

From the Department of Clinical Science and Education
Karolinska Institutet, Stockholm, Sweden; the Department of
Microbiology, Public Health Agency of Sweden, Solna, Sweden and
the Faculty of Veterinary, Eduardo Mondlane University, Maputo,
Mozambique

MOLECULAR CHARACTERIZATION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX ISOLATES IN MOZAMBIQUE

Sofia Omar Viegas



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ISOLATES IN MOZAMBIQUE

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Sofia Omar Viegas

Principal Supervisor:

Tuija, Koivula, Ph.D.

Karolinska Institutet
Department of Clinical Science and Education
Division of Research Center

The Public Health Agency of Sweden
Department of Microbiology

Co-supervisor(s):

Professor, Senior, Gunilla Källenius

Karolinska Institutet
Department of Clinical Science and Education
Division of Research Center

Ramona Groenheit, Ph.D.

The Public Health Agency of Sweden
Department of Microbiology
Division of Highly Pathogenic Bacteria

Opponent:

Ulf R. Dahle, Ph.D.

Norwegian Institute of Public Health
Department of Food-borne Infections

Examination Board:

Assoc. Professor, Håkan Mörner

Lund University
Department of Laboratory Medicine
Division of Medical Microbiology

Assoc. Professor, Asli Kulane

Karolinska Institutet
Department of Public Health Sciences
Equity and Health Policy Unit

Assoc. Professor, Charlotta Nilsson

Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Dedication

To my family.

ABSTRACT

Mozambique is one of the high burden tuberculosis (TB) and human immunodeficiency virus (HIV) countries with a prevalence of HIV infection in adults of 11.5% and an estimated TB prevalence of 559 per 100 000 population. Fifty six percent of the TB patients in Mozambique are estimated to be HIV positive. TB control strategies might significantly be affected by differences in virulence, epidemiologic characteristics and epidemiology of particular strains of the *Mycobacterium tuberculosis* complex. Molecular epidemiology studies allow the identification of circulating strain types, understanding of transmission dynamics, as well as investigations of the evolution of the *M. tuberculosis* complex.

The studies included in this thesis described the molecular epidemiology of *M. tuberculosis* complex in Mozambique, identified predominant genotypes responsible for TB transmission and prevalence and investigated the association between predominant spoligotypes and HIV sero-status. The prevalence and transmission of the Beijing genotype in Mozambique was also evaluated. With the aim to explore the public health risk for bovine TB, isolates from two sites were investigated, Maputo (tuberculous lymphadenitis or TBLN cases) and Govuro district (TBLN and pulmonary cases), the last site, Govuro, with known high prevalence of bovine TB in cattle (39.6%). Furthermore, a phylogenetic phylogeographic snapshot of worldwide *M. tuberculosis* complex diversity was created based on the classification of the Multiple-locus variable-number tandem repeat analysis (MLVA).

For the first time, the genetic diversity of circulating *M. tuberculosis* complex strains in Mozambique was described. It was found that the TB epidemic in Mozambique was caused by a wide diversity of spoligotypes with predominance of the Latin-American Mediterranean (LAM, n=165 or 37%); East African-Indian (EAI, n=132 or 29.7%); the evolutionary recent T clade (n=52 or 11.6%) and the globally-emerging Beijing clone (n=31 or 7%). The predominant lineages were also common in neighboring countries, indicating TB transmission by migration from one country to another.

The Beijing lineage, distributed worldwide and responsible for large epidemics was found to be particularly common in the Southern region of Mozambique, especially in Maputo City (17%) and associated with HIV infection (p=0.023). By combined use of region of difference (RD) analysis and spacer oligonucleotide typing (spoligotyping), a distinct group of four isolates had deletion of RD150, a signature of the “sublineage 7” recently emerging in South Africa. The same group was very similar to the South African “sublineage 7” by Restriction Fragment Length Polymorphism (RFLP) and Mycobacterial Interspersed Repetitive Units–Variable-Number Tandem Repeat (MIRU-VNTR), suggesting that this sublineage could have been recently introduced in Mozambique from South Africa.

No *M. bovis* was found in TBLN cases from Maputo. It was demonstrated that TBLN in Maputo was caused by a variety of *M. tuberculosis* genotypes, similar to the ones causing pulmonary TB, suggesting that in Maputo, cases of TBLN arise from the same source as pulmonary TB, rather than from an external zoonotic source.

For the first time, evidence of the occurrence of *M. bovis* in humans in Mozambique was revealed. In a study presently being conducted in the district of Govuro, among six *M. tuberculosis* complex isolates, one was *M. bovis*. Nevertheless, further research is needed on cases of abdominal TB and other forms of extrapulmonary TB, in Govuro and in other pastoral areas, where the prevalence of bovine TB in cattle is known to be high, in order to have a better answer about the public health importance of this zoonotic disease in Mozambique.

LIST OF SCIENTIFIC PAPERS

- I. **Viegas SO**, Machado A, Groenheit R, Ghebremichael S, Pennhag A, Gudo PS, Cuna Z, Miotto P, Hill V, Marrufo T, Cirillo DM, Rastogi N, Källenius G, Koivula T. Molecular diversity of *Mycobacterium tuberculosis* isolates from patients with pulmonary tuberculosis in Mozambique. BMC Microbiol. 2010;10:195.
 - II. **Viegas SO**, Machado A, Groenheit R, Ghebremichael S, Pennhag A, Gudo PS, Cuna Z, Langa E, Miotto P, Cirillo DM, Rastogi N, Warren RM, van Helden PD, Koivula T, Källenius G. *Mycobacterium tuberculosis* Beijing genotype is associated with HIV infection in Mozambique. PloS One. 2013;8(8):e71999.
 - III. Hill V, Zozio T, Sadikalay S, **Viegas S**, Streit E, Kallenius G, Rastogi N. MLVA Based Classification of *Mycobacterium tuberculosis* Complex Lineages for a Robust Phylogeographic Snapshot of Its Worldwide Molecular Diversity. PLoS ONE. 2012;7(9):e41991.
 - IV. **Viegas SO**, Ghebremichael S, Massawo L, Alberto M, Fernandes FC, Monteiro E, Couvin D, Matavele JM, Rastogi N, Neves MC, Machado A, Carrilho C, Groenheit R, Källenius G, Koivula T. *Mycobacterium tuberculosis* causing tuberculous lymphadenitis in Maputo, Mozambique. BMC Microbiol. (Submitted).
- **Study V** - Moiane I, **Viegas SO**, Ghebremichael S, Massawo L, Alberto M, Neves MC, Machado A, Carrilho C, Groenheit R, Källenius G, Koivula T. Investigating transmission of *Mycobacterium bovis* in a region with high prevalence of bovine tuberculosis in cattle in Mozambique (Preliminary results).

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
BCG	Bacillus Calmette-Guérin
bp	base pair
CAS	Central Asian
CRISPER	Clustered Regularly Interspaced Short Palindromic Repeats
DNA	Deoxyribonucleic Acid
DOTS	Directly Observed Treatment Short Course
DR	Direct Repeat
DST	Drug Susceptibility Testing
DVR	Direct Variable Repeat
EAI	East African Indian
EAI1_SOM	East African-Indian_Somalia
FNA	Fine Needle Aspiration
GDP	Gross Domestic Product
H	Haarlem
HIV	Human Immunodeficiency Virus
IS	Insertion Sequence
LAM	Latin American Mediterranean
LAM'	Lipoarabinomannan
LED	Light-Emitting Diodes
LJ	Lowenstein-Jensen
LSP	Large Sequence Polymorphism
MDR	Multidrug Resistant
MIRU	Mycobacterial Interspersed Repetitive Units
MIT	MIRU International Types
MLVA	Multiple-Locus Variable-number tandem repeat Analysis
MST	Minimum Spanning Tree
NTM	Non-Tuberculous Mycobacteria
PCR	Polymerase Chain Reaction
PGG	Principal Genetic Groups
RD	Region of Difference
RFLP	Restriction Fragment Length Polymorphism
rpoB	RNA polymerase beta
SIT	Shared International Type
SNPs	Single Nucleotide Polymorphisms
Spoligotyping	Spacer Oligonucleotide Typing
ST	Shared Type
TB	Tuberculosis
TbD1	Tuberculosis-specific deletion 1
TBLN	Tuberculous lymphadenitis
TCH	Thiophene-2-Carboxylic Acid Hydrazide
VNTR	Variable Numbers of Tandem Repeats
WHO	World Health Organization
XDR	Extensively Drug-Resistant

1 INTRODUCTION

1.1 GLOBAL BURDEN OF TUBERCULOSIS

Tuberculosis (TB) stands as a major global health problem, ranking as the second highest cause of death from an infectious disease globally, after the human immunodeficiency virus (HIV) (1). The World Health Organization (WHO) estimates that 9.0 million people developed TB in 2013, of whom, 13% were HIV positive individuals. Among the incident cases, 56% were from the South-East Asian and Western Pacific Regions and one quarter were from Africa. The African continent accounts for the highest rates of cases and deaths relative to population (1).

Figure 1 shows the estimated TB incidence for the top-ten countries in 2013.

In 2013, WHO estimates that 1.5 million deaths occurred due to TB (360 000 of whom were HIV positive). Among these deaths 210 000 were from multidrug resistance (MDR) patients, representing 43.75% of the total incident cases of MDR-TB.

