Mapping of the distribution of *Mycobacterium bovis* strains involved in bovine tuberculosis in Mozambique

by
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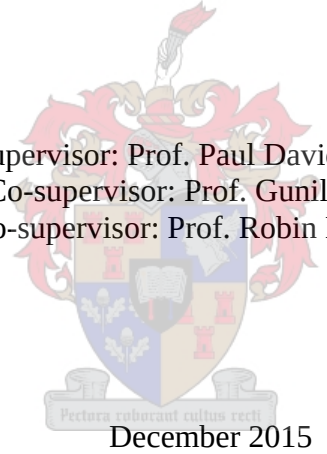

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Abstrak

Beestering (BTB), wat veroorsaak word deur bakterieë van die *Mycobacterium tuberculosis* kompleks, het ‘n negatiewe impak op die ekonomiese en publike gesondheid in lande waar dit voorkom. Die beheer van die siekte is ‘n moeilike taak wêreldwyd.

Die hoofdoel van hierdie tesis was om molekulêre toetse te gebruik om nuttige inligting te genereer wat sal bydra tot die ontwikkeling van toepaslike BTB beheermaatrëels in Mosambiek. Om dit te kon doen, was dit noodsaaklik om ‘n indiepte kennies te hê van BTB geskiedenis in Mosambiek. Die soektog was gebaseer op jaarlikse verslae van Veearts Dienste en ander beskikbare inligting. Ons het verslae gevind van BTB in Mosambiek so vroeg as 1940. Hierdie gevalle is hoofsaaklik geïdentifiseer as gevolg van roetine na-doodse inspeksie van vleis. Hoër getalle van sulke gevalle is geïdentifiseer in 8 distrikte, naamlik Maputo, Magude, Vilanculos, Beira, Chimoio, Tete, Quelimane en Nampula; en het gedien as ‘n basis vir die seleksie van studieareas vir die voorkoms studies.

Voorkoms studies is uitgevoer in 10 distrikte gekies op grond van die geskiedenis van 'n hoër aantal BTB gevalle in hierdie areas (doelbewus bevooroordeeld teenoor plekke vermoedelik met 'n hoër voorkoms), asook‘n hoë digtheid beeste, maar ook om distrikte in die suide, middel en noorde van Mosambiek te verteenwoordig. ‘n Verteenwoordigende steekproef is geïdentifiseer gebaseer op al die vee-gebiede of dorpe in Massingir and Govuro distrikte óf deur kleinskaalse en kommersiële kuddes lukraak te kies in 8 distrikte, spesifiek Manhica, Chibuto, Busi, Gondola, Mutarara, Mogovolas, Angoche en Mecanhelas. Resultate is verkry deur 6983 beeste te toets met behulp van die tuberkulien vel toets. Skynbare voorkoms het gewissel van 0,98 % in Massingir tot 39,6 % in Govuro, met voorkoms so hoog as 71,4 % in sommige vee gebiede/ kuddes. Die ontleding van risiko faktore het geen noemenswaardige verskil met betrekking tot die geslag van die dier gewys nie. Jonger ouderdom diere het ‘n aansienlike laer kans van infeksie gehad in vergelyking met die ouer ouderdom klas. Daar was 'n neiging van beeste van kleinskaalse kuddes om ‘n laer voorkoms te hê in vergelyking met die kommersiële kuddes.

Van die voorkoms studies, is 187 weefsel- en 41 melkmonsters van BTB reaktors ingesamel. ‘n Addisionele 220 weefselmonsters is verkry vanaf die Sentrale Veterinêre Laboratorium se roetine diagnostiese werk. Monsters was onderhewig aan bakteriologiese kweking en 'n versameling van 170 *M. bovis* isolate is verkry. Agt bykomende isolate is voorsien deur 'n

ander studie. Alle isolate was onderhewig aan molekulêre-tipering met behulp van spoligotipering en ‘n subgroep met behulp van MIRU-VNTR en analise van genomies diverse areas. Vyftien verskillende spoligotipering patrone is geïdentifiseer, waarvan 8 nie voorheen in die Mbovis.org databasis geregistreer is nie. Die SB0961 patroon is geïdentifiseer vir 61% van die isolate en gevind in alle dele van die land wat ondersoek was. Ons hipotese is dat hierdie een van die eerste klone was wat voorgestel is in Mosambiek. Nege en twintig isolate het die SB0140 patroon gehad wat spesifiek is aan die Europese 1 (EU1) klonale kompleks. Elf isolate met hierdie spoligotipering patroon is verder geanaliseer om genomies diverse areas te identifiseer, waarvan almal die Eu1 spesifieke delesie getoon het. Hierdie isolate is almal geïsoleer uit beeste van die suide van Mosambiek, asook beeste gevind op kommersiele plase wat hoofsaaklik vanuit Suid Afrika invoer- waar die EU1 klonale kompleks algemeen is. Daar is geen isolate van die Afrikaans 1 (AF1) of Afrikaans 2 (AF2) klonale komplekse nie, dikwels gevind in onderskeidelik Sentraal-Wes-Afrika en Oos-Afrika. Isolate wat in verskillende plase en distrikte geïdentifiser is dui roetes van transmissie en/ of a gemeenskaplike bron van infeksie aan.

Ten slotte, ons resultate dui op 'n moontlike toename in die voorkoms van BTB in Mosambiek, selfs met inagneming dat i) die keuse van areas in ons studie is bevooroordeeld teenoor areas met 'n geskiedenis van hoër BTB voorkoms en ii) die gebruik van 'n meer sensitiewe tegniek d.w.s. toetsing in die middel nekgebied i.p.v. toetsing in die stert vou soos gebruik in vorige studies.

Selfs al is geen bees-na-mens-oordrag gevind nie, is die bewys van *M. bovis* oordrag deur melk en die gebrek aan korrekte prosedures om dier-na-mens-oordrag te voorkom (verbruik van nie-gepasturiseerde melk), ‘n sterk bewys van die soönotiese risiko; ‘n onderwerp wat ondersoek moet word.

Die resultate van hierdie ondersoek beklemtoon die behoefte om die huidige regulasies wat ‘n negatiewe BTB toetsuitslag vereis voor beeste ingevoer word, te versterk. Dieselfde maatreëls moet ingestel word vir interne beweging van beeste, omdat die frekwensie van gedeelde genotipes (Spoligotipering en MIRU) tussen beeste met oorsprong uit verskillende dele van die land aandui dat interne oordrag van BTB plaasvind.

Abstract

Bovine tuberculosis (BTB), caused by bacteria of the *Mycobacterium tuberculosis* complex is reported to cause economic and public health negative impact in countries where it is prevalent. The control of the disease has been a difficult task worldwide.

The main object of this thesis was to use molecular tools to generate useful information to contribute to the design of appropriate BTB control measures in Mozambique. To do so we considered a deep knowledge of the BTB history in Mozambique to be essential. The search was largely based on the reports produced annually by the Veterinary Services and other available information. We found reports of BTB in Mozambique as early as 1940. These cases were mainly identified as a result of *post-mortem* meat inspection. The higher numbers of cases reported were from 8 locations, namely Maputo, Magude, Vilanculos, Beira, Chimoio, Tete, Quelimane and Nampula, and served as a basis to decide the locations to perform prevalence and molecular epidemiologic studies.

Prevalence studies were done in 10 districts selected based on the history of a high number of BTB case reports (intentionally biased towards locations presumably with higher prevalence), a high cattle density, but also to represent districts from the south, centre and north of Mozambique. A representative sample was defined, based on all livestock areas or villages in Massingir and Govuro Districts or by randomly selecting small-scale and commercial herds in 8 districts, specifically Manhiça, Chibuto, Buzi, Gondola, Mutarara, Mogovolas, Angoche and Mecanhelas. Results were obtained from 6983 cattle tested using tuberculin testing. Apparent prevalence varied from 0.98% in Massingir to 39.6% in the Govuro, with prevalence as high as 71.4% in some livestock areas/herds. The analysis of risk factors showed no noteworthy difference with respect to the sex of the animal. Younger age had significantly lower odds of infection compared to the older age class. There was a tendency of cattle from small-scale herds to have lower prevalence when compared to the commercial herds.

From the prevalence studies, 187 tissue and 41 milk samples from BTB reactors were collected. Additionally 220 tissue samples were obtained from the Central Veterinary Laboratory routine diagnostic work. Samples were subject to bacteriological culture and a collection of 170 *M. bovis* isolates were obtained. Eight additional isolates were supplied from another study. All isolates were subjected to molecular typing using spoligotyping, and a sub-sample using MIRU-VNTR and regions of difference (RD) analysis. Fifteen different spoligotype patterns

were identified of which 8 were not previously registered in the Mbovis.org database. The pattern SB0961 accounted for 61% of the isolates and was found in all areas of the country investigated. We hypothesize that this was one of the first clones to be introduced in Mozambique. Twenty-nine isolates had the pattern SB0140, which is specific for the European 1 (Eu1) clonal complex. Eleven isolates with this spoligotype were subjected to RD analysis, and all isolates had the Eu1 specific deletion. These were all isolated from cattle from the south of Mozambique and the majority from commercial farms that imported cattle, mainly from South Africa, where the Eu1 clonal complex is common. There were no isolates of the African 1 (Af1) or African 2 (Af2) clonal complexes that are frequent in Central-West Africa and East Africa, respectively. The clones identified from different farms and districts, strongly suggest routes of transmission and/or common source of infection.

In conclusion, our results show a potential increase in the prevalence of BTB in Mozambique even taking into consideration i) that the selection of locations in our study was biased towards locations with a history of higher BTB prevalence and ii) the use of a more sensitive technique i.e. the testing in the middle neck region as opposed to the testing in the caudal fold as used in previous studies.

Even if no cattle to human transmission was found in studies done in Mozambique so far, the evidence of *M. bovis* shedding through milk and the lack of correct practices to prevent animal to human transmission (consumption of raw milk), strongly suggests that there is zoonotic risk; a subject that needs to be investigated.

The results presented in this work also strengthen the need to reinforce the current regulations that require a negative BTB test result before cattle importation. The same should be enforced for the internal movements, as the frequency of shared genotypes (Spoligotype and MIRU) from cattle originating from different parts of the country strongly suggest intra-contry transmission of BTB.

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Dedication

I dedicate this work to the memory of my late husband Félix Mandlhate for his love, for inspiring me to join the academic career and for being always supportive.

I also dedicate to colleagues Proofs' Luis Neves, José Faftine and Custódio Bila for inspiring me to initiate my PhD studies when I believed it was the end.

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Chapter 1

General introduction

Bovine Tuberculosis a worldwide concern

Tuberculosis (TB) is an infectious and potentially chronic disease caused by bacteria of the *Mycobacterium tuberculosis* complex (MTC). Bovine tuberculosis (BTB), caused mainly by *M. bovis*, a member of the MTC, affects cattle, other domesticated animals and several free or captive wildlife species. Cross-over transmission between distinct animal species and from animals to man is not a rare event. For that reason BTB is one of the most prominent examples of a zoonotic disease (Michel *et al.*, 2010, Müller *et al.*, 2013).

BTB has a worldwide distribution, although there are claims for its eradication in humans and animals in some countries (e.g. Australia) as a result of organized established protocols involving test and slaughter of positive animals. Prevalence of BTB is known to be very low in the industrialized parts of the world although outbreaks still occur (e.g. deer in Sweden, USA and Canada) (Wahlström and Englund, 2006, Miller *et al.*, 2013). Unfortunately some of these countries still have persistent problems due to spread of bacilli between wildlife and livestock, e.g. badgers in U.K. (Byrne *et al.*, 2014) and possums in New Zealand (Kean *et al.*, 1999, Nugent *et al.*, 2015). Fortunately, due to implementation of milk pasteurization, transmission to humans seems to be proportionally lower (Michel *et al.*, 2010). The scenario is quite different for many developing countries, where BTB is still a significant economic problem for the agricultural sector. In the developing world BTB is an important disease of cattle and other domestic animals and several wildlife species. The transmission of the disease to humans still constitutes a public health problem (Müller *et al.*, 2013).

Main risk factors for BTB occurrence in cattle

The control of BTB requires a thorough knowledge of the risk factors involved. In many countries in Africa the relative importance of the different sources of transmission are still largely unknown (Cleaveland *et al.*, 2007, Etter *et al.*, 2006).

BTB is normally introduced in a free herd through acquisition of an infected animal or by close contact with an infected herd (Carrique-Mas *et al.*, 2008). In cattle, the exposure to aerosols containing *M. bovis* is considered the most frequent route of infection, however infection as a result of ingestion of foods (especially milk), water and contaminated material can also occur (Grange, 2001). The maintenance potential of the agent both in symptomatic and asymptomatic animals and on the environment creates conditions for its dissemination (Michel *et al.*, 2010).

Apparently some cattle breeds are more susceptible than others (Ameni *et al.*, 2007, Richardson *et al.*, 2014). This apparent susceptibility should be taken with caution has other study confounding factors can play a role e. g management, nevertheless it was supported in a study done under similar management conditions (Vordermeier *et al.*, 2012). Animals can get infected in an early age but the disease might show clinical signs only in the adult phase. Studies have shown that in a herd, older animals have a higher probability of being infected, not related to a higher susceptibility but to a long term exposure. In Africa oxen that tend to be longer time in the herd is normally the class where the prevalence is higher (Kazwala *et al.*, 2001).

Other important risk factors for BTB occurrence frequently identified in studies are herd size, cattle density, housing conditions (indoors) as they increase the probability of aerosol transmission and presence of maintenance reservoirs that increase the risk of re-infection (Skuce *et al.*, 2012). Katale and collaborators (Katale *et al.*, 2013) found that certain practices, common in the pastoralist communities in Tanzania, such as cattle movements and sharing of pasture and water points, increase the risk of BTB transmission in cattle while the consumption of undercooked meat and unpasteurized milk increased the risk of transmission to humans. The results obtained by Tschopp and collaborators (Tschopp *et al.*, 2009), showing no increased risk of cattle to cattle transmission with close contact in poorly ventilated housing, use of communal grazing and water points make it clear that the epidemiology of BTB varies not only between different African countries but also between different regions in Ethiopia, depending on livestock systems practices and geographical conditions.

As stated by Ayele and collaborators (Ayele *et al.*, 2004), culture and customs, illiteracy and socio-economic status of the families can be some of the factors responsible for the persistence of TB in Africa. In a study in Zambia it was found that 39.6% of the cattle owners knew about the existence of BTB but only 7% had basic knowledge about the disease (Munyeme *et al.*, 2010). A similar proportion was found in Tanzania (Katale *et al.*, 2013). Public knowledge about the disease is very important and an effort should be done to increase the design and implementation of appropriate awareness/education programs, which are important pieces on the strategy to control the disease.

Human health and economic impact of BTB

BTB is of special concern due to the susceptibility of humans to *M. bovis.* The disease in humans can occur in immune-competent individuals (Sunder *et al.*, 2009) and the widespread

immune-suppression caused by the rising epidemic of *HIV/AIDS* further increases the risk of developing clinical disease after exposure to this agent (Cosivi *et al*., 1998, Hlavsa *et al.*, 2008). In Africa this situation might also be aggravated because of malnutrition (Tesema *et al.*, 2015).

Precise information on the current prevalence of the infection caused by *M. bovis* in humans is limited. It is known that in industrialized countries the occurrence of zoonotic TB is negligible (Müller *et al*., 2013) however in developing countries a different scenario can be seen. Studies in Argentina reported prevalence's between 0.4 and 6% of the total cases of human TB (Ritacco and de Kantor, 1992) and even higher proportions (13.8%) have been reported from Mexico (de Kantor *et al.*, 2010). Analysing publications from Africa Muller and collaborators found a range from 0 to 37.7% of the total number of TB cases (Müller *et al*., 2013). There is increasing evidence that *M. bovis* infections may be more significant than is generally considered (Danker and Davis, 2000, de la Rua-Donemesh, 2006, Cleaveland *et al.*, 2007). De Kantor *et al*. (2010) discussing the barriers for complete and accurate information on *M. bovis* disease in humans referred to only 50% of the TB cases in the Latin America and Caribbean regions to be bacteriologically confirmed and in most of the cases the confirmation is only based on smear examination, so no species distinction is possible. When culture is performed, it is done using glycerol containing media that inhibit the development of *M. bovis*. They also referred to the fact that even in the United States where there is routine identification of isolates, it does not go beyond the level of MTC.

In the agricultural arena, the negative impact of BTB is multifold, due to the loss in production related to reduced fertility in animals, reduced milk production and rejection of contaminated milk, reduction of growth, poor body condition and condemnation of affected carcasses (Munyeme *et al.*, 2010, Katale *et al.*, 2013). It has also a negative impact on the market value of animals and international trade (Medeiros *et al.*, 2010). The spillover to a wide variety of species including the endangered species causes obvious negative consequences on conservation and tourism (Michel *et al.*, 2010).

This picture renders it clear that establishing correct control strategies to reduce the BTB prevalence is essential. Control measures require knowledge of the epidemiology of the disease, encompassing data on the routes of transmission, the prevalence, the risk factors and the effects of control measures.

Diagnostic tests for BTB screening

As BTB is often a sub-clinical disease, and when symptoms occur (in advanced cases weakness, anorexia, emaciation, dyspnoea, enlargement of lymph nodes and cough might be present) are not pathognomonic, the accurate detection and removal of infected cattle using immunodiagnostic tests has been the basis of control strategies for BTB worldwide. According to the International Organization for Animal Health (OIE), tuberculin skin test is the standard test for detection of BTB *in vivo* (OIE, 2015).

The results from the tuberculin skin test might not be conclusive and positive animals for this test are designated as reactors. The sensitivity and specificity are acceptable depending on the testing objectives and the prevailing conditions (Ameni *et al.*, 2008). The test performed by injection in the middle neck area is more sensitive than the same test in the caudal fold (Schiller *et al.*, 2010) and it has also the advantage that the resulting swelling cannot be misinterpreted with swellings resulting from tick bites, which are common at the caudal fold. False positive results can be caused by cross-reaction with *Mycobacterium avium* and other environmental mycobacteria. False negatives can be caused by anergy related to malnutrition, pregnancy, extensive disease and other causes of immune-suppression. To reduce the number of false positive results, a comparative test is used, with simultaneous inoculation of bovine and avian tuberculin. Tuberculin testing has an added drowback of being time and resource consuming since two visits are needed to test the animal.

Other methods developed to minimize these drawbacks are the ones based on *in vitro* assessment of interferon-gama (IFN-γ) production from activated T-cells, referred as Interferon Gamma Release Assays (IGRA). A positive result on IGRA test reflects, like a positive result on the skin test, that the immune system was previously exposed to *M. bovis*. The advantage of the IGRAS in comparison with the skin tests is that only one visit to the animals is needed. However, the logistics required to perform IGRAs are an important limitation especially for developing countries.

Antibody detecting tests have also been developed but have the disadvantage of lower sensitivity owing to the late and irregular development of humoral immune response during the disease in cattle (Bezos *et al.*, 2014). The cost of the commercially available tests is also a limitation. The tests are nevertheless reported to be able to detect early (IFN-γ) and late and anergic (antibody detection) infections, being for that reason recommended as complementary

rather than for replacing the tuberculin test. Therefore there is an urgent need for a rapid low-cost, field-adapted test to detect infected animals – both latently and those progressing to active disease.

Direct methods for BTB diagnosis

The confirmation of BTB can only be achived by evidence of the causal agent involvement that is possible using direct diagnostic test methods. The direct methods for BTB diagnosis have been mainly applied for *post-mortem* diagnosis, using mainly tissue samples with or without lesions, nevertheless there are references of use in live animals (Miller *et al.*, 2015). Microscopy for BTB detection is widely used for its simplicity and because it can give fast results. Two staining techniques are used, the Ziehl-Neelsen and Auramine-O, the last being more sensitive. However microscopy has a low sensitivity, because it only detects bacteria at concentrations higher than 10.000/mm (Wards *et al.*, 1995 citing Yeager *et al.*, 1967). In many samples the concentration can be lower, as mentioned by Wards *et al.* (1995), in a study where only 50% of the culture positive tissues samples, from various animal species, had a positive result using microscopy. Microscopy is also used after bacteriological culture to confirm that the bacteria isolated are acid-fast bacilli.

The isolation by culture of bacilli in a selective medium is more sensitive than direct microscopy but has some disadvantages, the most important being that the growth can take up to six to eight weeks and it requires level 3 bio-safety facilities. To achieve a diagnosis based on bacteriological culture one need to identify the bacteria obtained. The identification of species by biochemical tests has been for several decades the only available method. Although quite robust it renders the diagnosis procedure slower (Liebana *et al.*, 1995, Coetsier *et al.*, 2000). The biochemical tests are progressively being replaced by methods based on molecular biology. The culture also requires viable microorganisms and this can be a problem when the samples are not treated in an appropriate way. In human disease it is also important to identify the possible resistance to the antibiotics usually administered, as a way to begin a fast and effective treatment (Kulaga *et al.*, 1999).

Progress in the area of molecular biology has led to the development of methods based on the amplification of DNA, mostly by Polymerase Chain Reaction (PCR), and the identification of specific DNA sequences that enable both detection of the mycobacteria and identification of the different species among the various members of the MTC (Warren *et al.*, 2006). These

techniques might be applied using isolates obtained after microbiological culture or directly from the samples collected from animals; however, this last situation has been more difficult as it is negatively influenced by the existence of PCR inhibiting factors.

Typing using molecular biology techniques

Molecular biology techniques, Spoligotyping, MIRU/VNTR and IS*6110* Restriction Fragment Length Polymorphism (RFLP) are powerful tools for identification of individual isolates for molecular epidemiological studies. IS*6110* RFLP is now less used since it is labor intensive, it requires higher amounts of DNA than in PCR based methods and the comparison of results require complex data analysis (Allix *et al.*, 2006). While it can discriminate well *M. tuberculosis* isolates, in *M. bovis* it has a low discriminatory index because this pathogen has single or few IS*6110* copies.

Spoligotyping and MIRU/VNTR being PCR based techniques are easier and faster to perform and generate easily comparable numerical genotypes. However, the single locus nature of spoligotyping renders this technique less discriminatory. A further limiting factor of spoligotyping is the lack of its ability to detect mixed infections as the pattern obtained can cumulatively present the spacers of the different isolates. These disadvantages do not occur when using MIRU/VNTR, but the use of MIRU/VNTR in phylogenetic studies is not straightforward as in spoligotyping, because the evolution of the VNTR loci is not unidirectional. Repeat sequences can be lost but also acquired, rendering the direction of the evolution difficult to access (Gormley *et al.*, 2014).

Despite all the limitations, typing methods have been shown to be useful amongst other uses to identify the source of the infection (Kandume *et al.*, 2003), spatial and temporal trends (Haddad *et al.*, 2001, Shimuzu *et al.*, 2014), internal movements and the role of international trade (Diguimbaye-Djaibé *et al.*, 2006, Milian-Swazo, *et al*., 2008, Hauer *et al.*, 2015) and transmission between species (Ameni *et al*., 2013, Malama *et al.*, 2014,). Together with epidemiology data they were used to investigate de association between *M. bovis* genotypes with virulence and pathology (Wright *et. al.*, 2013). All these trends are important for the definition of BTB control strategies.

BTB in Africa

Despite of the recommendation for collection of accurate data on human TB due to *M. bovis* (Ayele *et al.*, 2004), very little is known about the prevalence and epidemiology of BTB in

Africa, and its impact on humans, on livestock and wildlife. While there is an increasing concern regarding BTB in Africa that can be measured by the development of networks of professionals working on this disease and the increasing number of research conducted and published both in animals and humans, related to diagnostics and epidemiology to guide control strategies (Michel, 2002, Ameni *et al*., 2008, Munyeme *et al*., 2008, Michel *et al*., 2007, Olaya *et al*., 2007 and Kazwala, 2006, Mucavele, 2008, Viegas *et al*., 2010), the information generated is evaluated as insufficient.

Muller and collaborators in a review on zoonotic tuberculosis in humans concluded with a suggestion that the incidence of zoonotic TB in Africa is low and that because of the lack of large scale population based studies was not possible to identify specific risk groups (Müller *et al*., 2013).

In an extensive reveiew de Gerrine-Wichatitsky and collaborators summarized the available information and presented the knowledge gaps that need to be addressed to be able to control BTB in the interface human-livestock-wildlife in Africa (De Garine-Wichatitsky *et al*., 2013). While they state that the ecological impact to wildlife populations was not demonstrated in Southern Africa, BTB is now recognized as one of the most important threats to wildlife in several African countries (OIE, 2015). It may be that BTB is today the most important threat to the lion population in Greater Limpopo Transfrontier Park/Conservation area in Southern Africa that includes Mozambique. Lions have recently been added to the IUCN red list.

BTB in Mozambique

An historical overview on BTB in Mozambique is presented in chapter 2.While there are no evidences of the disease presence in humans, it is confirmed both in livestock (cattle and pigs) and in wildlife. BTB is a concern in Mozambique and there is a need for updated data regarding the magnitude of the problem and other epidemiological information that can be used to guide control strategies.

