

**Study of the prevalence of bovine tuberculosis in Govuro District,
Inhambane Province, Mozambique**

By

Baltazar Antonio Macucule

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Declaration

I hereby declare that this dissertation, submitted by me to the University of Pretoria for the degree Master of Science has not previously been submitted for a degree at any other university.

Baltazar Antonio Macucule

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ABBREVIATIONS

BTB:	Bovine Tuberculosis
TB:	Tuberculosis
PCR:	Polymerase Chain Reaction
SE.:	Standard Error
VL:	Visible Lesions
INE:	Instituto Nacional de Estadística
MTC:	Mycobacterium Tuberculosis Complex
95% CI:	Ninety Five Percent Confidence interval
SIT:	Single Intradermal Test
SICTT:	Single Intradermal Comparative Tuberculin Test
OIE:	Office International des Epizooties
PARPA:	Plano para a Redução da Pobreza Absoluta
PPD:	Purified protein Derivative
PPD-b:	Purified Protein Derivative from <i>Mycobacterium bovis</i>
PPD-a	Purified Protein Derivative from <i>Mycobacterium avium</i>

VETAID: Veterinary Aid in Development, UK

DINAP: Direcção Nacional da Pecuária

SPP: Serviços Provinciais de Pecuária

HE: Hematoxylin/Eosin

ZN: Ziehl-Nielsen

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Supervisor : Prof Jacques Godfroid
Co-supervisor : Prof Peter Thompson
Co-supervisor : Dr Adelina Machado
Department : Veterinary Tropical Diseases
Degree : MSc

ABSTRACT

This study was conducted to confirm the presence of bovine tuberculosis (BTB) and determine its prevalence, based on skin test reactivity, in cattle reared under extensive farming conditions in the Govuro district, Inhambane province, Mozambique. The study was comprised of a primary screening test using the single intradermal test (SIT) in randomly selected animals from Colonato and Sede dip tanks in Govuro. Positive

reactors to the SIT were tested again with bovine and avian tuberculin using the single intradermal comparative test (SICTT) 7 weeks after the SIT.

The sample size was calculated using Win Episcopa 2.0 based on 95% confidence to detect a 2% expected prevalence using the SIT, with a 1% accepted error and accounting for a total population size of 7208. The calculated sample size was 682 animals. To compensate for the probability of 20% default in reading, the sample size was increased to 853.

During the testing process (SIT), it was evident from the first 3 reading days that the apparent prevalence (61, 94%) was higher than expected (2%), hence we decided to stop when the total number of cattle was 530. During the testing process (SIT), it was evident from the first 3 reading days that the apparent prevalence (61.94%) was far higher than expected (2%), hence we decided to stop when the total number of cattle was 530. This was due to the fact that, at such a high prevalence, it would not be necessary to achieve as high a precision as 1% accepted error. A sample size of 530 would be sufficient to achieve a precision of 4% accepted error, which was regarded as more than adequate.

The 530 cattle, 3 or more years of age, were selected using systematic random sampling from the two dip tanks (Colonato 371 and Sede 159 animals). All animals were identified by numbers painted, dorsally on the sacral region.

Out of 530 tested cattle by SIT, 268 were read, and 166/268 (61.94% with 95% confidence interval [CI]: 55.8 – 67.8%) were found positive, with visible swallow at the injection site. Apparent prevalence (AP) was found to be 61.94% while the true prevalence (TP) was 75.92%. The predictive value of a positive result (PV+) was found to be 87.9%. No significant difference in apparent prevalence between the two areas was detected by Fisher's exact test ($P = 0.11$). By SICTT, out of 28 animals positive reactors to SIT, 21 were possible to read, and 13/21 (61.9%; 95% CI: 55.1 – 89.3%) were found positive.

A three year old bull, positive reactor to the SIT, was slaughtered, and a detailed post mortem was carried out and organs with visible lesions were collected for further laboratory testing (histopathology, culture and isolation of *M. bovis* and PCR). Later on, 30 more positive reactors to the SIT test were slaughtered: 25/30 (83.3%) showed visible lesions compatible with BTB, and total condemnation of carcass was made in 3/25 (12%) due to generalized lesions.

The high prevalence rate of skin test positive animals as well as gross lesions and histopathology were confirmed to be BTB by the isolation and identification of *M. bovis* by culture and PCR. Our results suggest that bovine tuberculosis is highly prevalent in Govuro district and may thus represent a potential health problem of zoonotic tuberculosis in humans.

Our results suggest that BTB has reached the plateau phase of endemicity in cattle in Govuro district. In this context, the positive predictive value of the SIT is very high and thus the use of the SICTT as a confirmatory test has a limited value and should not be advocated. Our results further indicate that no other prevalence study of BTB should be conducted in the next few years in Govuro district, unless comprehensive control measures are implemented. The focus of further studies should be on the isolation and the molecular characterization of *M. bovis* from cattle and humans in order to assess transmission routes and the role played by BTB in human TB cases in Govuro district.

Chapter 1: Introduction

Background

Mozambique is a vast territory (area = 801,590 km²) and stretches for 2,470 km along Africa's southeast coast (Figure 1). The Republic of Mozambique is in Southern Africa, bordering South Africa and Swaziland to the south; Tanzania to the north; Malawi, Zambia and Zimbabwe to the west. The Comoros lie offshore to the north east and Madagascar lies across the Mozambican channel. The human population is 20,905,585 (INE: census 2007).

Most of the country possesses a favourable climate, fertile land and adequate rainfall with the exception of the south of the country where periods of drought, sometimes prolonged, occur. The climate ranges from tropical to subtropical. The rainy season coincides with the hot months (November to March) though most provinces have some rain over 7 to 9 months of the year. Annual precipitation varies from 500 to 900 mm, depending on the region, with an average of 590 mm. However, 80% of the rain falls from November to March. The cold/dry period is between July and September but in the coastal areas the average temperature does not fall below 12°C. Average temperature ranges are from 13 to 24°C in July to 22 to 31°C in February. Cyclones are common during the wet season.



Figure 1: Map of Mozambique and the neighboring countries (adapted from http://www.orphansinAfrica.org/mozambique_home.htm)

The Mozambican territory has a wide variety of agro-ecological conditions, food reserves and genetic animal resources that are extremely favourable for livestock activity. There is an age-old tradition of keeping animals and using draught animals in agriculture. About 80% of the Mozambican rural population are livestock keepers. The various species of animals kept by rural Mozambican families not only have an economic value, but also play significant social roles in the lives of these families. The extensive areas of natural pastures and the existence of a market in great need of livestock products are excellent development opportunities for the livestock sub-sector.

The prevalence of poverty in Mozambique is of 70% and it is most severe in the rural areas. It therefore means that if poverty is to be reduced, the target of the Mozambican government should mainly be directed to the rural communities, the great majority of them being livestock keepers (Bernard 2006, unpublished data).

One of the major challenges facing the government is to feed its population by encouraging more home-based food production and relying less and less on imported food products. The great potential of the livestock sub sector means that it can act as one of the key alternatives through which Government can fight poverty, with a resultant increase in economic growth (PARPA – Absolute Poverty Reduction Plan).

Presence of Diseases

The presence of diseases is the main limiting factor for livestock development on small rural farms. It affects productivity as well as impeding effective growth. Diseases such as foot-and-mouth disease, African swine fever, lumpy skin disease and Newcastle disease occur as epidemics in Mozambique and are thus strategically important diseases with regard to the economic impact on trade and food security in the country (DINAP, 1998). These diseases spread easily and can reach epidemic proportions that require regional cooperation. The existence of natural reservoir hosts and asymptomatic carriers contributes to the persistence of these diseases in the environment and threatens the family, national and regional economy (Bernard 2006 – unpublished data). An important livestock disease present in Mozambique is bovine

tuberculosis (BTB), a zoonosis, with a difficult control process due to lack of funds for compensation.

Study area

Govuro is one of the northern districts of Inhambane province with 34,809 inhabitants (6,474 families), according to INE (2007). It is bordered to the north by Machanga district of Sofala province across the Save River, to the west by Mabote district, to the south by Inhassoro district and to the east by the Indian Ocean (Figure 2). Of the 6,474 families, 453 keep ruminants, especially cattle, of which there are 7,208 head (Provincial Livestock Service Census 2006).

Similar to many other developing countries, the production system in Govuro district is a communal/pastoral system. Cattle are distributed in 19 zones or herds and the grazing is on natural pastures without any supplementation. There are two dip tanks (Colonato with 60% and Sede with 40% of the total cattle in the district), which serve as the concentration points for tick control as well as for the census and sanitary assistance in general. Each diptank is managed by a committee democratically elected by the community under the supervision of the District Directorate of Agriculture.

During the rainy season there are many natural water points throughout the grazing area, but during the dry season cattle drink water from Save River. The distance between the two dip tanks is about 15 km and between them there are many small farms growing crops.

