VIABILITY OF *MYCOBACTERIUM BOVIS* IN TRADITIONALLY PROCESSED SOUR MILK AND THE PREVALENCE OF BOVINE TUBERCULOSIS IN NAMWALA DISTRICT OF ZAMBIA.

BY

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DECLARATION

I, **Almond Casmir Madandu Sitima,** do here by declare that the dissertation represents my own work and that it has not previously been submitted for a degree at this or any other university.

Signature.....

Date 31/01/97

"to my late loving brother

ANTHONY HENRY KAMBEU

for the love and the family academic

standards set.

To Dad and Mum for the sacrifice

you have willingly and unselfishly chosen

for the love of your

eight children"

APPROVAL

This dissertation of **DR. ALMOND CASMIR MADANDU SITIMA** has been approved as fulfilling part of the requirements for the award of the degree of Master of Veterinary Medicine by the University of Zambia.

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ABSTRACT.

Tuberculosis is a debilitating disease caused by the bacteria of the genus Mycobacterium. The disease has been recognized for centuries and its causative agents have continued to be studied to date. Mycobacterium species cause disease in animals, birds and humans. They can cause cross infection between the hosts. The most important species in the genus are *Mycobacterium bovis* and *Mycobacterium tuberculosis*. These two cause zoonotic tuberculosis and can be excreted in milk of cows when udders are infected making the milk to be a very good vehicle for mycobacterium transmission.

Several authors have indicated that tuberculosis is transmitted by consuming contaminated milk. The Ila people of Namwala district in Zambia are cattle keepers and consume a lot of milk both as fresh and sour. Milk is allowed to sour in a bottle gourd (*Lagenaria siceraria*) commonly known as a calabash (*Insuwa*-Ila and Tonga, *Nsupa*-Chewa, *Insupa*-Bemba). The Ila people boil milk for immediate use but that meant for sour milk remains unboiled. The prevalence of bovine tuberculosis has been reported to be as high as 30 percent in their herds of cattle based on assumptions and abattoir returns. The risk of humans to be infected with mycobacterium may be seen to be high.

Therefore aims of the study were; to assess the viability of *M. bovis* in traditionally processed milk, to estimate the prevalence of bovine tuberculosis in cattle of Namwala district and to identify the flora in the calabash.

Within Namwala district data was collected from Baambwe and Maala Villages due to proximity and high cattle populations. Tuberculin test was used to estimate the prevalence of bovine tuberculosis. The tuberculin used was Purified Protein Derivative of *Mycobacterium bovis* and *Mycobacterium avium* manufactured in the Netherlands. Ten herds of cattle, totaling 507 cattle were tested. To assess the effect of traditional milk processing on *M. bovis*, a laboratory experiment was set to imitate the traditional method of souring milk. *M. bovis* was inoculated into fresh milk which was mixed with a cocktail of sour milk collected from calabashes . Samples of this milk were taken at intervals to measure the pH and for culturing on Lowenstein-Jensen medium, to determine the viability of the *M. bovis* and *M tuberculosis* . Samples of sour milk were taken from 14 calabashes for the identification of the flora in the calabashes. *M. bovis* was recovered at the various stages of the pH transition from 7.0 to 3.0 and at all sampling intervals from the milk in which it was inoculated experimentally. The obtained results indicate that raw milk containing *M. bovis* is not able to eliminate the organism in the process of souring the milk within the time frame of optimal consumption (24-48 hours).

The overall prevalence of bovine tuberculosis was found to be 12.8 percent. In the females it was found to be 13.5 percent while in the males 8.6 percent. It was

highest, (17.6 %), in the age group of 41-55 months old while the youngest 1-24 months had the lowest (6.3 %). The herd prevalence of the ten herds varied between 9.4 and 18.8 percent.

The high quantity of the lipids in the cell wall of the *M. bovis* was thought to account for the recovery of viable Mycobacterium in sour milk. Bacteria and yeast found to form the flora of the calabashes were mostly the Gram positive rods of the lactobacillus species and Candida species respectively.

It was concluded from the present study that the prevalence of bovine tuberculosis in Namwala district was high as compared to other districts in the country and that the traditional way of sour milk processing presents a public health risk.

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ABBREVIATIONS

DAPH	Department of Animal Production and Health.
DNA	Deoxyribonucleic Acid.
FAO	Food and Agriculture Organisation of the United Nations.
GMA	Game Management Area.
L-J	Lowenstein-Jensen Medium.*
NCSR	National Council for Scientific Research.
OIE	Office International des Epizooties
PBS	Phosphate Buffered Saline.
PCR	Polymerase Chain Reaction.
PM	Post Mortem.
T.B	Tuberculosis.
WHO	World Health Organisation.
Z-N	Ziehl-Neelsen (Stain)

* Löwenstein-Jensen Medium (Topley and Wilson's Systematic Bacteriology, Eighth Edition, Anold, London.) is read as Lowenstien -Jensen Medium in this text according to OIE Manual (1992).

Chapter One

INTRODUCTION

1.1. Tuberculosis in Zambia

The World Health Organisation (WHO) 20 years ago estimated that there were 15 million cases of tuberculosis in humans of which 70 to 80 percent (10.5 to 12 million) were in developing countries (Cruickshank, 1973). It is estimated that tuberculosis claims 3 million lives of humans per year (Kochi, 1990) However the extent of bovine tuberculosis is little known and is disregarded as a source of human infection in most of the rural areas of Africa and recent studies have indicated that *M. bovis* infection is still present in humans and animals in Africa (Cosivi, 1994) . The prevalence of *M. bovis* infection in Zambia may be higher than anticipated because patients infected with *M. bovis* are clinically indistinguishable from those infected by *M. tuberculosis* and coupled with lack of isolation or identification methods and facilities at most of the laboratories not only in Zambia but also in Africa as a whole. It is therefore important that any possible source of human infection with *M. bovis* should be seriously considered as it can lead to active infection especially in HIV sero-positive patients (Cook, *et al* , 1996).

Though tuberculosis occurs world wide, Shimao (1986) divides the world into following three groups on the grounds of prevalence and incidence of human tuberculosis.

1. Group 1.

The group includes the European countries and North American countries and Japan where tuberculosis is now a minor problem and continues to decline.

2. Group 2.

The group includes countries where the problem is greater than in the countries of Group 1, but is on the decline path, such as in Singapore, Argentina, Algeria and Korea.

3. Group 3.

The group includes countries where tuberculosis is still a major problem. These are mostly the developing countries, including Zambia.

The reported cases of human tuberculosis in Zambia starting from 1980 have been increasing as shown by the Ministry of Health Report. The increase in the reported cases may not be entirely attributed to high incidence rates but also to the level of awareness of the people. The country through the Ministry of Health has mounted a vigorous campaign against several diseases of which tuberculosis is one of them. The campaign involves immunisation

programmes, oral education, billboards, and television discussions, to mention a few. The predicted increase in the prevalence of the Human Immuno-deficiency Virus (HIV) infection will increase the risk of human infection with bovine source of tuberculosis progressing to clinical disease (Carlton and McGavin, 1995, Cook , *et al.,* 1996).

Judging from the provincial statistical reports of tuberculosis cases reported, it is noted that in 1988 Southern Province registered 2385 new cases, while Luapula, Central and Lusaka Provinces had lower figures of 704, 703 and 891 respectively although these provinces do not have similar population sizes to that in Southern province.

Southern Province has the highest traditional cattle population of 866 378, while Lusaka, Luapula and Central have 37 647, 10 031 and 322 732 respectively. In fact Southern Province has the highest number of abattoir carcass condemnation due to tuberculosis followed by Western Province (Anonymous, 1990). Further more, Lusaka and Central Province have well developed dairy products processing factories, while Southern Province does not.

It may be expected that human tuberculosis prevalence would be high in densely populated areas than the sparsely populated areas, but this is not the case in Zambia (Anonymous 1990). It is therefore, important to find out how the disease is spreading and what factors are

contributing to the apparently high reported cases including in populations having BCG introduced (Shimao, 1986)

1.2. Public Health Implication of Tuberculosis

As early as 1907, the British Royal Commission on Tuberculosis produced evidence that bovine tubercle bacillus can be responsible for the most serious cases of tuberculosis in human and that raw cow's milk containing the tubercle bacillus was the cause of some fatal cases and outbreaks of tuberculosis in man (Remain and Bryan, 1979).

Francis, (1950) and Kleeberg, (1975) emphasised that once *M. bovis* infection is established in man it behaves like human tubercle bacillus, infected human beings may remain as a constant source of re-infection for cattle and human (Karlson, 1960, Huitema, 1969, Black 1972). In countries where bovine tuberculosis has nearly been eradicated, human tuberculosis due to *M. bovis* is rare. However various reviews and reports emphasise that *M. bovis* infection in man is still fairly common where the infection is prevalent in cattle (Huitema, 1969).

Tubercle bacilli is an occupational hazard. *M tuberculosis* was isolated from a man on a French farm who was responsible for the feeding of cows These cows were positive reactors when they were subjected to tuberculin test (Lafont, 1985). Since cattle can be asymptomatic, and such infected cattle can be slaughtered with less attention of the abattoir workers. These would also be a source of infection to abattoir workers (Robinson , *et al.*, 1988).

In most African countries the prevalence of *M. bovis* is insufficiently unknown in either humans or bovine populations. There is little attention given to bovine tuberculosis in Africa. Of the 56 African countries 44 countries record the presence of bovine tuberculosis. Of these only 6 have implemented bovine tuberculosis control programmes, and 90 percent of this

whole cattle population falls in the countries which have not implemented any bovine tuberculosis control programmes (FAO/OIE/WHO 1993).