Aims and contents of the thesis

As an overall scientific goal, this study aims to bring knowledge on the species and strains involved in BTB and their distribution in Mozambique to provide clues concerning the dissemination of the disease in the country and to help guide control strategies. To achieve this purpose a review on the history of BTB in Mozambique was done to identify and summarize the relevant information on the prevalence of BTB in the country as well as control measures that have been used, as a means to understand the problem within the country, and its evolution

through the last few decades, to serve as a guide to relevant information on the topic and help to guide the prevalence studies. The findings are outlined in the chapter 2 of the thesis. The prevalence of the disease in various locations in districts of Mozambique, selected based on the history of notified BTB cases was evaluated and the results are discussed in chapter 3. The results obtained in chapters 2 and 3 served as a basis to perform the collection of samples for isolation, identification and characterisation of the species and strains of mycobacteria involved in TB in cattle and mapping of strain distribution in the locations collected that are presented in chapter 4. They served also as a basis for the interpretation of the results obtained in chapter 4.

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Chapter 2

Bovine tuberculosis in Mozambique: an historical overview

Bovine tuberculosis in Mozambique: an historical overview

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Introduction

Bovine tuberculosis (BTB) is caused by mycobacteria of the *Mycobacterium tuberculosis* complex (MTC), mostly *Mycobacterium bovis* but also other species, such as Mycobacterium *caprae. M bovis* has a wide range of hosts amongst domestic animals, causing losses in animal production; and amongst wildlife potentially endangering some species; and remains a public health problem due to the possibility of transmission from animals to humans. Where control strategies are effectively put into place, the negative impact of the disease is reduced. However, this is not the case in the vast majority of African countries, making the establishment of disease control strategies important for these countries (Good and Duignan, 2011).

In Mozambique, although there is no detailed and organized information on the magnitude of the problem, there is clear evidence that the disease is causing animal production losses in several regions, mainly from meat inspection rejections, illustrating the need for disease control. The design of successful and appropriately designed BTB control strategies is

presently crucial for the Mozambican growing livestock industry. It is thus important to learn from past experience, successes and failures to define the best strategy.

The aim of this work was to identify the sources and summarize the relevant information on the prevalence of BTB in the country, as well as control measures that have been used, in order to understand the problem within the country, its evolution along the last decades and to serve as a guide to source relevant information on the topic that was scattered in several publications and other documents.

BTB in Mozambique: relevant historical and geographical facts

Historical facts that could have influenced BTB in Mozambique

Human arrival in Mozambique can be briefly summarised thus: Bantu speakers migrated to Mozambique in the first millennium, Arab and Swahili traders settled the region thereafter, and the country was first colonized by Portugal in 1505.

During colonial time three different players in agriculture and animal production in Mozambique can be described: 1) the local farmers that were designated as “indigenas”; 2) the colonial peasants designated as “europeus” and; 3) the companies designated as “Companhias Majestáticas” or “Arrendatárias”. The first companies controlled large swaths of the Mozambican territory independently of the Portuguese government and the last rented well defined territories for economic exploitation and were responsible for the importation of a large number of cattle. One important company in particular had a powerful influence in animal production, viz. “Companhia do Buzi” that had its main focus in sugar production and was present in the areas of the actual Sofala and Inhambane provinces.

The guerrilla struggle for independence started in 1963 and ended with a cease-fire that was signed in September 1974. Mozambique became an independent country on June 25, 1975, after being under Portuguese colonial rule for almost 500 years.

In 1985, the country was paralyzed by a civil war perpetrated by an anti-government guerrilla movement, the Mozambique National Resistance (Renamo). A cease-fire agreement was signed in October 1992.

Before the civil war, the rural population was mainly dispersed, each family surrounded by their agricultural land. However, during the conflict the population was forced to aggregate in

defined areas designated as “aldeias comunais” (communal villages). The constitution of the “aldeias comunais” was intensified by natural calamities and civil war (Araujo, 1988). In addition, the government promoted “aldeias comunais” as a policy to encourage cooperative work and to facilitate assistance to the community in crucial areas e.g. health and education.

Mozambique: geography and demography

With an estimated human and cattle population of 24 and 1.6 million respectively (INE, 2014), Mozambique stretches for 2470 km along Africa's southeast coast, with an area of 801590 km^2 that is divided into 11 provinces (4 in the south, 4 in the centre and 3 in the north) and 128 districts (Fig. 1).

Possible sources of BTB to Mozambique

With respect to the history of BTB in Mozambique, an important question is: what were the main sources of the disease found in the country today. The cattle that were the first to be introduced to Mozambique were presumed to be from the Sanga group. These are animals with a hump between the shoulders, originally from Asia, and probably introduced to Africa c.a. 1500 BC. Descendants of these were brought by the Bantu community during their migration from Egypt, through Ethiopia (Mason and Maule, 1960 cited by Mukasa-Mugerwa, 1989).

There are several references to later introduction of well-known cattle breeds in the south of Mozambique, such as Afrikaner, Friesland, Hereford, Shorthorn, Jersey, Brow Swiss, Charolais, Simentaler, Brahman or crossbreeds imported from South Africa as well as Nelore and Gir imported from Brazil (Morgado, 2007). In the central part of Mozambique there are references to cattle imported from Europe, especially the breeds Aberdeen Angus and Hereford either directly or through Malawi and also from the United States and Brazil (Morgado, 2004). All these cattle introductions constitute potential sources of BTB introduction.

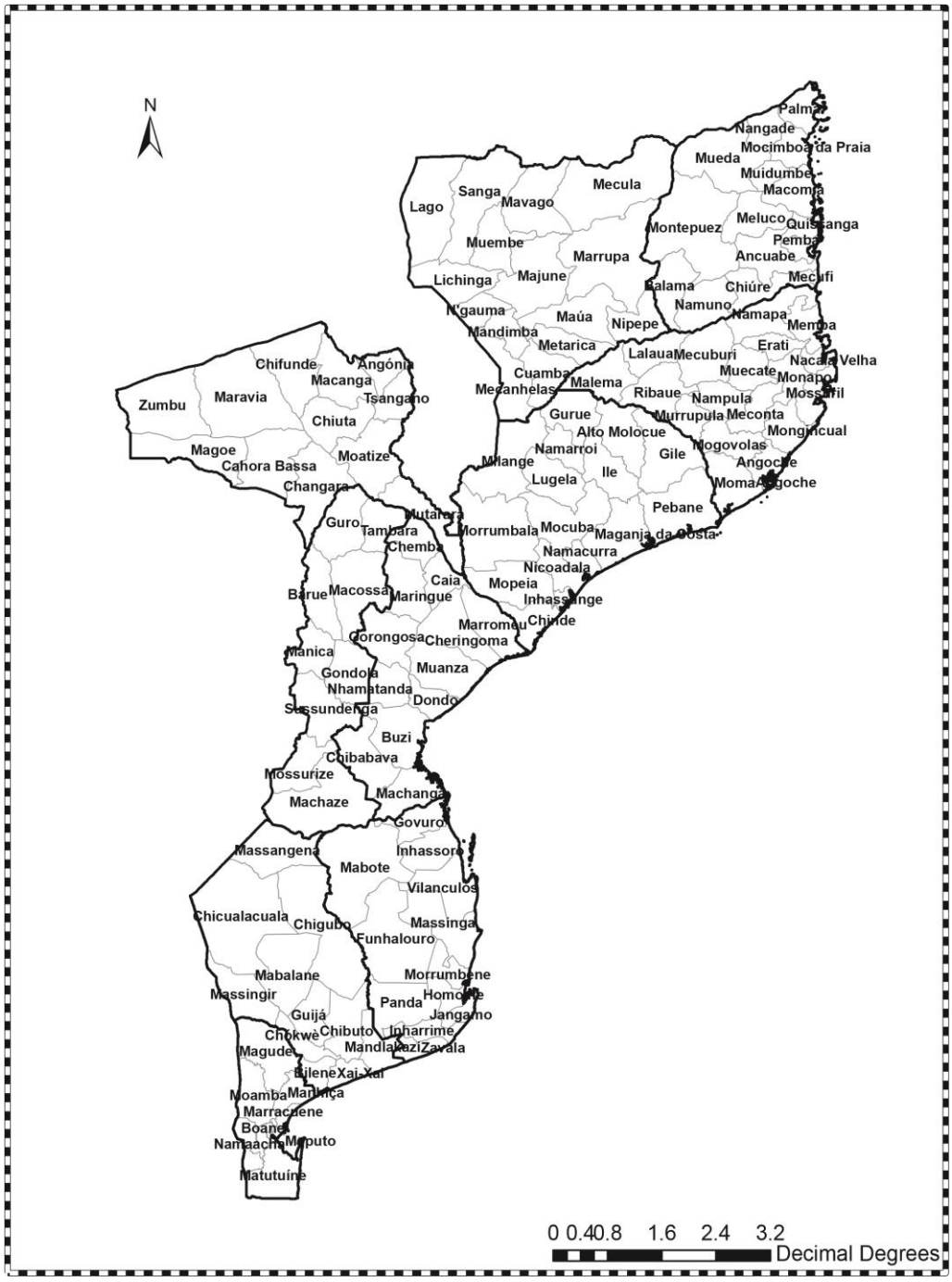

Fig. 1. Map of Mozambique with identification of the provinces and districts

The need for restocking was a constant issue throughout the history of Mozambique. For example, Morgado and Fevereiro (1961) reported their visit to Madagascar to study the possibility of acquiring cattle for restocking the north of the country. They concluded that despite the presence of BTB in some areas of Madagascar, the zebus of Madagascar could be a good source for cattle introduction in the north of Mozambique. This did not happen and

restocking was done by introduction of cattle brought from Lourenço Marques[1] (Morgado, 2000). It is of course possible that other introductions happened without the knowledge of the author and that some introductions could have occurred without knowledge of the government of the day, and also there will be no records of movement or introductions as for example, cross border land movement by isolated rural farmers.

The need for restocking is still current. A summary of information available concerning cattle population and importation over the years can be found in Fig. 2. Cattle were imported for restocking but also for direct slaughter; unfortunately the distinction between the two was not clearly reported in some occasions.

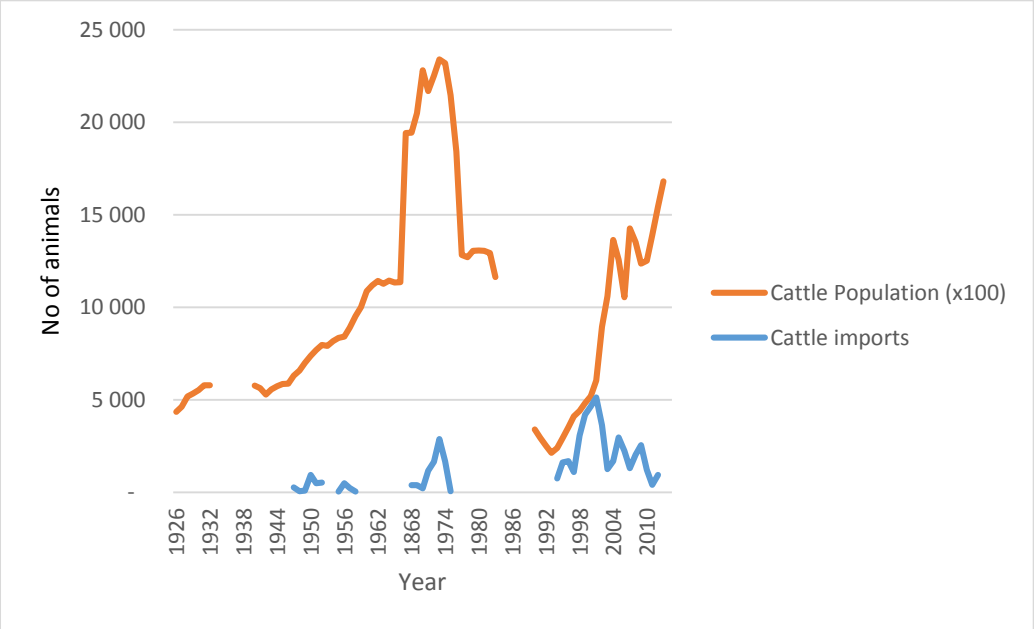

Fig. 2. Mozambique cattle population and total imports. The figure was constructed combining data from difference sources, namely: Agriculture and Animal Production Bulletin "Boletim Agrícola e Pecuário" from 1926 to 1932; Annual Reports of the Veterinary Services "Anais dos Serviços de Veterinária" from 1940 to 1973; Annual Reports of the Veterinary Services "Boletim da Direcção Nacional de Pecuária" from 1974 to 1976; Dionísio (1985) from 1977 to 1983 (might not include milk producing cattle) and Annual Reports of the Veterinary Services " Relatório Anual da Direcção Nacional de Pecuária" from 1990 to 2013.

[1] Lourenço Marques was during colonial the designation of the actual Maputo and Gaza provinces.

Early reports on BTB in Mozambique

The history of BTB in Mozambique can most probably only be relied on after the creation of the Veterinary services in 1908, when reports were written containing information regarding animal diseases. Based on above mentioned reports, apparently BTB was not an important disease during these early years. As an indication, a report published in June 1910 ("Boletim da Repartição de Agricultura No. 2"), refers almost exclusively to tick-borne diseases (Mendes, 2003). The bulletin contained information on diseases observed not only in cattle but also other species. The Veterinary Services operated as a division of the Agriculture Office until 1933, which implied a lack of autonomy and limited resources and manpower until 1939 (Câmara, 1949). Hence, the absence of reports on BTB does not guarantee absence of disease over this period.

One of the first regulations to be issued was the animal health regulation ("Portaria" No. 113 – 1908) recognising the need for eradication of the diseases that had considerably reduced the herds in Mozambique[2], to control the importation of diseases from the neighbouring countries and the need for cooperation with the governments of the neighbouring colonies. The regulation was evaluated by Mendes (2006), as very repressive. However, the policy set basic rules for the control of diseases, namely the control of animal imports and internal movements, quarantine and disease surveillance. Emphasis was placed on BTB since in the chapter dedicated to disease prevention, BTB was the only cattle disease explicitly mentioned with detailed procedures. BTB was included in the list of diseases that required reporting of occurrence.

The clear awareness of the presence and expansion of BTB in Mozambique came as a result of *post-mortem* findings in slaughterhouses and slabs (Aires, 1947). This was the first report found (reporting on information from 1940 to 1946) which referred to the presence of BTB in Mozambique, with the statement "it has been for many years that as a result of meat inspection total and partial rejections observed in slaughterhouses and slabs, we knew that tuberculosis was expanding to various regions of the colony[3]" (note direct translation from Portuguese with no grammatical correction), with no indication on numbers or locations involved.

[2] The original document refer to "province" since Mozambique was considered a province of Portugal since 1501

[3] Mozambique

In previous reports available, specifically the "Boletim Agrícola e Pecuário" of the years 1928, 1929 and 1933, no reference was made to TB, despite reports of other animal diseases.

Implementation of BTB control strategies in Mozambique

As a result of the recognition of the importance of BTB as a zoonotic disease, at that time transmitted mainly through milk consumption, the first control measures target this specific aspect of BTB. The compulsory tuberculin testing of cattle for milk production was established in 1941 through the "Diploma Legislativo" (Legislative diploma) No. 749/1941. Testing was determined to be performed every six months and the first two tests were at no cost to the animal owner. It was also specified that all positive cattle had to be removed from milk production and the fate of the positive animals should be decided by the veterinary authorities.

Even if compensation for slaughter of positive animals had already been regulated in the Animal Health Regulation, there were no funds to fulfil this need. Through the implementation of the "Diploma Legislativo" N. 918/1944 this problem was attenuated (Morgado, 2007). In this legislation, taxes were levied in order to accumulate funds for the animal production development fund "Fundo de fomento pecuário", which would be partially used to pay compensation for animals slaughtered or meat inspection rejections as a result of implementation of disease control measures.

The establishment of the Veterinary Pathology Laboratory (VPL) in 1933 came as a response to the recognized need to control animal diseases (Pereira & Garcês, 1985). The VPL was involved, from the early stages, in diagnostics, production of biologicals and research (Câmara, 1949). In terms of BTB control, reference is made to the local production of tuberculin. From 1954, heat-concentrated synthetic medium tuberculin started to be replaced by P.P.D. and its inclusion in the list of biologicals supplied by the VPL was announced in the "Boletim Oficial de Moçambique III serie" No. 36, 1954.

To create awareness about the disease, articles have been written in magazines such as "Gazeta do Agricultor", a magazine created to support farmers and to be a link between them and the agricultural services. In a series of articles on general information of the main diseases that affected cattle in Mozambique, BTB was the first to be presented by Valadão in 1949, where he reported the rejection of 2086 bovine carcasses between 1941 and 1945 in the Maputo slaughterhouse only (at that time called Lourenço Marques).

The relevant authorities noticed that the precautions to be adopted in the control of diseases in domestic animals were insufficient, therefore a new Animal Health Regulation was approved in 1948 (Portaria No. 7325). This was a more comprehensive legislation that added rules regarding centralization of slaughter for human consumption and compulsory meat inspection, declaration of infected areas and compensation in case of compulsory slaughter of livestock for sanitary reasons.

Data from the period 1947 to 1949 encompass a cattle population of 1057000 to 1205000 heads (increase in domestic herd), tuberculin production of 18913 to 23949 doses and BTB cases that varied from 1242 to 1713 a year. The areas that contributed with highest number of cases (in order of importance) were Chimoio (called at that time Vila Pery), Vilanculos, Maputo (called at that time Lourenço Marques), Beira, Quelimane and Magude. The total for compensation for positive animals slaughter was 1 862 077.10 Portuguese escudos in the 3 years (Repartição Técnica dos Serviços de Veterinária, 1950).

The implementation of the control regulations was considered timely, since they resulted in the reduction of the BTB positive animals from 12.7 % to 6.1% and to 3.2% in three consecutive test and slaughter campaigns in the Lourenco Marques district (area of the current Maputo and Gaza provinces). Similar results were obtained in other areas of the country, such as Manica and Sofala and Zambezia (Aires, 1947), but no figures were provided.

Even if the BTB control measures were compulsory only for milking cows, some farmers decided to use them for all animals, in order to clean their herds which were used for draft power and meat production ("Repartição Técnica dos Serviços de Veterinária", 1954).

The control measures established were generating some positive results but according to Napoles (1954), it was far from what would be desired. BTB from 1947 to 1949 was reported as continuing to be an important disease in Inhambane province, as presented by Morgado (2007) referring to the cattle of the Companhia do Buzi located at Mahave and Macovane[4] that presented extensive TB lesions on necropsy. That was also true for areas like Chimoio where a prevalence of 40% resulted in this being the subject of a publication in the newspaper "Notícias da Beira" (Paisana, 1953).

[4] These locations are part of Govuro District

At that time it was considered that the requirements for compensation for the slaughter of BTB positive animals needed actualization. In 1961, in response to this need, a new regulation was approved on this matter and others in respect to the function of the Veterinary services, the "Diploma legislativo 2057". It was determined that compensation would be attributable only to farmers with fenced properties, farmers that tested the entire herd (excluding calves) every six months for herd prevalence above 3%, every year for herd prevalence below 3% or every second year where no cases of positive reactors had been identified in the previous 3 years. All the positive reactors should be sent to public slaughterhouses up to 30 days after the test result was obtained. The value of compensation was to be determined by a commission established in every location. Pigs were the other species reported to be tested for BTB. In this regulation, conditions for compensation in pigs were also set.

From the years 1950 to 1976 there were reports of 2041 foci of BTB and 18415 cases. The higher number of cases (4038) were reported in 1953 (Fig. 3). From the 33 delegations[5] of the veterinary services that reported disease information, 5 did not report BTB cases. Adding all the cases for the period above, the delegations that presented the highest number of cases were (from the south to the north respectively), Lourenço Marques (2485), Magude (1426), Beira (1697), Vila Pery (3028), Tete (1906) Quelimane (2456) and Nampula (2720), the same locations mentioned by Morgado (2007) for the years 1947 to 1949, except Tete and Nampula that were not referred to then. For Vilanculos there was a reduction of notification of the number of cases from 970 to 208. The concept of disease focus was never presented but cases in some reports were presented as a mixture of necropsy or meat inspection findings and results of tuberculin testing. Of the total, 4 foci and 337 cases were in pigs, reported only between the years 1961 and 1974.

The highest number of cases reported did not correspond to the highest number of cattle tested: for example, in 1958 there were 77130 cattle tested (8.1% of the national herd) and 631 cases reported, whilst in 1953 only 46876 cattle were tested (5.9% of the national herd) and 4038 cases reported. It can therefore be assumed that in 1953 there were fewer animals tested but more TB positives. A more coherent result can be seen related to the higher compensation that was paid for slaughter of BTB positive cattle in 1954 (assuming that was relative to the higher number of BTB positive cattle identified in 1953), which amounted to 1 367 088.00 escudos.

[5] Delegations were representations of the Central Veterinary Services in the various locations of the country responsible to undertake the activities related to animal production and health.

The total amount of compensation paid in the years 1944 to 1960 amounted to 10 150 492.44 escudos, including 748 419.91 escudos paid for the compensation of slaughter of BTB infected pigs. In the following years the figures reported were not exclusively compensation for BTB cases but included compensation for brucellosis and funds attributed to farmers for other reasons such as for fencing properties.

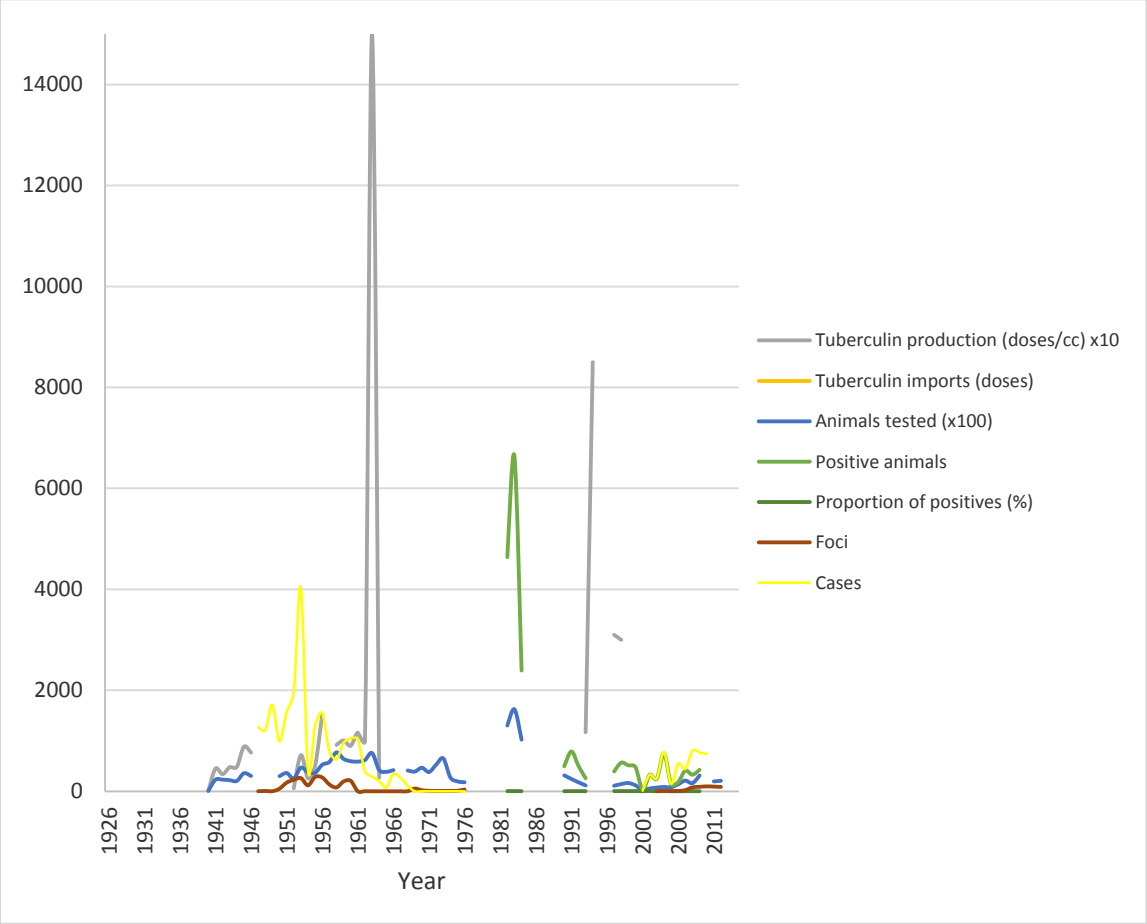

Fig. 3. Tuberculin production and imports; number of catle tested, BTB positive and proportion of positives and disease foci and cases reported. In the period before 1975 tuberculin production is presented in volume (cc), while after 1975 in number of doses. The sources of information were the same as in fig. 2 except for the period 1977 to 1983 where data was obtained from Garcês *et al.* (1985).

The Veterinary Research Institute of Mozambique (INIV), established in 1966, assumed the functions of the VPL and intensified research on BTB. In its bi-annual publication "Veterinária Moçambicana" there were a series of publications on BTB, aimed at providing relevant

information with respect to meat inspection (Petisca, 1969 a, b and c; Petisca, Atalaia & Serra, 1972). Another landmark in the history of BTB in Mozambique was the establishment of the task force for Animal Health, of which one of the missions was to perform activities to eradicate BTB in Manhiça and Chimoio (Portaria 23833, 1969).

BTB in Mozambique during the most recent decades

In general the cattle population in Mozambique grew until short after independence (Fig 2). In the first year after independence, state owned units were set up, consisting of the herds that were abandoned by the colonial owners. The veterinary assistance to the state owned farms contributed to the reduction of BTB prevalence. In a presentation from Garcês and collaborators in the Seminar of Animal production in 1985, it was shown that the tuberculin testing coverage had increased from 4% (52769 cattle tested over 1355613 existent) of the national herd in 1972 to 12% (162503 tested over 1163000 existent) in 1983. They also reported an increase of the national tuberculin production to cope with the demand, from 64900 in 1976 to 327500 doses in 1982 (Garcês *et al.*, 1985).