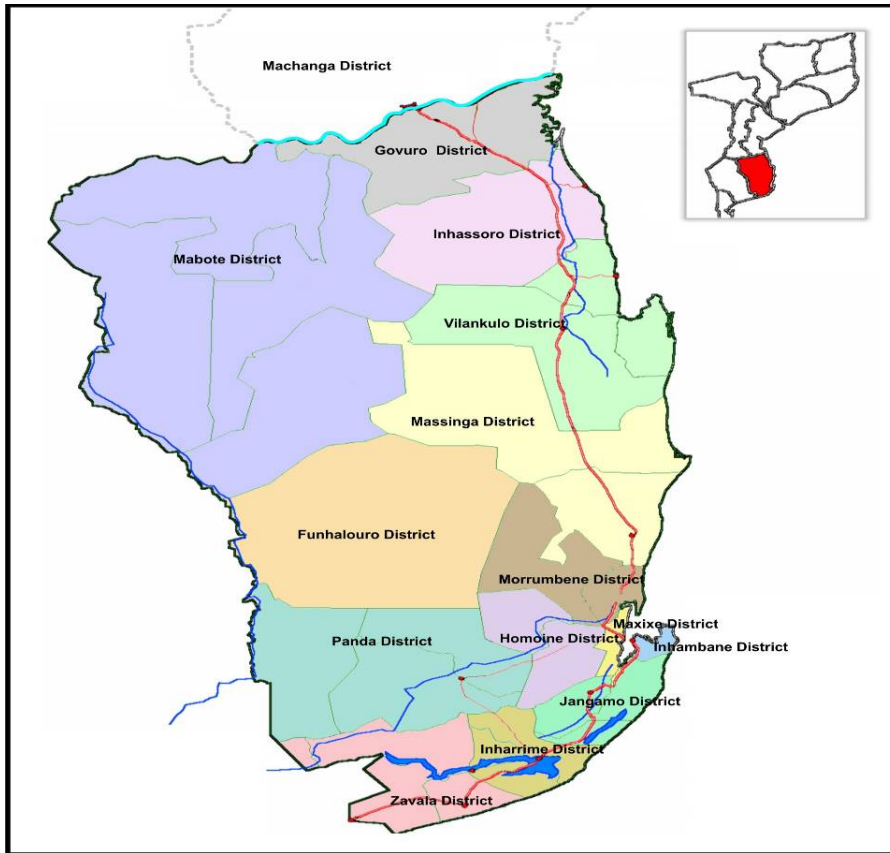


Figure 2: Location of Inhambane province and the district under study

Bovine tuberculosis in Mozambique

The monitoring of BTB prevalence in Mozambique was carried out up to 1981, when the Government was the primary provider of veterinary service delivery. After this period, as in many other developing countries, structural adjustment programmes were implemented, a financial crisis ensued, and the traditional veterinary systems began to experience severe difficulties. The result was a drastic reduction in the role of the state in veterinary service delivery. From 1981 to 1992, slaughter without compensation was enforced and more than 25% of the national herd was tested every year, with positive animals being sent to the abattoir. Thus the average prevalence of BTB in Mozambique declined from 11.9% to 2.9% by the end of this period (DINAP 2003).

Inhambane province had an average BTB prevalence of 10.2% in 1981 and achieved a reduction of 7.5% by the end of the period (Table 1), but the number of animals tested was considerably lower (1.6%) than the national herd. Govuro district, the prevalence of BTB was found to be 1.5% (n=869) in 1997 and 1.7% (n=517) in 2001 in an average population of approximately 5000 animals. In both surveys the testing was carried out in the caudal fold (SPP 2001).

Table 1 - BTB Testing results from Inhambane province, 1981-1986

Year	Cattle	Tested	Tested (%)	Test + (n)	Prev % (95% CI)*
1981	99949	1730	1.73	176	10.20 (8.8 – 11.7)
1982	89593	167	0.19	2	1.2 (0.15 – 4.3)
1983	79628	50	0.06	0	0.0 (0.0 – 5.8)
1984	45336	2821	6.22	115	4.1 (3.4 – 4.9)
1985	41777	611	1.46	6	1.0 (0.4 – 2.1)
1986	38215	331	0.87	9	2.7 (1.3 – 5.1)

* Binomial exact

Based on the results of the tuberculin skin tests associated with lesions compatible with TB found during inspections at slaughter houses, the Provincial Livestock Services (SPP), have considered Govuro as infected with BTB. In 1995 the district started the implementation of a movement control programme for cattle in an attempt to reduce the spread of the disease to the other parts of the province or country. The community was extensively informed about the problem and local committees were formed to aid in the control process. The task of the committees was to control the movement of cattle (Robbery and Disease Control). In reality, the committees and the community in general have been focused on preventing theft rather than on disease control.

In order to control the movement of cattle it was decided that no breeding animal should be moved out of the district. Only animals to be slaughtered were allowed to move out of the district under the control of both the community committee and district technician, with a permit issued by the District Officer. Only two selling points at the Colonato and Sede dip tanks were allowed once a week to enforce this control policy.

Machanga district, to the north of Govuro, was found to have the same prevalence of BTB as Govuro. The movement of cattle between the two districts is far more difficult to control, as the Save River's water level is reduced during the dry season. In addition to this, it was agreed that people from Machanga could market their cattle in Govuro as the latter has better trading conditions, hence the free movement of cattle between the two districts. The established control programme, rather than reducing the spread of disease, is complicating the livelihood of the producers because they face difficulties in selling their animals. Also, the test and slaughter policy is not enforced, as government lacks funds for compensation.

Cattle play an important role in the lives of smallholders in Govuro. The most common uses are for ploughing the land and for the transportation of people and goods. They are also used as a source of cash when sold for slaughter or for breeding. The multiple roles played by cattle in the area are an important contribution to the food security situation, which is periodically influenced by scarce and erratic rains.

As in many other districts of the country, apart from tuberculin skin testing and inspection at the slaughter houses, no other studies have been carried out in order to confirm the presence of BTB and to estimate its prevalence in Govuro district. For many years, based on the results from the tuberculin skin test, it has been assumed that BTB is present in Govuro district at a low prevalence. According to Corner (1994), bacteriological isolation of *M. bovis* from the lesion is the only way to make a definitive diagnosis. Isolated cases of organs with lesions compatible with BTB from cattle bought in Govuro district and slaughtered in three different slaughter houses in Inhambane province as annually reported, support a notion of an existing infection of BTB. Although the presence of BTB is suspected, its prevalence is unknown.

Objective of the study

The aim of this study, therefore, was to document the occurrence of BTB and estimate its prevalence with more accurate methods as the basis for the future National Programme of Prevention, Control and Eradication of Tuberculosis and Brucellosis (PNPCETB).

This study will be a crucial contribution on BTB control as well as on eventual investigation of the possible role played by bovine tuberculosis in humans under the National Programme of Prevention, Control and Eradication of Brucellosis and Tuberculosis (PNPCETB).

Chapter 2: Literature Review

Economic Impact

Bovine tuberculosis has been recognized as an important human and animal health issue for many years (Cousins and Roberts 2001; Ayele *et al.* 2004; Piran and James 2004; Moda 2006). According to the American Thoracic Society and Centers for Disease Control (1986, cited by Collins 1989), it have been estimated at more than half billion dollars a year, the amount incurred in identification, diagnosis, and treatment of tuberculosis worldwide, together with the associated loss in productivity. The susceptibility of man to tuberculosis due to *M. bovis* is one of the main reasons for interest in BTB (Grange and Yates 1994). According to Cosivi *et al.* (1998), more than 3.5 million people die annually from TB, with *M. bovis* being responsible for approximately 3% of the cases. In agriculture BTB causes severe economic losses in livestock due to low production, animal deaths and condemnation of carcasses. It is also an important constraint in international trade of animals and animal products (Cousins and Roberts 2001; Suazo *et al.* 2003). Despite intensive efforts towards freedom from the disease over a number of decades, BTB continues to be a significant local problem in many parts of the world. The impact of the disease varies, both by continent and by economic status within continents (Pollock *et al.* 2006).

Mycobacteria

According to Quinn, cited by Oloya (2006), and Biet *et al.* (2005), mycobacteria are gram-positive, slow growing and relatively resistant to chemical disinfectants, acid-fast, and non-sporing. *Mycobacterium bovis* is considered to be an obligate intracellular pathogen whose most efficient way of infection is direct animal contact (Pollock and Neill 2002). *Mycobacterium bovis* is a member of *Mycobacterium tuberculosis* (MTB) complex (Serraino *et al.* 1999; de la Rúa-Domenech *et al.* 2006). Experimental evidence has shown that *M. bovis* can survive for long periods outside an animal host in an environment directly or indirectly contaminated by discharges of infected animals, suggesting other possible ways of transmission (Biet *et al.* 2005). Some members of the genus *Mycobacterium* are major human and animal pathogens (de Kantor and Ritacco, 1994; O'Reill and Daborn 1995). According to Grange and Yates (1994) and Biet *et al.* (2005), human tuberculosis is caused mainly by *M. tuberculosis*, but *M. bovis* can also cause human disease, which makes this bacterium an important zoonotic agent.

