaction to bovine tuberculin PPD (of at least 2 mm) which is from 1 to 4 mm greater than the reaction to avian tuberculin PPD was considered inconclusive, while a reaction to bovine tuberculin PPD equal to or less than the reaction to avian tuberculin PPD was negative.

3.6 Bovine Interferon Gamma (BOVIGAM) Test

Blood in Heparinized vacutainer tube from each animal were mixed gently by inverting several times to dissolve the heparin and transported to the laboratory at ambient temperature (22 ± 5 ^{o}C). Then 1.5 ml of blood was dispensed into 2 ml eppendorf and labeled PBS, Avium and Bovine respectively. To 1.5 ml heparin blood, 100 µl of PBS (nil antigen control), avian PPD antigen and bovine PPB antigen were aseptically mixed and incubated at 37 ^{o}C for 16-30 h.

During incubation, if the animal were previously exposed to *M. bovis,* the T lymphocyte responds by releasing gamma interferon which is a macrophage activator. The released gamma interferon is measured by Bovigam ELISA kit (Prionics Lelystad BV, Lelystad, Netherlands). After incubation plasma was harvested and stored at -20 ^{o}C. Bovine gamma interferon (Bovigam) kits manufactured by Prionics were used for detecting gamma Interferon. The Multiscan reader (Multiscan RC, Fisher Scientific, Waltham, United States) was used to measure the optical density. The procedures and interpretation of results were followed as per manufacturer instructions. A microtitre plate is coated with

anti gamma interferon antibody which binds gamma interferon present in the plasma (Plate 1).

Plate 1: Principle behind Bovigam ELISA (picture from Manufacturer user manual)

3.7 Direct DNA Extraction

DNA extraction in milk was done using Qiagen DNeasy blood and Tissue kit (Qiagen, Hilden, Germany). Two millilitre of milk was centrifuged at 8 000 x g for 10 min. Supernatant were discarded and sediments were resuspended with 180 µl enzymatic lysis buffer and incubated at 37 °C for 30 min. After incubation 25 µl proteinase K were added followed by 200 µl Buffer AL, then mixed by vortexing and incubated at 56 °C for 30 min. Thereafter 200 µl ethanol (96–100%) was added to the sample and mixed thoroughly by vortexing. A mixed content of 600 µl were pipette into the DNeasy Mini spin column placed in a 2 ml collection tube and Centrifuged at 6 000 x g (8 000 rpm) for 1 min. The flow-through and collection tube were discarded. The DNeasy Mini spin column placed in a new 2 ml collection tube and 500 µl Buffer AW1 was added and centrifuged for 1 min at 6 000 x g (8 000 rpm). The flow-through and collection tube were discarded. DNeasy Mini spin column was placed into a new 2 ml collection tube

and 500 µl of Buffer AW2 was added and centrifuge for 3 min at 20 000 x g (14 000 rpm). To avoid the carryover of ethanol to occur, the collection tubes were empted and reused it in another centrifugation for 1 min at 20 000 x g (14 000 rpm). Then DNeasy Mini spin columns were placed in a clean 1.5 ml microcentrifuge tube and 100 µl Buffer AE was added directly onto the DNeasy membrane and incubated at room temperature for 1 min and then centrifuged for 1 min at 6 000 x g (8 000 rpm) to elude the DNA. The DNA eluted was stored at -20 ^{o}C.

DNA extraction in feces was done using Fast DNA spin kits (Mpbio, Illkirch, France) for soil. The capped 2 ml Lysing Matrix E Tubes were Pre-weighed and 0.2 g (± 0.03 g) of sample was added to the tube. Cap was replaced and re-weighed, and then 978 µl Sodium Phosphate Buffer were added followed by 122 µl MT Buffer. The tubes were closed very tight and secured in the Ribolyser (FastPrep-24, California, United States) and processed for 40 s at speed 6.0 (wibbles per minutes). Then lysing matrix E tube were centrifuged at 18 407 x g (Mikro 200, Hettick zentrifugen, Tuttlingen, Germany) for 10 min to pellet debris. Supernatant was transferred to a clean 2 ml tube and 250 µl PPS reagent was added and mixed by hand shaking the tubes 10 times and then centrifuged at 18 407 x g for 5 min to precipitate pellets.