The establishment of the meat trading company GAPECOM in 1977 contributed to the control of BTB. GAPECOM had transport to trade cattle all over the country and was also responsible for running the Lourenço Marques slaughterhouse that had facilities for preserving the meat after slaughter for later distribution/sale. This has allowed careful marketing and control of meat, making it easier for the government and the farmers to correctly remove positive animals from the herds affected.

In 1981 a BTB control program was outlined and implemented to some extent. The program was based on the testing on farms and well defined locations in 15 districts identified as BTB hot spots. It was defined that testing was compulsory in dairy farms and the slaughter of the positive reactors mandated. Compensation was planned for small-scale but not for commercial or state owned farms (OIE, 2004). A pilot program was implemented and failed in the small-scale sector since the animals provided for replacement of positive reactors soon died after acquiring tick-borne diseases (Cipriano, 2004).

With the start of the civil war, animal production was severely affected. The stock of state owned companies was soon depleted. Rural communities had to move from their dispersed locations to the villages designated as “aldeias comunais”, bringing along their stock. That situation created overgrazing and appropriate conditions for disease transmission (for example:

weakened animal movement control), while the government capacity for animal disease control was severely reduced. This is a possible explanation for the BTB prevalence increase that took place and can be still observed today in some locations. The cattle population was drastically reduced (Fig. 1.).

As a result of the peace agreement, the government in partnership with private sector and livestock civil society donors, started extensive restocking programs that resulted in increase of animal population, reaching 12507910 heads of cattle in 2010.

Reports of the veterinary services after independence make no reference to BTB, except for the data on test results and meat inspection rejections. However, in the report of 1999 a clear statement was made that rabies and BTB were public health concerns. In the report, concern regarding the lack of capacity of the government to support the financial burden related to the elimination of all positive reactors was presented. Reference was also made to the more seriously affected areas, viz. Buzi, Mambone and Quelimane (DINAP, 1999).

In 2000, tuberculin production was discontinued in Mozambique (Mata, 2004). Tuberculin used in the following years was imported from different laboratories and with different potencies rendering it difficult to compare BTB test results. A recommendation from the Seminar – Tuberculosis, interaction farm animals, wildlife and humans in Maputo, 2004 was made to correct that situation, which is actually corrected. Tuberculin is now purchased from a single company.

During early testing phases tuberculin testing was done in the right lower eyelid as can be seen in a picture presented in Morgado (2000), with no indication of when this occurred, but referring to the Veterinary Services activities in the years 1950). Now however, tuberculin testing is mostly done in the caudal fold. The performance of the comparative test in the mid-neck was reported for the first time by Macucule (2009).

The importance of BTB was reinforced in December 2003 in the workshop Applied Research in Animal Production and Health, “Investigação Aplicada em Saúde e Produção Animal”. This and a growing awareness of the problem discussed at the Veterinary Services National meeting 2004, led to a decision to prepare a disease control strategy. The strategy designated National Prevention, Control and Eradication of Tuberculosis and Brucellosis in Mozambique, “Programa Nacional Prevenção, Controlo e Erradicação da Tuberculose e Brucelose em Moçambique” (PNPCEBT) and a regulation for its implementation was written with an

expected outcome of reduction of the disease prevalence to 10% in a 10 year period, but this regulation was never approved. This expected outcome must have been erroneously defined as the national BTB positivity rate was estimated as 3.17% in the period 1981 to 1992 and as 4.06% in the period 1992 to 2003, based on series of surveys done in various parts of the country as presented in the Fig. 4. These estimates were calculated as a mean of the estimates of all provinces based on testing done in 25.49% and 11.04% of the national herd respectively.

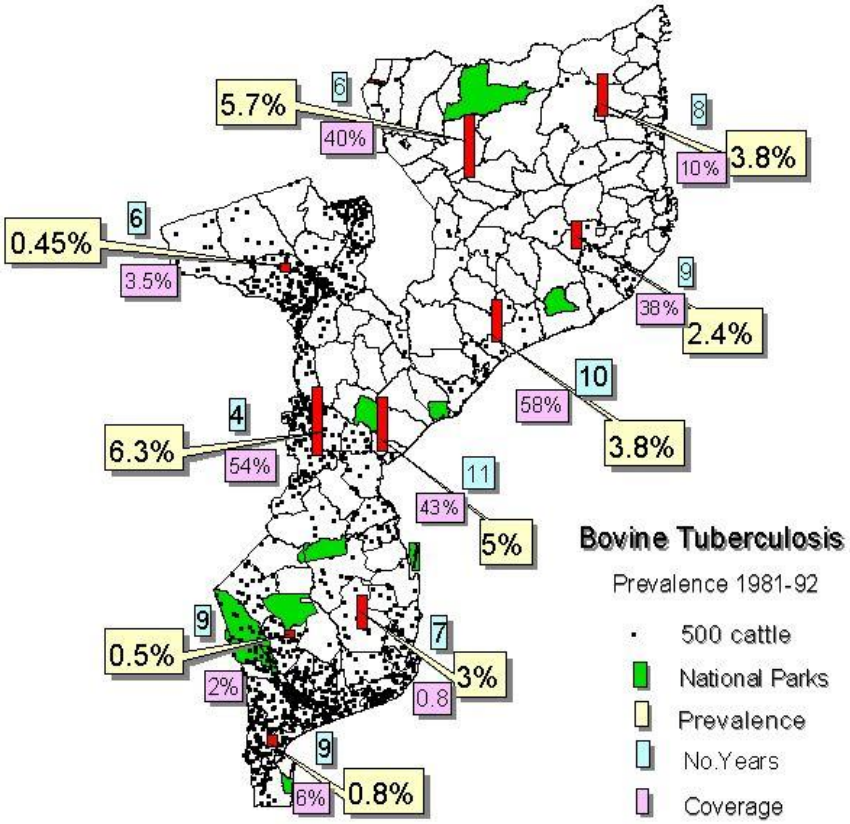

Fig. 4. Tuberculin testing results in the period 1981 to 1992. The figure was adopted from the PNPCEBT document produced by the National Directorate of the Veterinary Services, Mozambique.

Successive updates were written by the Veterinary Services but the failure to get approval from the Ministry of Agriculture has delayed full implementation. In the most recently revised document (2013), the aim is to reach a prevalence of 1%. No date was defined to reach that goal. The definition of this goal was based on the same assumption of national positivity rate as in the previous program.

The Veterinary Services are presently implementing parts of the strategy that do not require substantial financial input. As an example, tuberculin testing is compulsory when cattle are transferred from one location to another for breeding or rearing purposes. Only one negative test result is required, making this a weak measure to control disease dissemination. Other measures include testing animals from farmers that are willing to slaughter their stock even without compensation (mainly commercial farms).

The decentralization of the Veterinary Services (following the Mozambican Law of Local Government Institutions - LOLE) created difficulties in administration and management of sanitary programs in the country, since district Veterinary officers were no longer under direct supervision of the National Directorate of Veterinary Services and the resources to them for regular implementation of disease prospection and control activities were no longer allocated. For this reason tuberculin testing was no longer performed systematically and consequently information about BTB in the country is sporadic and inadequate. Furthermore, payment of compensation for slaughter of BTB positive animals had to be decided at provincial level as a consequence of decentralization (Cipriano, 2004) and local governments have in general not been able to generate funds for this activity.

With the creation of the Institute of Agricultural Research of Mozambique (IIAM) in 2004, the Veterinary Laboratories on the provincial level were no longer under the direction of them. This has almost certainly negatively affected the definition of priorities and technical assistance. In terms of BTB there was a discontinuation of a plan to establish TB diagnostics on provincial level (microscopy) and also culture in the Central Laboratory in Maputo. The decentralisation trough LOLE also affected the funds allocation to the laboratories at provincial level.

Sporadic surveys have been conducted as part of research projects from 2009 to 2014 as presented in the chapter 3. Results ranging from 0 to 71.4% positive reactors in some herds suggest a probable increase in the disease prevalence in the country in recent years.

For example, in the Govuro District in the Southeast of Mozambique 39.6% of cattle were positive skin test reactors for BTB (Moiane *et al.* 2014), while BTB was at very low levels (0.98%) in cattle in the Limpopo National Park (Tanner *et al*, 2014). Pilot projects to evaluate practices on compensation were undertaken as it occurred in Govuro, Inhambane province (Head of the Provincial Veterinary Services, personal communication). A total of 1400

tuberculin positive animals were removed and only 600 were replaced by the project. This is a clear indication that cattle owners are willing to cull positive animals even with no compensation. This willingness was also confirmed in a survey in which the cattle farmers of the district responded to a questionnaire (data not published). As for other events, as referred by Cipriano, the farmers were not satisfied with the compensation since the animals received by them to replace the BTB positive animals were not qualitatively equivalent to the ones culled, in terms of age and breed (Cipriano, 2004).

Animals selected for culling in Govuro were sent to slaughterhouses from 2008 and detailed meat inspection was performed. From the first 41 culled animals, 9 (22.95%) showed no visible lesions, 23 had lesions involving the respiratory tract (lung parenchyma and mediastinum and bronchial ganglions), 6 had concomitant lesions in the mesenteric ganglions and 9 only lesions at the head ganglia. Even if the majority of the meat was approved for human consumption the revenue obtained, according to the provincial veterinary services, was not sufficient to compensate for the costs of replacing the culled animals.

The situation continued to be alarming and BTB was again highlighted in the newspapers in June 2012, where the slaughtering of 250 dairy cows in Chimoio was reported. This compulsory slaughter was required by the veterinary services as a result of tuberculin testing that showed 69% (138/200) positive reactors in this investigation.

Since the case reports presented in the reports from 1990 to 2013 show 39 and 90 cases respectively, there is very clearly underreporting. The higher number presented in the Fig. 3 resulted from adding tuberculin positive cases, presented in the reports relative to the same years.

BTB as a problem in wildlife

In addition to livestock, BTB is also affecting wildlife. A comprehensive evaluation of BTB in buffalos was made during the “Operação buffalo”, a large culling operation based on the thinking of the time that there was overstocking in some National Parks. Apart from population reduction, there was also the rationale to evaluate the possibility of meat supply for human consumption, particularly for the military. In three operations, 6095 animals were culled but only 167 were *post-mortem* examined, 21 (100% of the total culled) in the Gorongosa National Park in 1978, plus 47 (2.35%) and 99 (2.43%) in Marromeu in the years 1978 and 1979 respectively. BTB compatible lesions were found in 6 (28.6%) of the animals studied in 1978

from Gorongosa and the lesions were found mainly in the lungs. From the six cases, only two were positive by culture and no typing was performed on the isolates. In Marromeu 7 (4.1% of the total examined) buffalos were found with BTB like lesions, 6 being identified in 1978 and 1 in 1979 (Ferreira & Rosinha, 1986). The same authors referred to this proportion being similar to that found in cattle in the Marromeu District in the same year. Presumably these are the first confirmed reports of BTB in wildlife in Mozambique.

A new concern related to a new risk of introduction of BTB into Mozambique arose with the creation of Greater Limpopo Transfrontier Conservation Area, commonly designated by GLTFCA that was established in November 2000, with the signature of a Memorandum of Understanding between the governments of South Africa, Mozambique and Zimbabwe, confirmed by a presidential agreement in 2002. To action this initiative, the removal of common fences was done to enable free migration routes for wildlife between the Limpopo National Park (LNP), the Kruger National Park (KNP) and the Gonarezhou National Park (GNP) and also migration to the Banhine and Zinave National Parks. The fence removal started in 2003 with a symbolic removal of 20 Km and it was planned that it would be concluded in 2013.

While all the surveys conducted before and shortly after the agreement in the LNP showed an undetectable prevalence of BTB in buffalo and a very low prevalence in cattle (Pereira *et. al*, 2007), in the KNP the prevalence in buffalos was estimated as 38.2% (±6.3%), in the south zone of the park, with prevalence declining towards the north (Rodwell *et al*, 2001).

The concern was and still is related to the presence of a human population (estimated at 27000), with livestock and living inside the LNP. Both animal and public health risks are therefore a concern (Working group, animal health for the environment and development – AHEAD, 2008).

A recent study in the LNP area showed that the prevalence of BTB is still very low in cattle (0.98% positive animals) and in buffalo herds (8,08% positive animals), although there is a suspicion that the buffalo positive reactors in this study might be false positive reactors (Tanner *et al.*, 2014). The future will tell what the health consequences of creation of the GLTFCA will be.

Is BTB a public health concern?

While the programs initially designed for control of BTB were concerned mainly with transmission to humans through milk consumption (compulsory testing only for milking herds), the awareness that meat could be a route of transmission was not discarded.

Various articles have been written by Petisca, on "general considerations on meat inspection", "pathogeny and anathomo pathology in tuberculosis of cattle and swine" and "evolution of the concepts about meat inspection of cattle with tuberculosis", all supporting meat inspectors in their decisions to avoid transmission of the agent to humans (Petisca, 1969 a, b and c) but this work also helped to show that the slaughterhouse could serve as an important source of epidemiological information in finding all possible cases.

A study on the presence of mycobacteria in the muscles of cattle with different extension of lesion distribution in the carcase and organs, was performed by Petisca *et al.*, 1972. They found the presence of mycobacteria in the meat of 9.1% (7/22) of the animals that according to legislation should be condemned and 7.1% (2/26) of the animals that according to legislation should be freely accepted for human consumption. The authors concluded that some of the meat that is condemned could be approved and the some that had been approved should have been condemned. They suggested that the infection potential of the meat was lower than in other studies (Gallo & Guercio, 1956, Romanelli, Asdrubaly & Baldelli, 1957, Montroni, La Placa & Mora, 1959, all cited by Petisca *et al.*, 1972). However, they noted a weakness of the study being that the number of animals studied was too small to draw clear conclusions.

The regulation on meat inspection approved in 1973 was clearly defined having in mind the need to guide inspectors on the judgment of meat derived from BTB infected or suspect animals. In an appendix with 31 pages, 20 were compiled with valuable information in that regard (Direcção Provincial dos Serviços de Veterinária, 1973).

With such high prevalence's as presented above in the study of Moiane *et al.*, 2014, the disease has most probably had some impact on public health. However, thus far in the two studies that were done no confirmation of the involvement of BTB on human health in Mozambique could be found (Mucavele, 2008; Viegas, *et al*., 2010). The studies had two main limitations, one being the fact that samples were from a population that does not have direct contact with BTB infected animals and the second limitation was that only cases with pulmonary infections were

investigated, whilst BTB may be more likely to be present in extra-pulmonary cases (Durr *et al.*, 2013). This subject requires further investigation.

BTB was and still is a concern in Mozambique and some of the recommendations produced in the Seminar – Tuberculosis, interaction farm animals, wildlife and humans in Maputo, 2004 (Table 2) are still current and their implementation may be a priority to control the disease in Mozambique.

Table 1. Comments to the recommendations of the Seminar – Tuberculosis, interaction farm animals, wildlife and humans in Maputo, 2004

	Recommendations	**Comments**
1	Improve commitment of government	More studies have to be conducted and data gathered to confirm the negative impact of BTB.
2	Update the BTB control policy	BTB control programme (PNPCEBT) and the regulation for implementation was updated but still not approved.
3	Identify and geo-reference herds	This recommendation is crucial and the basis for the implementation of other recommendations. Re-introduction of the farmer book and District Veterinary Services register is not equally applied all over the country. The regional (SADC) program for cattle marking was expected to have boosted this activity but did not gain ground.
4	Carry out comprehensive survey to study the situation in Mozambique	Done only as part of research projects or in some commercial farms. To amplify is necessary to implement the recommendation 1.
5	Establish testing scheme for movement of cattle between herds	Already defined in the BTB control programme. Not yet implemented.
6	Improve surveillance at abattoirs with laboratory confirmation of samples. Involve other institutions	Meat inspectors are regularly trained but registration of meat inspection results is not done using a uniform format. No compilation of results is done, resulting in the under-reporting seen in the annual reports of the Veterinary Services. In the proposed PNPCEBT laboratory confirmation is only necessary for herds under surveillance but should be for all cases.
7	Trace back from abattoirs to herds of origin	This requires the implementation of the recommendation 2 and correct issuing of transit certificate (correct identification of the stock sent to the slaughterhouse), what is not actually applied.

8	Train technical staff for standards of TB testing	A series of training opportunities are undertaken by technical staff all over the country.
9	Improve awareness of public	Not done and it is especially important for the farmers to increase the potential success of the control programme.
10	Improve communication between veterinary and health workers	The communication started but needs to be amplified.
11	Clean herds or farms used for restocking	Crucial. Not available.
12	Include risk analysis related to GLTP	Not done and is important
13	Increase the number of animals inspected at abattoirs	Requires control of cattle movement to guarantee that all are slaughtered in abattoirs or slabs under veterinary supervision
14	Solve the problem of compensation	Procedures for compensation are defined in the PNPCEBT. Needed and not available are funds and clean farms for stock for replacement of culled animals.
15	Isolate suspects from the rest of the herds	Difficult to implement especially if small-scale farmers
16	Test young animals (< 6 months) in herds of high prevalence	Not a requirement in the proposed PNPCEBT. Should be added.
17	Fence farms close to wildlife farms/conservation	Difficult to implement specially if small-scale farmers
18	Study risk/impact in humans such as *M. bovis* infection in Man and infection in Man due to unpasteurized milk	Studies have started but need to be amplified
19	Study risk/impact in wildlife and in cattle in the south;	Important all over the country and not only in the south, not yet started.
20	Establish cooperation with BTB lab in South Africa for Gama Interferon test;	Test difficult to implement under Mozambique conditions
21	Develop molecular tools to study BTB	Apply the existing tools is also important. It has started but needs to be amplified.

Methodology

To write the article on the overview in the history of BTB in Mozambique basic information as presented in three books by Morgado (Morgado, 2000; Morgado, 2004; Morgado, 2007), was used.

Additional records from the colonial times were collected from the annual reports of the Veterinary Services, the Official Bulletin of Mozambique where legislation was published, other publications of the veterinary and agricultural institutions and a newspaper that was retrieved from the Historical Archive of Mozambique and the National Bibliotheca.

Information corresponding to the years after independence was collected from the annual reports of the Veterinary Services, legislation, meetings, seminars and workshop reports that were found mainly at the technical archive of the Veterinary Services. Others were found with the support of veterinarians involved in various related activities.

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Chapter 3

Prevalence of bovine tuberculosis in selected districts of Mozambique

Two of the manuscripts of this chapter were published:

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Prevalence of Bovine Tuberculosis and Risk Factor Assessment in Cattle in Rural Livestock Areas of Govuro District in the Southeast of Mozambique was published in PLoS ONE 9 (3): e91527. March 2014.

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Conception and design of the experiments

Perform the experiments

Data analyses

Writing of manuscript

The text format is as required by the respective journals where published.

Bovine tuberculosis and brucellosis in cattle and African buffalo in the Limpopo National Park, Mozambique

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Summary

Bovine tuberculosis (BTB) and brucellosis are prevalent in buffaloes of the Kruger National Park (KNP, South Africa). Both diseases were considered to have no or a very low prevalence in wildlife and livestock in and around the Limpopo National Park (LNP, Mozambique). The same applies for tuberculosis in Gonarezhou National Park (GNP, Zimbabwe), but just recently, BTB was detected in buffaloes in the GNP and fears arose that the disease might also spread to the LNP as a result of the partial removal of the fences between the three parks to form the Great Limpopo Transfrontier Park. To assess the status of both diseases in and around LNP, 62 buffaloes were tested for bovine tuberculosis (BTB) and bovine brucellosis. The percentage of positive BTB reactors in buffalo was 8.06% using BovidTB Stat-Pak® and 0% with BOVIGAM® IFN-γ test and IDEXX ELISA. The brucellosis seroprevalence in buffalo was found to be 17.72% and 27.42% using Rose Bengal Test (RBT) and ELISA, respectively. In addition, 2445 cattle in and around the LNP were examined for BTB using the single intradermal cervical comparative tuberculin test (SICCT), and an apparent prevalence of 0.98% was found with no significant difference inside (0.5%) and outside (1.3%) the park. This is the first published report on the presence of positive reactors to BTB and bovine brucellosis in buffalo and cattle in and outside the LNP. Monitoring the wildlife–livestock–human interface of zoonotic high-impact diseases such as BTB and brucellosis is of outmost importance for the successful implementation and management of any transfrontier park that aims to improve the livelihoods of the local communities.

Introduction

In the Kruger National Park (KNP), bovine tuberculosis was detected for the first time in African buffalo (*Syncerus caffer*) in 1990; now being the major host in the park (Bengis et al., 1996; Michel et al., 2006; De Garine-Wichatitsky et al., 2013). The spillover from cattle to buffalo occurred most likely already in the 1960s (Michel (BTB) et al., 2009) when the southern border of the KNP was not fenced, allowing buffalo and cattle to mingle and to use the same pastures and water sources. Intense monitoring revealed that the disease was spreading northwards and reached the borders to Zimbabwe and Mozambique in 2006. Until the recent detection of BTB in two buffaloes in the southern part of the Gonarezhou National Park (GNP), Zimbabwe has been considered free of the disease (De Garine-Wichatitsky et

al., 2010). In some of the southern buffalo herds of the KNP, the infection prevalence is as high as 90% (De Vos et al., 2001; Rodwell et al., 2001).

Even though there are no reports of BTB in Mozambican wildlife species, annual reports of the veterinary services are stating varying prevalences of BTB in cattle of up to 30%, mainly in the provinces of Manica, Sofala, Niassa and Inhambane. A study in Govuro district of Inhambane province even revealed a soaring prevalence of 60% in cattle from small holding keepers, using single intradermal cervical comparative tuberculin test (SICCT) only (Macucule, 2009). However, no reports of BTB-positive animals have been found in relation to the LNP and surrounding areas. To our knowledge, only one study regarding the presence of BTB in buffalo and cattle (Pereira et al., 2007) was performed in this area prior to our investigation.

The first reliable record of bovine brucellosis in cattle in South Africa dates back to 1906 (Henning, 1956). In the same year, the presence of brucellosis in cattle was confirmed in Zimbabwe (Madsen and Anderson, 1995). Serological surveys have revealed that up to 23% of African buffaloes in KNP are positive for brucellosis (Herr and Marshall, 1981). In Mozambique, the first isolation of *Brucella abortus* was reported by Abreu (1967) investigating cases of abortions in cattle throughout the country. Manhica (2010) found 2–33% of cattle and small ruminants to be positive for specific antibodies using Rose Bengal, complement fixation, serum agglutination and ELISA tests. However, the prevalence of bovine brucellosis in the buffaloes of the LNP was estimated to be very low (Pereira et al., 2007). African buffaloes are considered a wildlife maintenance host for B. abortus (Godfroid et al., 2010).

The Great Limpopo Transfrontier Park (GLTP, 35 000 km²) was founded in December 2002 straddling the borders of South Africa, Mozambique and Zimbabwe, combining the KNP, the LNP and with a corridor the GNP, respectively. Almost 5000 animals have been translocated from KNP to LNP, and 50 km fence have been dropped, encouraging wildlife to cross borders. However, not only animals will cross borders, but also the pathogens they are carrying. Therefore, diseases that have been previously present only in one or two of the three countries of concern might spread over the entire territory of the recently established transfrontier park and might challenge the success of the peace park should a long-term impact threaten population dynamics of predators or endangered species or if wildlife populations are endangering livestock now free of this particular disease.

The main focus of this study was to assess the infection status of buffaloes in the LNP and cattle within and outside the LNP with regard to BTB and brucellosis and the spatial

distribution to evaluate the impact of the removal of the fences between the KNP and the LNP for the establishment of the GLTP.

Materials and methods

Study design and sampling of buffaloes

From three buffalo herds that show seasonal movements of limited range between KNP and LNP with an estimated total population size of 250 (B. Swanepoel, personal communication; Swanepoel, 2009), 62 buffaloes have been sampled to perform a tuberculosis field side antibody test based on lateral flow technology (BovidTB Stat-Pak®; Chembio Diagnostic Systems, Inc, Medford, NY, USA), a blood-based tuberculosis-specific interferon gamma (IFN-γ) assay (BOVIGAM®; Prionics, Zurich, Switzerland) and a IDEXX ELISA for specific antibodies directed against Mycobacterium bovis. For brucellosis, the Rose Bengal Test (RBT, serum agglutination test; Onderstepoort Biological Products, South Africa) and an ELISA for specific antibodies against B. abortus (Ingezin Brucella Multispecies Compac 2.0, SA, Spain) were performed.

The buffaloes were captured by darting from a helicopter using 6 mg of etorphine hydrochloride (M99; Novartis South Africa Pty Ltd, Novartis, Johannesburg, South Africa; 9.8 mg ml^{1}) combined with 30 mg of xylazine hydrochloride (Xylazil-100; Troy Laboratories Pty Ltd, Glendenning, Australia; 10% concentration) per animal in two different capture operations in 2011. Immobilization drugs were loaded into 1.5 ml Dan-inject® darts fitted with plain Dan-inject® needles (N2030, 2.0 9 30 mm) and delivered using Dan-inject® remote delivery system (dart gun). Jugular vein blood was collected in tubes with clot activator to separate serum and into vacutainer tubes with lithium heparin to be used for the IFN-γ test. Buffaloes were revived from anaesthesia after blood collection using 12 mg of diprenorphine hydrochloride (M5050, 12 mg ml^{1}; Novartis South Africa Pty Ltd) combined with 5 mg of atipamezole hydrochloride (Orion Pharma, Orion Corporation Espoo, Finland; 5 mg ml^{1}) per animal. Serum and whole blood (after stimulation) were transported in liquid nitrogen and stored at 20°C until further use.