1.3. Statement of the Problem

The Ila people in Namwala district consume most of the milk from their cows when it becomes sour. The prevalence of bovine tuberculosis was reported to be 30 percent in the examined areas (Anonymous, 1990) This may mean that milk consumed may be coming from a good number of infected cows. Most of these animals may be in sub-clinical state. Such animals, are used at all practical purposes in consideration as being normal in appearance by the local people. This in turn means that the risk of exposure to mycobacterium infection would be inevitably high in both humans and animal populations.

Whether the consumption of unpasteurised traditionally processed sour milk from unpasteurised milk serves as a source of infection to humans, or not, needs further investigation. This is because it is expected that all the milk that is meant for human consumption is supposed to be at least boiled before it is consumed. The Ila people boil the milk that is meant to be consumed just after milking, they do not boil the milk that is meant to be soured. They claim that sour milk made from boiled milk does not have a good taste. Because of this apparent difference in taste it is virtually not so easy to convince the Ila people to boil the milk before souring it.

A very small percentage of the liberal Ilas consume the commercially prepared sour milk. Some conservative Ilas, be it in town or in the villages, do not like the taste of the commercially prepared sour milk.

During the winter, when the pasture lands are over grazed, the Ila people take their cattle to the plains and remain there for months. During these periods the cattle attendants (herds workers) share the dusty dwelling with the cattle. This means that if the prevalence of bovine tuberculosis is high, this practice would serve as a source of infection to the herds workers as pulmonary airborne infection with the bovine bacilli occurs primarily in places where humans share dwellings with cattle. If sour milk is a source of infection the people at risk being infected will be high as the main component of their meals is sour milk. Herd workers also suck milk directly from the udders of the cows

1.4. Background of the Study Area.

Namwala District is located in the Southern Province, south west of Lusaka, the capital of Zambia (Appendix 1). The livelihood of the Ila people, the local tribe, is highly dependent on the cattle they keep.

Milk is a major source of protein and it constitutes a major component of their diet. Milk is commonly consumed as sour milk. Unboiled fresh milk is put in a calabash and allowed to sour. A single household may have several of such calabashes (plates 1-1, 1-2) which are replenished with unboiled fresh milk in sequence. Cases of human tuberculosis are apparently high in Namwala with records of 104 and 118 new case attended to by the local hospital in 1992 and 1993 respectively in an area of 35 000 people (Anonymous 1992 and 1993).

There are 6 Game Management Areas (GMA) in Namwala. A GMA is an area demarcated between the main National Park and the human settlements. People are allowed to settle in some parts of the GMAs. Wild animals and domestic animals are in constant contact in their grazing areas. As the Kafue river meanders eastwards, along its sides are the Kafue Flats, suitable flood lands for grazing. It is in these areas where cattle and the Kafue Lechwe (*Kobus lechwe kafuensis*) and other wildlife mingle. It is because of this contact that Dillmann, (1976), suggested that tuberculosis may have been introduced to the lechwes due to this contact, in turn the lechwes may introduce the disease to other game species and to uninfected cattle. *M. bovis* has been isolated from these lechwes (Gallagher, et *al.,* 1972). This mode of transmission may account for the 25 percent of cattle from the Kafue Flats that showed tuberculosis lesions on meat inspection (Anonymous 1965) Unlike the Kafue Lechwe, the Black Lechwe (*Kobus lechwe sinthemani*) despite inhabiting similar environment, Grimsdell and Bell (1975) speculate that tuberculosis is not a problem because these do not come into contact with cattle. This ties up with Bakunzi , *et al.,* (1995), who

found low bovine tuberculosis of 0.062 percent in a survey conducted in Molopo in South Africa, and attributed to the absence of mingling of wild life with cattle.

It was considered to be worth to study tuberculosis prevalence and survival of tubercle bacilli in sour milk in view of the high cattle population in Namwala District, high estimated prevalence of bovine and human tuberculosis and the sour milk consumption, produced from raw milk and to investigate transmission of tuberculosis from cattle to human population.

1.5. Objectives and the Scope of the Study.

The main objectives and scope of this study were threefold;

1. **To Examine the prevalence of bovine tuberculosis in Namwala.**

2. **To identify the flora in the traditional calabash used to sour the milk.**

3. **To examine the viability of *M. bovis* in traditionally processed sour milk.**

REVIEW OF LITERATURE

1.6. General Over View of Tuberculosis.

Tuberculosis is a chronic contagious disease caused by infection with certain species of acid fast bacteria of the genus Mycobacterium. It affects many vertebrate animals and is manifested in general by the formation of granulomas in various organs, but particularly in the lungs and lymph nodes and consists a caseous necrotic tissue surrounded by a zone of granulomatous inflammation. The term tubercle bacillus is still used for the organism that causes the development of the tubercle (Koch 1882). It is usually synonymous with *Mycobacterium tuberculosis* and *Mycobacterium bovis*. The characteristic appearance of lesion however differs according to animal species and the respective Mycobacterium with which it is infected. Before the effective control measures were adopted tuberculosis was one of the major diseases of humans and domestic animals. The disease in human still remains one of the most important notifiable infectious diseases and is one of the biggest public health problem in many countries of Africa including Zambia.

Robert Koch first described the causative agent in 1882 and tuberculin test which proved to be a valuable diagnostic tool for the detection of the disease was developed for its curative value (Koch 1891).

The genus Mycobacterium consists of about 50 species (Holt , *et al.,* 1994) many of which are environmental saprophytes. They exist and multiply in a wide variety of substrates such as soil, water and plants, domestic and wild mammals and birds. (Wolinsky, 1979, Nel, 1981, Grange, *et al.,* 1990). Some of these saprophytic mycobacterium may cause opportunistic infection in animals and human but others may cause disease (Grange and Yates 1986).

Certain members of this genus make up the *M. tuberculosis* complex (*M. tuberculosis, M. bovis, M. affricanum* and *M. microti*) which are also known as tubercle bacilli and cause tuberculosis in human and animals (Collins , *et al.*, 1994).

The two most important slowly growing species of the mycobacterium are *M. bovis* and *M. tuberculosis*. *M. tuberculosis* was one of the first mycobacterium to be described. *M. bovis* which is closely related to *M. tuberculosis* (Collins *et al.*, 1994). causes tuberculosis mainly in cattle but also affect other species including human if there is contact with tuberculous cattle and their products.

M. bovis and *M. tuberculosis* are so closely related that some authors regard them as mammalian strains and can only be differentiated when cultured . Infection of man by either strain cannot be differentiated clinically unless cultivated. *M tuberculosis* is usually susceptible to pyrazinamide and always resistant thiophene-2-carboxylate and can reduce nitrate, while growth of *M. bovis* is enhanced by the addition of pyruvate but suppressed by glycerol.

The primary aetiological agent of bovine tuberculosis, *M. bovis* was first clearly differentiated from other types of tubercle bacilli with a wider range of pathogenicity for different animal species than other species of the genus.

1.7. Classification of Mycobacterium

According to the latest edition of Bergy's Manual of Determinative Bacteriology, 9th edition (Holt , *et al.*, 1994) the genus Mycobacterium is classified into two groups;

1 Slow growing species of mycobacterium including 18 species and

2 Rapid growing species and subspecies of mycobacterium which including 33 subspecies.

These species and subspecies are listed in appendix 2.

1.8. Diagnosis of Bovine Tuberculosis.

Tuberculosis is diagnosed in live animals in several ways.

1. Clinical Examination

This involves examination of an animal. The animal may present itself in similar ways depending on the organ in which the organisms have localised. The most common symptoms are chronic soft cough, emaciation and swollen lymph nodes. These symptoms and coupled with the history of already confirmed cases in the area may lead to a tentative diagnosis of tuberculosis. This method can not be used to make a definitive diagnosis as the causative organism can not be confirmed. Other diseases, such as trypanosomiasis, and parasitic infection, can also manifest in the similar way.

2. Delayed Hypersensitivity

This is an allergic test. Kelly, (1984) defines an allergic response in an animal as one which elicits a sensitivity response following the injection, into its tissues, of an antigen in form of a protein derived from a specific micro-organisms with which the animal is or was infected. He

further describes allergy as a state of tissue hypersensitivity or immune responsiveness acquired by an animal as a result of exposure to an antigen.

For the diagnosis of bovine tuberculosis the delayed hypersensitivity test developed is called the tuberculin test. Though initially developed to be applied by intradermal, subcutaneous and ophthalmic routes, the latter two have generally been discarded. Currently only the first one is widely being used for diagnosis of subclinical tuberculosis.

3. Polymerase Chain Reaction (PCR) and Deoxyribonucleic acid (DNA) Probes (Persing, *et al.,* 1993).

These are some of the most recent diagnostic techniques that are being developed. These methods are being developed to a very high specificity and sensitivity. Results of work so far done indicate that a PCR assay combined with DNA probe is feasible for the direct detection of *M. bovis* in the blood of an infected animal. This method can detect the organism regardless of its viability status (Barry , *et al.,* 1993). The advantage is that they are being developed to very high sensitivity. They are able to detect organism regardless of its viability status and the results are ready within the same day. Unfortunately, the available PCR primers do not distinguish between *M. bovis and M. tuberculosis.*

4. Blood Test for Gamma Interferon Detection (Rothel, et al., 1992).

Enzyme immuno-assay (EIA) for detection of gamma-interferon has already been introduced in Australia for national tuberculosis control programmes. The advantage is that the animal is only handled once. The major disadvantage is that the processing of the specimens has to commence within eight hours of sampling the blood

5 Bacteriological Examination (Jarnagin , *et al.,* 1989).

Isolation of the organisms from specimens such as nasal swabs, faeces and other organs is for definitive diagnosis. The two disadvantages are, the inability to detect non viable organisms and the long time lapse before the results are ready.