Epidemiology

The epidemiology of *M. bovis* and other mycobacterial species infection in animals is complex with a dynamic interaction between host-agent-environment (Ryan *et al.* 2006). In most developing countries, BTB is enzootic, causing great economic losses (Rehren *et al.* 2007). The introduction of infected animals with BTB into a BTB free area constitutes the primary mode of spread of infection between herds (Goodchild and Clifton-Hadley 2001). Infected cattle with BTB pose an infectious risk to other cattle and to humans (McNair *et al.* 2007). Transmission of BTB through contact requires

infectious and susceptible animals to be present in the same epidemiological group (Goodchild and Clifton-Hardley 2001). According to Michel *et al.* (2006), it is most likely that the disease was introduced in South Africa by imported European cattle breeds during the 18th and 19th centuries. Tuberculosis in cattle is caused predominantly by *M. bovis* of which cattle are the maintenance hosts (Green and Cornell 2005). According to Biet *et al.* (2005), cattle, farmed buffalo and goats are considered reservoir hosts of *M. bovis* while pigs, cats, dogs, horses and sheep are considered spill over hosts. The routes for cattle to become infected are influenced by factors such as age, environment and farming practices (Neill *et al.* 2001). According to Weill *et al.* (1994) and Perez *et al.* (2002), alimentary route of infection is common in young calves ingesting infected milk from tuberculous udders. Airborne transmission is the principal route of bovine transmission (Buddle *et al.* 1994; Cassidy *et al.* 1998; Gannon *et al.* 2007). According to de la Rúa (2006), *M. bovis* is the causative agent of the vast majority of cases of tuberculosis in cattle and a large number of domestic and wild mammalian species, in which it causes a chronic, progressive respiratory disease. The course of the disease depends upon the dose, route and age at infection (O'Reilly and Costello 1988; Neill *et al.* 1989; Steger (1970, cited by Green and Cornell 2005). It may take several years for *M. bovis* infection within a herd to be clinically recognized as the cause of morbidity, mortality and decreased production (Perez 2002). African buffalo (*Syncerus caffer*) can act as maintenance host of *M. bovis* and propagate BTB in large ecosystems in the absence of cattle (de Vos *et al.* 2001). In sub-Saharan Africa, humans and animals share the same microenvironment and water holes, especially during droughts and the dry season, which makes the environment a potential factor in BTB transmission from

animals to humans (Gobena *et al.* 2006; Gobena *et al.* 2007). According to Neill, *et al.* (1994), it is agreed that cattle become infected with *M. bovis* by either oral or respiratory routes. Results of a study conducted by Palmer *et al.* (2004) have shown that infected deer can transmit *M. bovis* to cattle through sharing of feed. According to the definition of the International Animal Health Code of the Office International Des Epizooties (OIE), a region, state or country is considered free of BTB in cattle when the herd prevalence is less than 0.2% (Mitchell and Palmer 2006). In a number of developing countries where TB in cattle and other animals species is not controlled due to lack of national funds, the disease poses the same if not a greater threat to human health as it did a century ago in most developed countries (Gormley *et al.* 2006). According to Morris *et al.* (1994), infected animals if left unchecked or uncontrolled constitute a source of infection which spreads through the herd without involvement of other sources of infection. Spread of infection is facilitated through uncontrolled movement of cattle from infected to non-infected herds. Tuberculosis has also been found in a range of domestic and wild animal species. According to Collins (1981, cited by Collins 2006) and Brook and McLachlan (2006), the fact that TB is an infectious disease appears to be not sufficiently understood by herd owners.

Pathogenesis

Despite many studies conducted since the aetiology of BTB is known, there is still a gap in our understanding about its pathogenesis. The distributions of lesions and pathology have shown predominant involvement of the upper and lower respiratory tract and associated lymph nodes (Corner 1994, and Neill *et al.* 1994). The distribution and the

pattern of lesion observed in slaughtered animals can be an indicator of the route of transmission of *M. bovis* (Pollock and Neill 2002, and Gannon *et al.* 2007). According to Stamp and Francis (1948 and 1958, cited by Neill *et al.* 2001), the importance of respiratory transmission in cattle is demonstrated over the years by the reported pattern of lesion distribution primarily in lymph nodes associated with respiratory tract. Gannon and Hayes (2007) demonstrated that *M. bovis* is resistant to the stresses imposed immediately after becoming airborne with 94% surviving the first 10 min after aerosolisation, a fact that supports the hypothesis that the airborne route is an important route of transmission. The primary complex is initiated by infectious droplet nuclei involving lungs and associated lymph nodes. In the majority of cases very small lesions occur and the infection does not spread. According to Monaghan *et al.* (1994), a significant percentage of tuberculin reacting cattle have no macroscopic lesions detected at the abattoir inspections in many countries. According to Wiegshaas (1989, cited by Cousins *et al.* 2005), the primary complex develops in the lungs, and comprises the lesions in the lung parenchyma and in the mediastinal and/or bronchial lymph nodes. In a sensitized host, from the primary complex the infection may spread locally via lymph nodes and /or blood to involve other organs. A bacteraemia may develop as soon as 20 days after initial infection. Depending on the route of infection, primary foci of tuberculosis occur either in the gastrointestinal, infection in calves after consumption of *M. bovis* infected milk, or in the respiratory tract, infection via aerosolisation of droplet nuclei containing *M. bovis* (Thoen, *et al.* 1986, cited by Cousins *et al.* 2005). According to Grange and Yates (1994), milk-borne infection is the principal cause of abdominal tuberculosis and other non-pulmonary manifestations of the disease. Tuberculosis in

animals and man is mainly a respiratory disease (O'Reilly and Daborn 1995). About 90% of tuberculous infection in cattle occurs by respiratory route (Francis 1947, cited by O'Reill and Daborn 1995). *Mycobacterium bovis* is freely transmissible between cattle, as well as from cattle to man and from man to cattle (Grange and Yates 1994). According to Neill *et al.* (2001), respiratory excretion and inhalation of *M. bovis* is considered to be the main route by which cattle-to-cattle transmission occurs. Lesions of BTB in infected cattle may remain dormant for long period of time, progress or regress (Neill *et al.* 2001). According to Dannenberg (1989, cited by Cousins *et al.* 2005), the character of the lesions during the course of the tuberculous process depends on the fluctuation between cell-mediated immunity, associated with protective responses, and delayed type hypersensitivity that causes necrotic lesions which spread.

Diagnosis

According to Corner (1994), the traditional methods of post mortem examination and culture are very effective procedures for diagnosis of BTB. According to OIE (2004), clinical examination of an animal suspected to be suffering from BTB requires a thorough palpation of all the superficial lymph nodes, the udder in females, and percussion and auscultation of the pulmonary area. Investigation towards understanding of the history of the herd is most important. In cattle, clinical evidence of tuberculosis is usually lacking unless very extensive lesions have developed. For this reason, diagnosis in individual animals and subsequent eradication programmes were not possible before the development of tuberculin by Koch in 1890. According to Corner (1994) and Collins *et al.* (1994), post mortem examination and culture are the key steps

for the diagnosis of bovine tuberculosis. Lesions suspected to be tuberculous, detected at abattoir inspections, should be submitted for bacteriological and histological examination (Corner 1994). Confirmation of *M. bovis* infection is done through laboratory examination of lesions collected from the infected animals. Up to three lesions from each animal should be submitted for laboratory examination to ensure a correct diagnosis. A definitive diagnosis is dependent on the isolation of *M. bovis* (Corner 1994 and Al-Hajjaj *et al.* 1999). In the process of the diagnosis of BTB, the long time required to obtain a definitive diagnosis has been identified as the major problem. It was estimated that more than 12 weeks may be required for cultural confirmation of infection. In many laboratories, histopathology is used to overcome the long delay in making a bacteriological diagnosis (Corner 1994). However, cultural confirmation of BTB is rarely required when the frequency of the disease is high and the cost of misdiagnosis is negligible (Corner 1994).

Testing for infection in live animals is based on an intradermal reaction to bovine purified protein derivative (PPD), a crude extract of antigens from *M. bovis*. The test is convenient and inexpensive but lacks specificity because *M. bovis* has some antigens in common with other mycobacteria, such as *M. avium*, to which animals are often exposed (Lepper and Corner 1983; Adams 2001). According to de la Rua-Domenech *et al.* (2006), skin tests are the international standard for ante mortem diagnosis of BTB in cattle herds and individual animals. There are two types of tuberculin test in use: the single intradermal test (SIT) using bovine tuberculin and the single intradermal comparative tuberculin test (SICTT) using avian and bovine tuberculins. According to

Monaghan *et al.* (1978), the type of skin test to select depends on the prevalence of TB and on the prevalence of exposure to other sensitizing, environmental mycobacteria. According to Plum, and Stenius (1931 and 1938, cited by Monaghan *et al.* 1994), cattle infected with *M. avium* may have significant reactions to mammalian or bovine tuberculin and the SICTT was developed to discriminate between those sensitized by *M. bovis* and related organisms.

The tuberculin test is usually performed on the mid-neck (MCT), but can also be performed in the caudal fold of the tail. The skin of the neck is more sensitive to tuberculin than the skin of the caudal fold (Larsen *et al.* 1950, cited by Kanameda *et al.* 1999). Cervical skin test was also shown to be more sensitive in swamp buffaloes according to a study conducted by Kanameda *et al.* (1999). To compensate for this difference, higher doses of tuberculin may be used in the caudal fold with *M. bovis* and those sensitized to bovine tuberculin as a result of exposure to other mycobacteria. This sensitization can be attributed to the large antigenic cross-reactivity among mycobacterial species and related genera. The test involves the intradermal injection of bovine tuberculin and avian tuberculin into different sites, usually on the same side of the neck, and measuring the response 3 days later (Monaghan *et al.* 1994; OIE 2004). Bovine tuberculin injected into the skin of an animal that is not sensitized to tuberculin antigens does not produce a significant inflammatory response. However, if an animal is sensitized by infection with *M. bovis* or by exposure to cross-reacting antigens, develop an inflammatory response and swelling at the injection site that reaches its greatest

intensity 48-72 hours post-injection and regresses rapidly thereafter (Lepper *et al.* 1977; Francis *et al.* 1978; Pollock *et al.* 2003; Goodchild *et al.* 2006).