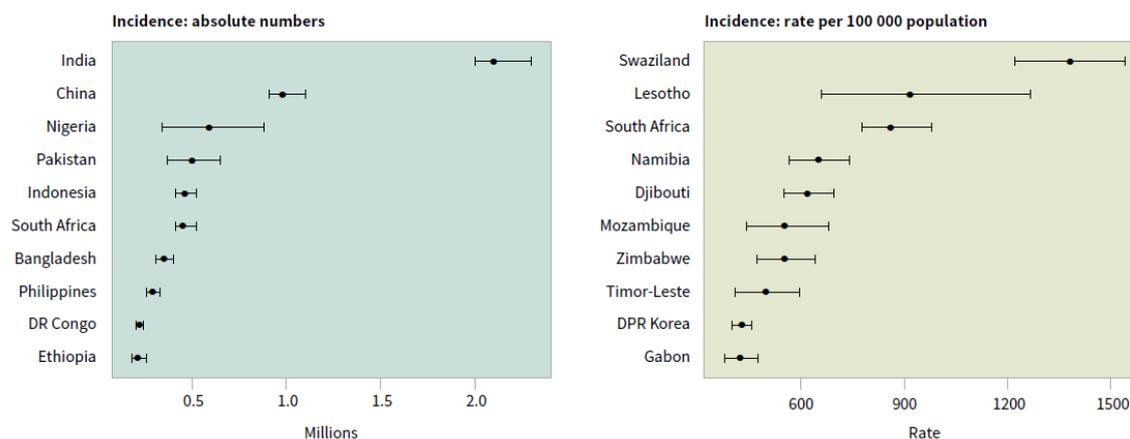


Figure 1. Estimated WHO TB incidence rate per 100 000 population: top-ten countries, 2013. Reproduced with permission from the World Health Organization (1).

In 2014, a post 2015 TB strategy was announced by the World Health Assembly, with the goal of ending the global TB epidemic with targets to reduce TB deaths by 95% and reduce incident cases by 90% until 2035 (2).

TB is a disease of poverty (3,4). A lack of basic health services, malnutrition, social disruption, tobacco consumption and inadequate living conditions all contribute to the dissemination of TB and its impact in the community. HIV infection and Acquired Immune Deficiency Syndrome (AIDS) amongst others are the strongest risk factor for TB (5). The observed increase in TB incidence in sub-Saharan Africa may have resulted from several of these factors.

1.1.1 Drug Resistant TB

The ability of a bacterial cell to survive the presence of a drug at a concentration that normally kills or inhibits growth is called resistance. Drug resistant TB is a particular problem because of the prolonged therapy of at least six months that makes patient compliance very difficult, frequently creating drug resistant *Mycobacterium tuberculosis* complex strains. Other factors that contribute to the development of resistance are the

inadequate use of antimicrobials, low compliance and completion of treatments, together with poor TB control programs and lack of access to drugs (6).

The emergence of drug resistance is a serious threat to global efforts to control TB (1,6–8).

Particular terminologies are used to define resistance in TB (9):

- Mono-resistance, defined as resistance to one first-line anti-TB drug only;
- Polydrug resistance, defined as resistance to more than one first-line anti-TB drug (other than both isoniazid and rifampicin);
- MDR, defined as resistance to at least both isoniazid and rifampicin;
- Extensively drug resistant (XDR), defined as a MDR strain which is also resistant to one of the three second line injectable drug (capreomycin, kanamycin or amikacin) and any fluoroquinolone;
- Rifampicin resistance, defined as resistance to rifampicin based on phenotypic or genotypic methods, with or without resistance to other anti-TB drugs. It includes any resistance to rifampicin, whether monoresistance, MDR, polydrug resistance or XDR.

Recently the term totally drug-resistant TB, although not clearly defined, has been used to define a strain resistant to a wider range of drugs than strains classified as XDR-TB (10). These types of strains have been reported in Italy (11), Iran (12), India (13,14) and South Africa (15).

Globally, 3.5% of new and 20.5% of previously treated TB cases were estimated to have had MDR-TB in 2013 and 9.0% of patients with MDR-TB had XDR-TB (1). In 2013, 55% of reported TB patients estimated to have MDR-TB were not detected (1).

To address the MDR-TB epidemic, the WHO considers five priority actions needed: 1) high-quality treatment of drug-susceptible TB to prevent MDR-TB; 2) expansion of rapid testing and detection of MDR-TB cases; 3) prompt access to quality care; 4) infection control; and 5) increased political commitment, including adequate funding for current interventions as well as research to develop new diagnostics, drugs and treatment regimens (1).

1.1.2 TB and HIV in Africa

The emergence of HIV had a unique impact on the epidemiology of infectious diseases in general and particularly on TB (16). Individuals with latent *M. tuberculosis* infection who contract HIV are at risk of developing active TB at a rate of 7 to 10% per year, compared to approximately 8% per lifetime for HIV negative individuals (17,18). Thus, the dissemination of the HIV infection has contributed to the expansion of TB, which is the main cause of mortality among HIV patients.

In Africa, the proportion of TB cases co-infected with HIV is the highest (1). WHO estimated that in 2013, 34% of TB cases were co-infected with HIV in the continent, accounting for 78% of TB cases among people living with HIV worldwide (Figure 2).

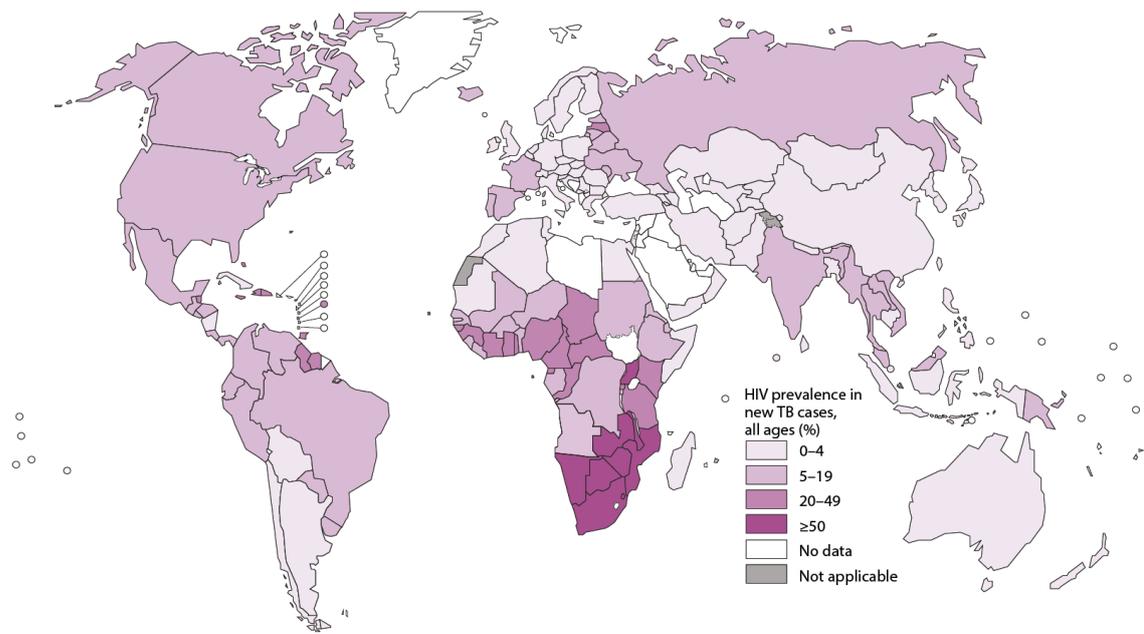


Figure 2. WHO estimated HIV prevalence in new and relapse TB cases, 2013. Reproduced with permission from the World Health Organization (1).

The emergence of HIV has not only increased TB incidence and TB associated mortality but it has also made the diagnostics of TB more problematic (16). Diagnosis of active TB disease in HIV-infected people is difficult, because patients with HIV associated TB are paucibacillary (i.e. have fewer bacilli in their sputum) when compared to HIV uninfected patients with pulmonary TB (19). Therefore, the WHO recommends the use of recent new diagnostic technologies, such as the GeneXpert, in order to increase case detection in that particular group and among MDR-TB suspects (20).

1.2 MOZAMBIQUE, THE COUNTRY

The Republic of Mozambique has a population of 25,727,911 inhabitants (21), it is located in Southern Africa and divided per 11 provinces and 128 districts. The country suffered almost five centuries of Portuguese colonization, a massive migration of skilled workers after the independence in 1975 and a terrible civil war that ended in 1990 where half of public health facilities and schools were destroyed.

Mozambique is now experiencing a period of political and economic transition, with a newly elected president and the expected promotion of natural gas projects that are expected to modify the country's economic and social scenery (22).

The economic situation of Mozambique has improved over the years, in 2014 the Gross Domestic Product (GDP) grew by 7.6% and growth is likely to remain strong, at 7.5% and 8.1% in 2015 and 2016, respectively, enhanced by the construction, transport and communications sectors (22). In Mozambique, the majority of the population is greatly dependent on natural resources for their livings and the primary sector plays a critical role in the country's economy. In addition, the country is rich with a variety of mineral resources, especially gas, coal, oil, heavy-sand deposits, gold, copper, titanium, graphite and other minerals in significant quantities (22).

While the economy has expanded strongly, its effect on poverty reduction has been minimal. The majority of Mozambicans (55%) still live below the consumption poverty line of USD 0.6 a day (22). The life expectancy is 53 years of age and access to health services remains low (21).

Inadequate financing, shortage of health professionals and essential medicines, all these historical, social and economic factors influence the present extreme poverty and health inadequacy in Mozambique (23,24).

1.2.1 TB and HIV in Mozambique

TB represents one of the principal causes of morbidity and mortality in Mozambique, affecting the main vulnerable groups, including young adults, children and people living with HIV/AIDS. This situation makes the early diagnosis and management of TB and MDR-TB cases a priority for the National TB Control Program (25). Since 1993, Mozambique stands on the list of the 22 high burden TB countries, where the prevalence rate is of 559 per 100,000 population (1).

In Mozambique, all Health Units have the capacity to perform Institutional DOTS (Directly Observed Treatment, Short-course), implying 100% coverage. However, many of the health facilities in the country, particularly at peripheral level, still have weaknesses, that can be observed in the number of screening and patients diagnosed. The major obstacle is the lack of human resources to perform preventive and curative care tasks (25).