Supernatant was transferred to a clean 15 ml falcon tube and mixed with 1 ml of binding matrix suspension by vortexing. The binding matrix were remixed periodically as the matrix tends to decant very fast by inverting falcon tubes by hand for 2 min to allow binding of DNA to matrix. The falcon tubes with the mixture were placed in the rack for 3 min to allow settling of the Silica Matrix and 500 µl of the supernatant removed careful to avoid disturbing settled binding matrix and discarded. Binding matrix was resuspend with the remaining amount of supernatant and 600 µl of the mixture was transferred to a

SPIN^{TM} Filter and centrifuged at 18 407 x g for 1 min. Catch tubes were empted and the remaining supernatant added to the SPIN^{TM} Filter and centrifuged again. SEWS-M of 500 µl was added to the SPIN^{TM} Filters and the pellets were gently resuspended using the force of the liquid from the pipette tip and centrifuged at 18 407 x g (14 000 rpm) for 1 min. the flow through were discarded and were placed again back in the catch tube and centrifuged at 18 407 x g (14 000 rpm) in Eppindorf 5 424 centrifuge for 2 min to dry the SPIN^{TM} filters. SPIN^{TM} filters were removed and placed in a fresh kit supplied catch tube and air-dried for 5 minutes at room temperature. Then, 100 µl DNase/RNase free water was added to the binding matrix above the spin filter and silica gently resuspended by finger flicking for efficient elution of DNA and incubated for 5 minutes at 55 °C in a heat block. After incubation SPIN^{TM} Filters were centrifuged at 18 407 x g (14 000 rpm) for 1 minute so as to transfer eluted DNA to the kit supplied catch tube. Then eluted DNA was stored at -20 ^{o}C.

3.8 Spiking

The term involves the introduction of a known number of cells into a sample that is free from those cells. The characterized isolates of *M. bovis* cells cultured in Lowenstein Jensen media with pyruvate were used. A 5 ml 10% Tween 20 solution containing glass spheres were prepared in a 15 ml falcon tube. Colonies *M. bovis* were added to 10% Tween 20 solution and the bacteria were subsequently mixed on a vortex mixer to destroy the hydrophobic clumps of *M. bovis*. Then optical density was measured by using Spectrophotometer (Spectrophotometer, Jenway, United Kingdom) and adjusted to give optical density of 0.25 nm which is equivalent to 10^{8} cells per ml as describe by Hughes *et al*. (2006). Ten falcon tubes with 9 ml 10% Tween 20 solution were prepared labeled 1 to 10. Then ten folds serial dilutions were made (Table 1).

Table 1: Summary of serial dilution of *M. bovis* cells per ml

Dilutions	Number of cells per ml
1	10 000 000
2	1 000 000
3	100 000
4	10 000
5	1 000
6	100
7	10
8	0.1
9	0.01
10	0.001

Then 1 ml of milk was aliquot into 20 Eppendorf tubes (2 ml). This milk samples were obtained from SCITT negative cows with negative culture and negative PCR results: free from any *Mycobacteria* species. Eppendorf were labeled according to serial dilutions. Then 100 µl of each serially diluted cell were added into appropriate 1 ml milk in the eppendorf and mixed very well to ensure equal distribution of cells. Then half (500 µl) of this milk was transferred into another eppendorf and four sets of this kind were made. These four sets were subjected to concentrating methods such as Centrifugation and IMC technique. Two sets were concentrated by centrifugation at 14 000 rpm (Mikro 200, Hettick zentrifugen, Tuttlingen, Germany) and another set by IMC method which employ the used of antibodies specific for capturing *M. bovis* in sample. Then DNA was extracted using Qiagen DNeasy blood and Tissue kit from centrifuged and immunocaptured cell from spiked milk.

3.9 Immunomagnetic Capture

The technique involves the use of Dynal Magnetic beads M-280 (Dynal Biotech ASA, Oslo, Norway) precoated with a secondary antibody that recognizes the primary *M. bovis* antibodies. A 200 μl of Dynabeads were washed twice with PBS using the magnetic capture device and mixed with 200 μl polyclonal antibody (Rabbit polyclonal antibody, Thermo scientific, Rockford, United States) and rolled overnight at 4 ^{o}C. Also 500 μl of milk sample of each dilution was blocked overnight at 4 ^{o}C mixing with 500 μl 3% Bovine serum albumin (BSA). After overnight incubation beads were washed twice with PBS and resuspended in 200 μl PBS. Then 100 μl beads were added to blocked samples and immunocaptured by rolling (Dynal Biotech, Oslo, Norway) at 4 ^{o}C for overnight. Beads were recovered with the magnetic device and washed twice with PBS. The recovered beads were resuspended in 100 μl PBS. DNA was extracted from recovered beads by using DNeasy Blood and Tissue kit and assayed by Real Time PCR.