Study design and sampling of cattle

From a total population of 34 507 cattle in the district of Massingir (Fig. 1), 2445 from 28

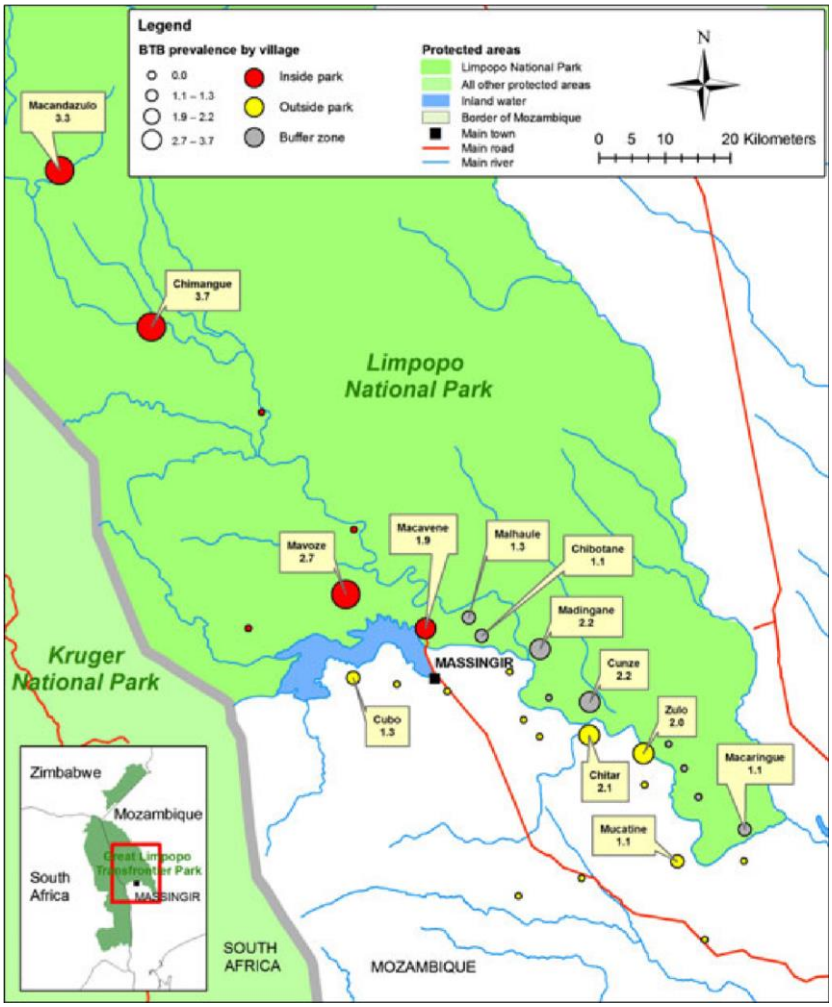

Fig. 1. Map of Massingir district showing the BTB prevalence's of the villages inside, outside and in the buffer zone of the LNP.

villages were tested by SICCT representing a sample proportion of 7.09%. The cattle belonged to 894 registered cattle keepers from the Massingir district, and 1337 of the sampled animals lived inside the LNP, whereas 1108 belonged to villages located in the vicinity, but outside of the park (Table 2). The number of animals per owner tested varied from 1 to 21 depending on the herd size. The sample size in each village was calculated based on the cattle population size, with an expected prevalence of 10%. Calculation was performed using the program EPICALC, 2000. The animals were driven into crushes and systematically sampled using fixed intervals determined by the proportion population size over sample size. All tested animals were marked with numbered metallic ear tags. While reading the tested animals after 72 h, jugular vein blood was collected from a subset of 133

animals from 18 villages, including 12 SICCT-positive, seven inconclusive and 114 negative reactors to compare the SICCT results as reference test with additional serological tests BTB BovidTB Stat-Pak® and IDEXX ELISA. The serum of the subset of 133 animals was also used to test for brucellosis using the RBT.

Diagnostic systems used to test for BTB

Single intradermal cervical comparative tuberculin test

The SICCT was applied according to the method recommended by the OIE. Tuberculin was purchased from Symbiotics (Lyon, France) with 2000 IU for the bovine PPD and 2500 units for the avian PPD. The date of tuberculin injection, breed, class (calf, steer, heifer, cow, bull, ox) and the owners name were recorded for each animal. The injected animals were then left to mix and graze freely with the rest of the herd. After 72 h, the skin test results were evaluated and recorded. Results were considered positive if the reaction in the skin fold at the site of injection of bovine tuberculin (B72-B0) was 4 mm or larger than the reaction at the injection site of avian PPD (A72-A0). The reaction was considered inconclusive, when the skin fold measurement was between 2 and 4 mm and negative if <2 mm.

The calculation of BTB prevalence was performed using the statistical program SPSS version 14 (Chicago, IL, USA). The comparison of prevalence's according to classes and locations was made using the chi-squared test.

Interferon gamma assay

For the BOVIGAM® test, blood samples were stimulated not later than 10 h after collection with avian and bovine PPD in order to induce lymphocytes to produce IFN-γ and PBS as a negative control. For cell viability control, an aliquot of the blood sample was stimulated with pokeweed mitogen. The incubation was performed overnight at 37°C. After incubation, the plasma was harvested and released IFN-γ was measured using a sandwich ELISA. Optical density in the reaction wells proportional to the amount of bound IFN-γ was quantified using a BIOTEK ELISA reader. Results were obtained by measuring the difference in stimulation (optical densities) between bovine tuberculin PPD and avian PPD following the manufacturer's instructions, and positive results were determined as OD450 of PPD-B minus OD450 of PPD-A >0.1.

BovidTB Stat-Pak®

To perform the test, the serum was brought to room temperature. The test was performed following the instructions of the manufacturer and the test was assessed positive if the blue bands of both control and test were readily visible.

Mycobacterium bovis antibody test (ELISA)

The M. bovis antibody test (IDEXX ELISA) was performed following the instructions of the manufacturer IDEXX Laboratories, Inc. Westbrook, ME, USA. Briefly, 100 µl of diluted controls (positive and negative) and serum samples were added into plates coated with M. bovis recombinant antigens in duplicated wells. After incubation for 1 h at room temperature (18–26°C), the plates were washed five times with the washing solution provided in the kit. Subsequently, 100 µl of the conjugate (antibody anti-bovine/ horseradish peroxidase conjugate) was added to each well and incubated for 30 min at room temperature. After washing as previously described, 100 µl of the substrate (Tetramethylbenzidine – TMB) was added to each well, and the plates kept for 15 min at room temperature. The reaction was stopped after 15 min by the addition of 100 µl stop solution to each well, and the plates read at 450 nm. The presence or absence of antibody to M. bovis is determined by calculating the sample to positive (S/P) ratio for each sample ($S/P = \frac{SampleA(450) - NC\bar{X}}{PC\bar{X} - NC\bar{X}}$). Samples with S/ P ratios ≥0.30 are considered positive for M. bovis antibodies.

Diagnostic systems used to test for bovine brucellosis

Rose Bengal Test

The test was performed following the instructions of the manufacturer. Briefly, Brucella abortus antigen (Onderstepoort Biological Products, South Africa) was used to screen sera for the presence of antibodies against Brucella spp. Twenty-five microliters of the serum was mixed with the same amount of stained Rose Bengal antigen. Distinct agglutination was considered a positive test.

Enzyme immunoassay (ELISA)

The blocking enzyme immunoassay (Ingezim Brucella Compac 2.0) was performed following the instructions of the manufacturer (Inmunologia y Genetica Aplicada, SA,

Spain). Briefly, 100 µl of controls and serum samples were added into plates coated with purified LPS of B. abortus in duplicate wells. After incubation for 1 h at room temperature, the plates were washed four times with the washing solution provided in the kit. Subsequently, 100 µl of the conjugate (monoclonal antibody against LPS antigen conjugated with peroxidase) was added to each well and incubated for 1 h at room temperature. After washing as previously described, 100 µl of TMB was added to each well and the plates kept for 10 min at room temperature. The reaction was stopped by the addition of 100ll stop solution to each well, and the plates read at 450 nm. Percentage inhibition (PI) calculated for each sample (PI = 100 9 [1 (OD sample/OD negative control)] >40% was considered positive for Brucella antibodies.

Results

Bovine tuberculosis in buffalo

The results of the tests performed in buffalo samples are presented in Table 1. Five of 62 buffaloes tested positive in the BovidTB Stat-Pak® test, whereas all animals tested negative with the BOVIGAM® gamma interferon and IDEXX ELISA antibody tests.

Table 1. Buffalo results of bovine tuberculosis and brucellosis in LNP

		BTB		Brucella	
Park section	Herd size	Stat-Pak	IFN-γ	RBT	ELISA
Macandazulu	103	1/25	0/25	0/25	3/25
Chiondziuene	68	1/25	0/25	6/25	9/25
Pafuri	Not known	2/8	#	4/8	4/8
Not known[a]	Not known	1/4	#	1/4	1/4
Positive/total number tested		5/62 (8.06%)	0/50 (0%)	11/62 (17.72%)	17/62 (27.42%)

[a]Animals found roaming outside the park, # test not performed.

Bovine tuberculosis in cattle

The apparent prevalence of BTB in the Massingir district was estimated as 0.98% at a 95% confidence interval (0.64–1.48) with 24 positive and 23 inconclusive skin test results (Table 2). Higher apparent prevalences were found in the villages of Chimangue with 3.7% (3/81), Macandazulo with 3.3% (3/91) and Mavodze with 2.7% (3/111). In 15 villages, no skin test-positive cattle were identified (Fig. 1). The comparison of the proportion of positive cattle

in the different villages showed no statistical significant differences between them (P > 0.05), and the same result was obtained when the proportion of positive cattle resident inside the LNP was compared with the proportion of positive cattle living outside the park (Table 3). From the animals tested, the majority were cows and no positive cases were found in young animals (Table 4).

Table 2. Bovine tuberculosis and brucellosis test positive results in cattle in Massingir district

	BTB SICCT	Brucella Rose Bengal
Positive	24/2445 (0.98%)	13/133 (9.77%)
Inconclusive	23/2445 (0.94%)	

Table 3. Results of single intradermal cervical comparative tuberculin test (SICCT) in cattle according to the location (within or outside LNP)

	Results			
Location	Negative	Positive	Inconclusive	Total
Outside LNP	1088 (98.2%)	6 (0.5%)	14 (1.3%)	1108 (100%)
Inside LNP	1310 (98.0%)	18 (1.3%)	9 (0,7%)	1337 (100%)

Table 4. Proportion of single intradermal cervical comparative tuberculin test (SICCT) results in cattle according to age and sex

	Results			
	Negative %	Positive %	Inconclusive %	Total %
Calf (male)	17 (100)	0 (0)	0 (0)	17 (100
Calf (female	34 (100)	0 (0	0 (0)	34 (100)
Steer	314 (98.7)	0 (0)	4 (1.3)	318 (100)
Heifer	310 (99.4)	0 (0)	2 (0.6)	312 (100)
Bull	343 (97.2)	4 (1.1)	6 (1.7)	353 (100)
Cow	1148 (98.0)	14 (1.2)	10 (0.9)	1172 (100)
Ox	219 (96.9)	6 (2.7)	1 (0.4)	226 (100)

From a subset of 133 cattle, 14 (10.52%), 5 (3.76%) and 12 (9.02%) tested positive using BovidTB Stat-Pak®, IDEXX ELISA and SICCT (reference test), respectively (Table 5). However, a comparison of the results in individual animals showed discrepancies. Of the 30

positive results in all tests, 11 (52%) were positive with the SICCT only, 13 (40%) with the BovidTB Stat-Pak® only and five by IDEXX ELISA only, whereas only one animal (1.4%) was positive in both the SICCT and BovidTB Stat-Pak tests and none was positive in all the three tests (Table 5).

Table 5. Results of a subset of 133 cattle using Stat-Pak® and IDEXX ELISA in comparison with single intradermal cervical comparative tuberculin test (SICCT) as reference test, including the table for Cohen's kappa calculation comparing the tests that are based on humoral immune response only (k = 0.059)

	No	SICCT	Stat-Pak	IDEXX
	1	+	+	-
	11	+	-	-
	13	-	+	-
	5	-	-	+
	103	-	-	-
Total	133			
		12	14	5

	Stat-Pak	
Kappa calculation	Positive	Negative
ELISA		
Positive	0	5
Negative	14	114

Bovine brucellosis in buffalo

Using the RBT, 11 of 62 (17.72%) animals yielded a positive result and 17 of 62 (27.42%) reacted positive in the ELISA. All positive animals in the RBT were concurrently positive in the ELISA test (Table 1).

Bovine brucellosis in cattle

Thirteen of 133 (9.77%) serum samples from cattle tested positive for bovine brucellosis using the RBT (Table 2). This group was tested for bovine tuberculosis as well by SICCT, BovidTB Stat-Pak® and IDEXX ELISA indicating a co-infection only in four animals.

Discussion

During the last two decades very high BTB prevalence's of up to 92% in buffalo herds of the southern KNP were reported (De Vos et al., 2001; Michel et al., 2009), and it was estimated that there was an increase of 1.6% per year from 1991/1992 to 1998 (Rodwell et al., 2001). Introduced to the south of KNP (Michel et al., 2009), BTB continued to move northwards, spilled over to various other wildlife species and was recently found in buffaloes of GNP in Zimbabwe. Following this trend of ongoing dissemination and transmission of the disease, it was expected to see an increase in BTB in the vicinity of the LNP over time as 50 km of buffalo-proof fences between RSA and Mozambique were dropped eleven years ago allowing buffalo and other wildlife to move across borders.

In our study, five of 62 (8.06%) buffaloes tested positive for BTB using Stat-Pak®, but both of the parallel used tests, BOVIGAM® and IDEXX ELISA, produced negative test results and point towards false-positive results of the StatPak test due to cross-reactions. On the other hand, the infection of the buffaloes in the LNP cannot be ruled out due to the low sensitivity (80–85%) of the BOVIGAM® test in buffalo (Michel et al., 2011), which might lead to false negative results and the moderate-to-high specificity of the Stat-Pak® test (90%) in buffalo (Michel and Simoes, 2009), which reduces the likelihood of false-positive test results.

In cattle, we found low BTB prevalence's within (1.3%) and outside (0.5%) the LNP, whereas a study carried out before in the same area found no cattle or buffaloes, which tested positive for BTB using the single intradermal tuberculin skin test and the BOVIGAM® test, respectively (Pereira et al., 2007). For 133 cattle, it was possible to compare skin test, BovidTB Stat-Pak® and IDEXX ELISA results, but even though the first two tests revealed similar numbers of positive results, they were not derived from the same animals (Table 5). Therefore, we speculate that the BovidTB Stat-Pak® might have more value concerning the assessment of the infection status of herds rather than for identifying individual animals infected. The low BTB prevalence in cattle might either indicate an endemic infection status or a recent infection. Acquisition of cattle for restocking from areas, where BTB is highly prevalent is another possibility for the introduction of the disease into the area. However, a recent questionnaire survey revealed no evidence of frequent cattle trade with districts of higher prevalence's (data not published). On the other hand, there is evidence of exchange of animals between farmers in the district as during the survey animals tested in one village were found in other village as a result of exchange or trade. There was no significant

difference in the prevalence of BTB between cattle resident inside or outside the LNP (Fig. 1, Table 2). The geographical distribution of the villages with higher BTB prevalence's (Fig. 1) did not show any tendency that would require a more detailed analysis. We found the highest proportion of positive reactors in oxen (Table 4). Similar results were reported from Tanzania (Kazwala et al., 2001) and from Ethiopia (Dinka and Duressa, 2011). The oxen are trained as draft animals, used in agriculture and transport; thus, they have longer lifespan resulting in increased probability of exposure and to develop the disease. The lack of identification of positive reactors in young animals is consistent with the studies in Tanzania and Ethiopia (Kazwala et al., 2001; Cleaveland et al., 2007; Dinka and Duressa, 2011), where it was evident that the duration of the exposure increased with the age of the animals. Pereira et al. (2007) studied the occurrence of bovine brucellosis in buffalo in LNP and found one positive case of 49 buffaloes tested by RBT (C.L. Pereira 2013, personal communication). Several wildlife species have been tested positive for brucellosis in the Kruger National Park with a prevalence of up to 23% in buffaloes (Herr and Marshall, 1981). In Zimbabwe, a prevalence of even 48% was found in African buffaloes (Madsen and Anderson, 1995). The results of this study of 17.72% and 27.42% test positives using the RBT and a competitive ELISA, respectively, are perfectly in line with these findings. Only an epidemiological study showing the same molecular well characterized brucella strains in all these countries would suggest also the transmission of the disease across borders.

Even though bovine brucellosis in cattle was found frequently in several Mozambican districts in the past, no surveys have been conducted in the Massingir district before our study, and therefore, the previous status of disease is unknown. We found 9.77% positive reactors in cattle using the RBT (Table 2). Even though the percentage found in buffalo is much higher than the percentage in cattle, it is impossible to predict the direction of spread based on the data currently available. The rate of brucella infections in humans is virtually unknown, and public awareness is extremely low.

Conclusions

This is the first published report on the possible presence of BTB and bovine brucellosis in buffalo and cattle in and outside the LNP. The infection status of the local communities living in and around the LNP is not yet known.

Zoonotic high impact diseases such as BTB and brucellosis may have a detrimental impact on public health, trade and the socio-economy of these countries. Monitoring the wildlife–

livestock–human interface is of outmost importance to authorities and decision-makers as there are people residing inside the park and there are no fences to control movements of possibly infected wildlife or livestock.

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Author contributions

MT, IM, OI and ACM, participated in the conception and design, the conduct of the study and drafting of the manuscript; ICM, JM, JF, BM, ZM, GM, CS and ASM participated in the testing and collection of samples; AP and FM participated in the analysis of data; AnM and PvH participated in the design of the study and drafting of the manuscript. All authors critically reviewed the manuscript, read and approved the final version.

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Prevalence of Bovine Tuberculosis and Risk Factor Assessment in Cattle in Rural Livestock Areas of Govuro District in the Southeast of Mozambique

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Abstract

Background: Bovine tuberculosis (bTB), caused by Mycobacterium bovis, is an infectious disease of cattle that also affects other domestic animals, free-ranging and farmed wildlife, and also humans. In Mozambique, scattered surveys have reported a wide variation of bTB prevalence rates in cattle from different regions. Due to direct economic repercussions on livestock and indirect consequences for human health and wildlife, knowing the prevalence rates of the disease is essential to define an effective control strategy.

Methodology/Principal findings: A cross-sectional study was conducted in Govuro district to determine bTB prevalence in cattle and identify associated risk factors. A representative sample of the cattle population was defined, stratified by livestock areas (n = 14). A total of 1136 cattle from 289 farmers were tested using the single comparative intradermal tuberculin test. The overall apparent prevalence was estimated at 39.6% (95% CI 36.8–42.5) using a diagnostic threshold cut-off according to the World Organization for Animal Health. bTB reactors were found in 13 livestock areas, with prevalence rates ranging from 8.1 to 65.8%. Age was the main risk factor; animals older than 4 years were more likely to be positive reactors (OR = 3.2, 95% CI: 2.2–4.7). Landim local breed showed a lower prevalence than crossbred animals (Landim X Brahman) (OR = 0.6, 95% CI: 0.4–0.8).

Conclusions/Significance: The findings reveal an urgent need for intervention with effective, area-based, control measures in order to reduce bTB prevalence and prevent its spread to the human population. In addition to the high prevalence, population habits in Govuro, particularly the consumption of raw milk, clearly may potentiate the transmission to humans. Thus, further studies on human tuberculosis and the molecular characterization of the predominant strain lineages that cause bTB in cattle and humans are urgently required to evaluate the impact on human health in the region.

Introduction

Bovine tuberculosis (bTB) is an infectious disease of cattle caused by Mycobacterium bovis, a member of the Mycobacterium tuberculosis complex. This chronic disease also affects a wide range of other domestic and wildlife animals and may also cause disease in humans [1].

Worldwide, bTB is considered one of the seven most neglected endemic zoonosis, presenting a complex epidemiological pattern and with the highest prevalence rates in cattle found in

African countries, part of Asia and of the Americas [2]. In affected countries, the disease has an important socio-economic and public health-related impact, and represents also a serious constraint in the trade of animals and their products [3]. In developed countries, bTB was regarded as one of the major diseases of domestic animals until the 1920s [4], when preventive and control measures based on tuberculin skin test and subsequent slaughter of positive reactors and sanitary surveillance in slaughterhouses, began to be systemically applied [5]. After implementation of the control programs, bTB in cattle populations was greatly reduced or even eradicated [3]. Nevertheless, wildlife species are still considered a significant source of infection and responsible for the failure of the complete eradication of livestock bTB in some developed countries [6].

Unfortunately, a vaccination strategy for animals is not available and present bTB control strategies are expensive and difficult to implement. Consequently, in developing countries, where bTB remains of economic and public health importance [7], these strategies are often not in use or not applied systematically [1,8]. In addition, it is estimated that in Africa 90% of the milk is consumed raw or fermented, increasing the risk of bTB transmission to humans [9].

In order to develop an effective national program for bTB surveillance and control in developing countries, accurate data on bTB prevalence is needed [10]. In Mozambique, data on bTB epidemiology is still scarce and mostly unpublished. However, bTB is estimated to be one of the most important causes of economic losses in cattle production, due to rejection of carcasses at the slaughterhouse and limitations on trade, both intracommunity and between districts [11]. Surveillance and control programs based on the tuberculin skin test in cattle at the farm and subsequent slaughter of positive reactors are not applied systematically and do not cover the small holder sector due to the costs with replacement of slaughtered animals [11]. Additionally, there are no effective measures for preventing the transmission of zoonotic diseases and a ‘‘bridge’’ between control programs of bTB and human tuberculosis has not been implemented.

In Mozambique’s Govuro district, a great proportion of the population holds livestock animals (especially cattle and goats). According to the findings of positive skin test reactors, associated with lesions compatible with bTB found at slaughterhouses, the Provincial Livestock Services (SPP) considered Govuro positive for bTB [11] but accurate information on the bTB prevalence and its role in human tuberculosis is missing. Control measures are

nowadays only based on compulsory test for bTB in cattle to be transferred for breeding or rearing purposes. While slaughter for local consumption is uncommon (only in traditional ceremonies), animals are frequently sold to be consumed in the south of the country. In Govuro there is extensive consumption of untreated milk, direct contact between people and livestock, together with malnutrition and a high prevalence rate of HIV infection, all of which constitute risk factors for this zoonosis. Also still unknown (but crucial to minimize disease propagation) are the main risk factors contributing to the spread of the disease between animals.

Previous studies conducted in Govuro in 2008, using the single intradermal tuberculin test (SITT) in the caudal fold and in the middle neck region of cattle, found prevalence values of 61.94% (n= 268) [11]. This represents the highest recorded prevalence in cattle in the country, however only two livestock areas of the district were analysed. In order to determine the prevalence rate of bTB in Govuro we conducted a cross-sectional survey covering a representative sample of the cattle population of all livestock areas within the district. The single comparative intradermal tuberculin test (SCITT) was used since its specificity is higher than the one from the SITT [12]. While the SITT is the standard diagnostic test used in the Mozambican Bovine Tuberculosis Control Program, the SCITT is the confirmatory test and can also be used as screening test in herds with a history of cross-reactivity. We also assessed intrinsic determinants of disease associated with SCITT positivity in the study area in order to define strategies suitable to bTB control in cattle in Govuro.

Materials and Methods

Ethics Statement

The purpose of this study was explained to the cattle owners and an informed consent was obtained. While the SITT is the standard diagnostic test used in the Mozambican Bovine Tuberculosis Control Program we used the SCITT due to its higher specificity and to the fact that using this more complete test the data for SITT was also obtained. The Mozambican National Animal Health authority (Direcção Nacional dos Serviços de Veterinária) approved the present study and provided the ethical clearance (Nota 162/ MINAG/DNSV/900/2013).

Study Area

A cross-sectional study was carried out in the Govuro district (Figure 1) located in the northern part of Inhambane province, south-eastern Mozambique. The region is bordered on

the north by the Machanga district of the Sofala province (across the Save River), on the east by the Indian Ocean, on the south by Inhassoro district and on the west by the Mabote district. The district covers an area of 3,960 km^2 with an estimated population of 35,500 inhabitants. The climate is tropical dry in the interior and humid close to the coast with an average temperature of 25.5uC (18– 33uC). The rainy and dry seasons generally occur around October to March and April to September, respectively [13]. Govuro comprises 2 administrative posts (Nova Mambone and Save), 5 localities, 14 livestock areas and 45 villages containing an estimated number of 773 farmers and 8,760 cattle heads.