The TB epidemiological distribution in the country varies from region to region. The Central and South Regions of the country have the highest burden of disease, with 41.6% and 38.3% of total notified cases, respectively (23).

MDR-TB remains one of the major challenges for the National TB Control Program, with a prevalence of 3.5% in new cases and 11% in previously-treated patients (26).

Presently Mozambique has 337 laboratories performing smear microscopy for TB diagnosis and three TB Reference Laboratories, located in Maputo, Beira and Nampula. All of the

reference laboratories are fully operational, performing cultures on solid and liquid media, rapid speciation and drug susceptibility testing (DST) of the *M. tuberculosis* complex, the first one also performing rapid detection of MDR-TB using Line Probe Assay (LPA). The National TB Reference Laboratory, located in the capital Maputo, recently achieved the ISO 15189 accreditation for fluorescent microscopy and cultures on solid and liquid media, representing the first clinical laboratory reaching international accreditation in Mozambique, an enormous achievement for patient care.

Recent molecular diagnostic tools as the GeneXpert are currently being implemented in the country, focusing on MDR-TB detection and diagnosing TB in HIV co-infected patients. At present 36 laboratories have the capacity to perform GeneXpert.

Regarding HIV, the civil war had two opposing effects, the first in protecting the country from the spread of HIV as it influenced population movements (27) and at the same time facilitating the spread of HIV by eroding traditional norms, destroying the health care infrastructure and influencing labor migration to and from neighboring countries with high HIV and TB prevalences (28). The actual prevalence of HIV in adults (15-49 years) in the country is 11.5% and more women are infected (13.1%) compared to men (9.2%) (29).

The prevalence of HIV among TB patients decreased from 58% in 2012 to 56% in 2013. In 2013, 91% of TB patients knew their HIV status (1).

1.3 THE IMPORTANCE OF STUDIES ON MOLECULAR EPIDEMIOLOGY OF TB

Molecular epidemiological studies of TB, based on molecular techniques enabled studies to address important epidemiological questions, such as outbreak investigations (30–34), describing transmission dynamics (35–38), estimates of recent-versus-reactivation disease and the extent of exogenous reinfection (39–41). Furthermore, molecular epidemiological studies have also enabled the understanding of spatiotemporal transmission and evolutionary dynamics (42–46) and generate evidence that different strains of the *M. tuberculosis* complex from distinct phylogenetic lineages may differ in virulence, pathogenesis, and epidemiologic characteristics, influencing TB control and vaccine development strategies (47).

Below are summarized some applications of molecular techniques in TB epidemiology described by Mathema and colleagues (47):

- Study of the *M. tuberculosis* complex transmission dynamics (outbreak, transmission, chains of transmission, risk factors and groups at risk of *M. tuberculosis* complex infection).
- Discriminating recurrent TB due to exogenous reinfection and reactivation.
- Detection of laboratory error/cross-contamination.
- Determination of geographic spread of strains.
- Monitoring transmission of drug-resistant strains.
- Investigation of the evolution of drug-resistant TB within and between patients.
- Detection of mixed infections among TB patients.
- Sampling of strain types for further studies.
- Evaluation of TB control programs (level of clustering).
- Identification of strain-specific transmission/infection rates.
- Identification of predominant strain types (clonal strains) in study populations.
- Identification of hypervirulent strains in populations.
- Investigation of the evolution of the *M. tuberculosis* complex.

1.4 THE *MYCOBACTERIUM TUBERCULOSIS* COMPLEX

TB is caused by bacteria belonging to the *M. tuberculosis* complex, which consists of highly related slow growing, acid-fast, aerobic, non-spore forming, non-motile bacteria. They form slightly curved or straight rods which may branch (0.2 to 0.6 µm by 1.0 to 10 µm) (48).

The *M. tuberculosis* complex comprises seven members, *M. tuberculosis*, *M. africanum*, *M. canettii* where the natural host are humans and *M. bovis*, *M. caprae*, *M. microti* and *M. pinnipedii* which usually have animals as their natural hosts. In addition, rare *M. tuberculosis* complex variants, standing within the *M. tuberculosis* complex are not yet completely described; the *M. suricattae*, *M. mungi* and the *Dassie* bacillus.

Although the mycobacterial species of the *M. tuberculosis* complex are highly similar to each other on Deoxyribonucleic Acid (DNA) level, *M. tuberculosis* complex members differ widely in terms of host tropism, phenotype and pathogenicity (42,49,50). Detection of the different species within the complex has mainly been based on the analysis of phenotypic characteristics such as acid-fast microscopy, colony morphology, growth rate and biochemical tests. Genotyping methods have currently made epidemiological studies and rapid species discrimination more promising, enlarging our understanding of phylogenetic relations and evolutionary origin of the members of the *M. tuberculosis* complex.

1.4.1 *Mycobacterium tuberculosis*

M. tuberculosis is the principal agent of TB in humans, first described by Robert Koch in 1882 (51). Regarding the origin of the *M. tuberculosis* complex strains, it was previously presumed that *M. tuberculosis* had evolved from *M. bovis* by specific adaptation of an animal pathogen to the human host (52–54). However, genomic analysis has shown that *M. bovis* has a smaller genome, suggesting that it is evolutionary younger (42).

Phenotypically, *M. tuberculosis* can be identified using analysis such as nitrate reductase, production of niacin, resistance to thiophene-2-carboxylic acid hydrazide (TCH) and sensitivity to pyrazinamidase (55,56). Genotypically, by Spacer Oligonucleotide Typing (spoligotyping), *M. tuberculosis* has been classified into different phylogenetic lineages (57).

1.4.2 *Mycobacterium bovis* and *Mycobacterium bovis* BCG

Bovine TB, caused by *M. bovis* is the main zoonotic disease caused by mycobacteria, affecting cattle, other domesticated animals and certain free or captive wildlife species. The disease is spread to humans, typically by ingestion of unpasteurized milk or contaminated meat, causing extrapulmonary TB, but can also be transmitted by inhalation of aerosols causing pulmonary TB (58,59).

TB caused by *M. bovis* and TB caused by *M. tuberculosis* cannot be distinguished clinically, radiographically, or pathologically in individual patients (60). Thus, the identification of these causative agents can only be through mycobacterial culture and subsequent use of biochemical or molecular methods (61). However, containment facilities to identify the causative agent of TB are largely absent in low income countries (61).

In high income countries, zoonotic TB accounted for a relevant proportion of the TB cases until the introduction of regular milk pasteurization programs (61). In our days, bovine TB is well controlled or eliminated, and zoonotic TB cases are rarely seen; however, reservoirs in wildlife can make complete eradication challenging (62,63).

In Africa, the situation is somehow more critical, as bovine TB is an economical and public health threat in low income countries (58). In most African countries, effective control of bovine TB is largely absent, including regular milk pasteurization and slaughterhouse meat inspection (58,59). Additionally, the presence of multiple risk factors such as human behaviour and HIV infection (58,59,62) makes the situation even worse. The reported median proportion of bovine TB in Africa is 2.8% (range 0%–37.7%) of human TB cases (58). Control policies have not been enforced due to cost implications, lack of capacity and infrastructure limitations (58,59).

There is also a non-virulent strain of *M. bovis* called Bacillus Calmette Guerin (BCG), which has its origin from a virulent *M. bovis* strain (64). Calmette and Guerin performed 230 *in vitro* passages of *M. bovis* until the organism lost its virulence. While this strain has been used worldwide as a live attenuated vaccine to immunize people against TB, with highly variable efficacy (65), it may cause disease in humans. In many high TB incident countries, the BCG vaccination is mandatory and free of charge, given on the first three days after birth, showing protection in children against more serious forms of TB (66,67), although in adults, protection varies from 0 to 80% (68).

1.4.3 *Mycobacterium africanum*

M. africanum was first described in 1968 in a Senegalese patient (69), after that it was found almost exclusively in West Africa. The prevalence of *M. africanum* varies from 5,3% in the Ivory Coast (70), 47,1% Guinea-Bissau (71) and 67,7% in Uganda (72).

M. africanum is phenotypically heterogeneous, with characteristics common to both *M. tuberculosis* and *M. bovis*. Based on their geographic origin and biochemical characteristics, two subgroups of *M. africanum* have been described, in western Africa (subtype I) and eastern Africa (subtype II) (73).

1.4.4 *Mycobacterium canettii*

M. canettii, a rare variant of the *M. tuberculosis* complex with smooth colony morphology was first isolated from a Somali-born patient in 1969 by Canetti (74). *M. canettii* differs from the other *M. tuberculosis* complex strains by having large amounts of lipooligosaccharides on the cell wall (75). The smooth and glossy colonies produced are highly exceptional for this species. This smooth phenotype is however unstable and can switch to a rough colony morphology (74).

1.4.5 *Mycobacterium microti*

M. microti, is the causative agent of TB in voles, wood mice, and shrews and can also cause disease in a limited number of other mammalian species.

It was described for the first time by Wells in 1946, in voles (*Microtus agrestis*) from Great Britain (76). In humans, it was first reported in 1998 in immunocompromised patients (77), although human to human transmission of *M. microti* infection seems to be rare (78).

Based on biochemical properties, this bacterium is difficult to distinguish from *M. tuberculosis*, *M. africanum*, or *M. bovis*, but *M. microti* strains display characteristic Insertion

sequence (IS)6110 Restriction Fragment Length Polymorphism (RFLP) banding patterns and spoligotypes, distinct from other *M. tuberculosis* complex strains (77).

1.4.6 *Mycobacterium pinnipedii*

In 1993, it was reported for the first time that isolates from seals captured on the coast of Argentina had a characteristic IS6110 RFLP pattern (79). This seal bacillus was later designated *M. pinnipedii* and appeared to have a unique position in the *M. tuberculosis* complex (79). Later on, reports have described *M. pinnipedii* infections in various marine mammals (80–82).

