EVALUATION OF MULTIPLEX PCR FOR DETECTION OF *MYCOBACTERIA TUBERCULOSIS* COMPLEX MICROORGANISMS ISOLATED FROM CATTLE TISSUES AND HUMAN TUBERCULOSIS CASES TANZANIA

BY

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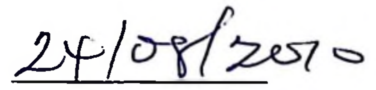
ABSTRACT

A study on the evaluation of multiplex PCR on detection on *Mycobacteria tuberculosis* complex was carried on samples obtained from Morogoro district. Specimen collected from abattoir in Morogoro Municipal and human cases from Muhimbili Research Station were analysed at the Faculty of Veterinary Medicine Laboratory by Multiplex PCR. A total of eight nine-pooled lymph nodes samples collected from none tuberculin tested slaughter cattle in the study area-Morogoro abattoir were cultured and Seventy two (80.89%) yielded mycobacteria. Seventeen were identified as *M.bovis* and three as *M.tuberculosis.* The remaining fifty two were mycobacteria other than tuberculosis (MOTTs). A total of forty five isolates from human sample were submitted for subculture and thirty eight (84%) were positive for mycobacteria. Twenty five isolated were identified as *M.tuberculosis*. The remaining thirteen were mycobacteria other than tuberculosis. The IS986 and mtp40 multiplex PCR was able to differentiate *M.bovis* from *M.tuberculosis.* The finding of this therefore necessitates the collaboration of veterinary and medical professions to the control transmission of *M.bovis* infection in human and animal populations.

DECLARATION

I, Harrison Sadiki Gabrieli, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work and has not been submitted for the degree award in any other university.



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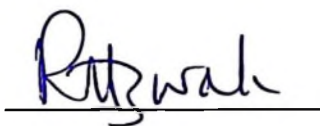

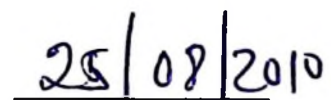

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To my lovely wife Paulina Paul Mosha and our two lovely son Harrison Jr. and Gordon. For their love and proper care during my course of study.

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LIST OF ABBREVIATIONS

AFB	-	Acid Fast Bacilli
bp	-	Base Pair
BTB	-	Bovine Tuberculosis
DNA	-	Deoxyribonucleic Acid
DNTP's	-	Deoxyribonucleoside Phosphate
ELISA	-	Enzyme Linked Immuno-Sorbent Assay
EP	-	Extra Pulmonary
IU	-	International Units
M	-	Mole
Mg	-	Milligram
mm	-	Millilitre
MOTTs	-	Mycobacteria Other Than Tuberculosis
M.b	-	*Mycobacterium Bovis*
M.tb	-	*Mycobacterium Tuberculosis*
Mtp40	-	Mycobacterium Tuberculosis Protein 40
PCR	-	Polymerase Chain Reaction
PPD	-	Purified Protein Derivatives
rmp	-	Revolution Per Minute
SCIT	-	Single Comparative Intradermal Test
SIT	-	Single Intradermal Test
SUA	-	Sokoine University of Agriculture
TBE	-	Tris-HCL, Boric Acid and EDTA

V	-	Volt
ZN	-	Ziehl-Neelsen
v/v	-	Volume By Volume
v/w	-	Volume By Weight
#	-	Number

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Bovine tuberculosis (BTB), caused by *Mycobacterium bovis*, is a highly prevalent disease in developing countries. In developed countries the disease is kept under control through rigorous eradication measures, though it is still under surveillance. In countries where BTB has been eradicated or brought under control in cattle, the test and slaughter control approach is being employed. Pasteurization of milk and strict meat inspection help facilitate the control of this disease in human (Caffrey, 1994; Grange and Collins, 1987).

Little is known about the prevalence of BTB in the human population in Tanzania. However, Kazwala *et al.* (2001) identified 16% of the mycobacterial isolates from human samples in Tanzania as *M. bovis*, whereas Mfinanga *et al.* (2004) demonstrated 10.8% of mycobacterial isolates from human samples to be *M. bovis*. On the other hand, more research has been carried out on the presence of BTB in cattle. This disease was demonstrated for the first time in Tanzania by the use of the single comparative intradermal tuberculin test (SCITT) by Markham in 1952. Other studies using SCITT showed a prevalence of 14% in pastoral cattle on the Usangu plains (Kazwala, 1996). 0.2% in the Lake Victoria zone Jiwa *et al.* (1997). In the eastern zone of Tanzania 1% and 2% in the pastoral and the intensive sector respectively Shirima *et al.* (2003). Mdegela *et al.* (2004) found a prevalence of 0.4% and 1.7% in Kibaha and Morogoro respectively, and Cleaveland *et al.* (2007) reported 0.9% of cattle tested in the Manyara region to be bovine tuberculosis reactors on SCITT.

M. bovis and atypical mycobacteria were isolated respectively from 2 and 31 out of 805 milk samples taken from pastoral cattle in villages in the Southern Highlands of Tanzania (Kazwala *et al.*, 1998) and atypical mycobacteria from 14% out of 109 milk samples in Kibaha and Morogoro (Mdegela *et al.*, 2004).

These studies have shown that BTB is still present in Tanzania and that control measures are required to control and prevent both livestock and human infections. Although the tuberculin tests can identify cattle with BTB effectively, the disease has not been eradicated from many countries (Amanfu, 2006). One of the main reasons for this is the lack of knowledge about the wildlife reservoir hosts (Gortazar *et al.*, 2007). The intradermal tuberculin test is based on the delayed type of hypersensitivity reaction developing at the site of intradermal inoculation with maximum sensitivity at 72 hours post injection (Francis, 1947; Ritchie, 1953).

Tuberculin inoculated simultaneously, but at separate sites in the neck, the agent causing the sensitization provokes the skin reaction. Is easy to perform on a large number of livestock but it has inconvenience of having a broad range of sensitivity and specificity (O' Reilly, 1992; Arend *et al.*, 2001), estimates of sensitivity of tuberculin tests in cattle range from 32 to 99% and specificity 75.5 to 99.9% (Oloya *et al.*, 2006).

Postmortem examination is very crucial in the diagnosis of bovine tuberculosis prior to bacteriological confirmation of the disease by isolation of *Mycobacterium bovis*. However, pathological lesions may resemble nocardiosis, actinomycosis and old parasite infection (Sewell and Brocklesby, 1990) hence, and making diagnosis by this method difficult. Culture is considered to be the gold standard and the definitive test for diagnosis of

tuberculosis, but isolation of *M.bovis* and *M. tuberculosis* complex is an extremely slow procedure which may take as long as 4 – 6 weeks (Marks, 1976). Furthermore, an addition of two to three weeks is required for the biochemical identification of the isolates. It is also known that the sensitivity of culture is not 100% and false negative results may occur (Duffield *et al.*, 1989).