3.10 RD4 real time PCR

The techniques uses fluorescent (TaqMan) probe which discriminates *M. bovis* from other *M. tuberculosis* complex members since it hybridizes with both the 5 and 3 prime RD4 deletion flanking sequences, which only occur directly adjacent to each other in *M. bovis.* The reagents used in this study includes; Applied Biosystems 2 x TaqMan Environmental Master Mix 2.0 (Applied Biosystems, Foster City, United States), BSA 25 mg/ml (Sigma), forward primer *M. bovis* F ($^{5'}$TGTGAATTCATACAAGCCGTAGTCG$^{3'}$0.02 μM/μl), reverse primer *M. bovis* R ($^{5'}$CCCGTAGCGTTACTGAGAAATTGC$^{3'}$ 0.02 μM/μl) and *M .bovis* TM probe (6 FAM-AGCGCAACACTCTTGGAGTGGCCTAC—TMR 0.02 μM/μl). BCG Standards 1 to 7 ($8.5x10^{5}$/μl serially diluted to $8.5x10^{-1}$/$μl^{-1}$ to give a range of standards, labeled Standard 1 to Standard 7 respectively) were used. Master Mix was prepared in a sterile 30 ml universal tube for PCR 96 well plate (Table 2).

Table 2: Master Mix composition of RD4 Rt PCR for BTB diagnosis using

Master Mix contents	Volume(µl)
Applied Biosystems 2x TaqMan Environmental Master Mix	12.5
M. bovis Forward primer	0.5
M. bovis Reverse primer	0.5
M. bovis TaqMan probe	0.5
BSA 25 mg/ml	1
Total	15

Then 15 µl of prepared master mix was added to each well in 96 well plates. Then 10 µl of the diluted (1:10) BCG standard samples (Standard 1 to Standard 7) was added into wells in triplicate. MonoQ water of 10 µl was added to each of the No Test Control (NTC) wells and 10 µl of sample was added to each well in triplicate. The plate was Sealed with optical adhesive film and centrifuged at 1 250 rpm for 1 min. The plate was secured in ABI 7 500 fast Real time machine and run using the Absolute Quantification application with the standard parameters with exceptions that the annealing temperature was set at 58° C and the reaction volume was 25 µl. The information was entered accordingly and the machine commanded to run for 50 cycles. The results interpretation was based on the threshold value of 0.02; any sample which gave a sigmoid curve which goes above 0.02 was considered as positive for *M. bovis* and below the 0.02 as negative for *M. bovis* (Fig. 3). The cycle at threshold value is called C_T value and the BCG standards are used by the machine to plot and standard curve, which is used to find the concentration of DNA of each sample.

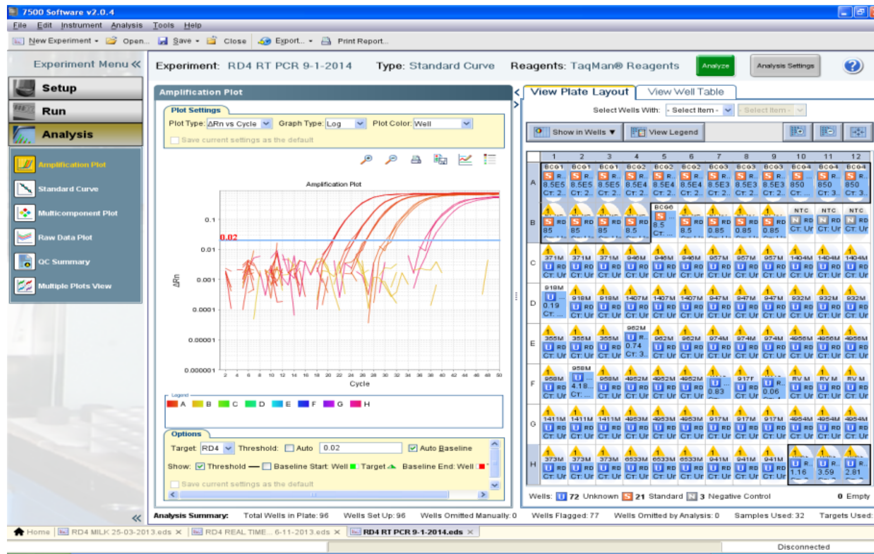

Figure 3: Results of Real time PCR amplification plot of milk sample.