Transmission of *M. pinnipedii* to humans has been reported in individuals who are in close contact with marine mammals (82,83).

M. pinnipedii isolates present a distinct spoligotype pattern when compared to other members of the *M. tuberculosis* complex (79).

1.4.7 *Mycobacterium caprae*

M. caprae was first isolated from goats in Spain (84), but has since been found in other animals in Europe, such as cattle (85–87), pigs (88), red deer (88) and wild boars (86). Its isolation from humans has also been described (86,89).

Based on biochemical tests, results are similar to *M. bovis* and *M. bovis* BCG. By spoligotyping, *M. caprae* species form a homogeneous cluster easily recognizable by the absence of spacers 1,3-16, 30-33 and 39-43. The lack of spacers 39-43 has also been described in *M. bovis* and *M. microti* (84,90).

1.4.8 Novel variants of the *M. tuberculosis* complex

The novel *M. mungi*, was identified as the causative agent of TB in banded mongooses (*Mungos mungo*), in Botswana (91). *M. mungi* was characterized as highly virulent, causing high numbers of deaths in a short period of time (2–3 months from clinical presentation to death), apparently through environmental transmission (nonrespiratory route) (91).

The *Dassie* bacillus, the causative agent of TB in the dassie (*Procapra capensis*), is considered an infrequent variant of the *M. tuberculosis* complex characterized as being similar to *M. microti*, based on morphology and growth requirements (92), although, they differ in growth preferences and bacillary morphology under microscopy. In terms of pathogenicity, the *Dassie* bacillus was reported to have a very low level of virulence in rabbits and guinea pigs (93). Genome comparison of nine regions of difference (RD) shows that five are shared with *M. microti* (RDs 3, 7, 8, 9, and 10). Although the *Dassie* bacillus does not share the other documented deletions in *M. microti* (RD1^{mic}, RD5^{mic}, MID1, MID2, and MID3) (94).

The *M. oryxis* or *Oryx* bacilli was identified to be the causative agent of TB in oryxes and gazelles (95), deer, antelope and waterbucks (50), although their exact host range remains uncertain. Combined findings based on Single nucleotide polymorphisms (SNP), RD, spoligotyping and 24-locus Mycobacterial Interspersed Repetitive Units–Variable-Number Tandem Repeat (MIRU-VNTR) analysis, placed *M. oryxis* at a distinct phylogenetic position

between the *Dassie* bacillus and *M. microti*. It was proposed by Ingen and colleagues that *M. orygis* was attributed a subspecies status (96).

M. suricattae, closely related to the *Dassie* bacillus was first isolated in meerkats from South Africa, it was proposed as a novel member of the *M. tuberculosis* complex (97). The deletion of the direct-repeat region spacers, i.e. no amplification of any spacer by spoligotyping, distinguishes this strain from all other *M. tuberculosis* complex members (97).

1.5 LABORATORY DIAGNOSIS OF TB

The bacteriological confirmation of TB and the determination of drug susceptibility are essential to ensure that a patient is correctly diagnosed with TB and started on the most effective treatment regimen (1). In 2013, among all new cases of TB, only 58% were bacteriologically confirmed worldwide (1).

1.5.1 Identification of the *M. tuberculosis* complex

1.5.1.1 Microscopy

Smear microscopy, based on the Ziehl-Neelsen (ZN) stain, is often the only diagnostic tool available in resource-limited settings for detection and diagnosis of TB. The purpose of this approach is to ensure detection of most infectious cases with minimal cost, which is essential for low income countries. However, the sensitivity is low when the bacterial load is less than 10,000 organisms/ml sputum sample (98). The sensitivity of smear microscopy is further reduced in diagnosing extrapulmonary TB, pediatric TB and TB in patients co-infected with HIV (99–101), based on the paucibacillary nature of TB disease in these patients. In addition it cannot distinguish *M. tuberculosis* complex from other mycobacteria.

Fluorescence microscopy, based on auramine O fluorescence acid fast stain, is 10% more sensitive than ZN staining (102). This method uses a lower power objectives lens (25x, while the ZN uses 100x), that makes the reading faster. The light-emitting diodes (LED) microscopy now being used for fluorescent microscopy, is less expensive and there is no need of a dark room, which means the same infrastructure as the one for conventional ZN staining, making the implementation much easier.

In order to improve the diagnostics by smear microscopy, the WHO have recommended the gradual substitution of the ZN microscopy to fluorescent microscopy (102).

1.5.1.2 Culture

Culture, considered the most accurate test due to high sensitivity and specificity, is labor-intensive and slow. Clinical laboratories hold cultures for 6 to 8 weeks to achieve maximum sensitivity on solid Lowenstein Jensen (LJ) media. Liquid culture (BACTEC MGIT 960) is the most sensitive culture technique for recovery of mycobacteria from clinical samples (103). The liquid culture it's not currently utilized by all laboratories, particularly in low income countries, as a result of limited funding, reduced number of trained and qualified personnel and proper biosafety management and equipment.

1.5.1.2.1 Phenotypic identification of the *M. tuberculosis* complex

Accurate species identification of the *M. tuberculosis* complex members is essential, particularly in countries with high HIV prevalence, where non-tuberculous mycobacteria (NTM) have been identified in human, and *M. bovis* remains a problem for cattle.

The traditional methods of species identification is relying on the phenotypic character, which is based on biochemical testing including growth characteristics on different media and colony morphology. The colony morphology varies among the *M. tuberculosis* complex species ranging from flat smooth, domed glossy colonies to dry and rough colonies.

Biochemical tests such as nitrate reductase, detection of niacin, growth in the presence of TCH, and catalase activity may be used for differentiation of *M. tuberculosis* complex species (104). All these tests, although simple and inexpensive to perform, require experienced personal to interpret the results and do not clearly differentiate between species (105,106).

1.5.1.2.2 Immunochromatographic identification of the *M. tuberculosis* complex

The immunochromatographic assays, also called lateral flow assays, have been developed to allow differentiation between the *M. tuberculosis* complex and NTM. It uses a monoclonal antibody to detect the MPB64 protein (Rv1980c; also termed as MPT64), which is specifically secreted during growth of *M. tuberculosis* complex bacteria (107). The immunogenic protein MPB64 is highly specific for *M. tuberculosis* complex, except some variants of *M. bovis* BCG (108,109)

Immunochromatographic assays mostly used are commercial kits, including the SD Bioline Ag MPT64 Rapid assay (Standard Diagnostics, Kyonggi-do, Korea), Capilia TB (TAUNS, Numazu, Japan), and the MGIT TBc Identification Test (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD).

1.5.1.2.3 Genotypic identification of the *M. tuberculosis* complex

In recent years, the identification of NTM has become a challenge for clinical laboratories since there are currently more than 150 NTM species catalogued (110).

Molecular biology techniques have been successfully used for identification of the *M. tuberculosis* complex, with the advantage of being more rapid and accurate than conventional methods.

The introduction of radioisotope-labelled DNA probes and acridinium ester-labelled DNA probes (AcuProbes; Gen.Probe) greatly facilitated the identification of commonly isolated mycobacteria. Subsequently, commercially available and in-house developed nucleic acid amplification tests were successfully used for early identification of *M. tuberculosis* complex grown in liquid cultures.

Commercially available systems such as the INNO-LiPA (Innogenetics NV, Ghent, Belgium) in which the 16S-23S rRNA spacer region of mycobacterial species is amplified and the GenoType MTBC (Hain Lifescience GmbH, Nehren, Germany) targeting the 23 rRNA have been successfully used to directly detect and identify *M. tuberculosis* complex. The GenoType MTBC, enables rapid differentiation of *M. tuberculosis* complex bacteria, with higher sensitivity compared to the AccuProbe assay (111).

1.5.1.3 *Recent diagnostic methods*

Although TB diagnosis in many countries is still reliant on smear microscopy and culture, recent techniques are changing the landscape of TB diagnostics, presenting a pipeline of various new tools, particularly molecular methods (112).

The GeneXpert

The Cepheid GeneXpert System's MTB/RIF assay is a single use cartridge-based semi-quantitative nested real-time Polymerase Chain Reaction (PCR) in-vitro diagnostic test that detects *M. tuberculosis* complex DNA and rifampicin resistance associated mutations of the RNA polymerase beta (rpoB) gene.

Based on a WHO meta-analysis of the sensitivity and specificity of Xpert MTB/RIF, the test has shown very high sensitivity in sputum samples, 98% in smear-positive, culture positive and 79% in people living with HIV. Regarding extrapulmonary samples, the test shows high sensitivity when compared to culture, in diagnosing extrapulmonary TB from lymph node tissues or aspirates (84.9%), gastric lavage (83.8%), cerebrospinal fluid (79.5%) and other tissue specimens (81.2%). By contrast pleural fluid samples did not demonstrate good sensitivity (43.7%). The specificity is notably high in all groups, more than 92.5% (113).

Detection of lipoarabinomannan (LAM) in urine sample

A number of mycobacterial antigens can be detected in the urine of patients with pulmonary TB, but the most promising of these to emerge is the cell wall lipopolysaccharide lipoarabinomannan (LAM) (114–117).

The commercial test (Allere-Determine TB LAM in urine) is simple to use, gives rapid results, there is no need of instruments and has low cost. The test is sensitive in patients with advanced HIV disease but not in HIV negative adults and HIV positive adults with CD4 counts higher than 100 cells per microliter. (118–121).

1.6 STRAIN IDENTIFICATION OF THE *M. TUBERCULOSIS* COMPLEX

Genotyping methods are based on the analysis of chromosomal DNA of the *M. tuberculosis* complex.