The prolonged time interval between exposures to an infectious agent to the diagnosis of the disease makes tracing of the source of infection difficult. Therefore, this creates a need for rapid, specific and sensitive techniques for the detection of mycobacterium in different types of specimens. Different conventional approaches on diagnosis of the positive individual have been used.

The advent of molecular biology techniques in recent years is reported to have provided rapid, sensitive and specific diagnosis of many diseases. The techniques are particularly useful for the rapid detection of fastidious or slowly growing pathogens like mycobacteria (Miller *et al.*, 1997; Sreevatsan *et al.*, 2000; Patnaik *et al.*, 2001). These methods involve DNA amplification of specific sequences in the genome of an organism by polymerase chain reaction (PCR). This has reduced the time taken for achieving the definitive diagnosis and so allowing early tracing of the source of infection. Multiplex PCR has been developed which is able to distinguish *M. tuberculosis* from other members of the *M. tuberculosis* complex (Sinclair *et al.*, 1998).

The assay is based on the simultaneous amplification of two different targets: a 396bp region from the mtp40 species-specific gene sequence of *M.tuberculosis* and a 245bp fragment from the *M.tuberculosis* complex insertion sequence IS986 (Sinclair *et al.*, 1995).

The purpose of this work was to apply multiplex PCR typing technique directly on DNA isolates from tissue specimens so as to detect the presence of *M.tuberculosis* complex organism.

1.2 Justification

Tuberculosis is an endemic disease affecting both humans and animals. In the past, differential diagnostic tools, methods and technique have been employed in the differentiation and typing of Mycobacteria. However, most of these methods lacked specificity and sensitivity. The role of Mycobacteria outside the tuberculosis complex (MOTTs) in causing human and animal diseases is also increasingly recognized especially as secondary to HIV infection in humans.

In this study a Multiplex PCR technique to distinguish species of *M.tuberculosis* complex was preferred, as it is rapid and sensitive technique. *M.bovis* is known to be among the causative agents of tuberculosis in humans. A selected collection of isolates from human cases cultured at Muhimbili Research Centre were included to provide knowledge regarding the sensitivity of detecting *M.bovis* in human samples. Hence the Multiplex PCR for typing Mycobacteria isolates from abattoir and human specimens to the species level will be carried out.

1.3 Objectives

1.3.1 Main objective

To detect the presence of *M.bovis* in cattle and human using molecular method.

1.3.2 Specific objectives

(i) To detect the presence of *M.bovis* from the human isolates using multiplex PC technique.

(ii) To detect *M.bovis* from *M.tuberculosis* complex isolates from the abattoir specimen using multiplex PCR.

(iii) Comparison of Multiplex PCR against conventional biochemical test, in distinguishing *M.tuberculosis* complex among mycobacterium genus organisms.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Bovine Tuberculosis

2.1.1 Definition

Bovine tuberculosis is a contagious and chronic bacterial disease caused by *M.bovis* and affects cattle and other animals. The bovine tubercle bacillus has broad host of ranges that include humans, domestic and wild animals (Grange and Collins, 1987; Pritchard, 1988; Radostits *et al.*, 1994). Human acquires infection through ingestion of raw products of animal origin and inhaling aerosols from infected animals.

2.1.2 Aetiology

M.bovis, the aetiological agent of bovine tuberculosis, is a slow-growing nonphotochromogenic acid-fast bacillus (Thoen *et al.*, 2004). It causes the disease in most warm-blooded vertebrates including man.

M.tuberculosis is the most common cause of human tuberculosis. It rarely causes progressive disease in lower animals other than non-human primates. *M.avium* complex is the only species pathogenic to birds but it has also been isolated from pigs, cattle sheep, mink, dog, cats and some cold-blooded animals (Inderlied *et al.*, 1993; Shinnick and Good, 1994; Wayne, 1994), and also from immuno-compromised human.

2.1.3 Classification

Bergey's manuals of determinative Bacteriology (9th Edition-1994), have been categorized species of mycobacteria into two main groups on the basis of their growth characteristics.

These groups are the slow growing species of mycobacterium and the rapid growers. The slow growing species are *M.tuberculosis* complex bacteria that comprise of *M.tuberculosis, M.bovis* and *M.microti* and *M.africanumi* (van Soolingen *et al.,* 1997). These organisms take 4-6 weeks to appear on Lowenstein-Jensen medium (Marks, 1976, Drobniewski, 1994). The majorities of mycobacteria other than tuberculosis (MOTTs) are rapid growers and will produce colonies on the surface of solid medium in two weeks (Marks, 1976). This group includes *M.avium* and all mycobacteria other than tuberculosis.

Various attempts have been made to group mycobacteria based on pigments production, growth rate, biochemistry, pathogenicity and genetic studies. Most mycobacteria are free living and only a small proportion causes disease. The MOTTs are genetically heterogenous and are distinguished into four groups based on pigment production and growth rate; Photochromogens, Scotochromogens, Achromogens and rapid growers. Members of this group can be isolated from environmental sources, such as pond water.

2.1.4 Bacteriological description

The bacteriological description of *Mycobacterium tuberculosis* complex bacteria is that they are fastidious, slow growing, strictly aerobic *(M.tuberculosis)* or microerophilic (*M.bovis*), lipid rich, hydrophobic and acid-fast (AFB) bacterial rods (Wayne, 1994).

The major causative agent of bovine tuberculosis is *Mycobacterium bovis*, which is an acid fast, non-motile and non-spore forming bacillus, which is either slender-rod shaped, straight or curved and relatively resistant to chemical disinfectants (Gracey, 1986).

It has thick cell wall containing mycolic acids that are responsible for maintaining basic dyes such as carbolfuschin and resisting de-colouration with dilute acids (Daniel *et al.*, 1994; Wayne, 1994). The latter characteristics feature forms the basis for the Ziehl-Neelsen stain used in the diagnosis of mycobacteria. However, other agents such as Nocardia, Corynebacterium and Rhodococcus spp also show some acid-fast features (Pritchard, 1988), and hence they can be confused with *M.bovis*. The surface layer consists of a diffused capsule, which enables the organisms to survive in adverse environments. For example, *M.bovis* can remain viable in meat stored at 10° C for over two years and 18 days in pickled meat (Gracey, 1986).

Although there is no direct evidence of the survival of *M.bovis* in cooled milk, studies on butter showed that the organism can remain viable at 4-6°C for a number of days (Sinha, 1994).

The growth of *M.bovis* is enhanced by pyruvate in Lowenstein-Jensen media under microerophilic environment. Biochemically, *M.bovis* strains have no ability to reduce nitrate into nitrite and, are unable to hydrolyse pyrazinamide into pyrazinoic and ammonia production (Collins and Grange, 1983).

2.2 Epidemiology

2.2.1 Distribution in livestock and wild animals

The available information shows that bovine tuberculosis affects 10 – 20% of cattle in majority of countries in Africa, Asia and Latin America (Cosivi *et al.*, 1995; Stella; 1995; Thoen and Stella, 1995).