After death of an animal bovine tuberculosis diagnosis is done by post-mortem examination, histopathological examination, and bacteriological methods.

1. Post-mortem Examination

The tuberculus lesion may be seen in all the visceral surfaces but frequently in lungs bronchial, pleura, mediastinal and restropharnygeal lymph nodes, mammary glands, liver, spleen, and intestines. The lesions are usually calcified and granulomatous. Older lesions are grey white nodules and localised in the tissue.

2. Histopathological Examination

This is used to support PM findings. This procedure involves fixing a section of an affected tissue in 10 percent buffered formalin, embedding in paraffin, sectioning and staining with haematoxylin and eosin.

3. Bacteriological Examination

It involves the isolation and demonstration of the organism from the tissues showing lesion. The most common medium recommended by OIE, (1992), for the growth of *M. bovis* is the Lowenstein-Jensen medium enriched with pyruvate. Colonies of the organism takes 5 to 8 weeks to emerge on the media. They do not produce any pigment and may be confirmed by Ziehl - Neelsen staining methods as being acid fast. The same method may be used to detect Mycobacterium presence by making an impression smear of a lesion and staining it with the Ziehl - Neelsen. Auramin staining method is also used for preliminary confirmation.

The above two method are the most reliable conservative methods for the diagnosis of *M. bovis* in cattle (Coner , *et al.*, 1990).

4. PCR and DNA Probes

This can also be used as a diagnostic method on the specimens from a suspected animal.

1.9. Transmission of Bovine Tuberculosis

Although infection can be readily set up by feeding contaminated material, inhalation is probably the most common route of infection in cattle and 80% - 90% of infections may be contracted by this route (Timoney , *et al.*, 1988, Morris , *et al.*, 1994).A small proportion of

infection has been shown to be congenital and sexually transmitted (Timoney , *et al.,* 1988). In calves, infected milk is a very important source of infection. The respiratory passage is also a common portal of entry. Infection occurs most commonly among housed and crowded animals and hence the higher incidence among housed dairy cattle than among beef cattle kept under range condition. When transmission occurs by ingestion the source of infection is usually infected milk, feed and water contaminated by discharges and faeces.

Human infection of *M. bovis* is usually of bovine origin, however other animals are also known to be infected with *M. bovis* and remain as a potential source of human infection. Infection from person to person is, however possible. Human population is more susceptible to develop disease when *M. bovis* or *M. tuberculosis* is transmitted through inhalation than ingestion (Cook , *et al.*, 1996).

It is generally agreed that the pathogenicity for cattle of the human type of tubercle bacillus is lower than that of avian type. Infection of bovine by *M. tuberculosis* is usually self limiting and is associated with small lesion of no clinical significance. It is possible for cattle with no gross lesion to excrete *M. tuberculosis* in milk just as they excrete *M. bovis*. However several cases of infection of cattle with human type tubercle bacilli have been reported (Kleeberg 1975). The bacilli may escape through the blood stream from the original lesion to lodge elsewhere. They form new tubercles in the new locations. When such systematically translocated bacilli lodge in the mammary glands it creates a serious zoonotic problem for milking cows. Active tubercles can contaminate milk meant for calves and humans (Remain and Bryan 1979, Carlton and McGavin, 1995,). This can go on for a long time because the disease is a chronic one. Other organs that are affected are uterus, lymph nodes, kidneys, spleen and meninges (Timoney , *et al.,* 1988). The infection of the superficial lymph nodes is important because it can contribute in detecting infected animals.

1.10. Epidemiology of Bovine Tuberculosis

M. bovis is present in cattle world wide but varies in prevalence in the different countries. Its prevalence in the developed countries is very low unlike the developing countries. (Thoen , *et al.,* 1979, Shimao 1986, Carlton and McGavin, 1995).

1.11. Contamination of Milk With Mycobacterium and other Microorganisms.

Milk mainly consists of protein, as such it is a good medium for mycobacterium and other microorganism to grow. These organisms can be pathogenic for both humans and calves and can gain entry to milk by any of the following routes described by Riemann and Bryan, (1979).

1. a cow can be infected and causative organism reach the milk.
2. a cow can be infected directly or indirectly through contamination of the udder by infected humans, and
3. milk can be contaminated after it leaves the cow.

1.12. Pathogenic Bacteria Commonly Found in Milk.

Bacteria like B*rucella melitensis M. bovis Coxiella burneti* have been isolated from milk and milk products (Riemann and Bryan 1979). These bacteria constitute important zoonoses and milk borne diseases. Milk can be contaminated with *M. bovis* from the mammary glands

(Riemann and Bryan 1979). Tuberculosis outbreaks in which milk has been incriminated as a vehicle dates as far back as early this century (Riemann and Bryan 1979).

Many other pathogenic micro-organisms are able to survive in milk while it is being processed to milk products such as cheese. These include Salmonella, *Escherichia coli, Brucella abortus,* B. *melitensis Campylobacter jejuni, Yersinia enterocolitica, Staphphylococcus aureus,* and some others. Even in the presence of inhibitory factors, such as lactic acid and nisin in sour milk, these bacteria remain viable in sour milk for at least four months (Northolt, 1983).

Faecal material and infected humans are also sources of milk contamination. It is indicated that faecal material clinging to the udder, tail, and flanks of cows is a major source of contamination of milk (Timoney, *et al.,* (1988).

1.13. Non Pathogenic Bacteria Commonly Found in Milk.

There are a lot of non pathogenic bacterial found in the flora of milk. They reach milk either by contamination the udder or reaches the milk after it leaves the cow. In fact diary factories have taken advantage of some of these bacteria to produce milk products such as sour milk, cheese and yoghurt.

1.14. Decontamination of Milk With Respect to Mycobacterium.

To prevent contaminating milk meant for human consumption milking has to be done in a clean environment and with clean equipment and handled by healthy personnel. It is imperative to follow the milking hygiene procedures so as to reduce the chances and the amount of bacterial milk contamination. Even in the most clean milk parlour, milk is expected to be contaminated with some micro-organisms. Therefore in order to reduce the risk for the consumer, it is necessary to decontaminate the milk which can be done as follows:

1 Pasteurisation

When bacterial cells are exposed to heat, they are inactivated and are reduce in total numbers to a large extent. Mild heating does not instantaneously kill the bacterial but instead causes varying degree of injury and as a result bacterial can die, or will recover depending on the degree of injury.

The process of elimination of non-spore forming pathogens in milk is called Pasteurisation. (Riemann and Bryan, 1979). Currently this is the most common method of decontamination of milk. In general the procedure involves heating milk to a specified temperature and holding it for at least a specified time followed by cooling. These heating conditions are specified to ensure safety for the consumers. For example heating milk to 62.8 degrees for 30 minutes or heating milk to 71.7 degrees for 15 seconds.

Other devices such as the infrared heating devices have been invented and are proving to be equally effective against Mycobacterium just as the conservative heating methods (Rienmann and Bryan, 1979).

2 Chemical Disinfecting

A good number of disinfectants can destroy *M. bovis* in milk. Experiments have indicated that *M. bovis* can be destroyed using chemicals such as a low concentration of phenol or formalin. These, however, can not be used on milk meant for human consumption (Richards and Thoen 1979).

CHAPTER TWO

MATERIALS AND METHODS

2.1. Tuberculin Test

An opportunistic sampling of ten herds from the study area was made comprising of three herds from Baambwe and seven from Maala. The total number of animals tested was 507 (Plate 2). At the agreed dates and times with the owners of the animals visits were made to these herds. All tests were done in the morning. In each herd at least ten percent of the milking cows were included in an opportunistic sample for tuberculin test. Each animal in the tuberculin test was first cast down (Plate 3). To cast down an animal, first a 30 millimetres square thick sisal rope was used to trap both legs and then the tail and the rope were pulled in opposite directions. An ear tag was then fixed for the purpose of identification. In certain cases owners of the animals objected to this type of identification. In these cases a number was written with a water proof ink felt pen on the shoulder.

A side of the neck was then exposed by pulling the horns either to the right or to the left depending on which side was easy to expose. Two sites of 2 cm in diameter in the mid third of the neck and approximately 12 cm apart, one up and one down, were shaved using a

surgical blade. The thickness of the skin at the shaved sites were then measured using a venires callipers (Plate 4). The measurements were recorded on a record sheet (Figure 2.1). A single comparative tuberculin test was carried out by using the Mclintoc Syringe to inject 0.1 ml of avian tuberculin into the upper site and 0.1 ml of mammalian tuberculin on the lower sites. Avian and bovine purified protein derivative antigens (The Central Veterinary Institute, Lelystad, The Netherlands.) were used.

Figure 2.1.

Chart used to record individual data of an animal.

HERD NUMBER

VILLAGE _____

DATE(FIRST VISIT) _____

DATE NEXT VISIT DUE _____

TAG #	SEX	AGE	DAY 0 AVIAN (UP)	DAY 3	DIFFERENCE BETWEEN DAY 0 & 3	DAY 0 M/LIAN* (DOWN)	DAY 3	DIFFERENCE BETWEEN DAY 0 & 3	DIAGNOSIS

* mammalian

The age of the tested animals were estimated using the teeth eruption method as described by Fraser , *et al.,* (1986).

Seventy two hours after inoculation of the tuberculin each animal was again cast for the observation of the results. The skin thickness at the sites of inoculation were measured and entered on the record sheet in the appropriate column. The results were interpreted by following the method described by Kelly, (1984).

A negative reaction to tuberculosis was indicated by increase in skin thickness less than 2 mm. An inconclusive reaction was indicated by an increase in skin thickness of between 2 mm and 4 mm. A positive reaction was indicated by an increase in skin thickness of more than 4 mm.