A large number of different molecular methods have been developed to measure the genetic relationship between different *M. tuberculosis* complex strains. Ideally, molecular genotyping tools should be inexpensive, highly discriminative, deliver rapid results, be straightforward to perform, and produce easily and interpretable results that allow for accurate comparison between laboratories (122). In order to discriminate between bacterial strains as much as possible, the best approach would possibly be whole genome sequencing for each strain. As this is at present relatively costly, and require specialized laboratories, only parts of the genome are being examined. Each molecular method provides specific genetic profiles referred to as fingerprints. When two or more strains have identical fingerprints they are referred to as the same cluster and may be epidemiologically linked (123).

1.6.1 Spoligotyping

Spoligotyping is a simple, rapid, reproducible and cost effective method for simultaneous detection and differentiating of the *M. tuberculosis* complex without the need of purified DNA. The method is based on the polymorphism in direct repeat (DR) locus which consists of multiple direct variable repeats (DVR). Each DVR is composed of 36 bp-DR and a non-repetitive short sequence also called spacer (124).

Spoligotyping can be applied directly to cultured cells and to clinical samples (125).

The results, expressed as positive or negative for each of the 43 spacers, can be readily digitalized. Polymorphism in the DR locus do not discriminate the *M. tuberculosis* complex as well as IS6110 does (i.e., strains with different IS6110 RFLP patterns may have the same spoligotype).

Spoligotypes can be assigned to the major phylogenetic lineages according to signatures provided in the international *M. tuberculosis* molecular markers database, SITVIT2 database, of the Pasteur Institute of Guadeloupe (<http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/>), which is an updated version of the previously released SpolDB4 database (57). This database defines 62 genetic lineages/sublineages (57), which are often named after regions, countries, cities or places of high prevalence. These include specific signatures for the various *M. tuberculosis* complex members, as well as rules defining major lineages/sublineages for *M. tuberculosis sensu stricto*.

The various spoligotyping-defined lineages fit well into three large phylogenetical groups: ancestral Tuberculosis-specific deletion 1 (TbD1)+/Principal Genetic Group (PGG)1 group (East African Indian, EAI), modern TbD1-/PGG1 group (Beijing and Central Asian or CAS), and evolutionary recent TbD1-/PGG2/3 group (Haarlem - H, X, S, T, and Latin American and Mediterranean or LAM) (126). However, proper epidemiologic and phylogenetic inferences are not always an easy task due to a lack of understanding of the mechanisms behind the mutations leading to the polymorphism of these genomic targets. It was demonstrated that phylogenetically unrelated *M. tuberculosis* complex strains could be found with the same spoligotype pattern as a result of independent mutational events (homoplasy) (127), an observation that corroborates the fact that spoligotyping is prone to homoplasy to a higher extent than the MIRU-VNTRs (128). Furthermore, spoligotyping has little discriminative power for families associated with the absence of large blocks of spacers, e.g., the Beijing lineage.

1.6.2 IS6110-RFLP

RFLP was the first standardized molecular typing method. This method is based on the detection of the IS6110. The IS6110 is present in different copy numbers and integrated at different chromosomal sites in *M. tuberculosis* complex isolates. The fragments based on the IS6110 are highly polymorphic but stable enough for epidemiological studies. Strains with fewer copies of IS6110 are more homogenous and the fingerprints are not as reliable concerning epidemiological links as of those containing multiple copies (95).

In this technique, a restriction enzyme, *PvuII* is used to digest *M. tuberculosis* DNA and southern blots of the DNA electrophoresed on agarose gel are probed with a fragment of IS6110 that lies upstream of *PvuII* site. The RFLP patterns are entered into a computerized database and analyzed with an image analysis system.

1.6.3 MIRU-VNTR

This is a PCR based method that analyses multiple independent loci containing variable numbers of tandem repeats (VNTR) of different families of interspersed genetic elements collectively called mycobacterial interspersed repetitive units (MIRU) (129). In its original format, the PCR primers were each run in separate reactions and the sizes of the products were analyzed by gel electrophoresis.

For the *M. tuberculosis* complex, the 24-loci MIRU-VNTR is the current reference method for surveying transmission events (130,131). A set of 24 MIRU-VNTR loci was standardized to increase the discrimination power (131).

The advantages of MIRU-VNTR analyses are that the results are intrinsically digital and analysis can be applied directly to culture without the need for DNA purification. The discriminatory power of MIRU-VNTR analysis is typically proportional to the number of loci evaluated; in general, when only the 12 loci are used, it is less discriminating relative to IS6110 RFLP genotyping for isolates with high-copy-number IS6110 insertions but more discriminating than IS6110 RFLP genotyping for isolates with low-copy-number IS6110 (47). When more than 12 loci are used, or MIRU analysis is combined with spoligotyping, the discriminatory power approximates that of IS6110 RFLP analysis (47). MIRU-VNTR genotyping has been used in a number of molecular epidemiologic studies, as well as to elucidate the phylogenetic relationships of clinical isolates (132–134) and evaluating *M. bovis* transmission (135).

The standard 24 loci MIRU-VNTR typing lacks resolution power for accurately discriminating closely related clones that often compose the Beijing strain populations, thus it was proposed a 4 hypervariable MIRU-VNTR loci set as a consensus for subtyping Beijing clonal complexes and clusters, after standard typing for epidemiologically relevant subtyping in order to ensure transition until whole-genome sequence analysis might become universally accessible for TB surveillance (136).

1.6.4 Genomic deletion analysis

Regions of difference (RD) are used to differentiate between species in the *M. tuberculosis* complex. It is a rapid, simple and reliable PCR-based *M. tuberculosis* complex typing method that makes the use of *M. tuberculosis* complex chromosomal region-of-difference deletion loci. Several specific primers are used to amplify specific loci which together formed a *M.*

tuberculosis complex PCR typing panel (49). The final pattern of amplification products of all reactions, given by failure or success, segregates the tested strains from NTM isolates and by *M. tuberculosis* complex subspecies identity. The panel not only provides an advanced approach to determine the subspecies of *M. tuberculosis* complex isolates but also differentiate them from clinically important NTM species (49).

1.6.5 Single nucleotide polymorphisms

Single nucleotide polymorphisms (SNPs) are a common form of genetic variation in *M. tuberculosis* complex. Generally, SNPs represent single nucleotide differences between at least two DNA sequences. To generate data on SNP, it requires comparative sequencing of multiple genes or whole genomes in two or more strains of interest (137). Thousands of SNPs have been discovered in clinical isolates of the *M. tuberculosis* complex thanks to advances in DNA sequencing (138).

SNPs represent robust markers for inferring phylogenies and for strain classification (139), because of the low frequency of SNPs and limited ongoing horizontal gene transfer in the *M. tuberculosis* complex, resulting in low levels of homoplasy (i.e. the independent occurrence of the same SNP in phylogenetically unrelated strains) (138,140). In addition, SNPs carry functional information, including drug resistance-conferring mutations and can also be used to construct transmission networks (141–143).

1.6.6 Whole genome sequencing

Genome sequencing, generates a complete information of a strain, including the evolutionary background, drug resistance mutations, virulence-associated polymorphisms, and assessment of TB transmission (144–146), distinguish between relapse and reinfection (147) and delineates outbreaks (143).

Large-scale DNA sequencing studies have usually been performed by specialized sequencing centers, but in the upcoming years, it is expected that standard laboratories will be able to perform it (148), making it more accessible and affordable. It's also predicted that whole genome sequencing will at least partially replace all previous genotyping methods for the *M. tuberculosis* complex (149).

2 THE PRESENT INVESTIGATION

2.1 STUDY RATIONALE

Mozambique is one of the high burden TB countries and little information was available regarding the genetic diversity of *M. tuberculosis* complex strains in the country.

Furthermore, there was no evidence available to define whether bovine TB represents a public health problem in Mozambique, especially in HIV infected individuals.

This thesis describes the molecular epidemiology of the *M. tuberculosis* complex in Mozambique, identifies the predominant genotypes responsible for TB transmission and prevalence, and investigates the association between predominant spoligotypes and HIV sero-status, prevalence and transmission in Mozambique.

Furthermore, with the aim to determine the occurrence of *M. bovis* in humans in Mozambique, extrapulmonary cases were investigated, including a region where bovine TB is a problem in cattle, in order to contribute to a better understanding of the importance of this zoonotic disease, what impact it has on the TB epidemic as a whole, and to provide clues on how to improve TB control programs with respect to human TB.

2.2 OBJECTIVES

2.2.1 General objective

- To characterize isolates of the *M. tuberculosis* complex and estimate the relative prevalence of bovine TB in humans in Mozambique.

2.2.2 Specific objectives

- By molecular genetic methods characterize *M. tuberculosis* complex isolates into sub-families and clones.
- To relate the findings from pulmonary and extrapulmonary cases.
- To correlate these findings with the findings in husbandry.
- To relate the obtained result with international databases and with the results of other studies accomplished in neighbouring countries.
- To study the transmission of TB in HIV co-infected patients in the community by assessing the degree of strain clustering.