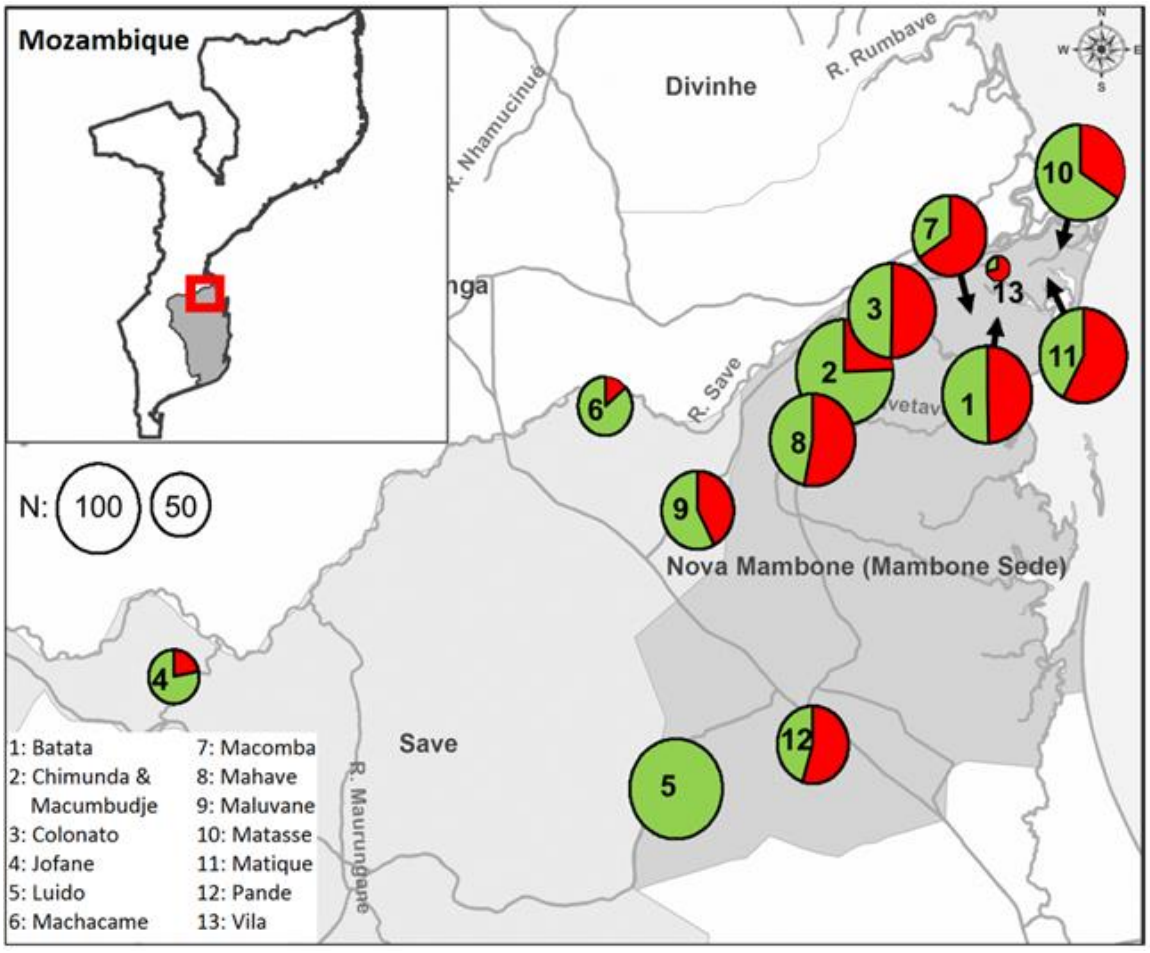

Figure 1. Location of the study district Govuro and spatial distribution of positive reactor cattle. The circle size is proportional to the number of animals tested in each location, and red area denotes the proportion of positive animals

Animals and Production Systems Tested for Bovine TB

The animals included in this study were from the small holder sector or from the commercial sector. In the small holder sector most animals were longhorn *Landim* cattle (local breed, mixed *Bos indicus* and *Bos taurus*) and crossbreeds (*Landim* x *Brahman*) and with herds typically comprised of both these cattle breeds. Cattle were kept traditionally in a free-range grazing system using communal grazing grounds (without supplementation) and watering points such as small puddles (formed throughout the grazing area during the rainy season) or the Save River (during the dry season) [11]. Animals received little veterinary assistance, mostly restricted to vaccination. Animals from the commercial sector were mostly of Simmental (*Bos taurus*), Brahman (*Bos indicus*) and Bonsmara (mixed *Bos indicus* and *Bos taurus*) breeds. They were reared under semi-intensive farming with limited grazing areas (maintained in fences separated from cattle of the small holder sector) and with established water sources. Veterinary assistance and supplementation were provided. Whilst in the commercial sector the livestock production was mainly market-oriented, in the small holder farming animals were frequently used to till the ground and to transport material and people. Livestock trading in the small holder sector is restricted to special occasions, essentially when there is scarcity in agriculture production; in case of diseases and medical assistance is needed; to raise money for children's school fees or other essential livelihood assets for the family such as food items, soap and clothes.

Sample Size Calculation and Study Animals

To obtain a sample size representative of the Govuro district cattle population, the number of animals to be tested was calculated with Epicalc 2000 (Brixton Books v.1 2), using an expected prevalence of 10% and precision measured as one-half length of the 95% confidence interval of 5%. Sample sizes were calculated for each livestock area and corrected for finite population sizes. The epidemiological unit of this study was the livestock area, which corresponds to the cattle from several owners belonging to the same village. Even belonging to distinct owners, animals have regular direct contact between them and share natural pasture areas and watering sites (grazing groups) or even cowsheds. All cattle of the district (estimated as n= 8,763) was included in the sampling frame. The required sample sizes for the livestock areas ranged from 45 to 139 animals and resulted in a total sample size of n =1,443 (Details are provided in Appendix S1).

All cattle owners from each livestock area were contacted to participate. The vast majority of the owners brought their animals to pre-defined locations. For the owners that could not bring the animals to the testing place we went to their home place. As a result more than 6,000 cattle participated, out of an estimated population of 8,760. Animals were selected randomly by systematic sampling according to the sample size previously determined and the number of cattle present on the day of the test. They were moved through a cattle chute and every k^{th} animal was selected for sampling, being k the number of animals presented for testing in that livestock area divided by the intended sample size for that same livestock area. At the time of SCITT testing, each animal was identified by a numbered ear tag and individual animal data on age, gender, breed, body condition score (BCS) and owner were registered. Information regarding the age of the animals was provided by the farmers and the breed was determined according to the phenotypic characteristics. The body condition was scored using the guidelines established by Nicholson and Butterworth [14]; all study animals were categorized in four groups: very poor (1), poor (2 to 3), reasonable (4 to 6) and good (score, 7 to 9).

Single Comparative Intradermal Tuberculin Test

The purpose of the study was explained to the owners with the assistance of local veterinary services (SDAE), community leaders, the local prosecutor and trusted intermediaries. SCITT was performed by intradermal injections of both avian and bovine purified protein derivates (PPD) in the middle neck region (usually on the right side) according to the method described by the World Organization for Animal Health standards [2]. Briefly, two sites of about 2 cm^2 diameter, approximately 12 to 15 cm apart, were shaved and the skin thickness was measured using a manual calliper. Aliquots of 0.1 ml containing 20,000 IU/ml of bovine PPD (Bovituber PPD, Synbiotics Europe, Lyon, France) and 0.1 ml with 25,000 IU/ml of avian PPD (Avituber PPD, Synbiotics Europe, Lyon, France) were injected using two different syringes into the dermis in the corresponding shaved area. Palpation of a small grain-like thickening at each site of injection was done to confirm the correct intradermal injection. Three days after injection, the tested animals were brought back for reading. The relative change in skin appearance was classified as swelling or induration followed by measurement of skin thickness at both injection sites. Skin thickness measurements on testing and reading day were performed by the same person to avoid errors related to individual variations in technical procedure.

The SCITT results were analysed and interpreted according to the recommendations of the World Organization for Animal Health standards [2]. The reaction was considered positive if the increase in skin thickness at the bovine PPD site of injection ($B_{72}B_0$) was at least 4 mm greater than the reaction at the avian PPD injection site (A_{72}-A_0). The livestock area was considered positive for bTB if at least one positive reactor was found.

Additionally we determined the SITT results by analysing the same dataset taking into consideration only the bovine PPD data, using the same cut-off. Also, to assess the prevalence of reactors to other sensitising organisms such as Mycobacterium avium, the skin reactions at the injection site of the avian PPD alone were analysed; animals that reacted to the avian PPD with an increase in skin thickness equal or superior to 4 mm were considered reactors to M. avium. Geographical coordinates were registered at the central point of each livestock area by a hand held global positioning system.

Data Analysis

All data at individual animal level were entered into a Microsoft Access database. Data analysis was performed in R statistical software (v2.15.1). Prevalence, odds ratios (OR) and their 95% confidence intervals were adjusted for correlation within livestock areas using generalized linear mixed models with binary outcome and livestock area as random effect.

Results

Sample Characteristics

Over the study period a total of 1,419 cattle were injected with PPDs and measurements were obtained from 1,136 animals (80%) belonging to 289 farmers. Table 1 shows the main characteristics of the sample tested. One hundred and twenty five (11%) animals came from the commercial sector (all from the Luido area) and 1,011 (89%) from the small holder sector. About two third of the cattle were female. The age distribution was as follows: 3% of the animals between 0 to 1 years old (calf - ‘‘< 1 year’’); 22% between 1 to 4 years old (steer - ‘‘1–4 years’’); and 75% were older than 4 years (bull, cow and ox - ‘‘> 4 years’’). Almost half (49%) of the animals were of crossbreeds (Landim x Brahman), 43% Landim and 8% Bonsmara. Simmental, Brahman and Limousine were tested only in one herd from the commercial sector in Luido representing 1% of the sample. Sixty-two percent of all animals tested were classified as having good BCS, 30% reasonable BCS, and a small proportion

presented poor (8%) and very poor (0.2%) BCS. Characteristic measures were not recorded for a few animals (Table 1).

Table 1. Basic characteristics of the sample.

Characteristics		n	%
Gender	Female	773	68.0
	Male	362	31.9
	Not recorded	1	0.1
Age	0-1 yr (calf)	38	3.3
	1-4 yrs (steer)	245	21.6
	>4 yrs (bull, cow, ox)	852	75.0
	Not recorded	1	0.1
Breed	*Landim*	468	43
	Crossbred (L*andim* x *Brahman*)	534	49
	Bonsmara	88	8
	Other	11	1
	Not recorded	35	3.1
Body condition score	Good	705	62.1
	Reasonable	337	29.7
	Poor	86	7.6
	Very poor	2	0.2
	Not recorded	8	0.5

Cattle bTB Prevalence in Govuro

The results of the SCITT are presented in Table 2 as prevalence per livestock area. The overall apparent prevalence of SCITT positive reactors was 39.6% (95% CI: 36.8–42.5). Except in Vila, where most of the PPD-inoculated animals failed the reading day, representative samples were obtained for each of the other 13 livestock areas. Among them, only in Luido, where the animals were all from the commercial sector, no SCITT positive reactors were detected (Table 2). In addition, data shows that bTB prevalence rates vary remarkably between livestock areas (ranging from undetectable up to 65.8%).

SCITT results showed that 137 (12%; 95% CI: 10.3–14.1) out of 1,136 cattle tested were positive reactors to avian PPD (Table 2). Among the 137 cattle with a positive reaction to

avian PPD, 49 (36%; 95% CI: 28.2–44.1) had an overall SCITT test also positive but 24 (18%; 95% CI: 12.1–24.8) showed a stronger response to avian PPD than to bovine PPD.

Table 2. Apparent prevalence of bTB in Govuro per livestock area.

Livestock area	Animals Tested		Bovine PPD positive SCITT reactors			Bovine PPD positive SITT reactors			Avian PPD positive SITT reactors		
	Total	Read	n	%	95% CI	n	%	95% CI	n	%	95% CI
Batata	133	117	58	49.6	40.7 – 58.5	78	66.7	57.7 – 74.6	17	14.5	9.3 – 22.0
Macomba	101	79	52	65.8	54.9 – 75.3	60	75.9	65.5 – 84.0	7	8.9	4.4 – 17.2
Colonato	173	111	56	50.5	42.3 – 59.6	70	63.1	53.8 – 71.5	12	10.8	6.3 – 18.0
Jofane	37	37	8	21.6	11.4 – 37.2	11	29.7	17.5 – 45.8	1	2.7	0.5 – 13.8
Maluvane	84	76	32	42.1	31.7 – 53.3	39	51.3	40.3 – 62.2	4	5.3	2.1 – 12.8
Matasse	123	115	39	33.9	25.9 – 43.0	53	46.1	37.2 – 55.2	16	13.9	8.6 – 21.4
Chimunda	75	62	27	43.5	31.9 – 56.0	37	59.7	47.2 – 71.0	16	25.8	16.6 – 37.9
Mahave	150	105	56	53.3	43.8 – 62.6	74	70.5	61.2 – 78.4	15	14.3	8.9 – 22.2
Matique	142	111	64	57.7	48.4 – 66.4	83	74.8	66.0 – 82.0	22	19.8	13.5 – 28.2
Pande	95	74	41	55.4	44.1 – 66.2	46	62.2	50.8 – 72.4	8	10.8	5.6 – 19.9
Luido	130	125	0	0.0	0.0 – 3.0	1	0.8	0.1 – 4.4	8	6.4	3.3 – 12.1
Vila	43	7	5	71.4	35.9 – 91.8	5	71.4	35.9 – 91.8	0	0.0	0.0 – 35.4
Machacame	45	43	6	14.0	6.6 – 27.3	9	20.9	11.4 – 35.2	3	7.0	2.4 – 18.6
Mucumbudje	90	74	6	8.1	3.8 – 16.6	20	27.0	18.2 – 38.1	8	10.8	5.6 – 19.9
Total	1421	1136	450	39.6	36.8 – 42.5	586	51.6	48.7 – 54.5	137	12.1	10.3 – 14.1

Risk Factors Associated with Positive Reaction to SCITT

Univariate and multivariate analysis showed that age and breed represented intrinsic risk factors associated with positive reaction to SCITT (Table 3). Animals older than 4 years were more likely to be infected compared to young animals (45.4% vs 21.9%; OR = 3.2, 95% CI 2.2–4.7) (Table 3), and this difference was statistically significant even when considering the age classes ‘‘< 1 year’’, ‘‘1–4 years’’ and ‘‘> 4 years’’ ($x^2 = 55.56$; d.f. = 2, P,0.001) (Figure 2). Male animals tended to show higher prevalence rates for bTB (42.7% vs 37.2% in females), but there was no statistically significant difference in reactivity to the SCITT test between gender ($x^2 = 2.20$; d.f. =1; P.0.05).

Table 3. Risk factors associated with positive reaction to SCITT.

Risk factor	Category	Positive/total	% positive	Univariate analysis		Multivariate analysis		
				OR	95% CI	OR	95% CI	P
Age	≤4 yrs	62/283	22		Reference		Reference	
	>4 yrs	388/854	45	2.9	2.0-4.1	3.2	2.2-4.7	<0.001
Gender	Female	259/773	38		Reference		Reference	
	Male	155/362	43	1.1	0.8-1.4	1.2	0.9-1.6	0.300
Breed	*Landim* x *Brahman*	265/534	50		Reference		Reference	
	Landim	167/468	36	0.7	0.5-0.9	0.6	0.4-0.8	0.002
	Bonsmara[a]	0/88	0	nd		Nd		
Body condition score	Good	250/705	35		Reference		Reference	
	Reasonable	153/337	45	1.0	0.8-1.4	1.0	0.7-1.4	0.820
	Bad and Very bad	44/88	50	1.4	0.8-2.3	1.3	0.7-2.1	0.410

[a] Excluded from the multivariate model to avoid quasi separation

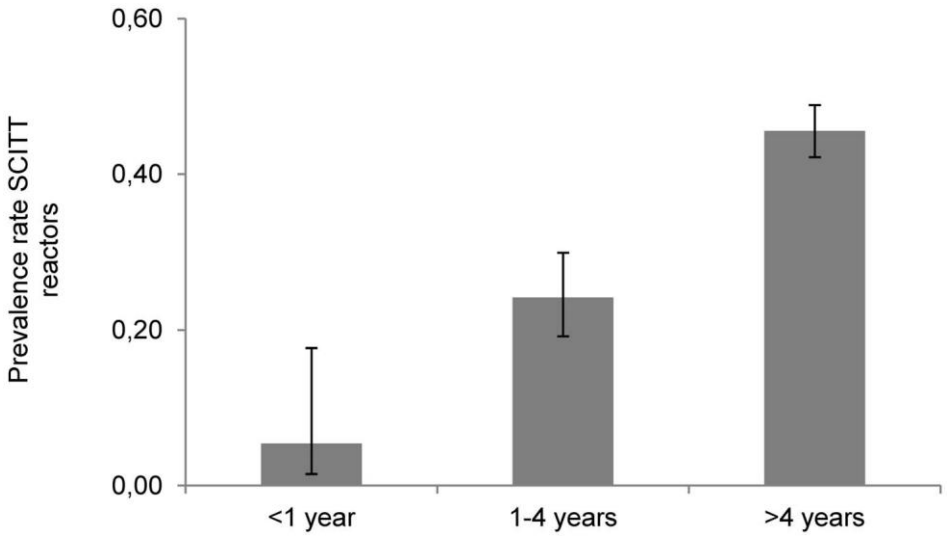

Figure 2. Prevalence rates among SICTT reactors (95% confidence intervals) by age classes

The rates of SCITT bTB reactors were higher in crossbreeds (Landim x Brahman) when compared to local Landim breed. Out of 468 of the Landim breed animals tested, 167 were found to be positive for the disease (35.7%; 95% CI 31.5–40.1) whereas 265 out of 534 (49.6%; 95% CI 45.4–53.9) in the crossbred (Landim x Brahman) cattle were positive reactors (Table 3). These data revealed a statistically significant association between the type of breeds and bTB prevalence, where the Landim breed seemed to be at lower risk for

infection (OR = 0.6; 95% CI 0.4–0.8). All animals of the breed Bonsmara belonged to two private farmers from the livestock area Luido, where no positive reactors were found (0/88).

Discussion

Our results show that bTB is highly prevalent in Govuro district, with an overall prevalence rate of 39.6%. The sample size in each livestock area was slightly lower than targeted and the observed prevalence was closer to 50%, consequently, the precision associated with the prevalence estimates for the single livestock areas was lower than planned in the sample size calculation. However, the overall prevalence and risk factors were associated with high precision and narrow interval estimates. The SCITT has a less than perfect sensitivity, with a range of 52.0– 95.5%, dependent on local factors [12]. Adjusting for the relatively low sensitivity of the SCITT, we estimated that the true prevalence in Govuro district is likely to be substantially higher than the apparent prevalence. The Rogan-Gladen estimator yielded a true prevalence of 65%, assuming a test specificity of 0.96 and sensitivity of 0.59, recalculated from data on Chadian cattle [15]. A high prevalence of bTB was observed in almost all livestock areas where small scale farming was practiced, in sharp contrast with what was observed in the commercial sector (only present in Luido), where no SCITT positive animals were detected. While in the commercial sector animals are normally tested for bTB and kept in quarantine before being introduced, trading of animals among breeders in the small holder sector is frequently performed without previous information about bTB status of the animals. In addition, the two tested farms in Luido were established in 2008, only four years before sampling. Interestingly, in the two livestock areas with the lowest bTB prevalence in the small holder sector (Mucumbudje and Machacame), livestock was just recently introduced (years 2007–2008). Our data show that age of the animals was an important intrinsic risk factor, most probably associated with increased exposure to M. bovis with lifetime. The type of management system applied in the small holder sector in Govuro, with sharing of water points and grazing areas, and close contact between animals from the same or different herds, promotes the spread of respiratory diseases such as bTB [4,16,17].

Additionally, during vaccination campaigns or external deworming, the animals from different farmers or herds use the same dip tanks. In contrast, animals from the commercial sector are kept inside fences and reared on a rotational grazing system with no contact with cattle from the small holder sector.

A study carried out in 2008 in the same region reported a bTB prevalence rate of 61.9% (95% CI: 55.8–67.8) [11]. Together with the present study, these data suggests that bTB is stable at an extremely high prevalence in the region. In the previous study the covered sample was limited to two livestock areas (Colonato and Vila), whereas in the present study all livestock areas were included. In addition, this study by Macucule et al. [11] made use of the single intradermal tuberculin test (SITT) while in the present study we made use of the SCITT. When our data were analysed taking in consideration only the bovine PPD result, which corresponds to the SITT, the prevalence rates obtained (63.6%, 95% CI: 0.55–0.72) were similar to what was reported from these two livestock areas in 2008.

The choice of the SCITT instead of the SITT has been shown to be of relevance to differentiate between animals infected with M. bovis and those responding to bovine PPD possibly as a result of exposure to other mycobacteria. In fact, in our study the overall prevalence of bTB, taking into consideration only the bovine PPD results, was 51.6% (95% CI: 48.7–54.5), clearly higher than the one determined using the SCITT. This higher rate of positive SITT reactors can be attributed to sensitization with cross-reactive antigens among mycobacterial species and related genera [2]. According to the definitions of positivity, the animals that reacted equally to both PPDs (avian and bovine) were classified as negative reactors to SCITT [2]. Reactivity to the avian PPD may indicate infection or simply exposure to species of the M. avium complex or other environmental mycobacteria. This reactivity, however, may indicate a mixed reaction to both agents and hence the classification of bTB negative might also lead to some false negatives. The equal reactivity in both sites of injections (avian and bovine PPD) could be related with a generalized sensitization in which the immune response is not specific to a particular mycobacteria species.

In our study we found 137 (12%) animals that reacted positively to the avian PPD, a finding that has also been previously described. In a cross-sectional study done in Uganda, Inangolet et al. [18] attributed the high number of avian reactors in cattle with the existence of large poultry population in the studied areas, where chicken production in a free-ranging system is common. Faecal contamination of the watering sources was indicated as the main route of transmission of M. avium to cattle. In our study area, poultry production is a common activity, nevertheless the system where cattle are kept in corrals (mainly during the night), away from residences, do not promote direct and frequent contact between these two species. The reactivity in the avian PPD in Govuro could be associated with the high population density of cattle egret (Bubulcus ibis) in the district. This species is usually found along grazing cattle

(removing ticks and flies from the animals). In fact, the presence of M. avium subsp. avium was already found in faecal samples of cattle egret [19] which could constitute a source of spread to the cattle. The causative agent of avian tuberculosis, M. avium subsp. avium, was the predominant MAC isolated from tuberculous lesion in cattle [20]. The role of small ruminants (goats and sheep) as vector of M. avium subsp. avium and Mycobacterium avium subsp. paratuberculosis has also been identified [3].

In Govuro, the predominant production system is the communal/pastoral system, where the small ruminants graze together with cattle. Sharing of pastures and watering points could represent a potential source of infection of M. avium to cattle. However, according to Okuni et al. [21], paratuberculosis in cattle was not reported in Mozambique. Further studies are necessary to clarify the source of the avian PPD reactions found in cattle in Govuro.

In accordance with findings from numerous cross-sectional studies conducted in both developed and developing countries [e.g. 18,22–25], our results show that age was the main individual risk factor. Some authors suggest that it could be related to increased duration of exposure with age, with older cattle being more likely to have been exposed than the younger [24,26]. Out of 38 calves tested only 2 (5.26%) had a positive result on SCITT. The low number of positive cases in young animals may be associated with the predominance of gamma delta (cd) T cells in calves that have been shown to play a relevant role in antimycobacterial immunity [27]. The positive calves (although in low number) could be due to congenital transmission in utero [28]. In addition, ingestion of contaminated colostrum has already been reported as another route of bTB transmission [29], as well as pseudo-vertical transmission (close contact between cow and its calf) [30].

The analysis of our bTB reactors according to gender showed that, although the reactivity among males was slightly higher (43% versus 38%) the difference was not statistically significant. Male cattle were identified as being the group at highest risk in other studies due to their particular longevity in the herd, given their use as draught oxen, facilitating maintenance of the infection in the herds [22]. Higher reactivity of females than males was previously reported in dairy cows [18,25] and associated with their maintenance in the same herd for several years [5]. In Govuro, however, male cattle tend to be maintained for longer periods in the herds since they are commonly used for ploughing the land and pull carts for transportation of people and goods.

Most of the cattle included in the present study were crossbreed (Landim x Brahman) and Landim local breed and bTB prevalence rates were found to be significantly higher in this later breed. The cattle recorded as Simmental and Bonsmara breed, from the two commercial sector farms in Luido, were too few to allow a relevant comparison of susceptibility. Several studies [31,32] have shown a variation in susceptibility to bTB among cattle breeds, with European breeds (*Bos taurus*) being less resistant compared to Zebu cattle (*Bos indicus*). Although crossbred cattle in Ethiopia (local Bos indicus breed Arsi x Holstein Bos taurus breed) has been suggested to exhibit intermediate levels of susceptibility [31], our data does not support this observation, as animals with more Zebu background (Landim x Brahman) showed higher bTB prevalence than Landim cattle. It should be mentioned that differences in bTB prevalence between breeds - as observed in several studies - can be influenced by different husbandry conditions; however, genetic variations among cattle breeds are also likely to have an influence on susceptibility to infection with M. bovis. The genetic variations among cattle breeds have an influence on susceptibility to infection with M. bovis. In diverse breeds of British cattle, the genomic regions INRA111 and BMS2753 were strongly associated with bTB infection status [33]. Two others loci have also been linked to susceptibility in Holstein cattle, namely, a variant in the TLR1 gene [34] and BTA 22 [35].