Following eradication programmes in many countries of Nothern America and Europe, the incidence of bovine tuberculosis have been reduced to the extent that some were declared free from the disease (Griffin and Dolan, 1995). Surveys in United States, Scandinavia and South England shows these countries altogether, have less than 1% tuberculosis cases (Grange and Yates, 1994). However, current data in Great Britain shows the increased herd incidence amongst unrestricted herds from 1.3% in 1999 to 3.5% in 2003 (DEFRA. 2004).

Epidemiological data on wildlife has shows that *M.bovis* infection are present throughout the world in any area where livestock are raised, and in most situations these cases have been considered to be spillover from infected domestic populations (Mclnerney, *et al.*, 1995 and Michel. 2002). Several cases of tuberculosis in wildlife have been reported from National parks and Game Reserves. In Kruger National Park in South Africa, *M.bovis* infection was reported to spread among African buffaloes (*Syncerus caffer*) (Keet *et al*,. 1996). A similar case of *M.bovis* infection in buffaloes was reported in Uganda in the Ruwenzori National Park where a prevalence of 10% was found among buffaloes (Woodford, 1982). In Zambia, a prevalence of 36% *M.bovis* infection was found in Kafue lechwe (*Kobus leche kafuensis*) entelope population (Gallagher *et al.*, 1972).

The presence of *M.bovis* infection in wildlife is not only confirmed to the wild ungulates, but also in other species of wild animals. In Narok district, Southwest Kenya, the wild Olive baboons (*Papio cynocephalus anubis*) were found to have tuberculosis due to *M.bovis* infection (Robert and James, 1987). In Michigan USA *M.bovis* was isolated from carnivores and omnivores. The organisms were also isolated from free-ranging cheetah (*Acinonyx jubatus*), Lions (*Panthera leo*) and Chacma baboon (*Papio ursinus*) in Kruger National Park (Keet *et al.*, 1996). A similar case of tuberculosis in carnivores was reported

in Spain where *M.bovis* was isolated from free-living Iberian lynx (*Lynx pardinus*) (Victor *et al.*, 2000). In recent years, free ranging wildlife reservoir hosts have been identified. These species include North American bison (*Bison bison)* (Nishi *et al.*, 2002), African buffalo (*Syncerus caffer*) (Michel, 2002).

While many species of animals can become infected with *M. bovis*, only a few wild animals may act as reservoirs for bovine tuberculosis which could complicate the control and eradication programmes (O'Rielly and Daborn, 1995). In United Kingdom and Ireland, badgers (*Meles meles*) were implicated in outbreak of tuberculosis (Nolan and Wilesmith, 1994). In New Zealand, bush tailed possum (*Trichosurus valpecula*) were said to be the source of infection in a number of tuberculosis free herds (Collins *et al.*, 1986).

In Africa, bovine tuberculosis is prevalent in 33 out of 40 countries, (Chillaud, 1995) but there is great variation in the prevalence of the disease between these countries in livestock. For example, in Uganda the prevalence reported was 27% in 1975 (Nyakuhama and Margaret, 1995) where as in Malawi a rate of 22.3% was encountered in 1980 (Chizonda, 1995) and lowest (0.6%) was reported in South Africa in1986 (Thoen and Steele, 1995).

In Tanzania, bovine tuberculosis was reported for the first time just after the First World War (WWI) among the animals imported from South Africa (Hornby, 1934). Further information was obtained in cattle in the Northern Province (now Kilimanjaro) where the prevalence of infection was found to be 1.8% (Cornell, 1934). The first report of tuberculosis in indigenous cattle (*Bos indicus*) was from an abattoir meat inspection report in Iringa. Studies carried in the same zone by (Kazwala, 1996) in pastoral cattle and (Maiseli *et al.*, 1989) in dairy cattle revealed the prevalence of 14% and less than two

percent, respectively. The disease has been observed in slaughter cattle in Morogoro abattoir (Matovelo, 1995).

2.2.2 Distribution in humans

In humans, *M.bovis* infection is the commonest cause of cervical lymphadenitis and other forms of extra pulmonary tuberculosis that occur in infancy and childhood age groups (Sjogren and Hillerdal, 1978).

Children acquire the infection through drinking infected raw milk from cattle and in adults; aerosol transmission is a common route of infection (Danker and Davis, 2000). A survey along the United States-Mexico border revealed that 10.8% of all cases of tuberculosis in children there were caused by *M.bovis* infection, and that 33.9% of 180 positive cultures from the individual patients proved to be *M.bovis* (Danker and Davis, 2000).

Current reports from developing countries indicate that the incidence of extra pulmonary tuberculosis is on the increase, and this is attributed to lack of or inadequate appropriate control measures and high rates of the HIV infection in places like Africa, where *M.bovis* is enzootic in several countries (LoBue, *et al*., 2003). For example, in Ethiopia of 29 cases of tuberculosis, 17.1% were caused by *M.bovis* infection (Dawit *et al*., 2004). South Africa the rate was 7.2% (Thoen and Steele, 1995).

Currently, human tuberculosis has been observed to be a secondary infection in immuno-compromised patients suffering from HIV/AIDS. Since extra pulmonary tuberculosis is often associated with livestock and livestock products, keeping infected animals could pose a great public health risk in the wake of the current HIV/AIDS pandemic. Increased human

populations and living in close proximity to wildlife has a great chance of transmitting the disease to livestock as they are sharing the pasture and water sources, also livestock keepers consumption habits on the raw blood, milk and undercooked meat can increase chances of infection to human. In Tanzania, regions with cattle population have high prevalence of extra-pulmonary tuberculosis than the national average, which stands at 15.9% (Kazwala *et al.,* 1993). Under the current situation therefore, (Darbon and Grange, 1993), urged more efforts to be made to assess the contribution of mycobacterial infection derived from cattle to human.

2.2.3 Transmission in cattle

In cattle, common routes of infection are respiratory and alimentary, while congenital, genital and percutaneous are less common. However, inhalation has been found to be the major route of infection in cattle; accounting for 80-90% of infections (Alhaji, 1976; Morris *et al.*, 1994).

Housed or crowded cattle are at risk of acquiring the disease through inhalation of air contaminated with droplets from infected cattle. Crowding pastoral cattle in the kraals during the night and in communal water points enhance the transmission through inhalation (Clay, 1971; Radostitis *et al.,* 1994). Further more, it has been speculated that trekking animals over a long distances especially in dustpaths to grazing areas and communal water points enhance aerosol transmission (Clay, 1971; Radostitis *et al.*, 1994; Griffin and Dolan, 1995).

Infection through ingestion is possible when pasture or water is contaminated with faeces or discharges from infected cattle (Clay, 1971; Radostitis *et al.,* 1994). In calves, the

principle source of infection is through ingestion of milk from tuberculous cattle (Gracey, 1986; Neill *et al.*, 1994).