2.3 MATERIAL AND METHODS

Table 1. Summary of the methods of all papers and study V

	Main aim	Study area	Sample size	Specimen	Diagnostic methods	Genotyping methods
Paper I	To identify the predominant spoligotypes and lineages responsible for pulmonary TB in Mozambique.	South and North regions of Mozambique	445	Sputum	Smear microscopy, ZN Culture DST	Spoligotyping RD105
Paper II	To investigate the prevalence and possible transmission of Beijing strains in Mozambique	Mozambique, all country	543 spoligotyped; 33 Beijing lineage isolates	Sputum	Smear microscopy, ZN Culture DST	Spoligotyping MIRU-VNTR RFLP RD 105, 142, 150, 181
Paper III	To develop a Multiple-locus variable-number tandem repeat analysis (MLVA) based classification of <i>M. tuberculosis</i> genotype lineages	Global	7793	NA	NA	Spoligotyping MIRU-VNTR IS6110 insertional events
Paper IV	To characterize the isolates from TBLN	Maputo, capital of Mozambique	110 recruited; 45 genotyped	Lymph node fluid	ZN cytology Culture Line Probe Assay	Spoligotyping MIRU-VNTR
Study V	To investigate the transmission of <i>M. bovis</i>	Govuro district, province of Inhambane, Mozambique	24	Sputum and lymph node fluid	Smear microscopy, ZN Culture	Spoligotyping MIRU-VNTR

2.3.1 Study area, patients and samples

2.3.1.1 Paper I and II

Study I and II included *M. tuberculosis* isolates collected during a one year (2007-2008) nationwide drug resistance surveillance study performed by the National TB Control Program of Mozambique in 40 randomly selected districts around the country.

A total of 445 isolates from patients older than ≥ 15 years, new pulmonary TB cases i.e. patients with pulmonary TB who had never been treated for TB or had been treated for less than 30 days, from seven provinces of Mozambique (Maputo City, Maputo Province, Gaza, Inhambane, Nampula, Cabo Delgado and Niassa) were included in study I.

In paper II, the study was extended to include isolates also from the Central Region of Mozambique, with the aim to investigate the prevalence and possible transmission of Beijing strains in Mozambique. A total of 543 *M. tuberculosis* isolates from Mozambique were spoligotyped. Of these, 33 belonging to the Beijing lineage, were included.

In order to compare with Mozambican Beijing strains, 13 previously characterised isolates from South Africa representing different Beijing sublineages were included in study II as reference strains and genotyped by IS6110-RFLP. To allow comparison with Mozambican strains by MIRU-VNTR, 54 previously described isolates from South Africa (122) were also included.

2.3.1.2 Paper III

Study III made use of available genotyping data or in-house typing of six different subsets of *M. tuberculosis* complex clinical isolates encompassing 7793 strains of diverse geographical origin as follows:

- 1) Spoligotyping and 12-loci MIRU-VNTR data on 7009 strains from the SITVIT2 proprietary database of Institut Pasteur de la Guadeloupe, n=5990 strains genotyped by various investigators, list available through http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE; n=1019 strains genotyped at Institut Pasteur de la Guadeloupe as follows: Guadeloupe n=203; Martinique n=88; French Guiana n=364; Dominican Republic n=88; Colombia n=134; and Turkey n=142.
- 2) Genotypic data on 176 *M. tuberculosis* complex isolates from the MIRU-VNTRplus database (<http://www.miru-vntrplus.org/MIRU/index.faces>). The aim of this selection was to compare the MLVA based classification of *M. tuberculosis* complex strains developed during this study versus previous labeling using SpolDB4 (57) and Large Sequence Polymorphism (LSP)-based classification (139,150).
- 3) The MIRU-VNTR rules were further evaluated on a subset of LAM strains to describe the novel RDrio lineage (151). This group was subdivided into two subgroups: 100 strains with RDrio deletion and 90 wildtype strains.
- 4) To test a hypothesis about an Asia-to-Africa back migration theory based on the study of Y-chromosome haplogroups at Neolithic times (152), published data on 154 *M. tuberculosis* complex strains from the north west of Iran (153) was also used.

5) To compensate the lack of MIRU-VNTR data on *M. tuberculosis* complex isolates from East-Africa in all published genotyping databases, 100 strains from Mozambique, from study I (154) were typed.

2.3.1.3 Paper IV

Study IV was conducted from July 2013 to July 2014 at the Pathology Service of Maputo Central Hospital. The Pathology Service of Maputo Central Hospital is the only referral site in Maputo for diagnosing TBLN, patients suspected of mycobacterial infection are referred from different health units for diagnosis. During the study period, they have received 677 patients suspected of TBLN, of whom, 110 (16.2%) were included in the study. Only patients suspected of TBLN that consented and could give at least 0,1ml of sample were recruited to participate in the study. That was applied in order to have enough material for the routine smear performed in the unit, direct microscopy using conventional ZN staining and cytology, and subsequent assays to be performed within the study.

The swellings observed were cervical, axillary or from other sites, either as a unilateral single or multiple mass or masses. Fistula formation could also be seen in certain cases.

Patients who also had pulmonary involvement were considered as extrapulmonary TB in our analysis.

2.3.1.4 Study V

Study V is still being conducted in the district of Govuro, province of Inhambane, Mozambique. Govuro is a region with known high prevalence of bovine TB in cattle.

Patients ≥ 18 years, suspected of pulmonary TB or TBLN, from the Donde health center or from the community were recruited to participate in the study. The sample collection process started in April 2015 and preliminary results of 24 patients are presented.

2.3.2 Sample processing

Clinical specimens were processed at the individual district laboratories for smear microscopy and sputum and sputum or lymph node fluid samples were then referred to the National TB Reference Laboratory for further testing.

Inactivated cultures were sent to the Center of Molecular Biology of Eduardo Mondlane University, in Maputo, for molecular characterization and extended analysis was performed at the Public Health Agency of Sweden (former Swedish Institute for Communicable Disease Control), in Stockholm.

2.3.3 HIV testing

All patients suspected of having TB were advised and tested voluntarily for HIV/AIDS. The patient had a right to refuse HIV testing.

HIV testing was performed at the Health Unit of enrolment, for patients who consented to undergo testing, according to the recommendations by the Ministry of Health of

Mozambique. Two rapid HIV tests were used sequentially, Unigold Recombinant HIV (Trinity Biotech, Wicklow, Ireland) and Determine HIV-1/2 (Abbot, Tokyo, Japan). Samples were tested first with Determine and reported only when negative. Positive samples were confirmed with Unigold. All tests were performed and interpreted according to the manufacturer's instructions.

2.3.4 Chromosomal DNA isolation

Briefly, mycobacteria were harvested, heat killed at 80°C for 20 minutes and then subjected to repeated freeze thawing. Bacteria were resuspended in TE (Tris; EDTA) buffer and lysed for two hours at 37°C. Incubation were made at 65°C with Sodium Dodecyl Sulphate, Proteinase K and finally with Cetyl Trimethyl Ammonium Bromide. A mixture of Chloroform-Isoamyl Alcohol was added and DNA was at last precipitated using isopropanol. The pellet was centrifuged, washed with 70% ethanol and re-dissolved in TE buffer.

2.3.5 Spoligotyping

Spoligotyping, previously described in the introduction, was performed to assign all isolates to lineages and sublineages. It was performed on genomic DNA according to the manufacture instructions (Isogen Bioscience BV, Maarsen, The Netherlands).

In brief, the DR region was amplified with specific primers and amplified DNA was hybridized with a set of 43 spacer oligonucleotides covalently linked to a membrane. A hybridization pattern was obtained and subsequently visualized by incubation with streptavidin peroxidase (Roche Diagnostics, Germany) followed by detection with Enhanced Chemiluminescent Detection system (Amersham Biosciences, UK). Appropriate controls; H37Rv, *M. bovis* BCG, and PCR mixture without DNA were used with each experiment.

Spoligotyping results were analyzed and dendograms created using the BioNumerics Software version 5.01 (Applied Maths, Kortrijk, Belgium) for papers I and II and version 7.5 (Applied Maths, Kortrijk, Belgium) for paper IV. Spoligotyping patterns were also compared with the ones existing in the international Spoligotyping database SITVIT2, which is an updated version of SITVITWEB (126).

2.3.6 RFLP

IS6110 RFLP genotyping, previously described in the introduction, was performed using the insertion sequence IS6110 as a probe and *PvuII* as the restriction enzyme. Visual bands were analyzed using the BioNumerics software version 5.01 (Applied Maths, Kortrijk, Belgium). Strains with identical RFLP patterns (100% similarity) and five or more hybridizing bands were judged to belong to a cluster. On the basis of the molecular sizes of the hybridizing fragments and the number of IS6110 copies of each isolate, fingerprint patterns were compared by the un-weighted pair-group method of arithmetic averaging using the Jaccard coefficient. Dendograms were constructed to show the degree of relatedness among strains according to a previously described algorithm (155) and similarity matrixes were generated to visualize the relatedness between the banding patterns of all isolates.

2.3.7 RD analysis

RD analysis was used in paper I and paper II, to investigate five Manu pattern isolates for the presence of genomic deletion of RD105 (deleted in the Beijing lineage) and for identification of the genomic deletions RD105, RD142, RD150 and RD181 in Beijing isolates, respectively.

The DNA was analyzed by PCR using primers previously described (156). PCR was carried out under the following conditions: 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, 0.5 mM primers, 0.2 mM deoxynucleoside triphosphates, 1U of Taq polymerase (Dynazyme) and 10ng DNA per 50ml of reaction mixture. PCR amplification was performed under the following conditions: 95°C for 15 min, followed by 35 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 3 min. 10µl aliquots of PCR products were analyzed using 2% agarose gel electrophoresis.

2.3.8 MIRU-VNTR

Standardized 24-locus MIRU-VNTR typing (131) 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 ABI3131xl 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.

2.3.9 Phylogenetic analysis

For paper III, phylogenetic inferences were drawn using two applications: BioNumerics (v. 3.5, Applied Maths, Sint-Marteen-Latem, Belgium), and MrBayes3 (available through <http://mrbayes.csit.fsu.edu/>) (157). BioNumerics v. 3.5 (Applied Maths, Sint-Marteen-Latem, Belgium) was used for phylogenetic reconstruction based on a "Minimum Spanning Tree" (MST) algorithm to draw MSTs. For this purpose, allele strings were imported into a BioNumerics software package and a MST was created based on categorical and the priority rules (<http://www.applied-maths.com/bionumerics/plugins/mlva.htm>) with highest number of single locus variants (SLV's). MrBayes3 was used to infer phylogeny relationships among the newly defined MIRU-VNTR lineages of *M. tuberculosis* sensu stricto using a bayesian approach that is particularly useful to reconfirm MST results (157).