An animal with any of the following reaction was considered to be a negative reactor;

- negative reaction to both tuberculin.
- negative reaction to bovine.
- inconclusive reaction to bovine tuberculin which was equal to, or less than a positive or inconclusive reaction to avian tuberculin.

A positive reactor was identified by a positive reaction in bovine tuberculin which was 4 m greater than the reaction to avian tuberculin.

2.2. pH at Which Most of the Sour Milk is Consumed.

A sample of 30 sour milk was chosen without specification from either a house hold (Plate 1, 1-2) or the market place. 50 ml of sour milk was poured from a container into a clean j A portion of each sample was given to three individuals to taste. Each sample was graded each individual as being Good sour, Not yet sour or bitter sour milk. A pH multi strip st with a range of 7.0 to 0.0 was then dipped in the remaining sour milk of that particu sample. One strip was used per sample. The results were recorded appropriately on a sh (Figure 2.2).

Figure 2.2

Chart used to record pH and the grading of the sampled sour milk.

CONSUMER	1	2	3	pH	GRADE
SAMPLE NO					
1					
2					
:					
:					
30					

2.3. Isolation of Calabash Flora.

A total of 14 milk samples were taken in duplicates from different house holds in the stu area. To two sterile plastic universal container 20 ml of sour milk was poured from a sing chosen calabash. The universal containers were then immediately put on ice for transportati to the laboratory in Lusaka. The following dilution's were done at the laboratory:

Nine ml of phosphate buffered saline (PBS) were put in 7 test tubes labelled 1, 2, 3, 4, 5 and 7. One ml of sour milk using a pipette and added to the first test tube. The test tube w then agitated at a high speed using a vortex machine. In this way a 10 fold dilution was mad This serial dilution was made to the last test tube. Diluted sour milk samples were subject to cultures onto the various media prepared in step 2.3.1. below.

2.3.1. Media Preparation for the Isolation of Calabash Flora and Inoculation of Sour Milk on the Media.

The following media were prepared and put in corresponding flasks. Distilled water was us and 150 ml of each media was prepared.

1. Bifidobacterium Agar Medium. (Nissui).

This medium is used for the selective isolation of anaerobes and differentiation Bifidobacterium. The dehydrated media was weighed at a ratio of 58g /1100 ml distil water.

1. Bifidobacterium Agar Medium. (Nissui).

This medium is used for the selective isolation of anaerobes and differentiation of Bifidobacterium. The dehydrated media was weighed at a ratio of 58g /1100 ml distilled water.

2. Lactic acid Bacteria Selective medium (BBL).

This medium is used for the selective isolation and enumeration of lactobacilli The dehydrated LBS powder was weighed at a rate of 84g/l This was first boiled for one minute. Then autoclaved.

3 Yeast Mod Agar medium.(Difcɔ).

This medium is used for the cultivation of yeast, moulds and other aciduric organisms. The powder was re-hydrated at a ratio of 41g/l.

4 DHL Medium.(Merck).

This medium is used for the detection and isolation of pathogenic Enterobacteriaeceae from all types of materials. This was prepared at a ratio 63.5g/l.

5 Triptic Soy Agar Medium.(Difco).

This medium is used for the isolation and cultivation of a variety of fastidious aerobic micro-organisms. The preparation ratio was 40g/l. It was first heated to dissolve. Sheep blood

was added aseptically upto 5% of the total volume after autoclaving and cooling to about 50°C.

6 M17 Medium. (Oxide).

This medium is used for the isolation of lactic streptococci and their bacteiophages and selective enumeration of *Streptococcus themophilus* from yoghurt.

The medium was prepared by weighing M17 powder at a ratio of 48.25g/950 ml. It was then boiled gently and autoclaved.

7 MacConkey Agar Medium (Oxoid).

This is a differential medium for the isolation of coliform and intestinal pathogens in water , diary products and biological specimens. This was prepared at a ratio of 51g/l This was then boiled to dissolve the powder and then autoclaved.

8 Tributyrin Agar Medium (Oxoid).

This medium is used for the detection and enumeration of lipolytic bacterial and moulds. It was prepared as follows:-

Peptone 5g

Yeast extract 3.0g

Tributyrin 10g

Agar 15g

Distilled Water 1000 ml.

The above where then dissolved in one litre of distilled water.

The above media were autoclaved at 121°C for 15 minutes and poured in petri dishes which were labelled according to the medium the petri dish contained.

Seven plates of each media were labelled 1 to 7. Each plate corresponded to a test tube with the same number. To all labelled medium plates, a millilitre of diluted sour milk was added. The milk was spread on the surface of the medium using an 'L' shaped glass rod. The culture media LBS, M17, tributyrin and BL (Blood Liver Agar medium, Nissui) were incubated anaerobically at 37 °C in BBL jars. The rest of the other cultured media were incubated aerobically at

37 °C.

After 24 to 48 hours of cultivation the colonies on each plate were differentiated on grounds of morphology , shape, size, and colour. They were picked and stored in Gifu Anaerobic Medium agar (Nissui) for identification.

2.4 Inoculation of Mycobacterium in Milk.

2.4.1. Media Preparation for the Isolation of Mycobacterium.

Only one type of medium was prepared for the isolation of *M. bovis* this was the L-J medium as recommended by OEI (1992).

The following steps were followed in the Preparation of L-J medium.

1 Mineral Solution preparation:

To prepare one litter of mineral solution the following chemicals were measured;

Potassium permanganate	4.0 g
Magnesium sulphate	0.4 g
Magnesium citrate	1.0 g
Asperagine	6.0 g
Glycerol	20 ml (when making L-J glycerol medium).
Sodium pyruvate	30 g

1 ml x1 000 000 IU of penicillin. (After autoclaving and cooling).

Distilled water	1000 ml

The above were dissolved by heating and then autoclaved at 121 °C for 30 minutes.

2 Malachite green solution.

To prepare one litre of malachite solution 20 g of malachite green was dissolved in 1000 ml of distilled water. The solution was then heated and filtered.

3 Complete Media

600 ml of mineral salts solution, 20 ml malachite green and 1000 ml of whole egg fluid were mixed thoroughly.

The above mixture was then dispensed into medium bottles and the medium was distributed into screw capped medium bottles with a volume of 20 ml in each and solidified at a slanting position to make slants.

2.4.2. Culture Preparation of the Stock *M. bovis* and *M. tuberculosis* .

M. bovis strain ATCC 35747 and *M. tuberculosis* strain H37Ra were obtained from The Zambia National Council for Scientific Research (NCSR) Laboratory , Luasaka. The two strains of organisms were revived by culturing them on Lowenstein-Jensen media. One glycerol medium bottle was labelled g-bovis and the another one g-tuberculosis . One pyruvate medium bottle was labelled p-bovis and another one was labelled p-tuberculosis('g' and 'p' denoting glycerol and pyruvate respectively). *M. bovis* is stimulated in the presence of pyruvate and inhibited in the presence of glycerol while growth of *M. tuberculosis* is stimulated in the presence of glycerol and inhibited in the presence of pyruvate.

To p-bovis and g-bovis a loop scoop each of stock *M. bovis* to be revived was inoculated. *M. tuberculosis* was inoculated into the bottles labelled p-tuberculosis and g-tuberculosis. All the four bottles were incubated aerobically at 37 °C in a light proof incubator for eight weeks.

2.4.3. Flask Preparation and Arrangement

2500 ml of fresh milk was obtained from tuberculin negative cows. 1140 ml of the milk was poured into a sterile 2-litres glass conical flask and labelled stock milk.

A cocktail of sour milk samples was made by pooling five of the sour milk samples used in step 2.3.1.. The 360 ml of cocktail sample was added to the stock milk flask to bring the total volume to 1500 ml.(1140 ml +360 ml = 1500 ml).

Twenty one 250 ml-glass flasks were sterilised and labelled AO, A2, A4, B0, B2, B4, C0, C2, C4, D0, D2, D4, E0, E2, E4, FO, F2, F4, G0, G2 and G4. The arrangement of the flask is shown in figure 2.3.

Figure 2.3.

Arrangement and labelling of the flasks.

A0	**B0**	**C0**	**D0**	**E0**	**F0**	**G0**
A2	**B2**	**C2**	**D2**	**E2**	**F2**	**G2**
A4	**B4**	**C4**	**D4**	**E4**	**F4**	**G4**

-Each square represents a flask.
-Each letter represents the label of the flask.
-The subscript number represents the dilution of *M. bovis* and *M. tuberculosis* (to be explained in the step below step 2.4.4).

100 ml of the stock milk was then measured and added to the first fifteen flask namely AO through to E4, and to the last three GO, G2, and G4. F0, F2 and F4, 100 ml of sterile PBS were added.

10 ml of the fresh milk and 10 ml of the resulting cocktail were taken and subjected to culturing of *M bovis* and *M. tuberculosis*(Procedure followed to be explained in step 2.4.7).

2.4.4. Harvesting and Dilution of *M. bovis* and *M. tuberculosis*

After eight weeks of incubation of the *M. bovis* and *M. tuberculosis* in step 2.4.2. The medium bottles were considered to be ready for the organisms to be harvested. A total of 66 colonies of *M. bovis* in the medium bottles labelled p-bovis and g-bovis from step 2.4.2. above were harvested by using a loop and added to the bottle labelled bovine containing 2 ml of PBS and glass beads. The bottle was agitated at a medium speed. A total of 45 colonies *M. tuberculosis* from step 2.4.2. were harvested and added to the other bottle labelled tuberculosis also containing 2 ml of PBS distilled water and glass beads. The bottle was agitated at a medium speed.