Hypersensitivity to tuberculin usually develops in cattle between 1 and 9 weeks after infection with *M. bovis*, depending on animal and test factors (Francis 1958; Kleeberg 1960), but for most animals a full response is likely to develop between 3 and 6 weeks post-infection (Goodchild *et al.* 2006). Newly infected cattle generally do not react to the intradermal injection of tuberculin. According to Monaghan *et al.* (1994), insufficient dose of tuberculin injected into the skin, and early or too late reading after injection can lead to false negative test results.

According to Monaghan *et al.* (1994), the sensitivity of the test is affected by the dose potency of tuberculin administered at the interval post-infection, desensitization, and post-partum immunosuppression. Specificity is influenced by sensitization as a result of exposure to *M. avium*, *M. paratuberculosis* and environmental mycobacteria and by skin tuberculosis. According to Doherty (1995), glucocorticoids administered topical or systemically can lead to a significant reduction in the size of the bovine tuberculin reaction in infected cattle. After the intradermal injection of tuberculin in infected cattle, skin reactivity to a second injection is depressed for some time. This phenomenon can result in failure to identify experimentally- and naturally-infected cattle as reactors (Radunz and Lepper 1985; Hoyle 1990; Monaghan 1994; Doherty *et al.* 1995)

Test procedure

Prior to injection, the sites must be clipped and cleansed. A fold of skin within each clip is measured with callipers and the site marked before the injection. A short and graduated syringe, bevel edge outwards, is used to inject tuberculin. It is inserted obliquely into the deeper layers of the skin. The dose of tuberculin injected must be no lower than 2000 International Units (IU) of bovine or avian tuberculin. A correct injection is confirmed by palpating a small peak-like swelling at each site of injection (OIE 2004). According to Monaghan *et al.* (1994) and OIE (2004) the distance between the two injections should be approximately 12-15 cm. In young animals in which there is not enough space to separate the sites sufficiently on one side of the neck, one injection must be made on each side of the neck at identical sites in the centre of the middle third of the neck (OIE 2004). The skin-fold thickness of each injection site is remeasured 72 hours (plus or minus 4-6 h) after injection (Griffin, and Mackintosh 2000; OIE 2004). The same person should measure the skin before the injection and when the test is read (OIE 2004).

Single Intradermal Test

The SIT can be carried out in the skin of the neck, as in Europe, or in the caudal fold, as in North America, Australia and New Zealand. The interpretation of results is based on observation, palpation and on measuring the skin fold thickness.

Single Intradermal Comparative Tuberculin Test

The SICTT is used in Ireland and the United Kingdom as a screening test and in other countries to help clarify the status of cattle which show reactions to the SIT (Monaghan *et al.* 1994). The interpretation of the SICTT test is based on the observation that *M. bovis*-infected cattle tend to show a greater response to bovine tuberculin than to avian tuberculin, whereas infections with other mycobacteria promote the reverse relationship (Karlson 1962; Francis *et al.* 1978; Kazda and Cook 1988; Pollock *et al.* 2003). The SICTT test allows better discrimination than the SIT between animals infected with *M. bovis* and those sensitized to tuberculin after exposure to organisms of the *M. avium* complex or to environmental non-pathogenic mycobacteria (Francis *et al.* 1978; Monaghan *et al.* 1994). The use of comparative tuberculin tests has resulted in improvements in specificity (Pollock *et al.* 2000; Griffin *et al.* 2004). According to Plum and Stenius (1931 and 1938, cited by Monaghan *et al.* 1994), cattle exposed to *M. avium* may have significant reactions to mammalian or bovine tuberculin as well as to avian tuberculin and the comparative intradermal test is used to discriminate between sensitization caused by *M. bovis* and *M. avium* and related organisms. The specificity of the SICTT using avian and bovine PPD tuberculin is high in cattle populations known to be free of tuberculosis (Leslie *et al.* 1975, cited by Monaghan *et al.* 1994). *M. avium* has frequently been recovered from cattle but generally causes only minor, non-progressive lesions in the mesenteric lymph nodes (Worthington 1967).

Specificity and sensitivity of the skin tests

According to Francis 1958, and Martin *et al.* (1987, cited by de la Rúa-Domenech *et al.* 2006), specificity (Sp) is the proportion of non-diseased (uninfected) animals that are correctly identified as negative by a diagnostic test while sensitivity (Se) is the proportion of diseased (infected) animals detected as positive in the diagnostic assay.

According to Monaghan *et al.* (1994), the sensitivity of the skin tests varies from 68 to 95% and these values may be reduced under field conditions. The specificity of the SICTT test is between 78.8% and 100% whereas sensitivity is between 52% and 100% while the SIT specificity ranges between 75.5% and 99% (median 96.8%). Imperfect test specificity leads to false positives, also known within the context of TB testing programmes as 'non-specific reactors' (NSRs) (Karlson 1962, cited by de la Rúa-Domenech *et al.* 2006). The SICTT is characterized by a low false positive rate, and the lack of complete specificity may be a problem in countries with low prevalence of disease (Monaghan *et al.* 1994).

Necropsy

According to Corner (1994), a tentative diagnosis of bovine tuberculosis can be made following the macroscopic detection at necropsy of typical lesions. Histo-pathological examination of the lesion is normally used to increase the confidence of the diagnosis but isolation of *M. bovis* from the lesion is the only way to make a definitive diagnosis.

The sensitivity of post mortem examination is affected by the method employed and the organs examined. Examination of as few as 6 pairs of lymph nodes, the lungs and the mesenteric lymph nodes can increase to 95% the probability of cattle with macroscopic lesions being identified. According to Corner (1994), between 70 and 90% of lesions are found in either the lymph nodes of the thoracic cavity or in the head, hence the wide range of lymph nodes that need to be examined.

Microscopic examination

Mycobacterium bovis is a difficult bacterium to isolate and identify. By microscopic examination, *M. bovis* can be demonstrated on direct smears from clinical samples and on prepared tissue materials (Corner 1994). The acid fastness of *M. bovis* is normally demonstrated with the classic Ziehl-Neelsen stain, but a fluorescent acid-fast stain may also be used. Immunoperoxidase techniques may also give satisfactory results. According to OIE (2004), a presumptive diagnosis of BTB can be made if the tissue has the following characteristic histological lesions: caseous necrosis, mineralization, epithelioid cells, multinucleated giant cells and macrophages. The presence of acid-fast organisms in histological sections may not be detected although *M. bovis* can be isolated in culture (OIE 2004).

Culture of *M. bovis*

Culture remains the gold standard method for detection of *M. bovis*, in clinical samples, (de la Rúa-Domenech *et al.* 2006). According to Corner (1994), *M. bovis* are characterized by slow growth, and on subculture require a minimum of 14 days for colonies to become visible on media of culture. For culture, the tissue is first homogenized using a pestle and mortar, stomacher or blender a blender followed by decontamination with either an acid or an alkali, (5% oxalic acid or 2-4% sodium hydroxid). The mixture is shaken for 10 minutes at room temperature and then neutralized. Subsequently, suspension is centrifuged, the supernatant is discharged, and the sediment is used for culture and microscopic examination. For primary isolation, the sediment is usually inoculated on to a set of solid egg-based media such as Löwenstein-Jensen, Coletsos base or Stornebrink media. Growth considered to be mycobacterial is subcultured on to egg-based and agar-based media or into tween albumin broth, and incubated until visible growth appears. In some laboratories, sterile ox bile is used before inoculation to facilitate the dispersion of the bacterial mass into small viable units. It is necessary to distinguish *M. bovis* from the other members of the tuberculosis complex (*M. tuberculosis*, *M. africanum*, *M. microti*). Sometimes, *M. avium* or other environmental mycobacteria may be isolated from tuberculosis-like lesions in cattle. In such cases careful identification is needed, and a mixed infection with *M. bovis* should be excluded. *Mycobacterium tuberculosis* may sensitise cattle to bovine tuberculin without causing distinct tuberculous lesions (OIE 2004).