Several studies reported a correlation between body condition and bTB [e.g. 10,36]. In our study animals in reasonable and poor or very poor body condition showed more positive skin test results than animals in good body condition, however this difference did not reach statistical significance. Following recommendations by Humblet et al. [5], this parameter should be analysed carefully, since while a poor BCS might be a cause of disease, it is also extremely influenced by the seasonal climatic changes (rain or dry season) and the consequently more or less availability of pasturage and/or prevalence of intestinal parasites (in the small scale small holder farming of Govuro deworming for internal parasites is uncommon). It was reported previously [10,36] that animals in very poor body condition could be non-responsive to the SCITT due to anergy caused by immune-suppression. Our results do not support this finding. In addition, in cross-sectional studies, the status of the animal before becoming infected is not known, and thereby it is impossible to distinguish if the poor body condition was a risk factor or if it is a consequence of advanced stage of bTB. This is the first systematic study on bTB prevalence encompassing a representative sampling of all livestock areas of a particular district in Mozambique. The data clearly show that bTB is a serious problem in Govuro district with extremely high prevalence rates being maintained

for several years. Our results strengthen the notion that if strong measures were undertaken, as was the case among the commercial sector, the disease might be controlled. It is of relevance to stress that drinking raw milk is a common habit in Mozambique, especially for young children that take in charge the livestock grazing. In addition, due to their stature, children that graze animals may be extremely exposed to M. bovis airborne transmission from infected animals. Taking all this into account and the fact that studies on human tuberculosis have not been systematically performed in Govuro (neither for M. tuberculosis nor M. bovis) our results reinforce the need not just to undertake bTB control measures in the region but also the urgency to investigate the prevalence of tuberculosis in humans, especially in children in Govuro. Supporting Information Appendix S1 Sample Size Calculation.(DOCX)

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Author Contributions

Conceived and designed the experiments: IM AM JH GK JZ MCN. Performed the experiments: IM AM AN OI MCN. Analysed the data: IM AM NS JH GK MCN. Contributed reagents/materials/analysis tools: AM JH GK MCN. Wrote the paper: IM AM NS JH GK MCN.

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Prevalence of bovine tuberculosis in cattle in selected districts of Mozambique

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Abstract

The occurrence of bovine tuberculosis (BTB) was already documented in Mozambique. Since systematic BTB control activities have not been implemented in the country, it is expected that the disease prevalence would have increased in Mozambique. The purpose of this study was to evaluate the prevalence and risk factors of BTB occurrence in cattle in locations with previous reports of high number of BTB cases including areas from the south, centre and north of Mozambique.

The study was conducted in 8 districts, selected for their high number of BTB cases reported and for their good logistic conditions for the accomplishment of the work. Five small-scale and commercial herds were randomly selected in each district aiming to test 100 animals in each

herd. Results were obtained from 3402 cattle from 39 herds (27 small-scale, 11 commercial and 1 with both small-scale and commercial herds).

The overall apparent prevalence was calculated as 14.7% (95% CI: 9.6-21.4) translating in a true prevalence of 17.9%. There was considerable variation among herds with prevalence's ranging from 0 to 66.3%. Our results confirm the presence of BTB in Mozambique in hotspots of high prevalence. The identification of theses hotspots is crucial to define the most adequate and feasible strategy to control BTB as they constitute potential sources of infection for the locations with low prevalence that are in close proximity e.g. the same district.

Introduction

Bovine tuberculosis (BTB) caused by *Mycobacterium bovis* and other bacteria of the *Mycobacterium tuberculosis* complex is a worldwide concern. In Africa in particular, where human's, domestic animals and wildlife share the same space, a higher concern is caused by the fact that apart from being a zoonotic disease and causing economical loses in animal production, this disease endangers wildlife species (Michel *et al.*, 2010, De Garine-Wichatitsky *et al.*, 2013).

The introduction and persistence of BTB in cattle herds depend on a wide variety of epidemiological factors, illustrating the complexity of the interactions between hosts, pathogen and environment in this disease. The contact with infected herds (Kaneene *et al.*, 2002) introduction of an infected animal (Carrique-Mas *et al.*, 2008) or spillover from wildlife reservoirs (Cleaveland *et al*., 2007, Munyeme *et al*., 2008) have been documented as source of infection to cattle herds. The disease persistence depends on various factors, being the most mentioned the previous infection status, cattle density and housing, persistence of the agent in the environment (Skuce *et al.*, 2012) and keeping animals in the herd for long time as it is the case for milk farming and in small-scale farming, specially oxen (Kazwala *et al*., 2001).

Studies on BTB prevalence have been used worlwide to check for the disease presence, describe the populations potentially at risk and the distribution of the disease, both in time and space, within populations. They have been also used for decision on the most appropriate methods to control the disease and in a monitoring phase to ensure that the strategies being applied were generating the desired effect on reducing disease incidence/prevalence.

The occurrence of BTB in Mozambique has been reported as early as in the year 1940 as result of meat inspection *post-mortem* (Aires, 1947). While *post-mortem* and field surveys results

obtained before 1975 indicated spots of high prevalence, in the period 1981-1992 the positivity rate was estimated as 3.2% and 4.1% in the period 1992-2003. These estimates were calculated as a mean of the estimates of all provinces based on testing in 25.5% and 11.0% of the national herd respectively (DNSV, 2013).

In the absence of systematic BTB control activities in Mozambique the disease prevalence is expected to increase. This assumption gains ground with the results obtained in a prevalence study done in Govuro district in the Southeast of Mozambique, where 39.6% of cattle was shown to be positive skin test reactors for BTB (Moiane *et al.* 2014). Nevertheless locations with low prevalence do occur in Mozambique as evidenced in a study in the Massingir district where a prevalence of 1.0% was estimated (Tanner *et al*., 2014).

The purpose of this study was to estimate the prevalence and to assess risk factors for occurrence of BTB in cattle in locations with previous reports of high number of BTB cases including areas from the south, centre and north of Mozambique.

Material and methods

Ethical considerations. Institutional permission to conduct the study was obtained from the National Directorate of Veterinary Services in Maputo, Mozambique (Nota 162/ MINAG/DNSV/900/2013) and the Animal Use Committee (AUC) of Stellenbosch University (SU-ACUM13-00009). The purpose of this study was explained to the cattle owners and an informed consent was obtained.

The animals tested were from the small-scale and commercial sectors of 8 selected districts, from the Provinces in the south (Manhiça and Chibuto), centre (Buzi, Mutarara and Gondola) and north (Mechanelas, Mogovolas and Angoche) of Mozambique. The districts were selected for having a reported high number of BTB cases and for offering good logistic conditions for the accomplishment of the work.

A list of all herds from the district, distributed by localities, was supplied by the provincial Veterinary Services department. Herds were defined as commercial farms or groups of small-scale farmers sharing the use of a dip tank or crush for the provision of regular veterinary assistance. Numbers were assigned to all localities and 3 (in districts with commercial farms) to 5 (in districts with no commercial farms) localities were randomly selected. In each of the localities selected one small-scale herd was randomly selected. In districts where commercial

farms were available a different set of numbers were assigned to these herds for independent selection of two commercial herds. Random numbers were generated using the application - Research randomizer - available at https://www.randomizer.org/. In Mecanhelas one herd could not be tested because no containment facilities were available; in Mogovolas animals from one commercial herd could not be separated from the small-scale herd so they were tested jointly.

All cattle owners from the herds selected were invited to be included in the study. Farmer's sensitization was performed to reduce default in reading of the skin test results. Animals aged 6 months and above were tested at fixed intervals. The interval was defined dividing the number of animals per herd by 100, the desired number per herd. One hundred was defined as the number of animals that the team was able to test in one day. In herds where the number of animals, available at the testing day, was superior to the expected, more than 100 were tested and the opposite occurred when the number was inferior to that expected. In commercial farms where the number of animals were inferior to 100 the whole herd was tested.

The *in vivo* testing of animals was performed according to standard procedures. Single intradermal tuberculin testing (SIT) was used in 2 districts (Gondola and Mecanhelas), and single comparative intradermal tuberculin testing (SCITT) was used in the remaining districts, both applied in the middle neck. Aliquots of 0.1 ml containing 20000 IU/ml of bovine PPD (Bovituber PPD, Synbiotics Europe, Lyon, France) were injected in SIT and additionally 0.1 ml with 25000 IU/ml of avian PPD (Avituber PPD, Synbiotics Europe, Lyon, France) were injected in SCITT using 2 distinct automatic syringes, into the dermis in the corresponding shaved area. Reading of the skin thickness was performed 72 h after the injection using a manual caliper. The test was considered negative when the difference in the skin thickness before and after the application of tuberculin was 2 mm or less, inconclusive when between 2 and 4 and positive when the difference was equal or greater than 4 mm (OIE, 2009).

True prevalence was estimated using the Rogan and Gladen formula, following the same approach as described by Katale *et al.* (2013). Uni- and multivariable odds ratios were calculated with generalized estimating equations with binomial responses to account for potential correlations within locations. Statistical analysis were conducted in R v3.0.2.

Results

A total number of 3925 animals were tested and reading was performed in 3402 animals from 39 herds, 27 being small-scale, 11 commercial and 1 with both small-scale and commercially owned cattle. Distribution of animals with reading result per herd is presented in Table 1. In total 2782 animals were negative, 142 inconclusive and 478 positive reactors, resulting in an apparent prevalence of 14.7% (95% CI: 9.6-21.4). The true prevalence was estimated at 17.9%. There was considerable variation among herds with prevalences ranging from 0 to 66.3%. We found also substantial variation among districts as presented in (Table 2). In Gondola district the positive results were consistently high in all the herds tested while in Chibuto and Mogovolas districts they were consistently low (Table 1 and Figure 1).

The analysis of risk factors showed no difference with respect to the sex of the animals (Table 3). Steers and heifers had a significantly lower odds of infection compared to the older age classes (11.8% vs. 15.3%, OR: 0.74, 95%CI: 0.58-0.96). Calves showed the highest prevalence, however they were not included in multivariate analysis as the number of animals was low and with one exception, all positive animals within this category were from a single commercial farm (H4) where the highest prevalence was identified. Surprisingly, animals with an average body score exhibited a lower prevalence compared to animals with a good body score (11.0% vs. 17.6%), although this result was not statistically significant in the multivariate analysis. There was some indication that small-scale farms had lower prevalence when compared to commercial farms, however, this association lacked statistical significance.

Table 1. Prevalence of BTB in cattle stratified by herd

District	Herds	n	Negative	Positive	Inconclusive	A. Prev.	Type of farm
Angoche	H1	81	75	3	3	3.9	Small-scale
	H2	97	93	2	2	2.1	Small-scale
	H3	65	63	1	1	1.6	Commercial
	H4	99	31	61	7	66.3	Commercial
	H5	85	82	0	3	0.0	Small-scale
Buzi	H6	84	79	4	1	4.8	Small-scale
	H7	101	91	8	2	8.1	Small-scale
	H8	90	61	23	6	27.4	Commercial
	H9	69	64	4	1	5.9	Commercial
	H10	77	73	2	2	2.7	Small-scale
Chibuto	H11	92	87	1	4	1.1	Small-scale
	H12	93	90	1	2	1.1	Small-scale
	H13	100	99	1	0	1.0	Commercial
	H14	70	67	1	2	1.4	Commercial
	H15	94	94	0	0	0.0	Small-scale
Gondola	H16	107	37	57	13	53.3	Commercial
	H17	38	28	6	4	15.8	Small-scale
	H18[a]	92	44	36	12	45.0	Commercial
	H19[b]	67	48	10	9	17.2	Small-scale
	H20	75	49	20	6	29.1	Commercial
Manhica	H21	99	97	1	1	1.0	Commercial
	H22	111	87	22	2	20.2	Small-scale
	H23	86	79	4	3	4.8	Small-scale
	H24	98	95	2	1	2.1	Commercial
	H25	110	88	16	6	15.4	Small-scale
Mecanhelas	H26	69	52	12	5	18.8	Small-scale
	H27	88	80	6	2	7.0	Small-scale
	H28	54	36	15	3	29.4	Small-scale
	H29	95	87	4	4	4.4	Small-scale
Mogovolas	H30	103	98	4	1	3.9	Small-scale
	H31	95	88	4	3	4.4	Small-scale
	H32	48	44	1	3	2.2	Small-scale
	H33	86	81	4	1	4.7	Small-scale
	H34	110	107	2	1	1.8	Both
Mutarara	H35	93	57	29	7	33.7	Small-scale
	H36	88	59	24	5	28.9	Small-scale
	H37	96	46	40	10	46.5	Small-scale
	H38	109	61	46	2	43.2	Small-scale
	H39	88	85	1	2	1.2	Small-scale

n = number of animals were results were read, H1 to 39 – designation of different herds, A. Prev. = apparent prevalence, a and b were analysed together with Gondola but are from Manica and Sussundenga districts respectivly all from Manica province.

Table 2. Prevalence of bovine TB in Cattle stratified by District.

District	N	Negative	Positive	Inconclusive	Apparent prevalence	95%CI
Angoche	427	344	67	16	16.3	12.9-20.3
Buzi	421	368	41	12	10.0	7.4-13.5
Chibuto	449	437	4	8	0.9	0.3-2.5
Gondola[ab]	379	206	129	44	38.5	33.3-44.0
Manhica	504	446	45	13	9.2	6.8-12.2
Mecanhelas[b]	306	255	37	14	12.7	9.2-17.2
Mogovolas	442	418	15	9	3.5	2.0-5.8
Mutarara	474	308	140	26	31.3	27.0-35.8

[a] including 142 animals from neighbouring districts Manica and Sossundenga from Manica province
[b] only SITT instead of SCITT was used

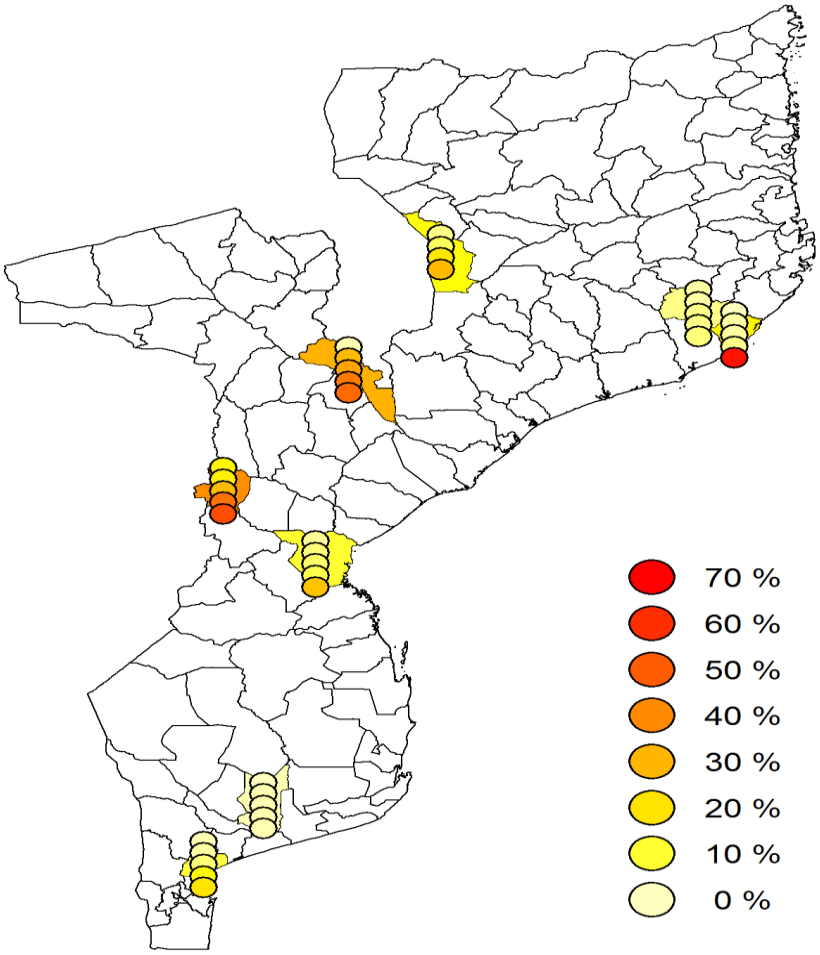

Fig. 1. Variation on BTB prevalence in the districts and herds tested. The prevalence in the individual herds from each district are presented as dots.

Table 3: Uni- and multivariable analysis of risk factors associated with BTB in cattle in selected districts in Mozambique. The analysis accounted for potential correlation within locations using Generalized Estimating Equations.

Factor	Positive	UniOR	95%CI	P	MultOR	95%CI	P
Sex							
Female (cow,heifer)	14.9% (369/2471)	Ref.			Ref.		
Male (bull,ox,steer)	13.1% (98/746)	0.86	0.60-1.24	0.42	0.75	0.58-1.45	0.71
Age category							
Bull,cow,ox	15.3% (383/2506)	Ref.			Ref.		
Steer,heifer	11.8% (84/711)	0.74	0.58-0.96	0.02	0.75	0.57-0.98	0.03
Calf[a]	32.3% (10/31)	2.64	1.04-6.72	0.04			
Body score							
Good	17.6% (284/1612)	Ref.			Ref.		
Average	11.0% (137/1242)	0.58	0.33-1.01	0.05	0.60	0.32-1.14	0.12
Bad	16.1% (37/230)	0.90	0.52-1.56	0.70	0.94	0.50-1.77	0.84
Breed							
Landim	14.2% (274/1925)	Ref.			Ref.		
Landin x Brahman	13.2% (137/1036)	0.92	0.38-2.23	0.85	0.74	0.31-1.81	0.51
Other	26.2% (62/237)	2.14	0.85-5.34	0.11	1.33	0.37-4.85	0.67
Farming type							
Commercial	21.9% (209/953)	Ref.			Ref.		
Small scale	11.7% (269/2307)	0.47	0.16-1.34	0.16	0.56	0.15-2.13	0.40

[a] excluded from multivariable analysis

Discussion

The apparent prevalence determined by tuberculin testing surveys conducted in our study varied from 0% in H5 and H15 to 66.3% in H4. Recent results of 65.8% in Macomba and 71.4% in Vila, both in Govuro district reported by Moiane and collaborators (Moiane *et al.*, 2014) and 61,9% reported by Macucule also in Govuro (2009) are examples of high BTB prevalence herds. These prevalence values are higher than ever reported before. The highest prevalence previously recorded was 40% in Chimoio (Paisana 1953). These results indicate an increase in the prevalence of BTB at least for some locations. The high BTB prevalence in cattle in some hotspots is a common trend in Africa (Munyeme, 2009, Mwakapuja, 2013) and has been linked in some studies to wildlife reservoirs, introduction of infected animals or long term persistence of infected animals associated with the absence of systematic control strategies. The influence of wildlife reservoirs on BTB prevalence in domestic animals in Mozambique was not yet documented, but a study by Ferreirra and Rosinha (1986), referred to comparable prevalences in buffalo and cattle in the Gorongosa district. A persistent infection can not be confirmed with our results, nevertheless the herd H16 where we found a prevalence

of 53.3% is from the same region where Paisana referred to a high prevalence in 1953 (Paisana, 1953). The same is true for the study of Moiane *et al.* (2014) where a prevalence of 53.3% was found in a location (Mahave) previously referred to by Morgado (2007) as having cattle with disseminated lesions on *post-mortem* examination in the years 1947-1949. One cannot discard the possibility that this results from re-introduction.

The analysis of risk factors for the occurrence of positive reactors showed no difference with respect to the sex of the animal. Younger animals (excluding calves) had significantly lower odds of infection compared to the older animals. This is a common finding in studies in Africa (Tschopp *et al.*, 2009, Munyeme *et al.*, 2009, Katale *et al.*, 2013). Calves had the highest prevalence but the numbers tested were too small and the positive animals were almost all from the same farm, with the highest prevalence in our study. As already discussed by Moiane *et al.* (2014) with citation to Ozyigit *et al*. (2007), Zanini *et al*. (1998) and Phillips *et al*. (2003), these infections can have various sources viz in utero, ingestion of contaminated colostrum or by close contact between cow and its calf. These sources of infection might be more relevant in farms with long term history of BTB infection (Demelash *et al*., 2009). Infection of calves trough milk consumption was evidenced in several occasions (Houlihan *et al.*, 2008, Doran *et al*., 2009).

In contrast to the study done in Govuro (Moiane *et al*., 2014) no difference was found in the prevalence when comparing Landim and crossbreeds (Landim x Brahman). This can be a result of crossbreeds not being a homogeneus population due to the different levels of crossbreeding, so their genetic heritage is not the same. Allen *et al.*, (2010) reported on studies where variation in prevalence was found even in daughters of different sires of the same breed.

There was an indication that cattle from small-scale farmers had fewer infections than those of commercial farmers. However, this association lacked statistical significance. Commercial farmers normally have larger herds and cattle density was already described as risk factor for BTB occurrence (Skuce *et al.*, 2012). It was expected that in a commercial setting better control strategies would be implemented but it was observed that management practices in some commercial farms in Mozambique do not differ from those of small-scale farming. Add to the fact that commercially farms acquire stock regularly, this could be another source of infection for commercial herds.

Our results confirm the occurrence of BTB in Mozambique in hotspots of high prevalence. The identification of these hotspots is crucial for the control of BTB as they constitute potential sources of infection to the locations with low prevalence located in close proximity. This identification could be done with better slaughterhouse surveillance. Some of the hotspots are commercial herds where contrary to the small-scale herds, removal of infected cattle could be done without compensation as they already extract the stock for commercial proposes.

Author's contributions

Conceived and designed the experiments: AM, GK, RW, PvH. Performed the experiments: AM, GM, ASM, JM, JB, FD. Analysed the data: AM, JH, MCN. Contributed reagents/materials/analysis tools: AM, MCN, JH, GK, RW, PvH. Wrote the paper: AM, MCN, JH, GK, RW, PvH.

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Chapter 4

Molecular diversity of Mycobacterium bovis isolates from cattle in Mozambique

Molecular diversity of *Mycobacterium bovis* isolates from cattle in Mozambique

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Abstract

Molecular typing of *Mycobacterium bovis* isolates has been widely used to support epidemiology studies that are essential to design disease control interventions. The aim of the present study was to apply these tools to provide novel information on how bovine tuberculosis caused by *M. bovis* is distributed in cattle in Mozambique and thereby provide relevant information for guiding policy on the strategies to be applied to contain the disease in livestock.

A collection of 178 *M. bovis* isolates was obtained from cattle from 8 of the 11 provinces in Mozambique. Fifteen different spoligotype patterns were identified. The pattern SB0961 accounted for 61% of the isolates. Twenty nine isolates had the pattern SB0140, which is specific for the European 1 (Eu1) clonal complex. Eleven isolates with this spoligotype were subjected to regions of difference (RD) analysis, and all isolates had the Eu1 specific deletion. There were no isolates of the African 1 (Af1) or African 2 (Af2) clonal complexes that are frequent in Central-West Africa and East Africa, respectively.

The dominant spoligotype SB0961 was found in all areas of the country investigated, while the twenty nine SB0140 isolates were all isolated from the South of Mozambique, the majority from commercial farms that import cattle, mainly from South Africa, where the Eu1 clonal complex is common.

With the MIRU-VNTR typing, clones shared by isolates originated from different farms and districts were identified in the two most frequent spoligotypes, strongly suggesting potential transmission and/or common source of infection.

Introduction

Bovine Tuberculosis (BTB) is an infectious disease caused mostly by *Mycobacterium bovis* that affects cattle, other domesticated animals and several free or captive wildlife species. BTB is of global concern at three socio-economic levels: the negative impact on animal production; the potential spread to wildlife species; and the risk of zoonotic tuberculosis in humans.

BTB is of particular importance for low income countries, as factors such as poor or no BTB veterinary control, consumption of uninspected raw meat and/or milk, difficult access to medical care, high prevalence of HIV/AIDS and malnutrition contribute to the increased risk for exposure and higher susceptibility of humans to *M. bovis* [1-3]. In the agricultural arena, the negative impact is multifactorial: loss in production related to reduced fertility; reduced

milk production and rejection of contaminated milk; poor body condition; and condemnation of affected carcasses. BTB therefore negatively impacts on the market value of animals and international trade [4].

The maintenance of the pathogen both in symptomatic and asymptomatic animals and in the environment creates conditions for spillover to a wide variety of species including endangered wildlife species with the obvious negative consequences on conservation and tourism [4].

BTB has a worldwide distribution, showing very low prevalence in most industrialized countries although eradication has been claimed only for a few countries. While BTB is known to be widespread in Africa, limited or outdated information exists on the precise distribution and incidence/prevalence of BTB in African countries/regions. In 1993, the World Health Organization (WHO), with the participation of the Food and Agriculture Organization (FAO), convened a meeting on zoonotic BTB, where the worldwide public health significance of *M. bovis* in humans and animals was discussed. It was then concluded that data collected from most African countries, mainly from sub-Saharan Africa, were insufficient to reveal the true epidemiological picture of the disease, and it was recommended that collection of data on BTB should be prioritized [5]. Several studies have been conducted, more in some countries (e.g. Ethiopia) than others, but the predominance of convenience (scattered) studies and the lack of detailed information in the vast majority of African countries is still of great concern [6].