Ten test tube were labelled BO, B1, B2, B3, B4, T0, T1, T2, T3 and T4. Five ml of sterile PBS were put in test tubes B0 and T0, while to the rest of the tubes 9 ml of sterile PBS were added. The letters 'B' and 'T' denoted *M. bovis* and *M. tuberculosis* respectively while the numbers denoted the negative logarithm of the dilution of the culture suspension.

The agitated 2 ml culture suspension in the bottle labelled bovine was then poured in the test tube B0 with a volume of 5 ml PBS to bring the total volume of the culture solution to 7 ml. This was homogenised by agitating. The contents of the tube labelled tuberculosis were

added to test tube T0 to make the total volume of 7 ml. This was also homogenised by agitating. One ml of the homogenate from B0 was pored into B1. Then tenfold serial dilution was made right through to B4. The homogenate in the bottle labelled tuberculosis was made in the same way as above T0 through to T4.

2.4.5. Inoculation of *M. bovis* and *M. tuberculosis* into Fresh Milk.

At this stage the equipment that was paid attention to was the test tube labelled B0 through to T4 and the flasks labelled A0 through to G4.

To flasks A0, B0, C0, D0, E0 and F0 one millilitre of the homogenate from the test tube B0 was added to each. To flasks A2, B2, C2, D2, E2 and F2 one millilitre of the homogenate from test tube B2 was added to each. To flasks A4, B4, C4, D4, E4 and F4 one millilitre of the homogenate from test tube B4 was added. To flasks G0, G2 and G4, homogenate from test tubes T0 T2 and T4 one millilitre each were added respectively.

The pH of the contents of all the flasks were measured using a multi pH strip per flask. The pH readings were recorded on an appropriate sheet. The mouths of all the flasks were then covered with sterile aluminium foils and incubated in a light proof incubator at 22 °C which is the ambient temperature of most of the hats in the village.

Flask F0 ,F2 and F4 were PBS control experiments while G0, G2 and G4 were used for *M. tuberculosis* experiment. (Figure 2).

Figure 2.4

Final arrangement of the flasks and their contents.

Flask label	**A0**	**BO**	**CO**	**DO**	**E0**	**F0**	**G0**
Organism	*M. bovis*	*M. bovis*	*M. bovis*	*M. bovis*	*M. bovis*	*M. bovis*	*M. tuberculosis*
Dilution	10^{0}	10^{0}	10^{0}	10^{0}	10^{0}	10^{0}	10^{0}
Flask label	**A2**	**B2**	**C2**	**D2**	**E2**	**E2**	**G2**
Organism	*M. bovis*	*M. bovis*	*M. bovis*	*M. bovis*	*M. bovis*	*M. bovis*	*M. tuberculosis*
Dilution	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}
Flask label	**A4**	**B4**	**C4**	**D4**	**E4**	**F4**	**G4**
Organism	*M. bovis*	*M. bovis*	*M. bovis*	*M. bovis*	*M. bovis*	*M. bovis*	*M. tuberculosis*
Dilution	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}

All the flasks contained milk except F0, F2 and F4 which contained PBS.

2.4.6. Preparation and Labelling of Media Bottles.

Eighty four bottles of L-J pyruvate culture were labelled as follows: A0*0, A0*12, A0*48 and A0*72. The letter was denoting the flask while the first number was denoting, the negative logarithm to the base 10, of the dilution of the culture suspension and the number after the star was denoting the number of hours after inoculation of *M. bovis* and *M. tuberculosis* into the milk flasks in step 2.4.5. above. The same was applied to the other flasks B, C, D, E, F and G. All the pyruvate medium bottles were labelled with a 'p' on top of the labels.

Another 84 bottles of L-J medium with glycerol were labelled as those above. 'g' was labelled on these bottles denoting glycerol.

2.4.7. Sampling of Sour Milk for Viability Test of *M. bovis and M tuberculosis.*

Milk samples were taken from all the flasks at 0, 12, 24 and 72 hours after inoculation. At each sampling interval 21 universal tubes were used. 10 ml of the contents of each flask were poured into a sterile universal container. The universal container was then labelled as the flask from which the sample it contained was taken.

All the universal tubes were centrifuged at 3000 rpm, (Kubota KS 5000P), for 15 minutes. The middle layer was sucked and discarded by using a Venturi pump. The cream (top layer) and the sediment were left in the container. Four ml of four percent sodium hydroxide was

added to the cream and the sediment of each sample. The mixtures were then incubated at 37 °C. After 15 minutes of incubation the universal containers were centrifuged again at 3000 rpm for 15 minutes. The supernatant was decanted and discarded. 18 ml of sterile distilled water was added to all the universal containers and mixed well. All the universal containers were again centrifuged at 3000 rpm for 15 minutes. The supernatant was decanted and discarded leaving only about 0.5 ml of the sediment.

A loopful of the sediment was scooped from each bottle and inoculated in corresponding cultured bottles of the Lowenstein-Jensen pyruvate and Lowenstein-Jensen glycerol medium. For example contents of flask C4 sampled 48 hours after inoculation were cultured in medium bottles labelled C4*48.

The bottles were incubated at 37 °C in a horizontal position for an hour after inoculation to allow the inoculum to be absorbed. The bottles were then placed on trays in an upright position and incubated for eight weeks.

2.5 Detection and Isolation Tubercle Bacilli From Lesions of the Sacrificed Tuberculin Positive Reactors.

Two tuberculin positive reactor cows test were sacrificed and detailed PM conducted. Mediastinal lymph were collected from both cows. A section of a tuberculous lymph node from cow 1 was cut into small pieces using a surgical blade. These small pieces were then put in a blender and minced at a high speed. Twenty ml of sterile water was added to the

blending jar. The resulting suspension at this stage was treated in the same way as the sour milk was treated in step 2.4.7. Inoculation was done onto Lowenstein-Jensen pyruvate and Lowenstein-Jensen glycerol media

Slide smears were made from the suspension of the minced lymph nodes and stained with auramine and Ziehl-Neelsen stains. Sections of the lymph nodes were taken for histopathological examination. All the staining procedures followed were as recommended by Baker and Silverton (1993).

CHAPTER THREE

RESULTS

3.1. Tuberculin Test.

There were a total of 507 cattle ranging from the age of six months to nine years old that were included in the tuberculin test. Below are the results regarding to sex, age and herd.

Results indicated 6 male reactors and 59 females reactors of the animals tested. However the prevalence observed in both male and female animals were not significantly different statistically (Table 3.1).

Table 3.1.

Tuberculin test results according to sex of the tested cattle.

SEX	REACTORS +	NON REACTORS −	DOUBT ±	TOTALS	PREVALENCE OF THE POSITIVE REACTORS (%)
MALE	6	61	3	70	8.6
FEMALE	59	344	34	437	13.5
TOTALS	**65**	**405**	**37**	**507**	**12.8**

The highest prevalence of bovine tuberculosis was in the age group ranging from 45-55 months. This is the group comprising of the productive females and the males that are used for draught power.

However prevalence of tuberculin positive animals were significantly higher in those age groups of greater than 25 months old in comparison to the younger ones in the age group of less than 24 months old ($P<0.005$). (Table 3.2).

Table 3.2

Tuberculin test results according to age of the tested cattle.

AGE IN MONTHS	REACTORS +	NON REACTORS −	DOUBTFUL ±	TOTALS	PREVALENCE OF THE POSITIVE REACTORS (%)
1-24	2	27	3	32	6.3
25-40	14	96	7	117	12.0
41-55	45	256	19	320	14.1
56 AND OVER	4	26	8	38	10.5
TOTALS	**65**	**405**	**37**	**507**	**12.8**

In the three herds of Baambwe the average prevalence was 13.1 percent while the herds from Maala had an average prevalence of 12.9 percent. The overall prevalence of the sample was 12.8 percent. There was no statistical difference in the prevalence amongst the herds (Table 3.3).

Table 3.3

Herd prevalence of bovine tuberculosis.

LOCATION OF HERD	HERD NUMBER	SAMPLE SIZE	REACTOR +	NON REACTORS −	DOUBTFUL ±	P (%)	P* (%)
Baambwe	1	35	5	28	2	14.3	
	2	64	6	55	3	9.4	
	3	51	8	40	3	15.7	13.1
Maala	4	40	4	34	2	10.0	
	5	32	6	22	4	18.8	
	6	28	4	19	5	14.3	
	7	53	6	45	2	11.3	
	8	30	3	22	5	10.0	
	9	67	9	54	4	13.4	
	10	107	14	86	7	13.1	12.9
TOTAL	**10**	**507**	**65 (12.8%)**	**405 (79.9%)**	**37 (7.3%)**		**12.8**

p herd prevalence.

p* overall prevalence.

3.2. Detection and Isolation of Tubercle Bacilli From Lesions of the Sacrificed Tuberculin Positive Reactors.

Of the two cows sacrificed tuberculous lesions were seen in mediastinal lymph nodes of cow number 1. The lesions appeared to be calcified.

The impression smear made from lymph node from cow number 1 showed the presence of organisms indistinguishable from Mycobacterium organisms while there were no macroscopic lesion or Mycobacterium like organisms seen from lymph node smears of cow number 2

After 6 weeks of incubation colonies appeared on the L-J pyruvate medium which was inoculated with the lymph node suspension. Smears of the colonies were acid fast and identified as *M. bovis*.

Histopathological examination revealed diffuse granulomatous lymphadenitis characterised by the presence of granulomas and effacement of normal architecture . Each granuloma consisted of a central necrosis, caseations, cellular debris and calcfication surrounded by a zone of scattered epithiliod cells and giant cells. In the periphery of the granulomas there was proliferation of mononuclear cells, mainly lymphocytes. The medullary sinuses were dilated with marked lymphocitic infiltration and diffuse hemosiderosis (Plate 5).