Polymerase chain reaction

According to Miller *et al.* (1997) and Miller *et al.* (2002), a PCR test can usually provide a rapid diagnosis of tuberculosis when it is applied to paraffin sections that have characteristic lesions and acid-fast organisms. PCR based diagnostic tests are able to detect in a few days DNA from a single organism of a pre-determined species (Wright and Wynford 1990, cited by Collins *et al.* 1994). PCR has initially been used for the detection of *M. tuberculosis* complex in clinical samples in human patients and has recently been used for the diagnosis of tuberculosis in animals (OIE 2004). There are a considerable number of kits and various methods used for the detection of the *M. tuberculosis* complex in fresh and fixed tissues. Various primers have been used, including those that have amplified sequences from 16S-23S rRNA, the insertion sequences IS6110 and IS1081, and genes coding for *M. tuberculosis* complex-specific proteins, of which MPB70 and Antigen85b are examples. Amplification of products has been analyzed by hybridization with probes or by gel electrophoresis (OIE, 2004). PCR provides the possibility of detecting the presence of *M. bovis* in samples even when organisms have become nonviable (Liebana *et al.* 1995). Optimal results are obtained when both PCR and isolation methods are used. DNA analysis techniques may prove to be faster and more reliable than biochemical methods for the differentiation of *M. bovis* from other members of the *M. tuberculosis* complex (Liebana *et al.* 1995).

Lack of sensitivity is the main problem of PCR when applied on field samples. The comparison of IS1081 PCR with the "gold standard" of culture showed a sensitivity of approximately 70%. The sensitivity of the RD4 PCR method was 50%. A series of further experiments indicated that the discrepancy between sensitivity of detection found with purified mycobacterial DNA and direct testing of field samples was due to limited mycobacterial DNA recovery from tissue homogenates rather than PCR inhibition (Taylor *et al.* 2007).

Blood-based laboratory tests

These tests are used to confirm or negate the results of an intra-dermal skin test. The lymphocyte proliferation assay and the gamma-interferon assay correspond to cellular immunity, while the enzyme-linked immunosorbent assay (ELISA) corresponds to humoral immunity (OIE, 2004). Coad *et al.* (2008), demonstrated that cattle with bovine tuberculosis, missed by SICTT, can be identified by in-vitro blood-based assays

Lymphocyte proliferation assay

This test is used to determine proliferation in lymphocytes by measuring the incorporation of ³H-thymidine. According to Buddle *et al.* (2001) the test compares the proliferation of peripheral blood lymphocytes to tuberculin PPD from *M. bovis* (PPD-b) and PPD from *M. avium* (PPD-a). The assay can be performed on whole blood or purified lymphocytes from peripheral blood samples (Griffen *et al.* 1994). These tests increase the specificity of the assay by removing the response of lymphocytes to non-

specific or cross-reactive agents associated with non-pathogenic species of mycobacteria to which the animal may have been exposed. Results are usually analyzed as the value obtained in response to PPD-b minus the value obtained in response to PPD-a. The B-A value must then be above a cut-off point that can be altered in order to maximize either specificity or sensitivity of the diagnosis (OIE 2004).

Gamma-interferon assay

The use of gamma-interferon (IFN- γ) can provide a means for the early identification of *M. bovis*, infected cattle, which might otherwise not be detected by tuberculin test until later, hence ensuring their removal from infected herd (Gormley *et al*, 2006). The test is based on the interferon gamma released in a whole-blood culture system which is measured. Gamma-interferon is released from sensitized lymphocytes during a 16-24 hour incubation period with specific antigen (PPD-tuberculin) (OIE, 2004). The test compares gamma-interferon produced after stimulation with avian and bovine PPD. The detection of bovine gamma-interferon is carried out with a sandwich ELISA that uses two monoclonal antibodies. The laboratory incubation of blood samples must be set up within 8 hours of collection. The test has proven to have a high sensitivity compared with the skin test, but on the other hand it is less specific in a number of trials. However, according to Buddle *et al*. (2001) the use of defined mycobacterial antigens promises to improve specificity. An additional advantage over the skin test is that the animals need only to be captured once.

Serology - Enzyme-linked immunosorbent assay (ELISA)

In the context of tests based on cellular immunity, ELISA has proven to be the best choice and can be a complement, rather than an alternative. It may be helpful in anergic cattle and deer. An advantage of the ELISA is its simplicity, but both specificity and sensitivity are limited in cattle, mostly due to the late and irregular development of the humoral immune response in cattle during the course of the disease. The ELISA may also be useful for detection of *M. bovis* in wildlife (OIE 2004). However, to date no serological test for the diagnosis of bovine tuberculosis in cattle has received wide acceptance and none of such tests is recommended by the OIE.

Chapter 3: Materials and Methods

This study consisted of two parts: a field study in March and May 2008, in Govuro district, followed by laboratory analysis in Mozambique and South Africa. The field study was composed of primary screening using the SIT test in randomly selected animals from Colonato and Sede dip tanks in Govuro. Animals reacting positively were tested again with bovine and avian tuberculin using the SICTT test.

Sampling procedures

Animals were sampled from the two dip tanks (Colonato and Sede). According to the previous surveys the prevalence was found to be 1.49% (n=869; 95% CI: 0.80 – 2.54%) in 1997 (SPP, 1997) and 1.7% (n=517; 95% CI: 0.67 – 3.03%) in 2001 (SPP 2001).

The required sample size of 682 animals was calculated using Win Episcope 2.0 based on 95% confidence to estimate a 2% expected prevalence using the SIT, with a 1% accepted error and a total population size of 7208 animals. To compensate for the probability of 20% default in reading, the sample size was increased to 853 animals. During the testing process (SIT), it was evident from the first 3 reading days that the apparent prevalence (61.94%) was far higher than expected (2%), hence we decided to stop when the total number of cattle was 530. This was due to the fact that, at such a high prevalence, it would not be necessary to achieve as high a precision as 1% accepted error. A sample size of 530 would be sufficient to achieve a precision of 4% accepted error, which was regarded as more than adequate.

The 530 cattle, 3 or more years of age, were selected using systematic random sampling from the two dip tanks (Colonato 371 and Sede 159 animals). All animals were identified by numbers painted dorsally on the sacral region.

Tuberculin skin testing

Single intradermal test

Injection sites were shaved and measured with callipers before inoculation and 72 hours after inoculation. The SIT was used as the primary test and carried out according to OIE standards (OIE 2004). Animals were injected intradermally with 0.1 ml of 1 mg/ml of *M. bovis* purified protein derivative (PPD-b), equivalent to 2 000 IU/ml (Onderstepoort Biological Products, South Africa). Positive reactors were identified by branding a “T” on the gluteal region using a hot iron.

The results were interpreted according to OIE standards (OIE 2004):

- negative result in the SIT was a swelling ≤ 2 mm;
- doubtful result was a swelling between 2 and 4 mm, and
- positive result was a swelling ≥ 4 mm

Single intradermal comparative tuberculin test

The comparative cervical test was performed 7 weeks after the SIT (Radunz and Lepper 1985; Hoyle 1990; Monaghan 1994; Doherty *et al.* 1995). Twenty eight cattle out of the positive reactors to the SIT were randomly selected from the two dip tanks (15 from Sede and 13 from Colonato) and subjected to SICTT. Bovine tuberculin (PPD-b), (0.1ml of 1mg/ml equivalent to 2000 I.U/ml) and avian tuberculin (PPD-a), (0.1 ml of 0.05mg/ml, equivalent to 2500 I.U/ml), (Onderstepoort Biological Products, South Africa), were simultaneously injected in the neck into shaved and clearly demarcated sites separated by 12 cm, according to the manufacturer recommendations and OIE (2004). Skin thickness was measured at both injection sites prior to intradermal injection of PPD and again 72 hours later (Griffin, and Mackintosh 2000; OIE 2004).

The tuberculin results were interpreted based on standard interpretation (OIE 2004). Results were obtained using the following formula: $[(Bov_{72} - Bov_0) - (Av_{72} - Av_0)]$. B_0 and Av_0 indicated skin thicknesses before injecting bovine and avian tuberculins, respectively and Bv_{72} and Av_{72} were the corresponding skin-fold thicknesses 72 hours post-injection. The differences in the increase in thickness between bovine and avian antigens were interpreted according to OIE (2004):

- ≤ 1 mm: negative
- 1-3 mm: doubtful reactor
- ≥ 4 mm: positive reactor

Post-mortem examination

A three year old positive bull, classified as a TB reactor after the SIT test, was slaughtered, and a detailed post mortem was carried out and samples collected according to Corner (1994) and Al-Hajjaj *et al.* (1999). Organs with visible lesions (VL) were collected for further laboratory testing.

Laboratory examinations

Analyses of field samples were conducted at the laboratories of the Instituto de Investigação Agraria de Moçambique (IIAM) in Mozambique and the Onderstepoort Veterinary Institute (OVI) and Vet Path, Faculty of Veterinary Science, South Africa. Laboratory studies included isolation and identification of mycobacterial species (Corner 1994 and Al-Hajjaj *et al.* 1999), PCR amplification of DNA, and histopathology using standard procedures (OIE 2004).

Culture, isolation and identification of mycobacterial species

Specimens of lung tissue and Lymph node tissue of a 3 year old bull, from Govuro district, were used in this study. The positive control was obtained from confirmed TB positive tissue, and the negative control comprised a sample from carcass declared fit for human consumption which underwent microbiological testing in the Feed and Food Laboratory and also a tissue confirmed TB negative from the tuberculosis laboratory.

The samples were defrosted in biohazard cabinet in Tuberculosis room # 013 for same day processing. The specimens were examined macroscopically for size, abnormal consistency, smell, the presence or absence of visible lesions and the color of the lesions. All fat was removed and cut ± 5 gm of tissue into small pieces (± 0.5 cm). Visible lesions or abnormalities observed formed part of the sample. Impression smears were made and stained as described under the Ziehl-Nielsen (ZN) staining method.