Concern is now being raised over the possibility of the so called 'reverse zoonoses' where cattle might be infected with human –derived (*M.bovis/M.tuberculosis*) in the wake of the HIV/AIDS pandemic (Daborn and Grange, 1993). Man to man transmission of *M.bovis* is also possible, only that in area where the disease occurs in cattle, it is difficult to ascertain the proportion of human *M.bovis* infection attributable to man to man transmission (Grange and Collins, 1987; Hardie and Watson, 1992).

2.2.4 Pathogenesis

All animals species including humans are susceptible to *M.bovis*, with cattle, goats and pigs being the most susceptible, whereas as sheep and horses show some resistance to the disease (Radostits *et al.*, 1994). The spread of *M.bovis* infection in cattle involves two stages namely, the primary complex formation and post-primary dissemination. The primary complex consists of lesions at the port of entry and in the local lymph nodes, and is found in the lungs and associated lymph nodes if the infection is through inhalation also in the pharyngeal and mesenteric lymph nodes for the oral route of infection (Gracey, 1986).

The primary complex develops within 8 days and becomes calcified within two weeks (Radostits *et al.*, 1994). The complex may resolve into the connective tissue or become encapsulated by fibrous tissue. (Jubb *et al.*, 1993) noted that the complex may remain unchanged for a long time in the animal.

Following primary complex stage, post-primary dissemination ensues which may take the form of acute military tuberculosis affecting other parts of the animal's body. The spread of infection is influenced by the virulence of the mycobacteria, the susceptibility of the host and the port of entry.

2.2.5 Clinical manifestation in cattle

Clinical disease in cattle is very difficult to diagnose because it takes the form of chronic disorder that is difficult to differentiate from other conditions. Clinical signs of loss of body condition, coughing, swelling of peripheral superficial lymphnodes and low-grade intermittent fever are indicative of tuberculosis in affected individuals (Radostits *et al.*, 1994).

However, these signs are not pathognomonic to tuberculosis as other conditions such as starvation, severe parasitism, trypanosomiasis, chronic metrirtis and the effects of old age may share the same diversity of symptoms (Radostitis *et al.*, 1994; Steele, 1995). Findings suggesting tuberculosis after carrying out the test, is achieved through auscultation, percussion, body temperature measurement and palpation of the mammary gland and superficial lymph nodes (Radostitis *et al.*, 1994).

2.2.6 Diagnosis in cattle

2.2.6.1 Tuberculin testing (antermortem diagnosis)

The intradermal tuberculin test is based on the delayed type of hypersensitivity reaction developing at the site of intradermal inoculation with maximum sensitivity at 72 hours post injection. Various forms of tuberculin have been used in the diagnosis and control of bovine tuberculosis (O' Reilly, 1995). There are two kinds of intradermal tuberculin tests

commonly used. The single intradermal test (SIT) and the single comparative intradermal test (SCIT). SIT uses bovine tuberculin alone, while SCITT uses both avian and bovine tuberculin (Francis, 1947; Ritchie, 1953).

Further advancement involved precipitation of tuberculo-protein with trichloro-acetic acid or ammonium sulphate to remove impurities and this resulted into a product referred as Purified protein derivatives (PPD), currently used in the field (Francis *et al.,* 1978). These are products from *M.bovis* strains (e.g.D4ER or TB56 strains).

2.2.6.2 Post-mortem diagnosis

Another way of achieving tuberculosis diagnosis in animals is by postmortem examination of carcasses. The postmortem findings of tubercle formations in various organs such as the lungs, lymph nodes, liver, and spleen are suggestive of tuberculosis infection (Sewell and Brocklesby, 1990; Radostits *et al.,* 1994).

2.2.6.3 Diagnosis of Mycobacterial spp. infections

2.2.6.3.1 Direct smear microscopy and culture

Direct detection of mycobacteria by microscopic examination of ZN stained smears of clinical specimens may reveal acid-fast organism but is not a sensitive technique (Sewell and Brocklesby, 1990). Hence, the confirmation of diagnosis is done by culture and biochemical tests. Lymphnodes and tuberculous organs are submitted for culture or animal inoculation (Wood *et al.,* 1992). The mycobacteria from primary culture can therefore be identified using a set of biochemical tests such as Niacin test, oxygen performance, nitratase activity and susceptibility of the organisms to pyrazinamide, para-nitrobenzoic acid (PNB) (Collins *et al.,* 1985).

2.2.6.3.2 Serological diagnosis

Serological tests that are widely used in detecting tuberculosis in live animals are those determining antibody responses in an animal. These tests include, Enzyme linked immunosorbent Assay (ELISA), Indirect fluorescent antibody test (IFAT), and Glutaraldehyde test (Lepper *et al.*, 1973; Lepper and Pearson, 1975; de Kantor, 1993).

Among serological tests, ELISA is more famous and has been widely used to detect antibodies (IgG) produced by *M.bovis* infection. In general serological tests suffer from low sensitivity and specificity (Wood *et al.,* 1992; Watt *et al.,* 1993).

2.2.6.3.3 Fluorescence Polarization Immunoassay

The fluorescence polarization assay (FPA) as an antermortem test, detects circulating antibodies against *M. bovis* in a one step procedure. The main advantages against the comparative intradermal tests (tuberculin test) are: The animals needs to be seen only once, fast and easy to perform, economical and is more accurate, also has been adapted for use in the field, and highly reproducible across laboratories and instruments, and reduces the human error and variability that occurs when reading test like- agglutination test, Elisa, etc.

The entire essay is done in solution, in a single tube with no precipitation or washing steps (Nielsen *et al.,* 1996). Current research is focused on the identification of antibodies such as secretory proteins of *M. bovis* for use in improved diagnostic test. However little is known about its comparative sensitivity against the intradermal test.

2.2.6.3.4 Molecular biology

The advent of molecular biology techniques in recent years is reported to have provided rapid, sensitive and specific diagnosis of many diseases. The techniques are especially useful for the rapid detection of fastidious or slowly growing pathogens like mycobacteria (Miller *et al.,* 1997; Sreevatsan *et al.,* 2000; Patnaik *et al.,* 2001). Mycogenus PCR technique has been developed to detect the Mycobacteria in the isolates more rapidly and also replaces the biochemical test (Personnel communication with Ally Kitime, Principle technician, Faculty of Veterinary Medicine, 2009)

Multiplex PCR has been developed which is able to distinguish *Mycobacterium tuberculosis* from other members of the *Mycobacteria tuberculosis* complex. The assay is based on the simultaneous amplification of two different targets: a 396bp region from the mtp40 species-specific gene sequence of *M.tuberculosis* and a 245bp fragment from the *M.tuberculosis* complex insertion sequence IS986. Multiplex PCR reaction combining amplification of the *M.tuberculosis* complex specific IS986 and the *M.tuberculosis* specific mtp40. The procedure detects all members of the *M.tuberculosis* complex and simultaneously identifies and discriminates *M.tuberculosis*, avoiding the needs for further tests. (Sinclair *et al.,* 1995).