Fifty five African countries have been reported to declare the occurrence of BTB; however, of these only 7 applied any BTB control measures, had a test and slaughter policy for cattle and considered BTB a notifiable disease. The remaining 48 countries either controlled the disease inadequately or not at all. Approximately 85% of cattle and 82% of the human population of Africa were estimated to live in areas where BTB is either not controlled or only partially controlled [7]. The OIE (International Organization for Animal Health) Animal health information from the years 2012 and 2013 revealed that this situation improved only slightly, with a report of 14 countries that apply test and slaughter strategies [8]. This is also the situation in Mozambique where recent efforts to gradually improve BTB control are being put into place. Implementation of a test and slaughter policy for cattle for the entire country would be the ideal strategy but it is unrealistic at present, given the limitations of human and financial resources. Attempts to obtain data concerning the prevalence of the disease in different areas of Mozambique have been initiated. Scattered information from the past and results from ongoing studies in specific regions show that the prevalence of BTB varies widely from region to region

and within each region. For example in the Govuro District, in the Southeast of Mozambique, 39.6% of cattle were skin test reactors for tuberculosis [9]; while BTB was practically absent (0.98%) in cattle in the Limpopo National Park [10]. While it is known that the differences in the BTB prevalence are associated with several parameters, our recent results from Govuro District clearly show that control of animals movement, that was almost exclusively undertaken in the commercial systems, appeared to be of extreme relevance [9]. Thus, to be able to define a realistic strategy to control BTB in cattle in Mozambique, information that will help understanding how the disease is contained or spread from one region to another is vital.

It is increasingly clear that genotyping of *M. bovis* strains is a useful tool to identify possible transmission routes of BTB, and that the genotype of *M. bovis* isolates is largely dependent on the geographical area. To date, three major clonal complexes of *M. bovis* have been defined by the presence of specific genomic deletions, each with distinct spoligotype signatures – two are geographically localized to Africa and the third has a worldwide distribution [11]. Genotyping tools have already been widely used in Africa [12-23] but to our knowledge not in Mozambique. The ideal situation would be to apply these tools to a representative sample from the whole Mozambique; however this is unrealistic now and will be for the upcoming years. *M. bovis* isolates from slaughtered animals can only be obtained when slaughter is performed under the supervision of the veterinary services, which only happens in some particular areas of Mozambique. In addition testing and slaughter of BTB positive animals is performed only occasionally. In spite of these limitations we obtained 178 *M. bovis* isolates from 8 out of the 11 Mozambican provinces and its genotyping provided interesting information to understand how BTB is being imported and is transmited within the country.

Materials and Methods

Ethical considerations

Institutional permission to conduct the study was obtained from the National Directorate of Veterinary Services in Maputo, Mozambique (Nota 162/ MINAG/DNSV/900/2013) and the AUC (Animal Use Committee) of Stellenbosch University (SU-ACUM13-00009). No cattle were slaughtered specifically to conduct this research and the sampling and culling was performed as part of the Veterinary Services regular activity for disease control, following the procedures determined by the Mozambican Animal Health Regulation. Animals were selected for slaughtering on the basis of signs and/or symptoms of BTB. The slaughter was done

in registered abattoirs according to abattoirs procedures as is done to process animals for human consumption by the abattoir staff.

Source of samples

Since a test and slaughter strategy is not in place in Mozambique we obtained the *M. bovis* isolates from all potential sources from 2007 to 2013, namely from samples collected from an on-going BTB prevalence research project (n=228) and from samples (n=220) sent to the Central Veterinary Laboratory in Maputo by district veterinary officers and farmers that suspected BTB at *post mortem*, either in the process of standard meat inspection or necropsy (Table 1). In the prevalence study the isolates were from the small-scale and commercial herds of 10 selected districts, from the Provinces in the south (Manhiça, Magude, Chibuto and Govuro), centre (Buzi, Mutarara and Gondola) and north (Mechanelas, Mogovolas and Angoche) of Mozambique. The districts were selected for having a reported high number of BTB cases and for offering good logistic conditions for the accomplishment of the work. Animals were purchased and slaughtered, selection criteria for these purchased animals being based on strong *M. bovis* tuberculin purified protein derivative (PPD) response, unproductive animal, and willingness of farmer to sell the animal. Tissue samples were taken from BTB test positive animals with and without suspected lesions. Each sample represented one animal. When more than one tissue was collected from each animal, tissues were pooled to constitute one sample prior to processing for microbiological culture. In two commercial farms (in Manhiça and Manica provinces), where skin test reactors cows could not be slaughtered, milk was retrieved and tested for the presence of *M. bovis* by microbiological culture.

Table 1. Sources of samples and number of isolates obtained.

Source of samples		**Total Samples**	**Samples Discarded**	**Negative samples**	**Isolates obtained**	**MTC positive**	**Isolates with spoligotype result**
Prevalence study	Tissue	187	15	74	98	90	86
	Milk	41	8	16	17	11	9
Laboratory samples		220	27	89	104	84	75
Other							8
							178

MTC – *Mycobacterium tuberculosis* complex

Sample data, such as the name of the owner and the origin (province and district) of the animals were recorded when available. In Mozambique, for commercial beef production or small-scale farming, the breeds that are kept are mainly a local breed (Landim) and Brahman or crossbreeds between the two, so for the samples collected in the prevalence study these breeds represent the majority of the cattle sampled. Eight isolates were supplied by another study, four of which were from a milk production farm with Holstein Friesian and Jersey breeds.

Tissue processing and microbiological culture

Maceration of pooled samples of each animal was performed in a *stomacher* apparatus (contained in duplicate sterile stomacher bags with added sterile distilled water). Samples were next decontaminated by adding 4% sodium hydroxide to the same volume of the macerate for 20 min. Supernatant was discarded after centrifugation. Distilled water was added to the sediment and after agitation and re-centrifugation the sediment obtained was used for inoculation of duplicate tubes with Löwenstein–Jensen medium with glycerol or with pyruvate. Incubation was done at 37 °C for up to 12 weeks. Fifty samples were discarded due to contamination (Table 1). Isolates were identified as acid-fast bacilli with Ziehl–Neelsen staining and as belonging to the *Mycobacterium tuberculosis* complex (MTC) by PCR [24]. Briefly the primers TB1-F 5'-GAA CAA TCC GGA GTT GAC AA-3' and TB1-R 5'-AGC ACG CTG TCA ATC ATG TA-3' were used, the PCR protocol started with an initial denaturation step of 95 °C for 10 min, followed by 35 cycles of 95 °C for 1 min, 61 °C for 30 s and 72 °C for 2 min ending with a final step of 72 °C for 10 min. The PCR products were analysed using 1,5% agarose gels. All isolates that generated a product of 372 bp were considered as belonging to the MTC. *M. bovis* was identified by spoligotyping [25] in 170 out of 185 MTC positive isolates tested (Table 1).

Genotyping

Genotyping was performed using spoligotyping, region of difference (RD) analysis and MIRU-VNTR typing. **Spoligotyping** was performed as described by Kamerbeek and colleagues [25], using membranes and equipment provided with a commercially available kit (Isogen Life Science B.V., Utrecht, The Netherlands). This method determines the presence or absence of 43 direct and variable repeat sequences within the direct repeat region, thereby generating spoligotype signatures which are characteristic of defined strains [26]. The spoligotype patterns of the respective isolates were entered into the *Mbovis.org* database. In this database each unique spoligotype pattern is named by 'SB' followed by a four integer number e.g. SB0120

[27]. To evaluate the relatedness of the spoligotypes identified the calculation of the minimum spanning tree was performed using the tool provided in the site http://www.MIRU-VNTRplus.org

RD analysis was based on PCR genomic deletion analysis to determine the presence or absence of specific regions of difference (RD). It was done by the assessment of the status of the RD Eu1, RD Af1 and RD Af2 regions. For RD Eu1, pairs of primers located at a suitable distance flanking the deletion boundary (RD Eu1 primer set A) were used as described elsewhere [12, 19, 28]. The forward primer was RD Eu1_FW (5'-CCGATGAACTTGGCCCACAG-3') and the reverse primer was RD Eu1_ Rv (5'– CGTGGTGGTGGGATGTCTTG-3'). PCR products were visualized after electrophoresis on a 1% agarose gels. A 1206 bp fragment was generated if the RD Eu1 region was intact and a 400 bp fragment if the region was deleted. The presence or absence of RD Af1 and RD Af2 was assessed by PCR as previously described [12, 19]. For RD Af1 two primers targeting the flanking regions of RD Af1 (FW, 5'-ACTGGACCGGC AACGACCTGG-3', and Rev, 5'-CGGGTGACCGTGAACTGCGAC-3') and one primer hybridizing with the internal region of RDAf1 (Int Rev, 5'-CGGATCGCGGTGATCGTCGA-3') were used. A 349-bp (RD Af1 intact) or a 531-bp (RD Af1 deleted) PCR product was identified by agarose gel electrophoresis [19]. For RD Af2 two primers targeting the flanking regions of RD Af2 (RD Af2_Fw, 5'- ACCGCCCTGTCCTATGTGAG -3', RD Af2_Rev, 5'-TGACGGTTGCCTTTCTTGAC-3') and one primer hybridizing with the internal region of RD Af2 (RD Af2_IntRev, 5'- CACTGTCTCCGCTCATCATG-3') were used. A PCR product of 458 bp (RD Af2 intact) or 707 bp (RD Af2 deleted) was identified by agarose gel electrophoresis [12].

MIRU-VNTR typing was done, in addition to spoligotyping, in 59 strains using a standardized 24-locus MIRU-VNTR typing [29]. The analysis was performed using the MIRU-VNTR typing kit (Genoscreen, Lille, France). The PCR-products were run with 1200 LIZ size standard (GeneScan, Applied Biosystems) on ABI3500 sequencers. Sizing of the PCR-fragments and assignments of MIRU-VNTR alleles were done with the GeneMapper software version 4.1 (Applied Biosystems) according to the manufacturers' instructions, generating a numerical profile for each strain. The clusters designation numbers and dendogram presenting the MIRU-VNTR results were produced using the site http://www.MIRU-VNTRplus.org.

The index of discrimination (D) [30, 31] was calculated to determine the overall discriminatory power of the Spoligotyping and MIRU-VNTR typing techniques using a tool provided in the

site http://insilico.ehu.es/mini_tools/discriminatory_power. The number of isolates assigned to each type (Spoligotyping and MIRU-VNTR) was introduced in the formula provided. Individual allelic diversity was calculated for all 24 MIRU-VNTR loci using the site http://www.MIRU-VNTRplus.org.

Results

Source of the isolates

The 178 *M. bovis* isolates were obtained from samples that originated from 8 out of the 11 Mozambican provinces from the Southern (n=113), Central (n=47) and Northern (n=18) parts of the country, covering 21 of the 128 districts of Mozambique. The overrepresentation of samples from the south is due to a pilot study using test and slaughter performed in one district (Govuro) from the Southern Provinces, two commercial farms that offered to cull the majority of the positive animals and the facility to send samples to the Central Veterinary Laboratory that is located also in the south. The isolates originated either from small-scale farms (n=43) or from commercial farms (n=11) (Table 2). Altogether 103 isolates were from small-scale farms (1-6 isolates per farm) and 68 isolates from commercial farms (1-17 isolates per farm). The type of farm was unknown for 7 isolates. The commercial farms were located in the south (n=5), in the centre (n=5) and in the north (n=1) of Mozambique (Table 2).

Table 2. Sources of *M. bovis* isolates.

Regions		**Number of farms**		**Number of isolates**			
	Number of Districts	**Small-scale**	**Commercial**	**Small-scale**	**Commercial**	**Type of farm not identified**	**Total**
South	14	24	5	69	38	6	113
Centre	5	10	4	25	21	1	47
North	2	0	1	9	9	0	18
	21	**34**	**10**	**103**	**68**	**7**	**178**

Spoligotype patterns identified

Fifteen individual spoligotype patterns were identified (Fig. 1). All isolates lacked spacers 3,

9, 16, and 39 to 43, which is the signature profile of the *M. bovis* BCG vaccine strain, the ancestral spoligotype pattern of *M. bovis*. Seven patterns were already registered in the Mbovis.org database while the other 8 patterns corresponded to spoligotypes that were not previously registered in the Mbovis.org database. The 8 new spoligotype patterns were incorporated into the database and received new SB numbers (SB2304 to SB2311).

Ref.	Spoligotyping Pattern	SB Number	Type	N. of isolates
1		SB0961	BCG –like derived	109
2		SB0140	Eu1	29
3		SB0120	BCG – like	17
4		SB0290	Eu1	1
5		SB2124	Eu1	1
6		SB1099	Af1	3
7		SB1272	BCG –like derived	1
8		SB2304	BCG –like derived	3
9		SB2305	Eu1	3
10		SB2306	Eu1	4
11		SB2307	BCG –like derived	2
12		SB2308	BCG –like derived	2
13		SB2309	BCG –like derived	1
14		SB2310	BCG –like derived	1
15		SB2311	Eu1	1
		TOTAL		178

Fig. 1. Frequency of the spoligotyping patterns identified in the study.

In respect to the distribution of isolates by the different spoligotype patterns, the most common spoligotype was SB0961, lacking spacer 2 in addition to the *M. bovis*-specific profile and accounting for more than half (n=109, 61.2%) of the isolates. Seventeen isolates (9.5%) had the SB0120 spoligotype which corresponds to the signature profile of *M.bovis* BCG. Three isolates had the SB1099 spoligotype, which is also similar to the BCG vaccine strain pattern by lacking spacers 2, and 30 in addition to spacers 3, 9, 16 and 39-43. This spoligotype is related to Af1 clonal complex i.e. lacking spacer 30 [19]. Ten isolates had spoligotype patterns similar to *M.bovis* BCG, differing in simple spacer deletions (Fig. 1).

Thirty-nine isolates had spoligotypes lacking spacer 11, which is the signature of the Eu1 clonal complex [28]. Of these isolates, 29 had the spoligotype SB0140, which has spacer 6 absent as well as spacers 8 to 12, in addition to spacers 3, 9, 16, and 39–43 (Fig 1). SB0140 was one of the most common Eu1 spoligotypes. One isolate had spoligotype SB0290, and one spoligotype

SB2124. Isolates with previously unknown spoligotypes (SB2305 - 3, SB2306 - 4 and SB2311 - 1) also had patterns with spacer 11 missing. None of the isolates had spoligotypes specific to the Af2 clonal complex i.e. lacking spacers 3-7 [12]. One sample in consecutive experiments gave different results, either SB0961 or SB0140 and for that reason was excluded from the analysis. The Discriminatory Index using spoligotyping was 0.5909.

With calculation of the minimum spanning tree based on the spoligotyping results, four clonal complexes and 4 singletons were identified (Fig. 2). Complexes 1 and 3 consisted of spoligotypes with "BCG-like derived" signatures, complex 1 also included the pattern with the BCG like signature, while complexes 2 and 4 included spoligotypes with Eu1 signature.

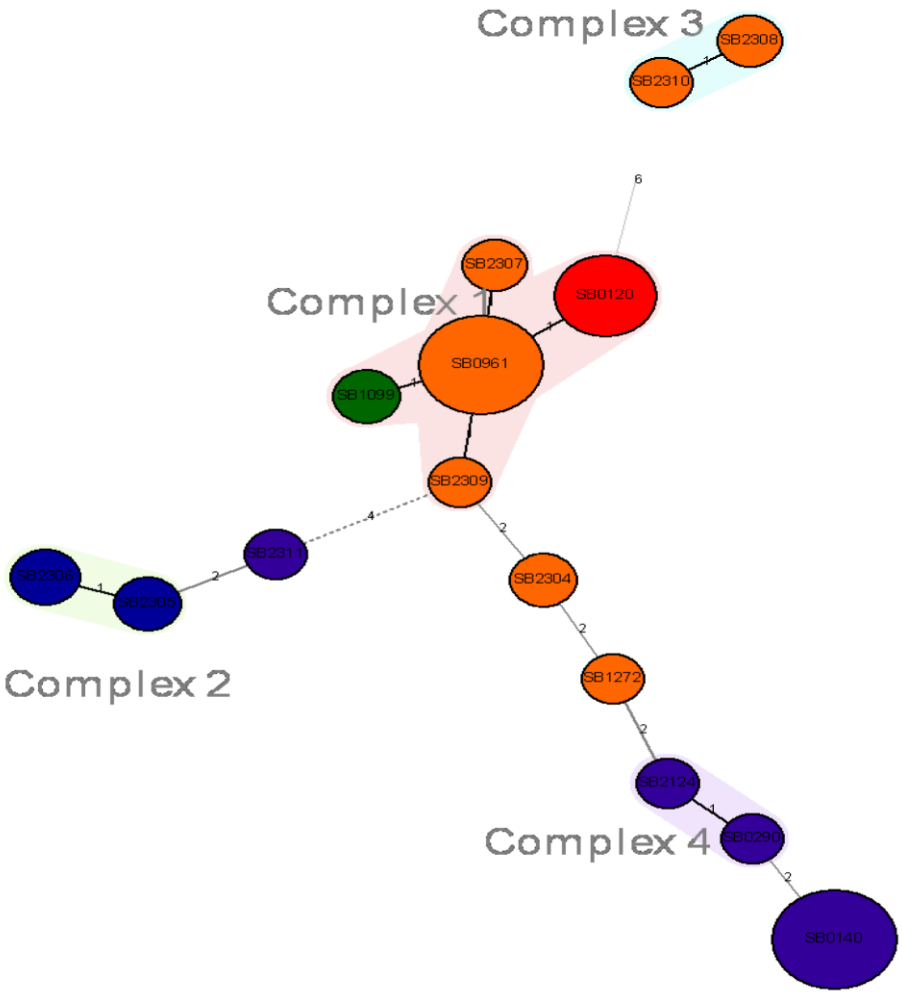

Fig. 2. Clonal complexes and singletons identified by the calculation of the minimum spanning tree based on sopligotyping results. In red de "BCG-like" spoligotype, in orange the "BCG-like derived" spoligotypes, in green the Af1 spoligotype and in blue the Eu1 spoligotypes.

Identification of members of Eu1, Af1 and Af2 clonal complexes

RD analyses were performed to identify possible members of actually well-established *M. bovis* clonal complexes. Eleven of the 29 isolates with the typical Eu1 spoligotype SB0140 were subjected to RD analysis, and all had the Eu1 specific deletion [28]. One of the isolates with a typical Af1 spoligotype pattern (absence of spacer 30) was subjected to RD analysis and did not have the Af1 specific deletion.

Additionally 42 isolates with spoligotypes SB0120, SB0140, SB0961, SB2305, SB2308 and SB2310 were tested for the Af1 specific deletion, 23 were tested for the Af2 deletion (SB0120, SB0140, SB0961, SB2305, SB2308 and SB2310) [12, 19] and 14 for the Eu1 deletion (SB0961, SB1099, SB2305 and SB2306). All the isolates tested were intact in the three regions.

Geographical and type of farm distribution of *M. bovis* spoligotypes

The *M. bovis* clonal complexes distribution (based on spoligotyping results) according to the provinces is illustrated in Fig. 3. The most common spoligotype, SB0961, was detected in all of the provinces of Mozambique investigated. Similarly, isolates with the spoligotype SB0120 were present in most provinces both in commercial and small-scale farms.

The 29 isolates with the spoligotype SB0140, which is specific for the Eu1 clonal complex, were obtained from animals from the south of Mozambique only, [Maputo (n= 22), Inhambane (n=2) and Gaza (n=5)]. Of these 29 isolates, 23 were from 3 different commercial farms. The type of farm was unknown for 4 isolates (one from Inhambane, one from Maputo and two from Gaza provinces). Two isolates were from two different small-scale farms, one from Inhambane province and one from Maputo province. The 10 isolates with Eu1 signature other than SB0140 were also all from the south of Mozambique, 4 from commercial farms (SB0290, SB2124, and SB2311) and 6 from small-scale farms from Maputo and Inhambane, except one (SB2305), that was isolated from one sample originated from Sofala province (Centre of Mozambique).

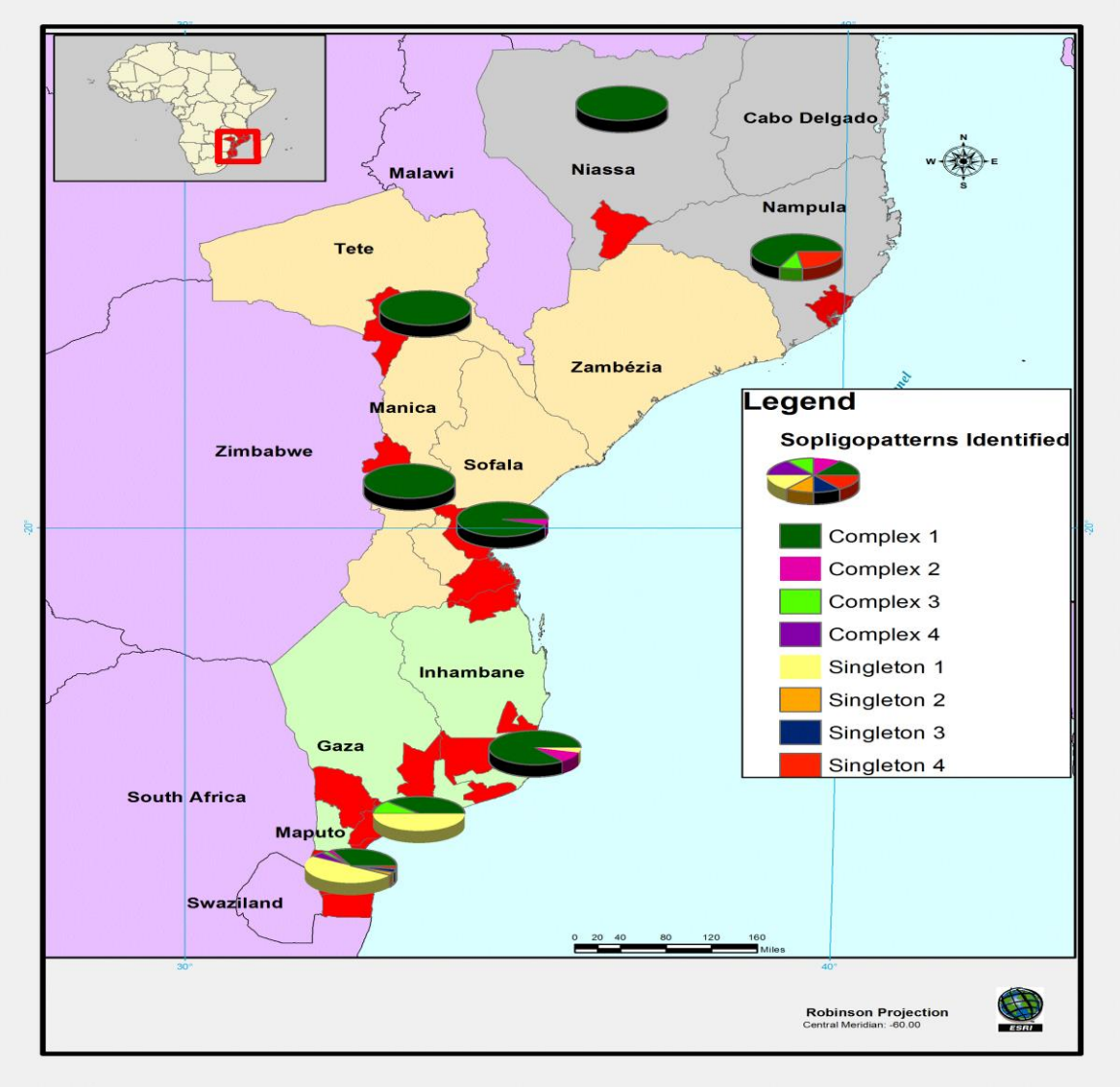

Fig. 3. Distribution of clonal complexes identified per province. Singleton 1 corresponds to spoligopattern SB0140, singleton 2 to SB1272, singleton 3 to SB2311 and singleton 4 to SB2304.

Only one of the small-scale farms generated 6 isolates collected from its stock. Five of these isolates had the pattern SB0961 and one SB0120. The other farms originated only 1 or 2 isolates, that in some cases had identical patterns and in others different. In the commercial farms there was a variability of patterns, ranging from 1 to 6 different patterns per farm.

Subtyping of spoligotypes with MIRU-VNTR analysis

To further discriminate the *M. bovis* isolates defined by spoligotyping and in an attempt to define potential links between farms (due to transmission or common source of infection) we

performed 24-locus MIRU-VNTR typing. MIRU-VNTR results were generated for 59 isolates. The loci MIRU 2, 10, 20, 23, 31, 39 and 40, and Mtub 4, ETR-B, Mtub 30, Mtub 34, Mtub 39 and QUB 4156 showed no variation in all the isolates analysed. From the loci that presented variability, ETR-A, MIRU 24, MIRU 26, QUB 26, MIRU 27 and Mtub 29 presented higher variability, with allelic diversities ranging from 0.50 to 0.43.

Among the isolates tested we found a fairly high degree of diversity, which resulted in the split of the 59 isolates into 23 different MIRU-VNTR types (Fig.4). Thirty-six isolates with spoligotype SB0961 could be further differentiated into 15 new types, 17 isolates with SB0140 into 5 types and 3 isolates with SB1099 into 2 different MIRU-VNTR types. Isolates with spoligotypes SB2305 and SB2306 shared the same MIRU-VNTR type with one of the SB0961 clusters.