3. 11 Data Analysis

Cohen's Kappa test was used to measure the agreement between SCITT and Bovigam and between shedding of *M. bovis* in milk and shedding in feces. Bellow is the formular for calculating the *Kappa* value:

$$\kappa = \frac{\Pr(a) - \Pr(e)}{1 - \Pr(e)}, \quad \ldots\ldots(3)$$

Where; Pr (a) is the agreement between compared techniques and Pr (e) the likelihood of random agreement. If techniques were in complete agreement $\kappa = 1$, If they disagree (or agree due to chance alone) $\kappa \leq 0$ (Cohen *et al*., 1960).

CHAPTER FOUR

4.0 RESULTS

4.1 SCITT, Bovigam and RD4 Rt PCR

A total of 63 cattle were tested for BTB using SCITT and Bovigam. RD4 Rt PCR was used to detect *M. bovis* DNA in feces of all 63 cattle and in milk from 35 cattle of 63 cattle. Two of 63 (3.17%) tested positive on both the SCITT and Bovigam, whereas 13/35 (37.14%) and 6/63 (9.52%) tested positive with RD4 Rt PCR in milk and feces respectively (Table 5). The two SCITT and Bovigam positive cattle were not lactating, but one was shedding *M. bovis* in feces. Two cattle were classified as inconclusive on the SCITT test. Two of these were shedding *M. bovis* in milk and one was shedding in feces (Table 4) but not in the milk of the SCITT negative cattle. Two tested positive in milk and feces, 8 tested positive in milk and negative in feces, 2 tested positive in feces but negative in milk, and 1 non-lactating cow tested positive in feces. The calculated *Kappa* value (*K*) (agreement) when SCITT and Bovigam were used together was 1 (Table 3) and the calculated Kappa value (K) (agreement) when RD4 Rt PCR was used in milk and feces was 0.07.

Table 3: Summary of agreement between SCITT and Bovigam

		SCITT		
		Positive	Negative	Total
Bovigam	Positive	2	0	2
	Negative	0	61	61
	Total	2	61	63

Table 4: Summary of agreement between RD4 Rt PCR results in milk and feces sample

		RD4 Rt PCR in milk		
		Positive	Negative	Total
RD4 Rt PCR in feces	Positive	2	2	4
	Negative	11	20	31
	Total	13	22	35

Table 5: Summary of results of SCITT, Bovigam, RD4 Rt PCR in milk and RD4 Rt PCR in feces

Diagnostic technique	No of cattle tested	Positive cattle detected	Percentage
SCITT	63	2	3.17
Bovigam	63	2	3.17
RD4 Rt PCR in milk	35	13	37.14
RD4 Rt PCR in faeces	63	6	9.52

4.2 Immunomagnetic Capture and Centrifugation

The IMC and Centrifugation were able to detect *M. bovis* from spiked milk samples. The minimum detection limit IMC was 100 to 1 000 *M. bovis* cells in 500 µl and by Centrifugation were 10 to 100 *M. bovis* cells 500 µl (Table 6).

Table 6: Summary of detection limit of *M. bovis* cells by centrifugation and IMC method coupled RD4 Rt PCR

Replicate 1	Cells concentration per 500 ul	Results C Rt PCR	Results IMC Rt PCR
1	100 000	detected	detected
2	10 000	detected	detected
3	1 000	detected	detected
4	100	detected	detected
5	10	detected	failed
6	1	failed	failed
7	0.1	failed	failed
8	0.01	failed	failed
9	0.001	failed	failed
10	0.0001	failed	failed
Replicate 2	Cells concentration per 500ul	Results Centrifugation	Results IMC
1	100 000	detected	detected
2	10 000	detected	detected
3	1 000	detected	detected
4	100	detected	failed
5	10	failed	failed
6	1	failed	failed
7	0.1	failed	failed
8	0.01	failed	failed
9	0.001	failed	failed
10	0.0001	failed	failed