For paper IV, phylogenetic relationships were calculated using MLVA Compare software v. 1.03 (Genoscreen and Ridom Bioinformatics). MSTs were drawn from spoligotyping and 24-loci MIRU-VNTR typing, to better visualize probable relationships and dependencies between isolates.

2.4 RESULTS AND DISCUSSION

2.4.1 Paper I

The primary objective of paper I was to use spoligotyping to characterize isolates from the South and North Regions of Mozambique, to assign all the strains in the study to the major clades in the SITVIT2 database and to describe the geographical distribution of predominant lineages. Additionally, the association between predominant strains and age, HIV status and geographical location were investigated.

In total we studied 445 isolates from new pulmonary TB cases from seven provinces of Mozambique. Of these, 282 were from the South region of the country and 163 were from the North. Of all patients, 98 (22%) were HIV positive, 122 (27.4%) HIV negative and 225 (50.6%) were not tested for HIV.

The predominant lineage was the LAM with 37% of all isolates; followed by the East African Indian (EAI), an evolutionary recent T clade, and the globally-emerging Beijing clone (Figure 3). The predominance of the LAM lineage was not surprising as it is believed that this lineage is globally disseminated, causing about 15% of TB worldwide (158).

M. tuberculosis genotype distribution of the predominant lineages from the South and North regions of Mozambique is illustrated in Figure 3. A comparison of spoligotype distribution among the two regions indicates that the LAM, EAI and T lineages were common across the country, while the Beijing lineage was found to be more common in the South 27/282 (9.6%) compared to the North 4/163 (2.5%).

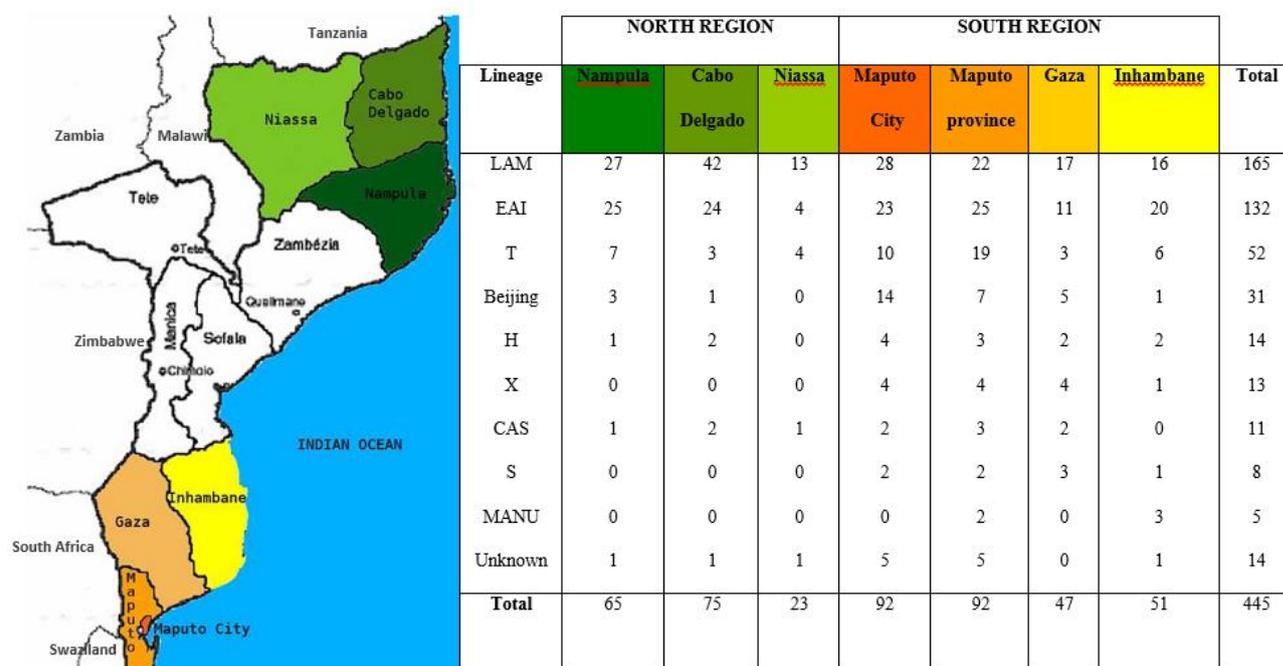


Figure 3. Geographical distribution of *M. tuberculosis* predominant spoligotype lineages in 7 provinces of Mozambique. The map describes the geographical distribution of predominant spoligotype lineages in Maputo city, Maputo province, Gaza, Inhambane, Nampula, Cabo Delgado and Niassa. The number of isolates per lineage in each province is depicted.

When the spoligotyping results and clade definitions were linked to the distribution of clinical isolates within PGG 1 versus PGG2/3 (characterized by the lack of spacers 33-36), it was evident that 185 or 41.6% of the isolates belonged to PGG1 (ancient lineages) as compared to

260 or 58.4% to the PGG2/3 (modern lineages), with a high spoligotype diversity documented for EAI, LAM and T lineages (Figure 4). The wide diversity found may be attributed to the extensive human movement in the country mainly due to Mozambican migration to neighboring countries and internal migration to look for better life conditions, since the structure of the TB population is determined by geography, demography and human migration. A great part of Mozambican migrants are miners working in South Africa where the incidence of HIV among miners is known to be high, varying from 10 to 20% (151); Being a mineworker is also considered a risk factor for HIV in Mozambique (159).

Paper I also attempted to describe the worldwide distribution of predominant Shared International Types (SITs) i.e. an identical pattern shared by two or more patients worldwide (within this study, or matching another strain in the SITVIT2 database). It was observed that many of the predominant SITs were more frequently present in Eastern and Southern Africa (mostly among its immediate neighbours Zimbabwe, Zambia, South Africa, Malawi, and to a lesser extent to Tanzania, Namibia, and Somalia) (57).

At the time of this comparison, in the SITVIT2 database no Manu strains were reported from Mozambique, and with the exception of three Manu1 lineage strains isolated in Tanzania, all the remaining *M. tuberculosis* Manu strains isolated from Africa belonged to the Manu2 sublineage. Hence, study I constitutes the first evidence of the presence of the Manu lineage in Mozambique. With both Beijing and Euro-American strains (lacking spacers 33-36) circulating in Mozambique, some of the Manu2 patterns on the other hand appear to result from mixed infections of Beijing and Euro-American TB. Such a mixture has been described in adjacent South Africa (134).

No *M. africanum* isolates were detected. *M. africanum* is highly prevalent in West African countries, with its epicenter in Guinea Bissau (71,160,161) but is rarely seen in East and Southern Africa (162,163).

The total absence of *M. bovis* in this one year study is noteworthy. Although bovine TB is an important disease of cattle and other domestic animals in Mozambique, no *M. bovis*, the causative agent of bovine TB, was found. One reason could be that only sputum isolates were studied. *M. bovis* is thought to spread through unpasteurized milk, and hence would mainly cause abdominal or disseminated TB. Another reason could be that we did not study areas with high prevalence of bovine TB in cattle as this information was not accessible at that time. In study V, with the aim to evaluate the public health importance of this zoonosis, preliminary results from a study performed in a region with known high prevalence of bovine TB in cattle are presented.

M. tuberculosis genotype distribution of the predominant lineages from the South and North regions of Mozambique indicates that the LAM, EAI and T lineages were common across the country, while the Beijing lineage was found to be more common in the South 27/282 (9.6%) compared to the North 4/163 (2.5%). Because of the strong relationship, migration history and geographic proximity of Mozambique and South Africa we further investigated the Beijing isolates found in this study and compared our findings with the ones from South Africa as described in paper II.