3.3 Calabash Flora

There were nine major bacterial genera isolated from the sour milk. The predominant ones being of Gram positive rods. Three genera of yeast were also isolated (Table 3.4).

Table 3.4

Bacteria and yeast isolated from the sour milk.

Bacteria/ Yeast Isolated*	Number of mycro-organisms** (Log/ml)
Gram Positive rods Lactobacillus species Carnobacterium species .	7.9
Gram Negative rods Serratia species Kluyvera (Escherichia) species and others.	3.9
Gram Positive cocci Lactococcus species Streptococcus species. Staphylococcus species. Enterococcus species Gemella species and others.	5.8
Yeast Saccharomyces species Candida species Tricosporon species and others.	6.9

* A total of 14 sample were investigated

** average number of the 14 samples

3.4. The pH at Which Most of the Sour Milk is Consumed

A total of 30 samples of sour milk were collected and grade. The final grade of the milk sample was considered to be the grading of at least two of the individuals agreed (Table 3.5).

Table 3.5

The pH and the grade of the sampled sour milk.

CONSUMER	1	2	3	pH	GRADE
SAMPLE NO					
1	G	G	G	3.5	G
2	G	G	G	3.5	G
3	B	G/B	B	3	B
4	N	N	G	4.5	N
5	G	G	G	3.5	G
6	G	G	G	3.5	G
7	G	N/G	G	4	G
8	B	B	G/B	3.5	B
9	B	B	N	3	B
10	N	N	N	6	N
11	N	N	G	6.5	N
12	G	G	G	3.5	G
13	G	G	G	3.5	G
14	G	G/N	N	4	G
15	N	N	N	4.5	N
16	N	N	G	4.5	N
17	G	G	G	3.5	G
18	G	G	B	3.5	G
19	B	B	B	3	B
20	B	B	B	3	B
21	G	G/B	G/B	3	B
22	B	G	N	3.5	B
23	N	N	G	4.5	N
24	G	G	G	3.5	G
25	G	G	G/B	3.5	G
26	B	G	N	3.5	B
27	N	N	G	4.5	N
28	G	G	B	3.5	G
29	B	B	G/B	3	B
30	B	B	G/B	3.5	B

G --- good sour milk.
N --- not yet sour.
B --- bitter sour milk.

There were seven samples graded as not yet being sour, on the average these samples had a pH above 4. Thirteen samples were graded as being good sour milk. These had a pH range of 3.5 to 3. There was no sample that was found to have a pH below 3. The average pH of the milk in the group that was graded as being good sour milk was 3.5. The most frequent pH was 3.5. Note worthy is the variation in with the taster themselves. Out of the 4 samples that had a pH of 4.5 (samples number 4, 15, 16, 27) three of the samples (4, 16, 27) were graded as being good sour milk, while only one was graded as not yet being sour. The other two graded 4.5. as not yet being sour. The same taster graded samples number 8. 22, and 28, with a pH of 3.5 as bitter contrary to the grading of the same samples as being good sour milk by the others. The same taster turns out to be inconsistent by grading sample number 14 with a pH of 4 as not yet being sour and number seven with the same pH as being good. (Table 3.5)

The results of the tasters as individuals may seem to vary yet as a group there is consistency. (Table 3.6).

Table 3.6

The total of graded sour milk and the average pH values.

GRADE	TOTAL NUMBER OF SAMPLES	AVERAGE pH OF THE GRADE	pH MODE OF THE GRADE
NOT YET SOUR	7	5.1	4.5
GOOD SOUR MILK	13	3.6	3.5
BITTER SOUR MILK	10	3.2	3.0

3.5. Tubercle Bacilli From Fresh Milk and the Sour Milk Cocktail.

There were no colonies of mycobacterium seen on the L-J (pyruvate and glycerol). media inoculated with the fresh milk and the cocktail used in steps 2.3 and 2.4.3 respectively.

3.6. Viability of *M. bovis* and *M tuberculosis* in Sour Milk.

M. bovis colonies were recovered from 37 culture bottles amongst 56 (66.1 %) with varying culture dilution (10^0, 10^{-2} and 10^{-4}) incubated in sour milk for varying intervals (0, 12, 24 and 72 hours). *M. tuberculosis* colonies were also recovered from nine culture bottles amongst 12 (75.0 %) with varying culture dilution incubated in souring milk in similar manner as *M. bovis*. All the colonies were confirmed as acid fast by Ziehl-Neelsen stain. Colonies of *M. bovis* and *M. tuberculosis* were observed from all the culture suspension dilution from the lowest to the highest concentrations (Tables 3.7 and 3.8).

As a result *M. bovis* and *M. tuberculosis* colonies were recovered on L-J (pyruvate or glycerol) medium even after 72 hours of inoculation in sour milk samples with the lowest to highest dilation of culture (10^0 to 10^{-4}).

Table 3.7

Recovery of *M. bovis* and *M. tuberculosis* in the Lowenstein -Jensen media after 8 weeks.

Hrs after inocula-tion	*M. bovis*						*M.tuber-culosis*
	A0	**B0**	**C0**	**D0**	**E0**	**F0**	**G0**
0	+	**FLASK**	**L**	+	**L**	+	+
12	+	**LOST**	-	+	+	+	+
24	**L**	**(L)**	+	-	+	-	+
72	+		-	+	**L**	-	-
	A2	**B2**	**C2**	**D2**	**E2**	**F2**	**G2**
0	+	**L**	+	+	-	-	+
12	+	-	+	-	-	+	+
24	-	+	+	-	**L**	+	-
72	+	+	**L**	-	-	+	+
	A4	**B4**	**C4**	**D4**	**E4**	**F4**	**G4**
0	+	+	+	-	+	**L**	-
12	+	-	+	+	-	-	+
24	-	-	**L**	+	-	+	+
72	+	+	-	**L**	+	+	+

+, indicates growth was confirmed with colonies
-, indicates no growth was confirmed
L, indicates medium lost due to contamination.

Table 3.8.

Summary of Growth of *M. bovis.* and *M. tuberculosis*

SAMPLING INTERVAL	0 hours		12 hours		24 hours		72 hours	
DILUTION	*M. bovis*	*M. tub*	*M. bovis*	*M. tub*	*M. bovis*	*M. tub*	*M. bovis*	*M. tub*
10^0								
+	2	1	3	1	2	1	2	0
-	0	0	1	0	1	0	1	0
L	2	0	0	0	1	0	3	0
T	2	0	4	0	3	0	0	0
F	2/2	1/1	¾	1/1	2/3	1/1	2/3	0
	(100%)	(100%)	(75%)	(100%)	(67%)	(100%)	(67%)	0%
10^{-2}		1						
+	3	0	2	1	2	0	2	1
-	1	0	3	0	2	0	2	0
L	1	0	0	0	1	0	1	0
T	4	1	5	0	4	0	4	0
F	3/4	1/1	2/5	1/1	2/4	0	2/4	1/1
	(75%)	(100%)	(40%)	(100%)	(50%)	0%	(50%)	(100%)
10^{-4}						t		
+	4	0	3	1	1	1	3	1
-	1	0	2	0	3	0	1	0
L	0	0	0	0	1	0	1	0
T	5	0	5	0	3	0	4	0
F	4/5	0	3/5	1/1	1/4	1/1	3/4	1/1
	(80%)	(0%)	(60%)	100	(25%)	(100%)	(75%)	(100%)

+ indicates the total number of culture bottles that showed growth of *M. bovis* at the end of the experiment.
- indicates the total number of culture bottles that showed no growth of *M. bovis* at the end of the experiment.
T indicates the total number of culture bottles examined at the end of the experiment.
F indicates the total number of culture bottles that showed growth of *M. bovis* and *M. tuberculosis* at the end of the experime
expressed as a fraction
L the total number of media bottles lost up to the end of the experiment.
M tub *M. tuberculosis*

The pH of the milk samples was reduced from pH 7 at the beginning of the experiment to p
3 after 72 hours, the end of the experiment (Table 3.9). The average pH value at the sampli
intervals of 0, 12, 24 and 72 hours were 7, 6.5, 3.7 and 3 respectively (Table 3.10).

Table 3.9.

pH of the milk at sampling intervals.

	Hrs. after inoculation	pH	pH	pH	pH	pH	pH	pH
Flask label		**A0**	**B0***	**C0**	**D0**	**E0**	**F0**	**G0**
	0	7		7	7	7	7	7
	12	6.5		6.5	6.5	6.5	7	6.5
	24	3.5		3.5	4	4	7	3.5
	72	3.		3	3	3	7	3
Flask label		**A2**	**B2**	**C2**	**D2**	**E2**	**F2**	**G2**
	0	7	7	7	7	7	7	7
	12	6.5	6.5	6.5	6.5	6.5	7	6.5
	24	4	4.5	3.5	3.5	4.5	7	3.5
	72	3	3	3	3	3	7	3
Flask label		**A4**	**B4**	**C4**	**D4**	**E4**	**F4**	**G4**
	0	7	7	7	7	7	7	7
	12	6.5	6.5	6.5	6.5	6.5	7	6.5
	24	4	3.5	4	3.5	3.5	7	4
	72	3	3	3	3	3	7	3

B_0^* the beaker broke just after inoculation.

F0, F2 and F4; buffered PBS

On the average there was a rapid decrease in pH from 7.0 to 3.8 in the first 24 hours and a very gradual decrease to 3.0 in the last 48 hours.