2.3.1 Control in human

Tuberculosis control in humans relies on interventions that will both cure patients and interrupt disease transmission. As with other contagious diseases, specific tuberculosis control measures is based on our understanding of the mode of transmission has to be applied depending on the route of infection. When tuberculosis in human is caused by *M.bovis* the goals of the health department are the same curing patients and interrupting

transmission. However, investigative procedures and interventions are more varied because *M.bovis* can be transmitted to humans from a number of mammals, including other humans, and because there are several potential routes of transmission, including gastrointestinal (Wilkins *et al.,* 2003) airborne and direct inoculation (Grange, 2001).

From the public health perspective, eradication programs in cattle and universal pasteurization of milk remain the mainstays of prevention the disease in humans that is caused by transmission from cows. These measures should be augmented by public education efforts explaining the dangers of consuming un-pastureurized dairy products in areas where *M.bovis* disease in humans is more common.

2.3.2 Control in cattle

Control of disease depends on several factors such as incidence of the disease; husbandry methods, public health implications and economic capacity of the country to incur losses from test and slaughter programmes (Radostits *et al.,* 1994). The test and slaughter programme has been proved to be an effective method of controlling bovine tuberculosis (Pritchard, 1988; Radostits *et al.,* 1994). Control in an infected herd is based on removal of positive reactors through test and slaughter coupled with prevention of re-introduction of infected animals. In herds where positive reactors are too numerous during the eradication programme, separation of non-reactors from reactors animals should be done. All positive reactors should be slaughtered and non-reactors re-tested after six months (Radostits *et al.,* 1994). Along with this, the separations of calves from reactors animals at birth and use of milk free from tuberculosis (Pritchard, 1988) have been helpful in the control of the disease.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Population

Cattle destined for slaughter at the Morogoro municipal abattoir, the selection of cattle and samples for this study was based on co-operation with the businessmen deals with butchers in Morogoro for the permission to collect samples from the carcasses post-slaughter and co-operation with meat inspectors during routine postmortem gross inspections.

3.2 Sampling Technique and Sample Size

Sampling technique was purposive sampling, collecting all organs with visible and palpable tuberculous lesion, and all condemned organs due to tuberculosis. Samples were collected only from the carcasses/organs with visible and palpable tuberculous lesion. The goal was to collect tuberculous lesion samples to yield at least one hundred and fifty (150) *M. bovis* for molecular studies.

3.3 Source and Type of Specimens

Animal specimens of tuberculous lungs, lymph nodes, spleen and liver collected from Morogoro Municipal abattoir. Other samples were cultured mycobacterial species of human tuberculosis cases from Muhimbili National Hospital, in Dar es Salaam. These isolates were cultured, sub cultured and subjected to biochemical tests, and then followed by DNA extraction for multiplex PCR at the Mycobacterium Laboratory Faculty of Veterinary Medicine, Sokoine University of Agriculture.

3.4 Collection of Specimens

3.4.1 Lymph nodes

The major lymph nodes of the respiratory and alimentary system were collected from slaughtered cattle at routine postmortem gross inspection. The lymph nodes involved were retropharyngeal, submandibular, bronchial, mediastinal, mesenteric lymph nodes. The pooled lymph nodes from each animal were placed in a 25ml screw capped plastic universal container. The containers were labeled properly with the tag number or roman letter of the respective animal, and then stored in the freezers for two months at –20°C prior to processing and inoculation.

3.5 Bacteriology

3.5.1 Sample processing

Samples received from Morogoro Municipality abattoir were stored in freezers at –20° C up to the time of processing. All the processing of specimen from cattle and human culture was carried in the class one safety cabinet.

After one hour of thawing, a pool of lymph nodes from abattoir where taken from the containers aseptically using sterilized forceps and placed in sterile containers. Using sterile scalpel blades, samples were further macerated to obtain fine pieces, which where divided into two portions. One portion was stored at –20°C for future reference, while the other portion of chopped lymph nodes was placed in stomacher bag containing about 5ml of distilled water and homogenized for two minutes using a stomacher 80 Lab blender (Seward Laboratory-London, U.K).

The lymph nodes homogenate was then put into a universal container and 3% oxalic acid was added equal volume to the universal bottle for decontamination. Decontamination was carried out for 30minutes with intermittent shaking. The duration of the decontamination step was increased depending to the level of contamination. After the allotted decontamination time, the lymph node homogenate was centrifuged at 1500g for 10minutes and the supernatant discarded into disinfectant. Then 2% NaOH mixed with an indicator-phenol red was added drop by drop to neutralize the pH of the suspension.

Neutralization was achieved when the suspension colour changed from purple to pink. Suspension were then centrifuged and the supernatant discarded to leave the sediment were by 2ml were used as inoculum for the cultivation of mycobacteria.

3.5.2 Microscopy

A loopful of the sediment from the above procedure was spread on a glass slide and heat fixed using hotplate within the safety cabinet. Glass slides were then placed on a rack over a sink for ZN staining in a similar manner to that described by Grange (1988). The observation of acid-fast-bacilli (AFB) was carried out at X100 magnification and results recorded. As a quality control for the staining technique, the stored isolate from previous study were subjected to the staining before staining the isolates for this study.

3.5.3 Culture

3.5.3.1 Media preparation

For primary isolation of mycobacteria, the egg media namely; International Union against Tuberculosis (IUT) and Lowenstein Jensen with added pyruvate (L-J pyruvate) were used. The media compositions were as follows: i) International Union against Tuberculosis (IUT)

medium was made up of 61.0% whole egg, 36.6%(v/v) IUT buffer salt solution (50Mm K_2PO_4; 25Mm $Na_2HPO_4.2H_2O$; 1.6Mm $MgSO_4.7H_2O$; 14Mm citric acid; 67mM L-Asparagine; 0.2% glycerol) and 2.4% (v/v) of 1% (w/v) malachite green and ii) L-J pyruvate medium was made of 64.6% whole egg, 32.3% (v/v) pyruvate medium buffer salt solution (50Mm K_2PO_4; 25Mm $Na_2HPO_4.2H_2O$; 114Mm sodium pyruvate; 14Mm citric acid), 2.6% v/v of 1% (w/v) malachite green and 0.6% (v/v) of 1% (w/v) trypan blue.

After mixing all the components, 4ml of the medium was dispensed into 30ml glass universals and then insipissated at 85°C for one hour to solidify. IUT medium had a pale green colour, while L-J pyruvate medium was pale blue. As a quality control for the media before inoculation the media were left in a room temperature for two days to check for any sign of contamination.

3.5.4 Cultivation of Mycobacterium

About 0.1ml of the sediments from each sample was spread on the surface of each media using a sterile disposable pipette and, in order to avoid sedimentation of inoculum at the bottom of the slope, all the slopes were laid horizontally overnight before being placed vertical for continued incubation. Cultures were incubated at 37°C for at least six weeks, with weekly observation for any signs of growth.