CHAPTER FIVE

5.0 DISCUSSIONS

The BTB infection diagnosis in cattle in Tanzania is always performed by SCITT and meat inspection in abattoir and very little with bacteriological culture (Katale *et al.*, 2012). This study focuses on detection of *M. bovis* DNA in milk and feces using molecular methods. A total of 63 cattle from LITA farm were involved in this study. SCITT, Bovigam in blood and RD4 Rt PCR in faeces were applied together in all 63 cattle. Additional RD4 Rt PCR in milk was applied to 35 lactating cattle of 63 cattle. The study was able to detect shedding of *M. bovis* using RD4 real time PCR from milk (37.14%) and feces (9.52%) cattle. The study also found shedding of *M. bovis* in both milk and feces (3.17%).

Therefore, the findings of this study show that it is not necessary that shedding of *M. bovis* in milk and feces by cattle should occur simultaneously; cattle can shed in milk and not in feces and vise versa or can shed both in milk and feces (Appendix 1). The study also found that reactor positive by SCITT/ Bovigam does not indicate shedding of *M. bovis* in milk and feces. Furthermore, the study reveals that conventional methods used are not definitive methods if used in alone, so they are supposed to be used in conjunction to complement each other.

Several studies were conducted in Morogoro to establish prevalence of BTB using SCITT. A close individual prevalence of 3.7% was established using SCITT in Morogoro (Mwakapuja *et al.*, 2013) and another study also used SCITT in Morogoro and the prevalence was 3.0% (Shirima *et al.*, 2003). BTB prevalence study conducted at Gairo in Morogoro district using Bovigam test was 3.0% (Mariki *et al.*, 2013). In this study the

prevalence of BTB obtained using SCITT and Bovigam is comparable to previously mentioned studies. SCITT and Bovigam are all immunological techniques which elicit the cellular immune response. SCITT and Bovigam use PPD and the only difference is that, SCITT is an *in vivo* test which involves injection of PPD intradermal that induces delayed-types hypersentivity response. On the other hand, Bovigam is as well an *in vitro* test but involves incubation of PPD with the collected blood sample (de la Rua Domenech *et al.,* 2006). In this study, SCITT and Bovigam tests were found to agree with each other on diagnosis of BTB (Table 3). In some countries skin tuberculin test is used in conjunction with Bovigam test to improve sensitivity and specificity in chronic infected herds (de la Rua Domenech *et al*., 2006). Therefore in case of little fund one of each can be used for BTB diagnosis. However, the study found very little agreement between shedding of *M. bovis* cells in milk and feces (Table 4), indicating that shedding may occur in both milk and feces simultaneously or in either of them,

The low number of BTB cattle identified by SCITT is due to its sensitivity and specificity which lies between 52.0% and 100% and 78.8% and 100% respectively (de la Rua-Domenech *et al*., 2006). A previous study in Tanzania on 805 raw milk samples isolated 31 (3.9%) mycobacteria of which 29 (87%) were atypical mycobacteria and 2 (6.5%) were confirmed to be *M. bovis*. Other studies reported the recovering of *M. bovis* from milk of infected herds (Medeiros and others 2010); still there is a lack of studies directed towards the examination of the SCITT negative test cow's milk (Zarden *et al*., 2013). Studies in India isolated *M. bovis* in milk from six cattle where four cattle were SCITT negative (Srivastava *et al*., 2008). Another study in Brazil reported the identification of specific *M. bovis* DNA in 12 per cent of the milk samples obtained from SCITT positive test cows (Figueiredo *et al*., 2012). In Argentina, Zumárraga and others (2012) reported positive results employing PCR in milk samples from bulk tanks, from TB-suspected and also from

certificated TB-free herds. In those samples, no culture was obtained. In Brazil, Five (62.5%) out of the eight milk samples collected from negative SCITT tested cows were positive to *M. bovis*; four of them only by PCR, and only one by culture(Zarden *et al*., 2013). Hence, this study and other mentioned studies showed that there is a high chance of recovering *M. bovis* from milk samples.