Table 3.10

The average pH values in the group flask at sampling intervals

GROUP OF FLASKS	A	B	C	D	E	AVERAGE pH (A,B,C,D,E.)	F	G
SAMPLING INTERVALS (hours)								
0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
12	6.5	6.5	6.5	6.5	6.5	6.5	7.0	6.5
24	3.8	4.0	3.6	3.6	4.0	3.8	7.0	3.8
72	3.0	3.0	3.0	3.0	3.0	3.0	7.0	3.0

CHAPTER FOUR

DISCUSSION.

4.1. Epidemiology of *M. bovis* in Zambia.

Bovine tuberculosis is an important disease of cattle in Zambia. The disease is widely distributed throughout the country. There are reports of high, (346 in 1995) abattoir carcass condemnation of cattle from the Southern Province due to tuberculosis (Anonymous 1995). In an abattoir survey of bovine pulmonary disease carried out at the Cold Storage Board of Zambia in Lusaka, Pandey and Sharma (1987), found a prevalence of bovine tuberculosis of 7.46 percent.

The overall prevalence of bovine tuberculosis in the tested herds was 12.8 percent . The prevalence in the female cows was 13.5 percent while in the males was 8.6 percent. The prevalence among the herds is not significantly different in studied areas . This is well reflected from the fact that all the cattle from the area mix when grazing on the pasture for more than 6 months throughout the year It is on these plains that these cattle inter mingle with game more especially free range lechews making the control of *M bovis* extremely hard (Stafford, 1991). There are, however no specific reports showing the isolation, identification and distribution of *M bovis* in Zambia. Although some Mycobacterium have been isolated from cattle. The data has not been presented in this work.

There are more accurate and fast methods, such as PCR, DNA probes have been developed for the identification of Mycobacterium species, and are being introduced in this country). Currently in most developing countries even conventional methods are hard to practice for diagnosis of bovine tuberculosis (Jarnagin and Payeur, 1989).

4.2. Public Health Importance of Bovine Tuberculosis in Zambia

Bovine tuberculosis is a zoonosis and is transmitted from cattle to human population (Acha and Szfres 1987). However *M. bovis* transmission from bovine and humans can markedly reduced in line with successful bovine tuberculosis control programmes (Shimao 1986).

Tuberculosis both in man and animals is subclinical in many cases. This means that one species can remain as a source of infection for the other directly or indirectly for long periods. The tubercle bacilli can survive in humid environment for years or even decades. Thus the contaminated environment (water, housing, soil, pastures etc.) can equally remain for long periods as a source of infection.

Whereas many developing countries are committed to eradication of human tuberculosis by chemotherapy, BCG immunisation and legislature in favour of breaking the transmission cycle, very little is being done to eradicate bovine tuberculosis. This is due to the fact that there is no vaccine, identification of infected herds and immediate slaughter of positive reactors is not practical due to economical constraints. The higher the cases of bovine tuberculosis the greater the chances of transmitting the disease not only to other animals but

also to humans. The chain of transmission of tuberculosis from bovine to humans has to be broken. This can effectively be achieved by pasteurisation of milk.

While *M. bovis* is the main species in the Mycobacterium genus that has been identified to be transmitted in milk, though other species are known to be excreted in milk . Acha and Szfres, (1987), described that there is some evidence of *M. tuberculosis* having been isolated from milk of cows. Tubercle bacilli were isolated from milk of buffaloes too ,that were commercially milked. This indicates that buffaloes can also be a source of infection. (Batish , *et al.,* 1989).

It is worthy to note that other atypical forms of Mycobacterium have been isolated from milk *M. paratuberculosis* has been isolated from milk and lymph nodes of asymptomatic cows. (Sweeney , *et al.,* 1992).

In certain communities where cattle infection is low this low prevalence is considered not to significantly contribute to public health problem (Bakunzi , *et al.,* 1995). Although in the authors opinion this is equally significant as a source of infection especially in those countries with reduced prevalence or practising the control programmes. Several decades ago however there has been a correlation between the prevalence of bovine tuberculosis and human tuberculosis, for every 10 percent increase in bovine tuberculosis there was a 5 percent increase in proportion of people with a positive tuberculin reaction (Hedvall, 1941). Such a correlation of human tuberculosis and tuberculin positive animals has also been demonstrated

in Zambia (Cook , *et al.*, 1996) Thus compulsory pasteurisation has been conducted even in those countries where bovine tuberculosis has become well controlled.

4.3. Public Health Significance of the Results Obtained.

In the present experiment the author elucidated that *M. bovis* was isolated from sour milk introduced experimentally at any dilution of 10^0, 10^{-2}, and 10^{-4} of the culture suspension . At all these concentration it was also isolated at 0, 12, 24 and 72 hours after inoculating it in the sour milk. Comparison of the *M. bovis* recovery results zero hours and 72 hours after being introduced in sour milk reveals that *M bovis* is not eliminated in the process of souring milk.

This is an evidence that there is an indication for remaining viability of *M. bovis* in sour milk processed even 72 hours. The organisms remained viable in the optimal consumption time (24-48 hours). A major implication to be drawn here is that, *M. bovis* excreted in milk by a tuberculous cow remain viable even 72 hours of souring. Since most of the sour milk is consumed 24 hours after souring it, the organism would still remain viable in sour milk contaminated with *M. bovis* at the time of consumption although pH goes down to 3.0.

As far a *M tuberculosis* is concerned, this micro-organism also remained viable and was recovered from sour milk processed for 72 hours. Therefore public health significance examined in *M bovis* infection in souring milk can also be applied to *M. tuberculosis* in case of milk infected with the micro-organism.

In a similar experiment done Lafont, (1985), it is reported that viable Mycobacterium organisms were recovered four months later from cheese made from milk that was inoculated with bovine stains of tubercle bacilli although tubercle bacilli decreased in number in the first and second weeks.

At 24 hour after milking and souring milk the pH is expected to be 3.5. The ability of Mycobacterium be viable even under the conditions of as low pH and the inhibitory factors of nisin is attributed to the high quantity of lipids and mycolic acids in the cell wall of the organisms (Jawetz , *et al.*, 1984).

Although consumption of unpasteurised milk, drinking habits with special interests to sour milk may contribute to the risk of human infections and the prevalence of bovine tuberculosis in the herds is an important factor in transmitting the disease.

Bovine tuberculosis via milk in natural transmission cycle can only be transmitted by cows with infected udders. It is estimated that infection of the udder is found in one percent of tuberculous cows (Timoney, *et al.*, 1993). For the organism to cause disease a sufficient number has to be excreted so that of these a number above the minimum infectious dose has to remain viable in the processed sour milk when it is consumed by the susceptible host.

This study therefore only elucidates that *M. bovis* which was experimentally inoculated in milk and then traditionally processed into sour milk remained viable within the optimal time (24-48 hours) of souring milk.

4.4. Future Work.

1) Zambia is a wide country with a varying cattle population from one province to the other. Though bovine tuberculosis has been reported mostly from Southern and Western Provinces more accurate characterisation of tuberculosis should be done with routine diagnostic methods in combination with other modern diagnostic methods including PCR as described by Barry , *et al.,* (1993), and using the methods described by Morris , *et al,. (*1994*)*.

2) The study was done on milk that was not naturally infected. This study therefore provides with an experimental model how M. bovis would behave in the process of souring milk. It would therefore contribute to the interpretation of the viability of *M. bovis* in naturally infected milk.

3) It is required to study the prevalence of milking cows that excrete *M. bovis* in milk and to examine the amounts of the organisms excreted to identify the magnitude.

4) It would be essential to isolate M. bovis from human populations suffering from tuberculosis and to identify the source of infection with special interest to whether the infection is caused by contaminated sour milk or other sources.

5) A safer way of souring milk that would preserve the taste but eliminate the tubercle bacilli should be sought and encouraged among the Ilas and other tribes which consume sour milk processed in a similar manner as the Ila people do. Bovine tuberculosis control programme have to be introduced to the area to protect human population at risk. Education of the general public is also needed to be urged in view of the present results obtained.

CHAPTER FIVE

CONCLUSION

The overall prevalence of bovine tuberculosis in the study area was 12.8%. This is high as compared with other parts of Zambia, Senanga and Mongu with 1.8 and 7.4 percent respectively. Unless drastic control measures against the spread both human and in cattle are taken, tuberculosis will for a long time remain not only as a zoonotic menace but also a threat to the diary production in Namwala.

The bacteria flora of the sour milk included some bacteria genera that have pathogenic species. The lactose fermenting bacteria were mainly isolated from the flora.

Viable *M. bovis* were isolated throughout the time intervals in the process of souring milk. This means that the traditional method of milk souring does not eliminate *M. bovis* at the time it is consumed. The present experiment showed that the results obtained with *M bovis* can also be applied to *M tuberculosis* in case of milk being infected with this mycobacterium species. The general tradition of close association of cattle and the consumption of traditionally processed sour milk in Namwala provides a conducive situation of transmission of tuberculosis as a zoonotic disease.

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PLATES:

Plate 1-1

Different sizes and shapes of calabashes

Note the stick on top of the calabash. This is used to close the mouth of the calabash (left) and are transferred from old ones to virgin ones. By doing so the flora is transferred from the old ones to the virgin ones.

Plate 1-2

Make shift homes of the herd workers on the plains with several calabashes. A child and a wife of a herd worker sharing dwellings with the cattle.