For cultures suspected to be contaminated, stored sediments were re-treated for a longer duration and streaked onto a blood agar plate. Blood agar plates were incubated overnight, and if any signs of contamination were observed, the sample was treated further by increasing the decontamination time. The positive cultures were those that provided colony morphology similar to that described by (Vestal and Kubica, 1966).

3.5.5 Identification

Positive cultures were subsequently sub cultured onto another set of media and incubated for another four weeks. The first step towards species identification was the visual observation of growth on IUT and L-J pyruvate media.

According to (Marks, 1976), *M.tuberculosis* produces eugonic growth on both media, while *M.bovis* only grows well on L-J pyruvate medium. Colonies were stained with ZN as described above and examined for acid-fast bacilli (AFB). The presence of AFB and cording of bacilli were indicative of presence of mycobacteria species. Due to limitation of resources, only fewer additional identification tests were performed. These were i) Temperature test to differentiate *M.tuberculosis* complex from other species, particularly the *M.avium* group, ii). Growth on medium containing para- nitro benzoic acid (PNB), the medium supporting the growth of other mycobacteria species but not *M.tuberculosis* complex iii) Nitrate test iv) Niacin test.

Confirmed mycobacteria strains as AFB+ were sub-cultured in IUT and L-J pyruvate medium to obtain enough cells for molecular analysis. When enough colonies have grown on the surface of the solid media these were harvested by scraping using sterile disposable plastic loop. The harvested cells were placed in a sterile 1.5ml eppendorf containing 20ul of distilled water and mixed thoroughly. The sealed eppendorf were placed in water bath and boiled at 72 ^{0}C for 10 minutes in order to kill the cell, and also as extraction method for the DNA. After heat treatment the eppendorf were transferred directly in the tray containing ice read for PCR, and the remaining part was then stored in refrigerator at 4 ^{0}C for further use.

3.6 Molecular Biology Techniques

All isolates of mycobacteria were subjected to multiplex polymerase chain reaction (PCR). In order to carry out multiplex PCR the following steps were undertaken:-

3.6.1 DNA extraction from tissue

The reagents and equipment used for extraction of DNA were autoclaved to make them sterile before carrying out any molecular process. Fresh culture of mycobacterium was suspended in 25ul of distilled water and then boiled at 98 ^{0}C for 10minutes. After mixing, 2ul was then used for PCR procedures.

3.6.2 Determination of quality and quantity of the extracted DNA

The quality and quantity of the extracted DNA was determined by subjecting the DNA to electrophoresis on 0.8% agarose gel.

3.6.3 Preparation of agarose gel

Agarose gel was made by mixing 0.4g of agarose powder (Promega U.S.A) with 1 x TBE buffer filling to 50ml to obtain 0.8% concentration of the gel. Agarose was dissolved by heating the solution on a hot plate.

A volume of 2.4ul of ethidium bromide (0.05%v/v) solution was added to every 50ml at 60°C of molten agarose to obtain the final concentration to 0.5ug/ml. Molten agarose was then poured into the electrophoresis gel casting equipment and left for half an hour to set.

3.6.4 Loading of DNA into agarose wells

Before loading, 2ul of loading dye blue/orange 6X (Promega MADISON, WI USA) was added and mixed to every 2ul of the DNA to be analyzed. For each analysis, the first well of the gel was loaded with DNA molecular marker 1kb DNA ladder (Promega MADISON, WI USA). The molecular weight marker was run parallel with the DNA sample in 1 x TBE buffer in a horizontal gel electrophoresis apparatus at a constant voltage of 80V/60minutes.

3.6.5 Evaluation of quality and quantity of the extracted DNA

Visualization of bands was done by medium wavelength ultra-violet (UV) light transluminator (STX-20, Jencons Ltd., USA). The quality and quantity of the DNA was determined by visual comparison of the intensity and conformation of bands of template DNA with those of molecular weight marker. A single clean band of the DNA with high intensity indicated that the DNA extracted was good in quality, while smearing signified poor quality.

3.7 Molecular Identification of Tuberculous Mycobacteria by PCR

A multiplex PCR as described by Sinclair *et al.*, (1995) was used in this study. Briefly the assay is based on simultaneous amplification of two different targets: a 369bp region from mtp40 species-specific gene sequence of *M.tuberculosis* and a 245bp fragment from the *M.tuberculosis* complex insertion sequence IS986.

3.7.1 Optimization of PCR conditions

The laboratory work was done in 4 different laboratory rooms to prevent contamination of reagents with other template DNA and amplicon from previous PCR works. Extraction of DNA was done in the Tuberculosis laboratory; preparation of the DNA for PCR

amplification in the molecular biology laboratory 2; PCR reagents master-mixing was carried out in the molecular biology laboratory 3, and the PCR was set in the molecular biology laboratory 1. Amplification of the DNA by PCR was carried out after adjusting stepwise scaling up and down the concentration of magnesium salts and template DNA in the reaction mixture.

3.7.2 Preparation of DNA templates

A loopful of cells/growth (template DNA), was mixed with 20μl of distilled water in a 1.5ml- eppendorf tube was first denatured by incubation in boiling water for 10 minutes. Then immediately, quenched on ice before adding 2μl of it in the 48μl of the master mix in 0.2μl PCR tube to make a final volume of 50μl of the reaction mixture.

3.7.3 Preparation of the master-mix

The PCR amplification was performed in a final volume of 50ul containing 5 X reaction buffer, Promega; (50Mm KCl, 2.5Mm $MgCl_2$, 10mM Tris-HCl pH 8.0, 0.01% gelatin, 100Um each dNTP, 0.5U Taq polymerase, 0.05μM of each of the primers for IS986, and 0.1μM of each of the primers for Mtp 40.

Table 1: Showing 50µl reaction mixture

Reagent	1X reaction
PCR buffer	10 µl
MgCl2	5 µl
DNTPs	0.5 µl
IS 986 forward	0.25 µl
Reverse	0.25 µl
Mtp40 forward	0.5 µl
Reverse	0.5 µl
Taq	0.25µl
Nuclease free water	30.75µl
Total	48µl
Template DNA	2µl
Total reaction mixture	50µl

3.7.4 Setting of the PCR

A PCR cycle comprised of denaturation, annealing, and extension steps. The reactions were subjected to 30 cycles of 95°C/2minutes denaturation, 62°C/30sec primer annealing, and 72°C/60sec for primer extension in a (Mastercycler personal-eppendorf AG-GERMANY). The primers used in the PCR for the amplification of DNA were as in (Table. 3).