The specimen was put into a sterile homogenizing jar, and covered with 100 ml ± 10 ml distilled, sterile water. The blades of the element were immersed in the sample mixture. By using an Ultra Turrax homogenizer at 17 500 rpm the mixture was completely mixed.

The homogenate was divided into 2 x 15 ml-centrifuge tubes, 7 ml each and the remainder was poured into 50 ml centrifuge tube for storage at -20°C in the Tuberculosis laboratory room # 017 as reference sample. The rest of the homogenate was sealed in the jar and put in the waste container and autoclaved.

The sample was decontaminated by using 7 ml ± 0.5 ml 2% HCl (final concentration 1% HCl) in one tube and 7 ml ± 0.5 ml 4% NaOH (final concentration 2%) in the other. After 10 minutes, the sample was centrifuged at 3500 rpm for 10 minutes.

Supernatant was poured off and neutralized with 7 ml ± 0.5 ml sterile distilled water. The sample was centrifuged again for 10 minutes at 3500 rpm. Most of the supernatant was poured off keeping approximately 0.5 ml. The pellet was mixed using an inoculating loop, which was then discarded into a beaker with 10% sodium hypochlorite. One loop full of each of the pellets was spread onto LJ (Löwenstein-Jensen) -pyruvate and LJ-

glycerol slants, respectively and incubated at $37 \pm 1^\circ\text{C}$ for 10 weeks or until visible growth appeared (OIE 2004).

Identification of *M. bovis* by PCR

Isolates from lung tissue and lymph node tissue of a 3 year old bull from Govuro district were used in this study. Thermolysates were prepared by heating pure colonies at 80°C for 30 minutes and then centrifuged at 12,000 rpm to remove the cellular debris. Ethanol precipitation was performed on the supernatant and the resultant pellet was then dissolved in TE buffer and stored at -80°C for future use.

A multiplex PCR was done with primers which were obtained from previously published data (Warren *et al.* 2006). The primers used targeted RD4 and RD9 genomic regions of difference. Each primer set consists of forward, reverse and internal primers.

The PCR mix contained $12.5\ \mu\text{l}$ of the Hotstart Taq multiplex master mix (Qiagen), $5\ \mu\text{l}$ of the Q-solution (Qiagen), $3.5\ \mu\text{l}$ of the RNase free water and $0.5\ \mu\text{l}$ of each 50 pM primer. The reaction was run at a denaturation temperature of 95°C for 15 minutes, and 40 cycles of 94°C for 1 minute, 62°C for 1 minute and 72°C for 1 minute, a final elongation step at 72°C for 10 mins and a holding step at 4°C until used. The PCR products were then separated by electrophoresis using a 3% agarose gel at 10 V/cm for 2 hours.

The positive controls included a known *M. bovis* isolate (AN5) provided by the Medical Research Council, Center for Molecular and Cellular Biology, University of Stellenbosch, whilst the negative control was water. The resulting gel images were

analyzed on the basis of their alignment on the gel, i.e. same band size with either of the controls.

Histopathology

For the present study, tissue of lung and lymph node from a 3 year old bull from Govuro district were used. These tissues were fixed in Bouin's solution for at least two weeks until they could be processed for histology. Before histological processing the specimens were washed several times with 50% ethanol and kept for three days in 70% ethanol. The specimens were halved sagittally, trimmed to only include their relevant parts, and then processed using standard histological techniques. Serial sections 6 μm thick were cut on an Anglia AS500 Universal Microtome. The sections were mounted on glass slides and stained with haematoxylin and counter stained with eosin. All the sections were examined using an Olympus BH-2 light microscope. Photographs of relevant areas were taken using a Leitz Wetzlar Research Microscope that is equipped for photography. The film used was Kodak TP 135-36 film.

Statistical analysis

The true prevalence, with 95% confidence limits, was calculated for each dip tank using Survey Toolbox version 1.0, based on an assumed sensitivity of 80% and specificity of 75%, with 95% confidence intervals. The Fisher exact test was used to compare apparent prevalence between the two areas (Colonato and Sede dip tanks). In order to

calculate the predictive value of a positive result test (diagnosability), the following formula was used:

$$PV_{+} = \frac{P \times Se}{(P \times Se) + (1 - P)(1 - Sp)}$$

Chapter 4: Results

Tuberculin testing and statistical analysis

Of the 530 cattle tested by SIT, 268 were read, and 166/268 (61.9%; 95% CI: 55.83 – 67.78%) was found positive, with visible swelling at the injection site (Fig 3). Overall apparent prevalence (AP) was found to be 61.9% while the estimated true prevalence (TP) was 67.2%. Apparent and estimated true prevalences, overall and for each dip tank, are shown in Table 2). At this true prevalence, the predictive value of a positive result (PV+) was found to be 86.8%. No significant difference in apparent prevalence between the two areas (Colonato and Sede dip tanks) was detected using the Fisher exact test ($P = 0.11$). By SICTT, out of 28 positive reactors to SIT, 21 were possible to read, and 13/21 (61.9%; 95% CI: 38.4 – 81.9%) were found to be positive.



Figure 3: Skin reaction (arrow) 72 hours after inoculation of PPD-b

Table 2: Results of SIT

Dip Tank	Pos	Neg	Doub	Total	AP % (95% CI)*	TP %	95% CI
Sede	62	23	5	90	68.9 (58.3 – 78.2)	79.8	73.6 – 86.0
Colonato	104	61	13	178	58.4 (50.8 – 65.8)	60.8	56.1 – 65.5
Total	166	84	18	268	61.9 (55.8 – 67.8)	67.2	63.4 – 70.9

* Binomial exact

Pos: Positive AP: Apparent prevalence

Neg: Negative TP: True prevalence

Doub: Doubtful

Table 3 – Results of SICTT

Dip Tank	Pos	Neg	Doub	Total
<i>Sede</i>	8	4	0	12
<i>Colonato</i>	5	3	1	9
Total	13	7	1	21

Post-mortem

Following a comprehensive examination of the carcass at the slaughter house, visible lesions were found in the lung tissue – a granuloma of 5 cm in diameter. On cut surface, the granuloma was gritty and contained central yellow caseonecrotic material (Fig 4). The left bronchial lymphnode showed significant change in colour, making it suspected to be infected.

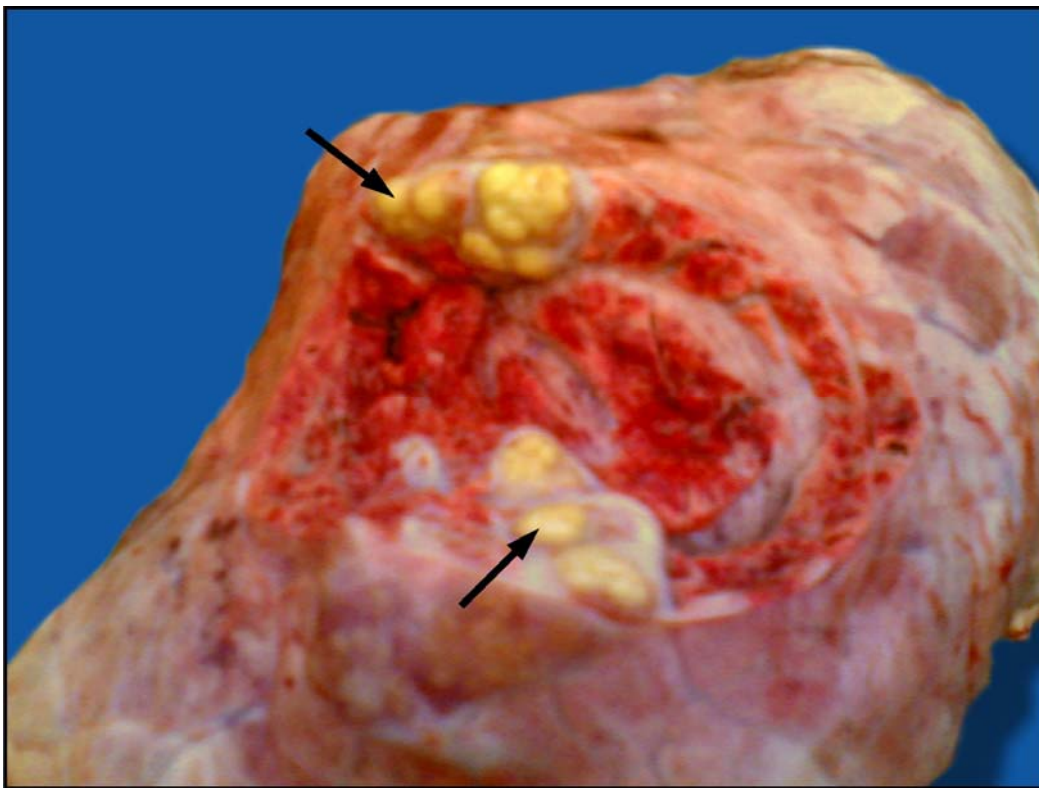


Figure 4: Lung presenting granulomatous lesions compatible with BTB

Later on, 30 positive reactors to the SIT test were slaughtered. Of these, 25 (83.3%; 95% CI: 65.3 – 94.4%) showed visible lesions compatible with BTB, and total condemnation of the carcass was required in 3/25 due to generalized lesions.