The major MIRU-VNTR clusters were “16919 – 270” shared by 11 isolates of the spoligo pattern SB0140 from two different commercial farms located in two different districts (Manhiça and Magude) and “16911 – 1358” shared by 15 isolates with spoligo pattern SB0961, 12 being from various small-scale farms and 1 from a commercial farm from 3 neighbouring districts (Machanga and Buzi from Sofala Province and Govuro from Inhambane province) and the remaining 2 from 2 different districts (Zavala and Angoche). Another cluster of interest was “16917-270” (SB0140) shared by 3 isolates all from different districts (Boane, Macia and Magude), 1 isolate was from a commercial farm, 1 from a small-scale farm and the third was of unknown origin. The cluster “? – 1358” was shared by 4 isolates from the same farm, a commercial farm from Gondola district. The Discriminatory Index using MIRU-VNTR was 0.8708.

VNTR_vals (· VNTR_vals

154 MIRU02	424 Mtub04	577 ETRC	580 MIRU04	802 MIRU40	960 MIRU10	1644 MIRU16	1955 Mtub21	2059 MIRU20	2163b QUB11b	2165 ETRA	2347 Mtub29	2401 Mtub30	2461 ETRB	2531 MIRU23	2687 MIRU24	2996 MIRU26	3007 MIRU27	3171 Mtub34	3192 MIRU31	3690 Mtub39	4052 QUB26	4156 QUB4156	4348 MIRU39			
2	2	5	3	2	2	2	3	2	4	6	3	4	5	4	1	1	3	3	3	2	2	1	2	16917-270	MpBoSs1	SB0140
2	2	5	3	2	2	2	3	2	4	6	3	4	5	4	1	1	3	3	3	2	2	1	2	16917-270	GzMcNIX	SB0140
2	2	5	3	2	2	2	3	2	4		3	4	5	4	1	1	3	3	3	2	2	1	2	?-270	MpMgCm1	SB0140
2	2	5	3	2	2	2	3	2	4	6	3	4	5	4	1	1	3	3	3	2	2	1	2	16917-270	MpMgCm1	SB0140
2	2	5	3	2	2	2	3	2	4	5	3	4	5	4	1	1	3	3	3	2	2	1	2	16924-270	MpMhCm1	SB0140
2	2	5	3	2	2	2	3	2	4	7	3	4	5	4	1	1	3	3	3	2	2	1	2	16919-270	MpMhCm1	SB0140
2	2	5	3	2	2	2	3	2	4	7	3	4	5	4	1	1	3	3	3	2	2	1	2	16919-270	MpMgCm1	SB0140
2	2	5	3	2	2	2	3	2	4	7	3	4	5	4	1	1	3	3	3	2	2	1	2	16919-270	MpMgCm1	SB0140
2	2	5	3	2	2	2	3	2	4	7	3	4	5	4	1	1	3	3	3	2	2	1	2	16919-270	MpMgCm1	SB0140
2	2	5	3	2	2	2	3	2	4	7	3	4	5	4	1	1	3	3	3	2	2	1	2	16919-270	MpMgCm1	SB0140
2	2	5	3	2	2	2	3	2	4	7	3	4	5	4	1	1	3	3	3	2	2	1	2	16919-270	MpMgCm1	SB0140
2	2		3	2	2	2	3	2	4	7	3	4	5	4		1		3	3	2	2	1	2	?-?	MpMgCm1	SB0140
2	2	5	3	2	2	2	3	2	4	7	3	4	5	4	1	1	3	3	3	2	2	1	2	16919-270	MpMgCm1	SB0140
2	2	5	3	2	2	2	3	2	4	7	3	4	5	4	1	1	3	3	3	2	2	1	2	16919-270	MpMhCm1	SB0140
2	2	5	3	2	2	2	3	2	4	7	3	4	5	4	1	1	3	3	3	2	2	1	2	16919-270	MpMhCm1	SB0140
2	2	5	3	2	2	2	3	2	4	7	3	4	5	4	1	1	3	3	3	2	2	1	2	16919-270	MpMhCm1	SB0140
2	2	5	3	2	2	2	3	2	4	7	3	4	5	4	1	1	3	3	3	2	2	1	2	16919-270	MpMhCm1	SB0140
2	2			2	2	2		2	4	3	1	4	5	4		1	2	3	3	2	2	1	2	?-?	NiCuSsX	SB1099
2	2	5	3	2	2	3	3	2	4	7	1	4	5	4	2	4	2	3	3	2	5	1	2	16911-1358	SfBzSsX	SB0961
2	2	5	3	2	2	3	3	2	4	7	1	4	5	4	2	4	2	3	3	2	5	1	2	16911-1358	SfBzSsX	SB0961
2	2	5	3	2	2	3	3	2	4	7	1	4	5	4	2	4	2	3	3	2	5	1	2	16911-1358	SfBzCm1	SB0961
2	2	5	3	2	2	3	3	2	4	7	1	4	5	4	2	4	2	3	3	2	5	1	2	16911-1358	IhGvSsX	SB0961
2	2	5	3	2	2	3	3	2	4	7	1	4	5	4	2	4	2	3	3	2	5	1	2	16911-1358	IhGvSsX	SB0961
2	2	5	3	2	2	3	3	2	4	7	1	4	5	4	2	4	2	3	3	2	5	1	2	16911-1358	IhGvSsX	SB2306
2	2	5	3	2	2	3	3	2	4	7	1	4	5	4	2	4	2	3	3	2	5	1	2	16911-1358	IhGvSsX	SB0961
2	2	5	3	2	2	3	3	2	4	7	1	4	5	4	2	4	2	3	3	2	5	1	2	16911-1358	IhGvSsX	SB0961
2	2	5	3	2	2	3	3	2	4	7	1	4	5	4	2	4	2	3	3	2	5	1	2	16911-1358	IhGvSsX	SB0961
2	2	5	3	2	2	3	3	2	4	7	1	4	5	4	2	4	2	3	3	2	5	1	2	16911-1358	IhGvSsX	SB0961
2	2	5	3	2	2	3	3	2	4	7	1	4	5	4	2	4	2	3	3	2	5	1	2	16911-1358	IhGvSs1	SB0961
2	2	5	3	2	2	3	3	2	4	7	1	4	5	4	2	4	2	3	3	2	5	1	2	16911-1358	IhPaSs1	SB2306
2	2	5	3	2	2	3	3	2	4	7	1	4	5	4	2	4	2	3	3	2	5	1	2	16911-1358	IhZvSs1	SB0961
2	2	5	3	2	2	3	3	2	4	7	1	4	5	4	2	4	2	3	3	2	5	1	2	16911-1358	InGvSs1	SB0961
2	2	5	3	2	2	3	3	2	4	7	1	4	5	4	2	4	2	3	3	2	5	1	2	16911-1358	SfMcSs1	SB0961
2	2	5	3	2	2	3	3	2	4	7	1	4	5	4	2	4	2	3	3	2	5	1	2	16911-1358	SfMcSs1	SB0961
2	2	5	3	2	2	3	3	2	4	7	1	4	5	4	2	4	2	3	3	2	5	1	2	16911-1358	IhGvSsX	SB2305
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2	2	5	3	2	2	3	3	2	4	8	1	4	5	4	2	4	2	3	3	2	5	1	2	16910-1358	SfBzSsX	SB0961
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2	2	5	3	2	2	3	3	2	4	8	1	4	5	4	2	4	2	3	3	2	5	1	2	16910-1358	InGvSs2	SB0961
2	2	5	3	2	2	3	3	2	4	5	1	4	5	4	2	4	2	3	3	2	5	1	2	16920-1358	IhMrSs1	SB0961
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2	2	5	3	2	2	3	3	2	4	7	1	4	4	4	2	4	2	3	3	2	5	1	2	16911-1359	InGvSsX	SB0961
2	2	5	3	2	2	3	3	2	4	7	1	4	4	4	2	4	2	3	3	2	5	1	2	16911-1359	MpMpNIX	SB0961
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Fig. 4. Dendogram displaying the MIRU-VNTR results of the 59 isolates evaluated using 29 loci with the designation numbers obtained using the site http://www.MIRU-VNTRplus.org, spoligotypes and the source of the samples (Xx00000 – province, 00Xx000 – district, 0000Xx – type of farm and 000000X – the farm: Mp – Maputo, Gz – Gaza, Ih –

Inhambane, Sf- Sofala, Mn – Manica, Np – Nampula, Ni – Niassa; Bo – Boane, Mt – Matutuine, Mh – Manhiça, Mg – Magude, Mc – Macia, Zv – Zavala, Mb – Morrumbene, Pa – Panda, Gv – Govuro; Ma - Machanga, Bz – Buzi; Gd – Gondola, Mn – Manica, Ag – Angoche, Cu – Cumba; Cm – comercial, Ss – small-scale, NI - Not identified, X – Not known).

Discussion

This is the first extensive study on genetic diversity of *M. bovis* isolates from Mozambique and will serve as a relevant basis for future studies. In summary these results show a diversity of *M. bovis* isolates that indicate multiple introductions of *M. bovis* in Mozambique. We found a dominance of isolates with "BCG-like" and "BCG-like derived" spoligotype patterns, which are widely spread in Mozambique.

In this study 39 isolates were lacking spacer 11, which is the signature of the **Eu1 clonal complex**. This was the second most common group of strains isolated. Eleven of the isolates with SB0140 spoligotype were investigated for the RD Eu1 deletion and all were RD Eu1 deleted. This group of strains is common in the British Isles, The New World, as well as UK' former colonies and trading partners [28]. The global distribution of these strains suggests a complex history involving historically recent international cattle trade [11]. Eu1 is rare or absent in the other African countries so far surveyed, except South Africa [15]. In South Africa Eu1 strains (SB0140) were identified in samples from cattle [15] and from wildlife [32].

All except one (SB2305) of the 39 isolates with the Eu1 spoligotype pattern were isolated in the South of Mozambique, and the majority were from commercial farms (n= 26), which have imported cattle, mainly from South Africa, where the Eu1 clonal complex is common in cattle [28]. It is thus reasonable to speculate that SB0140 strains were relatively recently imported from neighbouring South Africa or from cattle brought in from Europe as was done in South Africa [28]. Information collected from the reports of the veterinary services showed that cattle importation to the south of Mozambique in the years 1950 to 1957 were mainly from South Africa (467 head) and Portugal (59 head) while to the Centre of the Country cattle were mainly imported from Zimbabwe (previous Southern Rhodesia) with an importation of 917 head, but also from South Africa (116 head), and to the North mainly from Malawi (previously Nyasaland) with an import of 5 head in the same period. In more recent reports reference is made to the number of heads imported but no information is given on the source of imports.

It would be expected that the newly identified patterns lacking spacer 11 would have evolved from stains of the Eu1 clonal complex, nevertheless 2 isolates with spoligotype SB2305 and 2 with spoligotype SB2306 were tested for Eu1 deletion and all 4 were Eu1 intact, while all the SB0140 isolates tested had Eu1 deletion. Three isolates with the signatures SB2305 and SB2306 shared a MIRU-VNTR cluster with isolates with the SB0961 pattern ("16911-1358"), unlike SB0140 isolates that formed a well-defined cluster.

Three isolates (SB1099) had a spoligotype pattern lacking spacer 30, a marker of isolates of the **Af1 clonal complex** [19]. However, one of this isolates was tested and it was intact in the RDAf1 region, indicating that it is not a member of the Af1 clonal complex. This is not surprising since lack of spacer 30 is not specific for the Af1 clonal complex. Other isolates lacking spacer 30, for example strains with spoligotype pattern SB1103 from Chad, had an intact RDAf1 region [19] and there is also reference of isolates non RDAf1 and lacking spacer 30 in a non-African countries [33]. The Af1 clonal complex is geographically localised to Central-West Africa [19] and isolates belonging to that complex have, to our knowledge, not yet been found in cattle outside of this region [11].

No isolates with the spoligotype marker of the **Af2 clonal complex** (absence of spacers 3 to 7) were identified in our sample. Isolates belonging to the Af2 clonal complex have been recovered from cattle in East Africa [12], Uganda, Burundi, Tanzania and Ethiopia. Since Tanzania borders Mozambique, our data suggest that cattle movement between the two countries is limited, or that the BTB prevalence is sufficiently low in Tanzania that there has been no transmission across the border between the two countries at this stage. Alternatively, it is present in Mozambique but was not detected in our survey.

The "BCG-like" and derived strains are dominant in Mozambique. In addition to these well-defined clonal complexes, "BCG-like" and "BCG-like derived" strains have been identified, with spoligotype patterns similar to the vaccine strain BCG [11]. In the present study we found that the majority of *M. bovis* spoligotypes were of this type. Eigth of the 15 spoligotypes (SB0961, SB0120, SB1272 and five new spoligotypes, SB2304, SB2307, SB2308, SB2309 and SB2310), i.e. 136 (76.4%) of the 178 isolates were of the "BCG-like" or "BCG-like derived" type, with a dominance of spoligotype SB0961, which was present in all areas of Mozambique.

The “BCG-like” and “BCG-like derived” strains dominate in most of mainland Europe [11]. They were also found to dominate in *M. bovis* isolated from slaughter cattle in Algeria [34]. The 3 most frequently detected spoligotypes in the Algerian sample were also the 3 most frequently observed types in France. It was concluded that since importation of live cattle from Europe (mostly France) to Algeria has been frequent, during the last century, the similarities in spoligotypes may reflect the introduction of *M. bovis* from mainland Europe to Algeria, and variable Number of Tandem Repeat (VNTR) typing supported a link between *M. bovis* strains from Algeria and France.

In Zambia the “BCG-like” spoligotype is also dominant [35]. In 5 of 6 districts the ancestral spoligotype SB0120 was predominant in *M. bovis* isolates from cattle [35, 36] and wildlife [36]. Thus it suggests that *M. bovis* was introduced from Europe into the two neighbouring countries, Mozambique and Zambia, most probably by the same route. Alternatively trade between the two countries, or possibly introduction via wildlife could cause the spread of “BCG-like” and “BCG-like derived” strains between the countries. Subsequent in-depth genetic analysis of the isolates may elucidate possible connections between isolates within Mozambique and between the Mozambique and Zambia. Interestingly, the spoligotype SB0120 was found in a small percentage of isolates from cattle in South Africa [15] but “BCG-like derived” spoligotypes are also dominant in cattle [15]. SB0961, the predominant spoligotyping pattern identified in our study was also isolated in Argentina [37], in the Czech Republic and Slovakia [38], but in a very low proportion of the total number of the isolates identified. Following the conclusions of Smith and collaborators in a study of population structure of *M. bovis* in Great Britain [39], we can put forward the hypothesis that this was one of the first clones to be introduced in Mozambique, which became common over the time.

The identification of 2 different spoligotype patterns in a single sample, as found in one instance in our study, can represent the presence of two isolates (representing multiple infection) as a result of using pooled samples from each animal for culture, as it was done in our study. Navarro and collaborators found two different strains infecting independent lymph nodes in the same animal in 10.9 % of the cases they investigated [40]. This should be taken into consideration when culture is done for molecular epidemiology studies.

The genetic diversity of the strains as defined by spoligotyping and MIRU-VNTR was fairly high compared to other studies from Africa [41, 42], the reason probably being that this was a study encompassing a nationwide distribution. The diversity of MIRU-VNTR patterns within

individual spoligotypes illustrates that spoligotyping alone is not sufficient for studies of the transmission of BTB; it may be sufficient to exclude but not to suggest epidemiological links.

The results from our study supported findings from other studies that some MIRU-VNTR loci do not show variability in the strains from the same region [43, 44], so after extensive investigation it is possible to eliminate some loci from the analysis of the strains of a specific region [45]. Despite the non-use of a standardized set of loci investigated in various studies, that complicate the comparison of the results, it is clear that regional differences occur even in neighbouring countries like Mozambique and South Africa. Hlokwe and collaborators in a study in South Africa identified ETR-B, ETR-E, Mtub 21 and QUB 11b amongst the loci that showed higher allelic diversity [44], while in our study low or no variability was found in these loci. On the other hand we found higher allelic diversity in ETR-A, Mtub 12 and MIRU 27 also not corresponding to the results from the study mentioned above. Nevertheless similar allelic diversity was found in MIRU 16, MIRU 26 and QUB 26 in both our and their studies [44]. Our MIRU-VNTR results also support the hypothesis that the SB0140 isolates identified in our study are related to South African isolates, even if there is no 100% similarity. In isolates with this spoligotype from Mpumalanga and Western Cape, South Africa, 8 loci were identical to those from isolates in our study, while 2 loci (QUB 26 and MIRU 26) were not in agreement [44]. Further investigation is required to clarify this issue.

Since tuberculosis has been reported in goats, pigs (reports of the Veterinary Services for the years 64 and 67) and wildlife in Mozambique [46], isolation and typing of the bacteria involved would help to clarify the relations of the disease in the various species as done in studies elsewhere [47-49]

It was found that analysis of a single milk sample was sufficient to obtain a *M. bovis* isolate from as many as 9 out of 41 (22%) skin test positive cows was culture positive for *M. bovis*. Thus milk may be an important and easily available source of isolates from infected herds for molecular epidemiological studies. The evidence of *M. bovis* shedding through milk and the application of incorrect practices (consumption of raw milk), show a possible increased risk of BTB transmission to humans. *M. bovis* infection in humans was so far not confirmed in Mozambique [50, 51]

In terms of BTB control policy, and keeping in mind that test a slaughter for all animals is presently unrealistic in Mozambique, the results strengthen the need to reinforce the current regulations that require a negative BTB test result before cattle importation. The same should be enforced for the internal movements, as the frequency of shared genotype (Spoligotype and MIRU) from cattle originating from different parts of the country strongly suggest internal transmission of BTB.

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Author contributions

AM participated in the design and conduct the study, culture and isolation of mycobacteria, molecular assay, data analysis and drafting of manuscript; CM, ASM, BM, JB, GM participated in the testing and collection of samples, SG, EMS, AnM, ASM, JM, NM, IM, GM participated in isolation and molecular assay, MC-N in the design and drafting the manuscript; RMW, GK, TK, PvH participated in the conception and design of the study, and general supervision of the research. All authors critically reviewed de manuscript, read and approved the final version.

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Chapter 5

General discussion and conclusions

Bovine tuberculosis was identified in Mozambique decades ago and was reported at least from as far back as the year 1940 in the annual reports of the Veterinary Services (Aires, 1947). The lack of earlier reports may simply be an indication of the perception of a relatively low importance of the disease at that time. However, the emphasis given to it in the first Animal Health Regulation ("Portaria" No. 113 – 1908) suggests a different perception, since this regulation was probably introduced as a measure to control the entrance of the disease from neighbouring countries. Before that time, BTB had been diagnosed in at least one neighbouring country, for example Renwick *et al.* (2007) citing Raviglione *et al.* (1929) referred to the first diagnosis of BTB in cattle in South Africa in 1880.

From the first reports, BTB cases were identified mainly as a result of *post-mortem* meat inspection (Aires, 1947). This surveillance approach is presently not effectively used in Mozambique, as seen based on the extremely low number of cases reported by the Veterinary Services in the past few decades. However, the approach has been evaluated as the most sensitive and cost effective surveillance method (Hadorn and Stärk, 2008, Rossi *et al*., 2015). For this reason, in addition to other surveillance strategies, the recommendations in Table 2, Chapter 2 should be implemented: viz to improve surveillance at abattoirs with laboratory confirmation of samples, involving all required institutions and to trace back positive cases from abattoirs to herds of origin (recommendation 6 and 7). However, the sensitivity can be negatively impacted by the low extraction rates in small-scale farming in Mozambique, for example, Dionisio, (1985) reported extraction rates from 0.4 to 1.7% in cattle small-scale farming as opposed to 11.5 to 15.2% in commercial farming in Mozambique in the period 1967 to 1974. Slaughterhouse surveillance can also serve as a basis to indicate where targeted surveys should be conducted (Hadorn and Stärk, 2008).

Prevalence determined by tuberculin testing surveys conducted in our study varied from 0.98% in Massingir to 39.6% in the Govuro District, with prevalence as high as 71.4% in some livestock areas/farms. A work done also recently (2008) in Govuro reported a BTB prevalence of 61.9%. This study was done using SIT in only two livestock areas/herds (Macucule, 2009), that in our study from Govuro were also the ones with higher prevalence. These prevalence rates are higher than ever reported before. The highest prevalence in previous decades recorded was 40% in Chimoio (Paisana 1953), indicating an increase in the prevalence in some locations. The increase in BTB prevalence in cattle in some hotspots is a common trend in Africa (Munyeme, 2009a, Mwakapuja, 2013a) and has been linked in some studies to wildlife

reservoirs, introduction of infected animals or long term persistent infection in the absence of systematic control.

Averaging the results of all the surveys conducted in this study gives an overall prevalence of 13.6% (952/6983), which is significantly different from the result obtained in 2005 of 8.8% (751/8545), also not using a representative sample (DINAP, 2005). However, it is important to emphasize that in our surveys SCITT and SIT in the middle neck region was used, compared to the test in the caudal fold (CFT) used in previous surveys, a method that was confirmed to be less sensitive (Good & Duignan, 2011). Also the herds in our study have been intentionally selected from areas with history of high numbers of BTB cases. Thus direct comparisons carry risk of over interpretation.

The analysis of risk factors for the occurrence of positive reactors showed no difference with respect to the sex of the animal. In respect to age, younger animals had significantly lower odds of infection compared to the older animals. This is a common finding in studies in Africa (Tschopp *et al.*, 2009, Munyeme *et al*., 2009a, Katale *et al*., 2013). Our results suggest that cattle from small-scale farmers had lower prevalence than those of commercial farmers. However, this association lacked statistical significance. Commercial farmers normally have larger herd sizes and cattle density was already described as risk factor for BTB occurrence. It was expected that in a commercial setting better control strategies could be implemented but it was observed that management practices in some commercial farms in Mozambique does not differ to those of small-scale farming.

A collection of 178 *M. bovis* isolates from positive reactors were genotyped. The strains obtained by spoligotyping were quite diverse, with 15 different patterns identified, of which 8 were not previously recorded in the Mbovis.org database. Identification of new patterns is often encountered in studies from locations where no previous studies have been performed (Rodríguez *et al.,* 2010). High diversity of strains has been associated with high BTB prevalence, cattle movement and presence of wildlife reservoirs (Acevedo *et al.*, 2013). In accordance high BTB prevalence and both controlled and uncontrolled movements of livestock and, to a lesser extent, wildlife (internal and imports) are a reality in Mozambique. The potential role of the last factor has still to be investigated in Mozambique since two studies involving cattle-wildlife interfaces did not include typing of isolates (Ferreirra and Rosinha, 1986, Tanner *et al*., 2014).

The spoligopattern SB0961 accounted for 61% of the isolates and was found in all areas investigated. This pattern was not identified in other studies from neighbouring countries (Munyeme *et al.*, 2009b, Hlokwe *et al.*, 2011, Hlokwe *et al.*, 2013, Mwakapuja, 2013b). No studies on genotypes of *M. bovis* from Zimbabwe and Malawi were found. The reasons for this strain dominance could be hypothesized to be owing to a higher virulence (Wright *et al.*, 2013) but in other countries where isolates with this spoligopattern were identified, it is present at a low frequency and therefore higher virulence may not be a suitable explanation (Pavlik *et al*. 2002, Shimizu *et al*, 2014). In addition, on 2 commercial farms from our study where other spoligopatterns are prevalent, SB0961 is present at a low frequency (1/17 in the farm MpMgCm1, 5/16 in MpMhCm1 and 2/9 in NpAgCm1). However, there is a farm where SB0961 isolates were predominant (11/14 in MnMnCm1). We suggest that SB0961 might have been one of the first clones to be introduced in Mozambique (Smith *et al.*, 2003).

The 29 isolates identified with the spoligotype pattern SB0140, a European 1 (Eu1) common spoligotype pattern, were all isolated from cattle from the South of Mozambique and the majority from commercial farms. There were no isolates of the African 1 (Af1) or African 2 (Af2) clonal complexes that are frequent in Central-West Africa and East Africa, respectively. While international cattle trade with European countries and South Africa where SB0140/Eu1 clones are prevalent are registered and can be considered as a potential source of the SB0140 isolates identified in Mozambique. Trade with Zimbabwe and Malawi is also documented but from these countries there is no information on typing of *M. bovis* isolates, There are no reports of trade with countries from Central-West Africa and East Africa. This is a likely explanation for the absence of Af1 and Af2 isolates in our study. Uncontrolled movement of cattle from South Africa, Swaziland, Zimbabwe and Malawi is common, as reported by the outbreaks of Foot and Mouth Disease, but is limited in the case of Tanzania by a natural barrier, the Rovuma River.

Using MIRU-VNTR typing, clonal variants shared by isolates originating from different farms and districts were identified amongst isolates with the two most frequent spoligotypes (SB0961 and SB0140), strongly suggesting potential transmission and/or common source of infection. While transmission in neighbouring districts (Govuro, Machaga and Buzi), can be justified by free movement of the herds, the occurrence in locations far apart is most probably a consequence of trade (acquisition of infected animals) both internal and imports.

In conclusion, our results show a potential increase in the prevalence of BTB in the country despite taking into consideration that the selection of locations studied was biased, viz towards locations with a history of higher numbers of BTB case reports and the use of a more sensitive technique, i.e. testing in the middle neck region as opposed to the testing in the caudal fold as done in previous studies.

Even if no cattle to human transmission was found in previous work done in Mozambique and this was not the subject of this study, the evidence of *M. bovis* shedding through milk and the application of incorrect practices (consumption of raw milk), indicate that this is a subject to be addressed in the disease control strategy.

The results also strengthen the need to reinforce the current regulations that require a negative BTB test result before cattle importation. The same should be enforced for internal movements, since the frequency of shared genotypes (spoligotype and MIRU) from cattle originated from different parts of the country strongly suggest internal transmission of BTB.

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