Table 2: The actual program sequence for the multiplex PCR

Sequence	Temperature	Time	Number of cycles
Initial denaturation	95°C	2minutes	1
Actual denaturation	94°C	30secs	30
Annealing	62°C	30secs	30
Extension/Elongation	72°C	60secs	30
Final elongation	72°C	60secs	1
Cooling	22°C	4minutes	1

Table 3: Oligonucleotide primers used in the IS986/Mtp40 multiplex PCR and their targets sites

Primers	Sequence
IS986 (245bp)	
Forward	5'-CGT GAG GGC ATC GAG GTG GC-3'
Reverse	3'-AAA CAG TGG CTG CGG ATG CG -5'
Mtp40 (396bp)	
Forward	5'-CGG CAA CGC GCC GTC GGT GG-3'
Reverse	3'-GGG CCG CCA CGG CAC CCC CC-5'

3.7.5 Analysis of PCR products

(i) Electrophoresis

The PCR products were analyzed by using 2%(this was calculated based on the volume of the casting tank e.g. 120ml , 2% was equivalent to 2.4gm) Agarose gel electrophoresis.

(ii) Loading of PCR products in wells of agarose gel

A volume of 5µl PCR product (amplicon) was mixed with 2µl of loading buffer. After mixing the PCR products were loaded in the wells according to number of amplicon and to one of the wells, 1kb molecular weight marker was added and run in a parallel track at 80 volts for 90 minutes.

(iii) Visualization of PCR products

Visualization of bands was done by medium wavelength ultra-violet (UV) light transluminator (STX-20, Jencons Ltd., USA).

(iv) Recording of the results

Results after running the gel were recorded using the digital camera and drawing the patterns in the note book.

3.8 Analysis of the Results

The results of the gel were analyzed quantitatively based on the conformation of bands of template DNA with those of molecular weight marker.

CHAPTER FOUR

4.0 RESULTS

4.1 Collection and culture of abattoir cattle sample

Lymph nodes collected from individual animals included retropharyngeal, submandibular, bronchial, mediasternal, and mesenteric lymph nodes. These lymph nodes were pooled to make a sample for each animal involved. 89 pooled lymph nodes samples were collected at Morogoro municipal abattoir. A total of 72 (80%) samples showed positive growth upon primary culture (Table 4), of the 72 positive culture it is only 59 (81.9%) were positive for Acid fast bacilli up to ZN stain (Table 5).

4.2 Collection of cultures from Human tuberculosis cases.

A total of 45 cultures kindly provided by NIMR Muhimbili Station's Tuberculosis Laboratory for DNA analysis were confirmed to belong to the *Mycobacterium tuberculosis* complex. Thirty eight (84.4%) isolates showed positive growth on culture and 29 (76.3%) isolates showing positive when subjected to PNB.

Table 4: Total sample collected and isolates after primary culture

Specimen Source	Total number of Specimen	Growth	Percentage growth
Animal/Cattle	89	72	80%

Table 5: Status acid fast-bacilli (AFB) under microscopy

Specimen source	Number of Isolates/growth	AFB (+ve) on growth	AFB (-ve) on growth	% of AFB (+ve)
Cattle	72	59	13	81.9%

Biochemical test carried out on 72 positive cultures revealed that 9.7% and 41.7% were positive for nitrate and PNB tests respectively (Table 6).

Table 6: Biochemical tests for identification of Mycobacterium tuberculosis complex

Total number of isolates/growth in %	AFB+	Nitrate test + %	PNB + %
72	59	7	30
	81.9%	9.7	41.7

4.3 Multiplex PCR

The results from PCR performed on 104 DNA samples after extraction from clinical specimens and human cultures using IS986 region and mtp40 gene specific for *M.tuberculosis* were 245bp and 396bp fragments are summarized in Table (7). These products were obtained from amplification reaction and visualized on ethidium stained agarose gel. (Fig. are 1 and 2). Occurrence of two PCR products at 245bp and 396bp fragments signified presence of *M.tuberculosis* strains in the specimen and a single product at 245bp indicated presence of *M.bovis* strains. Those specimens which showed neither 396bp nor 245bp products were considered to be negative.

Table 7: PCR identified strains of *M.tuberculosis* and *M.bovis* from animal and human isolates

Specimen/culture source	*M. bovis*	*M. tb*	-ve	Total
Human	0	25	20	45
Animal	17	3	69	89
Total	17	28	89	**134**

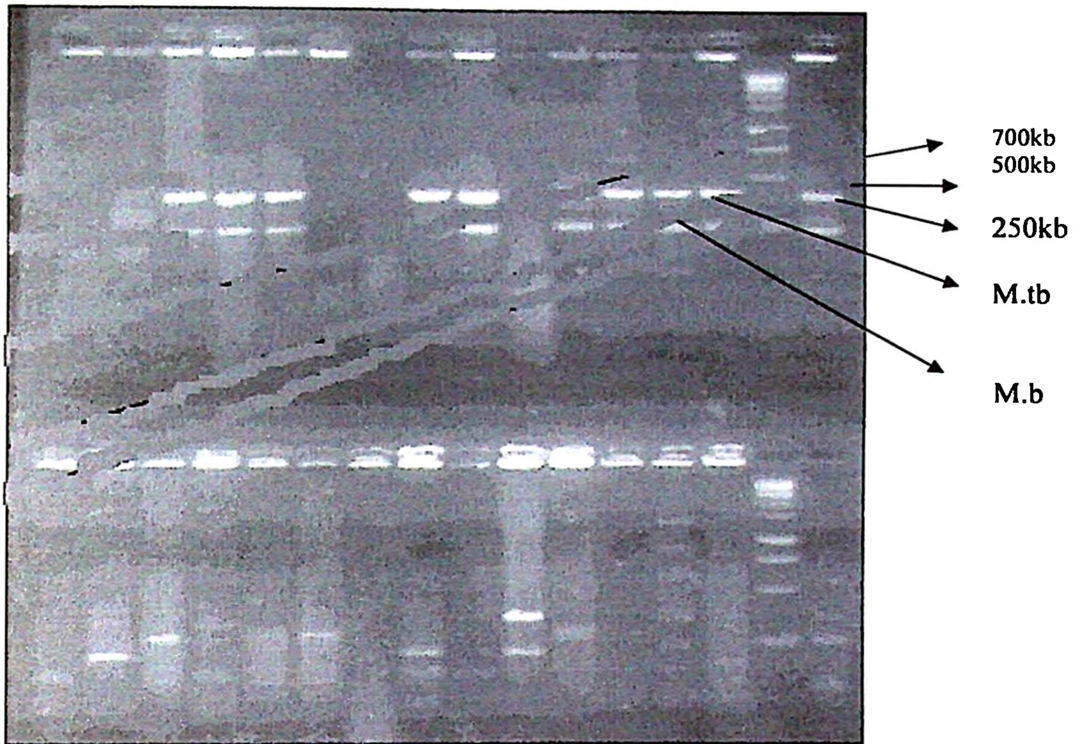

Figure 1: Agarose gel- showing the amplifications products *M.tb* positive (245 and 396kb), and *M.bovis* (245kb).