Laboratory analysis

Histological examination

Multifocal, well-demarcated, inflammatory granulomas consisting of central areas of necrosis were found, in which calcification/mineralization was also visible (Fig 5). These necrotic areas contained amorphous eosinophilic material and nuclear debris and were surrounded by a granulomatous inflammatory reaction in which lymphocytes, plasma cells and macrophages were present in large numbers (Fig 6). Fibroplasia was also evident. There was moderate activity of the lymphoid tissue. The tissues showed some autolytic changes.

A single acid-fast bacillus was visible in the centre of one of the necrogranulomas, embedded in the necrotic and calcified material. This positive result is consistent with a mycobacterial infection as the cause of the necrogranulomatous pneumonia.

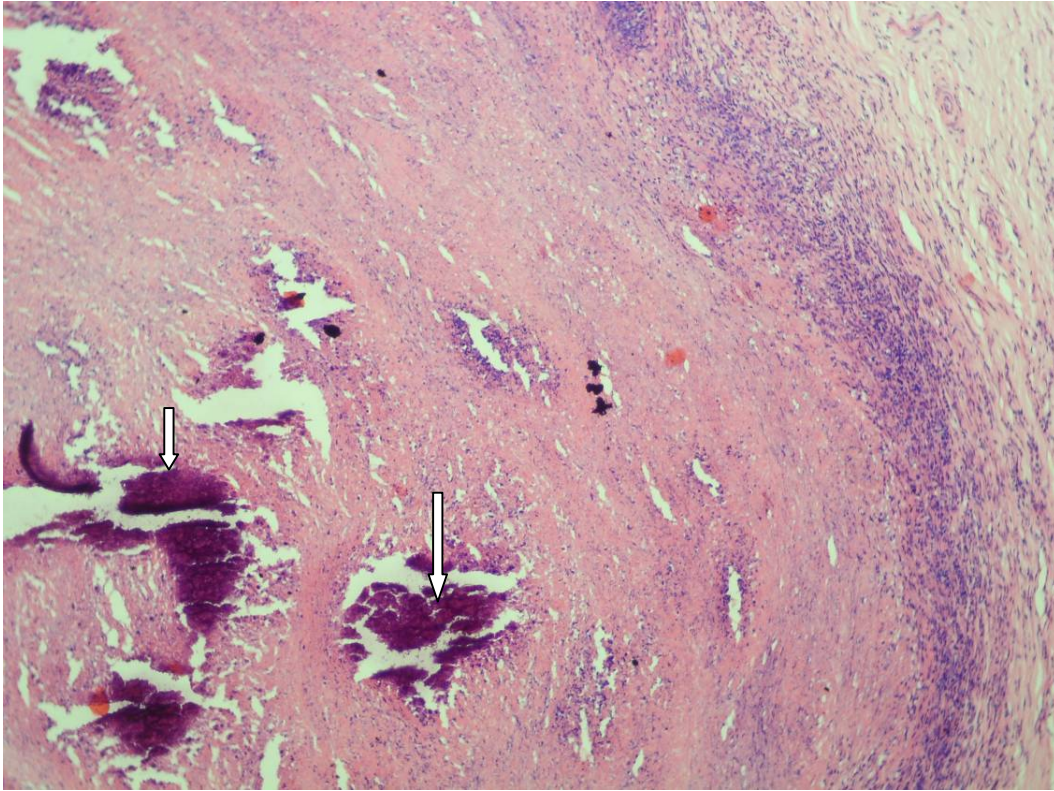


Figure 5: Micrograph of lung showing necrosis and calcification/mineralization (arrows). (X10)

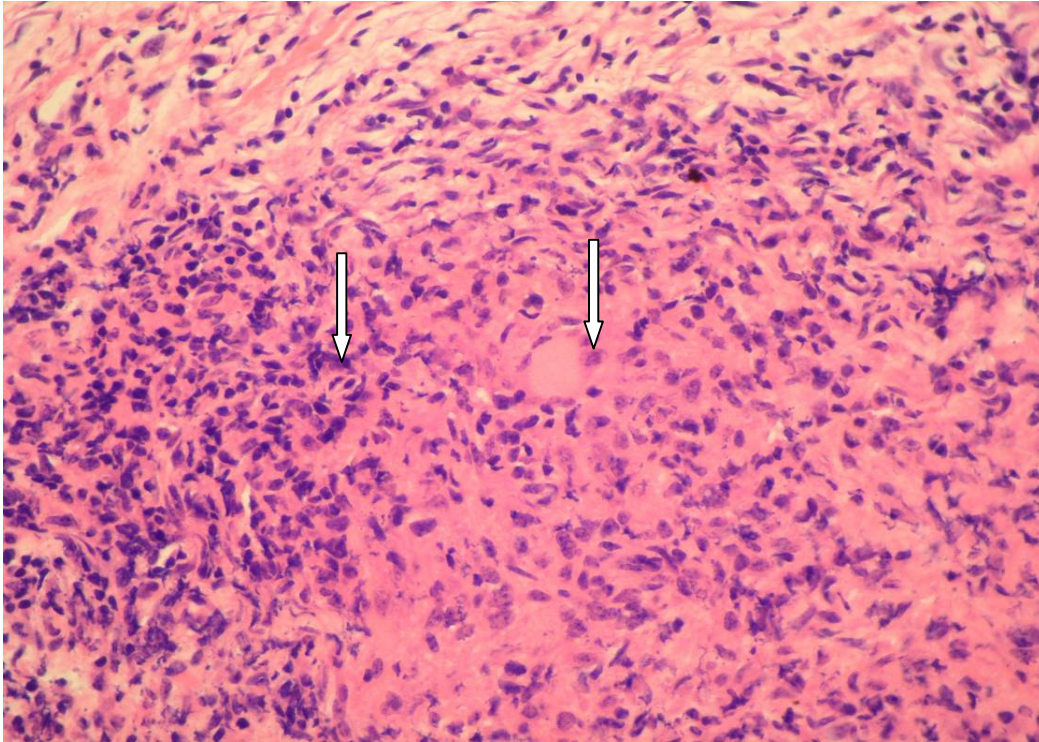


Figure 6: Micrograph of lymph node showing lymphocytes and macrophages. (X40)

Mycobacterial examination

The result of mycobacterial examination confirmed *M. bovis*.

Polymerase chain reaction method

Mycobacterium bovis species was confirmed from the isolates obtained from the lung tissue and lymph node tissue of a 3 year old bull using deletion analysis (multiplex PCR) as indicated by two band sizes (108bp and 268bp) corresponding to the deletions of the RD9 and RD4 regions respectively (Fig. 7). The reference strain used here was *M. bovis* AN5.

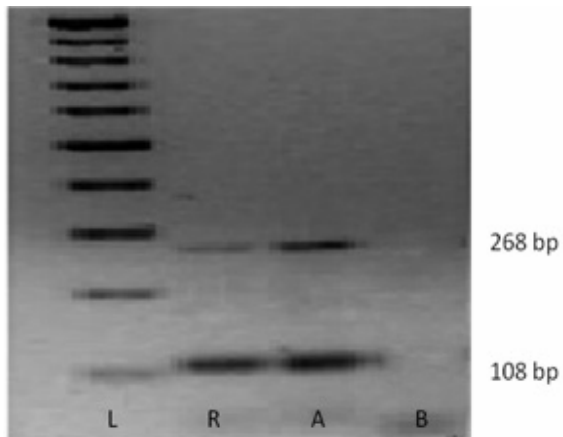


Figure 7: Species confirmation of the isolates obtained from the lung tissue (A) and lymph node tissue (B) of a 3 year old bull. L is 100 bp molecular ladder, R is a known *M. bovis* strain

Chapter 5: Discussion and Conclusions

The aim of this study was to confirm the presence of BTB and estimate its prevalence based on skin test reactivity, in cattle reared under extensive farming conditions in the Govuro district, Inhambane province, Mozambique. All animals were tested by PPDs, according to the international standard (OIE 2004).

Based on SIT results, AP was found to be 61.9% while the TP was 67.2% assuming Se (80%) and Sp (75%). Results of the SICTT seemed to indicate that many (up to 38%) of the positive reactors to the SIT were false positive. This would indicate that our initial assumption of the specificity of SIT (75%) was too high. However, the fact that 25/30 positive reactors to the SIT showed visible lesions at slaughter (suggesting a positive predictive value of at least 83.3%), is consistent with the assumption of 80% sensitivity and 75% specificity, which gave us a positive predictive value of 87.9%. These results therefore suggest that the sensitivity of the SICTT, when applied to the positive reactors from the SIT, was low.