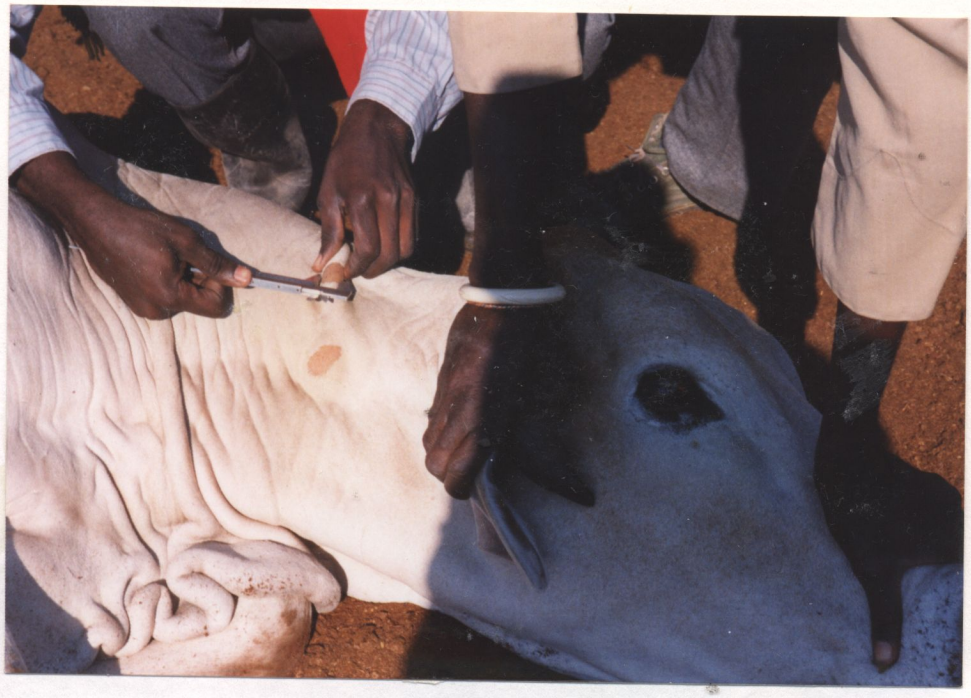
Plate 2

Part of the herd that was tested for bovine tuberculosis.

Plate 3

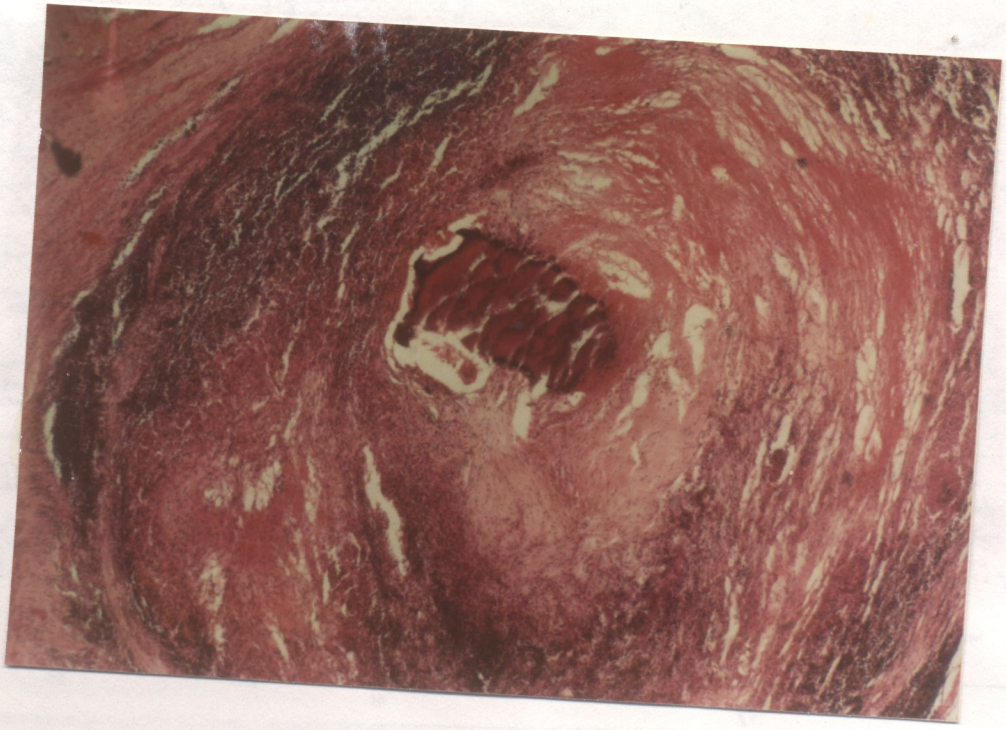
A cow cast down for tuberculin test. Note the two sites that are shaved for inoculation of the tuberculin.

Plate 4



Skin measuring of a reactor. Note that the breed is a cross breed.

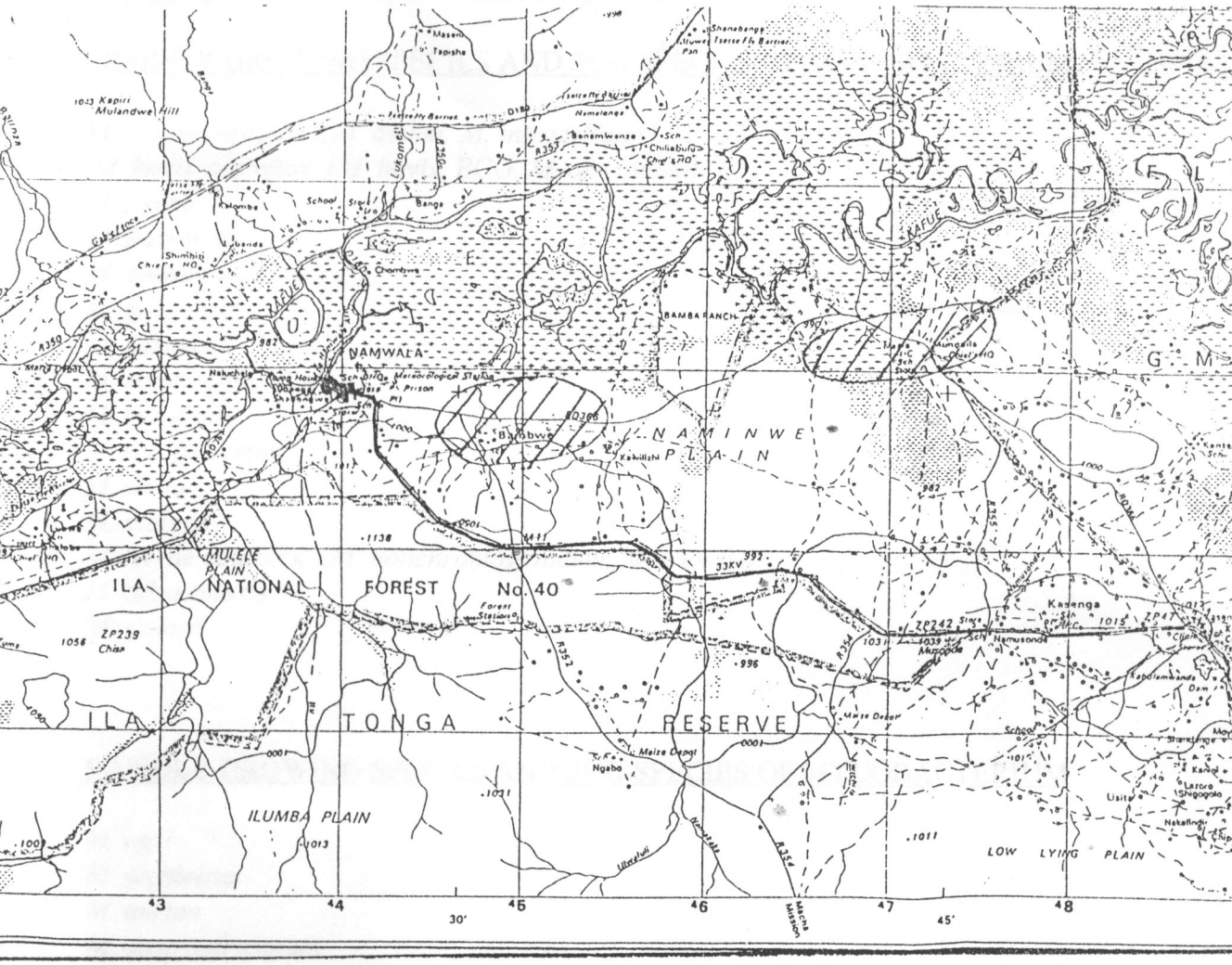
Plate 5



Histopathological picture of a lymph node from a tuberculin reactor.

APPENDIX I

MAP OF NAMWALA



STUDY AREA

APPENDIX: 2

List of Mycobacterium species and sub-species.

SLOWLY GROWING SPECIES AND SUB-SPECIES OF MYCOBACTERIUM

M. avium complex .(*M. avium, M. intracellular).*
M. bovis complex .(*M. bovis, BCG, M. africanum).*
M. cookii
M. flavescens
M. gastri
M. gordonae
M. kamsasii
M. malmoense
M. marinum
M. scrofulaceum
M. simiae
M. szulgai
M. terrae complex .(*M. nonchromogenicum, M. triviale).*
M. tuberculosis
M. xenopi

RAPIDLY GROWING SPECIES AND SUB-SPECIES OF MYCOBACTERIUM

M. agri
M. aichiense
M. aurum
M. anstroafricanum
M. chelonae abscessus
M. chelonae chelonae
M. chitae
M. chubuense
M. diesrnhoferi
M. duvalii
M. fallax
M. flavescens
M. fortuitum acetamidolyticum
M. fortuitum fortuitum

M. fortuitum peregrinum
M. gadium
M. gilvum
M. komossense
M. moriokaense
M. noearum
M. obuense
M. parafortuitum
M. phlei
M. porcinum
M. poriferae
M. pulveris
M. rhodesica
M. senegalense
M. smegmatis
M. sphagni
M. thermoresistibile
M. tokaiense
M. vaccae

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