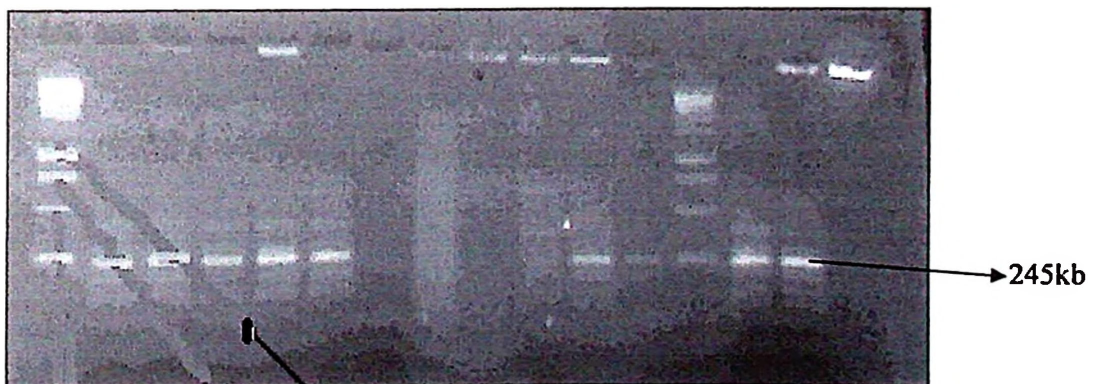

Figure 2: Agarose Gel showing the *M.bovis* at 245kb

CHAPTER FIVE

5.0 DISCUSSION

5.1 Multiplex PCR Result in comparison with animal and confirmed human culture

The use of the multiplex PCR in this study has shown its potential in identifying *M bovis* and *M. tuberculosis* in cattle and identifying presence of *M. tuberculosis* in human strains of the Mycobacterium complex organisms studied in this work. The 100 (72 animal and 38 human isolates) growth of mycobacteria in L-J medium with glycerol and that with pyruvate is not always synonymous to presence of *M. tuberculosis* and *M. bovis*. In this study only 45 (45%) isolates were *M.tuberculosis* complex organisms. This observation agrees with other studies (Patnaik *et al.*, 2001) in which conventional methods of identification of *M.tuberculosis* complex were poor when compared with PCR.

5.2 Detection of *M.tuberculosis* complex species by PCR amplification.

In recent years, development of PCR based techniques for the identification and differentiation of *M.tuberculosis* complex bacteria has offered a good alternative to conventional methods. These techniques are rapid and more reliable with increased sensitivities and specificities for identification of these organisms (Liebana *et al.*, 1995; Herrera Segovia, 1996; Rodriguez *et al.*, 1999). The *Mycobactreium bovis* is known to be among causative agent of tuberculosis in human. A collection of selected isolates from human cases cultured and confirmed to belong on *M.tuberculosis* complex at Muhimbili Research Centre were also included to provide knowledge regarding the involvement of *M.bovis*. Hence the Multiplex PCR for typing Mycobacteria isolates from abattoir and human specimens to the species level was carried out. These results are in agreement with those obtained by other workers (Sinclair *et al.*, 1995; Kazwala, 2006).

Previous studies (Sinclair *et al.*, 1995; Kazwala 1996; del Portillo *et al.*, 1996; Weil *et al.*, 1996) have shown that isolation of *M.microti* and *M.africanum* in culture is very rare. *Mycobacterium microti* is also rarely isolated from human and cattle specimens except in voles while the role of *M.africanum* in animal specimens needs further investigation (Castets *et al.*, 1969).

5.3 Comparison of Multiplex PCR against conventional biochemical test.

When the PCR results were compared with those obtained from biochemical test results performed on the same specimens. In this study the Multiplex PCR confirmed only 45 (46%) isolates were *M.tuberculosis* complex organisms. This observation agrees with other studies (Patnaik *et al.*, 2001) in which conventional methods of identification of *M.tuberculosis* complex were poor when compared with PCR.

CHAPTER SIX

6.0 CONCLUSION

The results of the present study suggest the application of Multiplex PCR to animal and human isolates following extraction of mycobacterial DNA is effective in the diagnosis of tuberculosis. The use of this multiplex PCR in routine diagnosis of tuberculosis would provide a rapid, sensitive and more specific means for the detection of *M. tuberculosis* complex strains in specimens.

However, its potential use for discriminating *M.tuberculosis* complex needs a critical review to validate its ability to identify other species belonging to *M. tuberculosis* complex apart from *M. bovis* and *M. tuberculosis*.

In this study, results shows the cattle slaughtered at the abattoir have been infected with the mycobacteria, and some of those were coming from Kilosa District, Morogoro Region (Personal communication with businessmen (Godlisten Nkondya) at the abattoir, 2007).

This observation requires surveillance studies in Kilosa District to validate the information we are getting and also requires efforts to trace back the source of these cattle (traceability is a big obstacles as is difficult to establish the origin of the most of the cattle due to poor recording systems in local cattle auction markets).

The abattoir in average slaughters 60 cattle and five goats per day, this has big impact on the public health for those who are processing meat at the abattoir, meat inspectors, animal keepers and the consumer using animal products and by products.

In this country where livestock industry is less developed and intensive, and cattle farming is a family affair for milk consumption or retail commercialization, on the other hand, if the total number of cattle is highly reduced, people live near the animal folds can practice on consuming pasteurized milk rather than consume raw milk and animal products, this has the impacts on controlling transmission ecology on both ends.

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APPENDIX

Appendix 1: Isolates from human sputum from Muhimbili Research Centre

SAMPLE NUMBER	GRWOTH	PNB	PCR
19LRT1554	+	+	Mb+
BP549	+	+	Mtb+
BR04	+	+	Mtb+
FR155	+	+	Mtb+
8RT420/5	+	-	Mb+
C1052SP	+	-	-
KR243	+	-	Mtb+
RT150	+	+	Mtb+
4RT772	+	+	Mtb+
LOP 1839	+	+	Mb+
16LOP 1580	+	+	Mtb+
27RT472	+	+	-
BR 106	+	-	Mtb+
RT 304/4	+	+	Mtb+
RT 129	+	+	Mtb+
13KR241	+	+	Mtb+
9RT 458	+	+	Mtb+
OP 131/5	+	-	-
RT 296	+	+	-
RT 223	+	+	-
22 RT 472	+	-	-
SLOP 1694	+	+	Mb+
BR 320	+	+	Mb+
25C 061	+	-	-

BR 32	+	-	-
RT 253	+	+	-

SAMPLE NUMBER	GRWOTH	PNB	PCR
BR 202	+	+	-
BR 280	+	+	-
BR 251	+	+	-
RT 116	+	+	Mtb+
18LOP 1393	+	+	Mtb+
BR 318	+	+	Mtb+
LOP 1852	+	+	Mtb+
BR 315	+	-	Mtb+
LOP 1837	+	+	-
RT 554	+	+	Mtb+
KP 1490	+	+	Mtb+
27BAL 242	+	+	Mb+