The results of this study have also shown that the estimated true prevalence of BTB, (67.2%, n=268, SIT) by mid cervical test (MCT), is much higher than reported in previous surveys (1.49%, n=869, DINAP 1997, unpublished data) in the same area by SIT caudal fold. Unless the prevalence of BTB has risen dramatically over the last 10 years, the reliability of the previous results must therefore be questioned. A proper

methodology should have been implemented in terms of sampling and tests to be used. Importantly, as demonstrated, confirmation of tuberculosis is always needed. The SIT in the skin of the neck is considered to be more sensitive than that of the caudal fold (Peterson 1959; Suther *et al.* 1974). The results of field trials in Australia suggested that, while the specificity of the caudal fold test is high (96-98.8%), its sensitivity is only moderate at 72% (Francis *et al.* 1978) and 68% (Wood *et al.* 1991). The SIT on the neck was thought to give high sensitivity (Francis *et al.* 1978; de Kantor *et al.* 1984). Bang (1892, cited by Monaghan *et al.* 1994), concluded that the tuberculin test is not perfect, but it would be a great mistake to reject the method because of this. The discrepancies between previous enquiries and our study cannot be explained by a difference in sensitivity of the respective techniques used. It is also very unlikely that in the period of time between the last previous enquiry and this study, BTB got into an epizootic-like phase in order to reach the prevalence rates we found. Altogether, our results question the validity of previous enquiries.

Using Fisher's exact test ($P = 0.11$), there was no significant difference in the prevalence of skin test positive animals between the two dip tanks (104/178 compared to 62/90) using the SIT test. There is thus insufficient evidence to suggest that the risk of infection differs between the two epidemiological areas.

The response rate (number of animals brought for the reading day) was very low, hence out of 530 tested cattle, we could only read 268 animals because of the following

possible reasons: the animals graze far (10-20 km) from the concentration points (Colonato and Sede dip tanks); some herds take a whole day to go to the dip tanks and return, hence, do not adequately feed on that particular day; there are no defined roads for cattle to go to the dip tanks; the roads used pass through cultivated agricultural fields, hence there is a permanent conflict between field and livestock owners as the animals graze on the crops during the trip to the dip tanks. This results in reluctance by the herdsmen to bring cattle for dipping unless the number of ticks becomes a problem to their animals; the majority of livestock owners do not stay with their animals, and are entirely dependent on the herdsmen to take care of the cattle in those remote areas; communication between the herdsmen and livestock owners is sometimes a problem. Another difficulty faced by the herdsmen is the collection of the animals from the bush, where they graze freely, into the holding facilities on the night before the trip to the dip tank. Thus, it is unlikely that the low response rate of the farmers on the reading days is related to the test result (i.e. the farmer's fear that the positive reactors would be taken without compensation – something that would have biased the prevalence estimate downwards), hence the animals read can still be considered a representative, if not strictly random, sample.

For the last 20 years, a skin test and slaughter policy has not been in place in the country due to economic reasons, a factor that might have contributed to the increase of the number of infected animals (source of infection) and its important contribution to the spread of the disease in the herds. According to Morris *et al.* (1994), if infection in a herd or group of animals is left unchecked or uncontrolled, it spreads through the herd

without involvement of other sources of infection. Spread of infection is facilitated through uncontrolled movement of cattle from infected to non-infected herds.

During the last 8 years, close contact between animals, at least 30 days per year in the study area, caused by annual floods, where animals compete for the limited available grazing area, to a large extent might have contributed to the spread of bovine tuberculosis in the herds and the subsequent increase in prevalence. According to Goodchild and Clifton-Hardley (2001), transmission of BTB occurs during contact which requires infectious and susceptible animals to be present in the same epidemiological group. The cattle from the two epidemiological units in this study, for more than 3 years have been using the dip tank Colonato for dipping when the Sede dip tank was not working, due to lack of maintenance after damages caused by floods in 1999. The effects of natural disasters in the last 8 years have been permanent not only in the district but all over the country. Movement of live animals between the two epidemiological zones is not controlled, nor are the slaughter of infected animals and movement of meat between the two zones. According to Goodchild *et al.* (2001), cattle-to-cattle transmission plays a part in the entry of infection into herds through purchased infected animals or contiguous spread, closeness of contact and ventilation. On the other hand, the fact that TB is an infectious disease with serious economic consequences appears to be not fully understood by livestock owners in Govuro district.

The network coverage of district veterinary services is very weak due to the presence of only one technician, associated with the limited funds for fuel and maintenance of his motorcycle. As a result, large numbers of animals are slaughtered for sale without involving a district technician for inspection, which may result in a human health problem. Furthermore, organs with visible lesions are often not properly destroyed, and may be scavenged by stray dogs or other wildlife, extending the animal reservoir of *M. bovis*. It may also contribute for the environmental contamination and the consequent increase in risk of infection of cattle. Indeed, according to experiments conducted by Biet *et al.* (2005), *M. bovis* can survive for long periods outside an animal host in an environment directly or indirectly contaminated by discharges of infected animals, suggesting a possible route of transmission.

The high prevalence rate of skin test positive animals as well as gross lesions and histopathology were confirmed to be BTB by the isolation and identification of *M. bovis* by culture and PCR. Our results therefore show that bovine tuberculosis is highly prevalent in Govuro district and may thus represent a potential health problem of zoonotic tuberculosis in humans.

According to Gibson *et al.* (2004), bovine tuberculosis in humans should be monitored, especially in those who are at high risk of primary infection such as agricultural and abattoir workers and to identify any transmission between animals and humans. In

immune-compromised people, zoonotic tuberculosis should be considered a health risk factor (Michel, *et al.* 2006).

Chapter 6: Recommendations

Our results suggest that BTB has reached the plateau phase of endemicity in cattle in Govuro district. In this context, the positive predictive value of the SIT is very high and therefore the use of the SICTT as a confirmatory test has a limited value and should not be advocated. Our results further indicate that there is no need for further prevalence studies of BTB in the next few years in Govuro district, unless comprehensive control measures are implemented. The focus of further studies should be on the isolation and the molecular characterization of *M. bovis* from cattle and humans in order to assess transmission routes and the role played by BTB in human TB cases in Govuro district.

Prevention and management of BTB in cattle at farm level require permanent understanding and attention of both farmers and their advisors as a fundamental component of the control programme (Gormley *et al.* 2006). Hence a widespread understanding of the importance of the disease by the livestock keepers is the prerequisite for a successful control strategy of BTB in Govuro district. The high prevalence demonstrated by this study suggests that strategic control should be designed and applied in order to control the spread of BTB to the rest of the province as well as to the other provinces. The BTB status in the surrounding districts such as Machanga should be investigated as a part of the control programme.

The use of skin tests (SIT and SICTT) and laboratory analysis (histopathology, PCR, isolation and identification of mycobacterial species) will be useful in identifying truly infected animals, with the benefit of increasing epidemiologic information, and differentiating causative species. These are the basic steps to be taken into account towards the understanding of the zoonotic consequences of BTB, and the definition and implementation of the most efficient control measures.

Test and Slaughter Policy

The implementation of regular tuberculin skin testing and removal of reactors is the most important TB control measure. According to de la Rúa-Domenech *et al.* (2006), and Gormley *et al.* (2006), the accurate detection and removal of animals infected with *M. bovis* constitutes the cornerstone of disease control in cattle and other species. The success of a BTB control programme relies upon the removal of the infected animal before it becomes a source of infection for other animals and contamination of their environment (Gormley *et al.* 2006). Establishing effective control within herds may not presents major technical problems, but may fail due to non-cooperation of the farmer with procedures when they refuse to present all animals for testing on all occasions (Morris *et al.* 1994). The livestock keepers should understand that BTB is their real problem so that they can fully participate in the control strategy process. On the other hand, this process can only be successfully implemented if livestock keepers are adequately compensated. Compensation is the cornerstone for the success of the process as it will ensure the replacement of the infected animals. Livestock keepers can only participate if they can gain some benefit in the process. Thus, mobilization and

demonstration of social and economic impact yield by BTB and the benefit gain from the process of its control should be emphasized before the implementation of the control strategy takes place. Thus, there should be a concerted effort from government to provide testing as a public good and with compensation to farmers.

Control of Animal Movements

According to Morris *et al.* (1994), spread of infection is facilitated through uncontrolled movement of cattle from infected to non-infected herds. The established movement control in Govuro district is poorly implemented due to poor veterinary service delivery. Prior to purchase, there is a need to subject cattle to the screening tests (skin tests) in order to minimize the spread of the disease between herds. Local committees and livestock owners can play an important role in the control of BTB in the context of community participation principles. The approach should be based on complementary relationships between the district veterinary service and the community. The community should actively participate in the process of movement control of cattle. District government has a key role to play in the process as it should provide financial resources and security to control cattle raids in order to minimize forced movement between districts and provinces.

Constraints

The Govuro district lacks cattle handling facilities and other infrastructure necessary for active surveillance to be performed. The District Veterinary Service is run by only one

medium level technician supported by the local committee of livestock owners at the dip tank level. The livestock extension service is currently very weak, with limited coverage particularly in terms of advice and training. Government is still economically weak and imports remain almost 40% greater than exports. Hence there is a gap between what is needed for a successful BTB control program and what can government offer. Thus, the veterinary service delivery in Govuro district is very weak. It is, therefore, particularly important to address these constraints and to implement changes in order to establish an effective district veterinary service delivery as a base for the control of bovine tuberculosis.

Research needs

The results of this study suggest that there is a need to address the following research questions:

- Study of the prevalence of BTB in surrounding districts of Govuro
- Genotyping of isolates to determine if the same or various strains are present in different foci
- Observational studies to investigate risk factors for BTB infection in the province
- Investigation of the prevalence of and risk factors for infection of humans with *M. bovis*.

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