In detection of *M. bovis* in biological samples, requires concentrating the bacteria cell, examples of concentrating methods includes Centrifugation and Immunomagnetic capture. A study was conducted on *M. avium* subsp. *Paratuberculosis* in spiked milk sample using centrifuge at 8 000 x g for 10 minutes to concentrate bacteria and Immunomagnetic capture. Samples were spiked with *M. avium* subsp. *paratuberculosis* organisms, which bound to Immunomagnetic beads and DNA was then analyzed by conventional and real time PCR in order to detect *M. avium* subsp. *paratuberculosis* in faeces and milk. Ten or fewer *M. avium* subsp. *paratuberculosis* organisms were consistently detected in milk (2 ml) and faeces (200 mg) samples (Khare *et al*., 2004). Another study was reported to concentrate *M. bovis* from soil, faeces and urine samples by centrifugation and Immunomagnetic capture coupled with RD4 real time PCR (Sweeney *et al*., 2006). In this study, centrifugation method and Immunomagnetic capture was used to concentrate *M. bovis* in spiked milk samples. This study determined the minimum detectable concentration of *M. bovis* by IMC and Centrifugation concentrating method in a spiked milk samples. The findings ranged from 100 to 1 000 *M. bovis* cells per 500 µl for IMC and 10 to 100 *M. bovis* cells per 500 µl for centrifugation method.

Other previous studies on using IMC have been successful in the selective recovery of *Streptomyces lividans* (Wipat *et al*., 1994) and *Streptosporangium fragile* (Mullins *et al*., 1995). Few studies using traditional culture techniques have successfully isolated

members of the *M. tuberculosis* complex from soil (Donoghue *et al*., 1997), whereas IMC has been used to isolate Mycobacterium paratuberculosis from milk (Grant *et al*., 1998) and to capture *M. tuberculosis* from cerebrospinal fluid (Mazurek *et al*., 1996). Finding from this study shows that centrifugation is a better technique for concentrating *M. bovis* cells in milk sample; however IMC can also be used for concentrating *M. bovis* cells for detection of BTB (Table 6). Molecular techniques such as the Real time PCR they are rapid, reliable, and highly sensitive alternative tools for the detection of many infectious agents **(**Malhotra-Kumar *et al*., 2008). Milk and feces samples can be obtained easily in live animal compared to tissue samples so application of molecular techniques will help for rapid disease diagnosis. SCITT and Molecular techniques such as Real time PCR applied in conjunction enhanced the efficacy of BTB diagnosis for better control and management of tuberculosis.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

i. This study showed the need of using molecular techniques such as Real time PCR in the diagnosis of BTB infection in cattle in Tanzania.

ii. The study has shown SCITT and Bovigam correspond to each other in diagnosis of BTB infection in cattle, so either test can be used for the diagnosis of BTB infection in animals but considering their own merits like cost and repeat access to animals.

iii. The study revealed that SCITT does not indicate *M. bovis* shedding in milk and feces.

iv. *M. bovis* in feces indicates the possible contamination of the environment and cattle carcasses during slaughter process to *M. bovis*, thus molecular techniques such as Real time PCR can be used to monitor environmental and carcasses contamination in slaughter houses.

v. Confirmation of *M. bovis* in milk also indicates the potential root of transmission of *M. bovis* from cow to calf also to human who consume raw milk.

6.2 Recommendations

i. Based on the difference in BTB prevalence detected by each test, this study recommends the use of SCITT or Bovigam in parallel with Real time PCR where

possible, it is rather new technology and cost of machine and reagents make it not easily adaptable in developing countries.

ii. *M. bovis* in milk is of public health importance, the study recommends more studies should be done to investigate the transmission of BTB to young calves and animal handlers.

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APPENDIX

Appendix 1: Summary of cattle IDs which were positive by either one or more test

S/N	Sample ID	SCITT	RD4 RT PCR in milk	RD4 RT PCR in faeces
1	941	Negative	Positive	Positive
2	925	Inconclusive	Positive	Negative
3	408	Inconclusive	Positive	Negative
4	917	Negative	Negative	Positive
5	355	Negative	Positive	Negative
6	909	Positive	No milk	Positive
7	138	Positive	No milk	Negative
8	945	Negative	Positive	Positive
9	395	Negative	Positive	Negative
10	MF 439	Inconclusive	Negative	Positive
11	340	Negative	Positive	Negative
12	4951	Negative	No milk	Positive
13	438	Negative	Positive	Negative
14	141	Negative	Positive	Negative
15	371	Negative	Positive	Negative
16	975	Negative	Positive	Negative
17	488	Negative	Positive	Negative
18	395	Negative	Positive	Negative