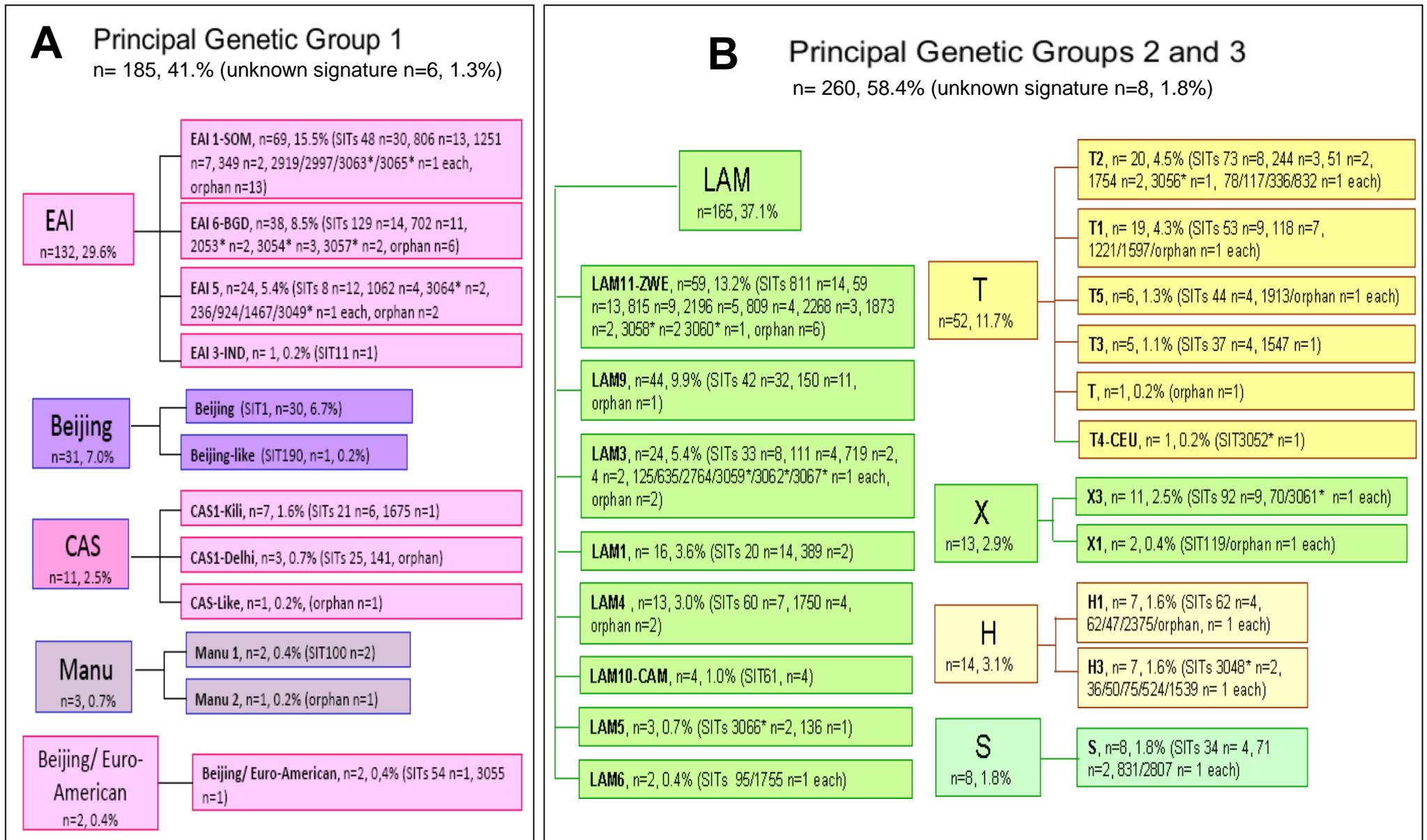


Figure 4. The principal genetic groups (PGG) in Mozambique.

2.4.2 Paper II

Paper I reported the genotypic lineages of 445 *M. tuberculosis* isolates collected from the North and South regions of Mozambique. The Beijing family was found to be the fourth predominant lineage, and the Beijing SIT1 was the third most frequent single spoligotype in Mozambique. In study II, we investigated in depth the isolates belonging to the Beijing lineage, by extending the study to include isolates also from the Central Region of Mozambique, with the aim to investigate the prevalence and possible transmission of Beijing strains in Mozambique.

The Beijing genotype has a worldwide distribution (47,164) and it was reported to be associated with HIV infection (165) and MDR-TB (164,166,167). In this study, 14 (10.9%) of the 129 HIV positive patients had Beijing strains while 6/141 (4.3%) of HIV negative patients had Beijing strains. Thus HIV positive serostatus was significantly ($p=0.049$) more common in patients with Beijing strains than in patients with non-Beijing strains in a univariate analysis. In a multivariate analysis (adjusted for age, sex and province) the correlation remained significant ($p=0.023$). The fact that a significant number of patients with Beijing strains were HIV positive is worrying. Beijing strains have recently been reported to be associated with HIV positive serostatus also in South Africa (165), a connection which is further supported here. This association can be due to a combination of increased virulence of the strains and an increased susceptibility of HIV infected patients to these strains.

When we analyzed the association between HIV infection and the other most prevalent lineages in the country, an association with HIV was found for the LAM lineage, but for the EAI and T lineages no association with HIV was observed, this finding warrants further investigation.

Although Beijing strains have been associated with MDR-TB, only one case was identified in this study.

In relation to the geographic distribution of Beijing strains, we found that the majority of the Beijing strains were from the Southern region ($n=29$), with a prevalence of 10.1% (29/288) while in the North the prevalence was 2.4%, (4/164). In the Central region, none of 91 isolates were of Beijing genotype (Figure 5). In the Southern region we found that, although present in the four provinces, the Beijing lineage was most common in Maputo City 16/95 (16.8%), compared to the other provinces ($p<0.001$). In a multivariate analysis the correlation remained significant between Beijing genotype isolates and Maputo City ($p=0.004$), suggesting that Maputo City and its surroundings is the likely origin.

Based on spoligotyping, Beijing genotype isolates were identified by the deletion of spacers 1–34, and the presence of at least three of the nine spacers 35–43 in the DR locus of the *M. tuberculosis* genome.

Of the 33 strains that were defined by spoligotyping to be of the Beijing genotype, 32 had all the characteristic spacers 35–43, corresponding to the shared type SIT1 as defined in SITVIT2 (Table 2). One strain (isolate 35) in addition lacked spacer 40, corresponding to SIT190.

Analysis of large sequence polymorphisms has shown that the Beijing lineage has evolved into distinct branches defined by specific RD deletions. The large deletion of RD105 is considered to be a marker for Beijing strains (156,168,169), although deletion of the RD105 was also found in ancestral strains with non-Beijing spoligoprofiles (170). From the present investigation, 32 of the Beijing genotype strains (31 SIT1 isolates and the SIT190 isolate) were analyzed for RD deletions. One isolate was not analyzed because there was insufficient

DNA. The majority of the isolates (n=28) had the RD105 and RD181 deletions, while RD150 and RD142 were intact. Four strains lacked RD105, RD181 and RD150 while RD142 was intact (Table 2).

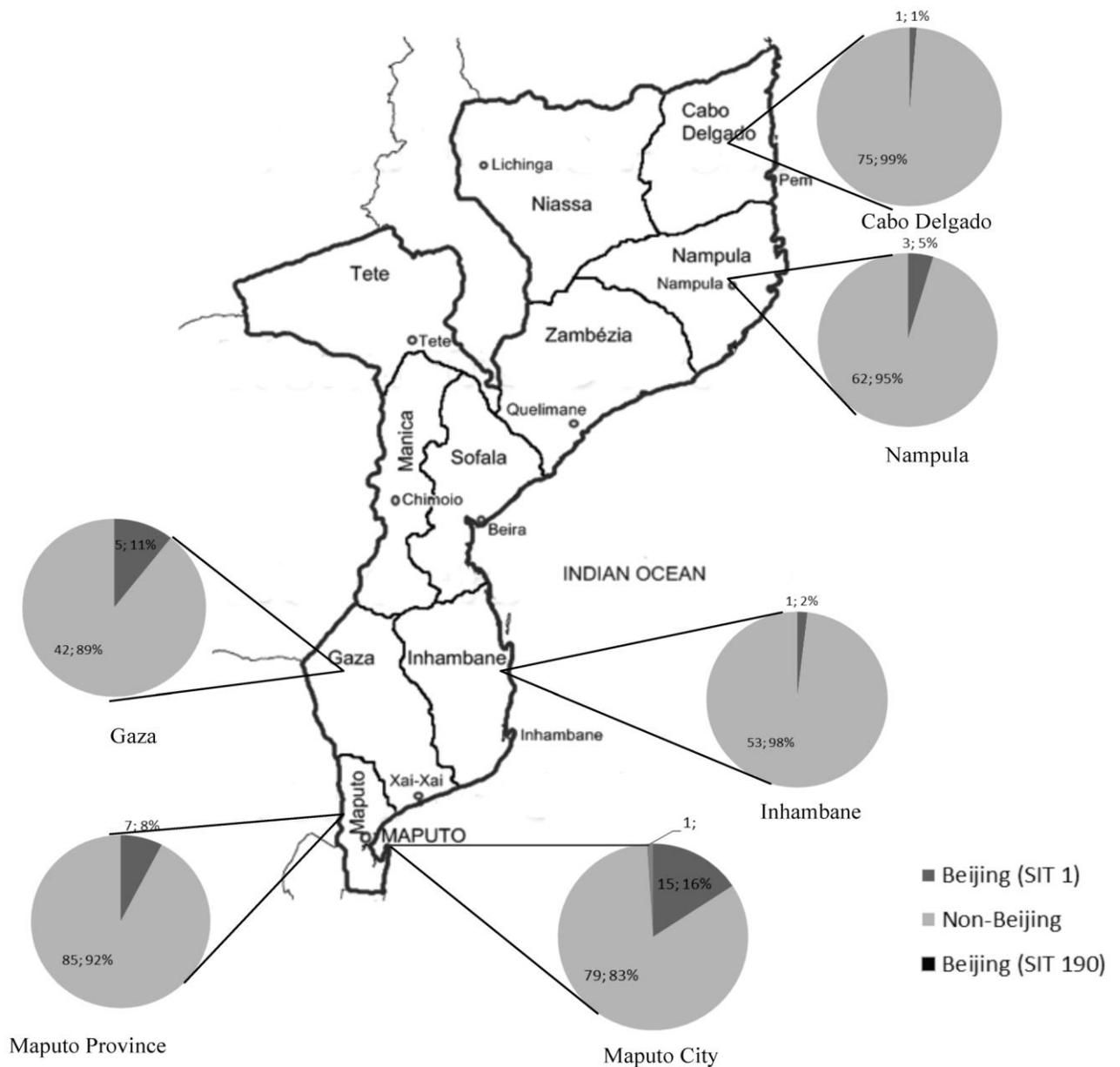


Figure 5. Distribution of Beijing genotype in Mozambique.

By a combined use of RD deletions and spoligotyping the 32 Beijing strains could be tentatively divided into three genetic sublineages, A, B and C (Table 2).

All Beijing genotype isolates (defined by spoligotyping) in this study had the RD105 deletion. Additional deletions of RD142, RD150 and RD181 may further divide this family into different sublineages (156). Interestingly, four out of 32 isolates (sublineage C) lacked RD150, a relatively rare deletion, present in a recently evolved sublineage, “sublineage 7”, in South Africa, reported to be associated with increased transmissibility and/or pathogenicity (168). Moreover the sublineage C isolates were very similar to South African sublineage 7 by 24-loci MIRU-VNTR and RFLP.

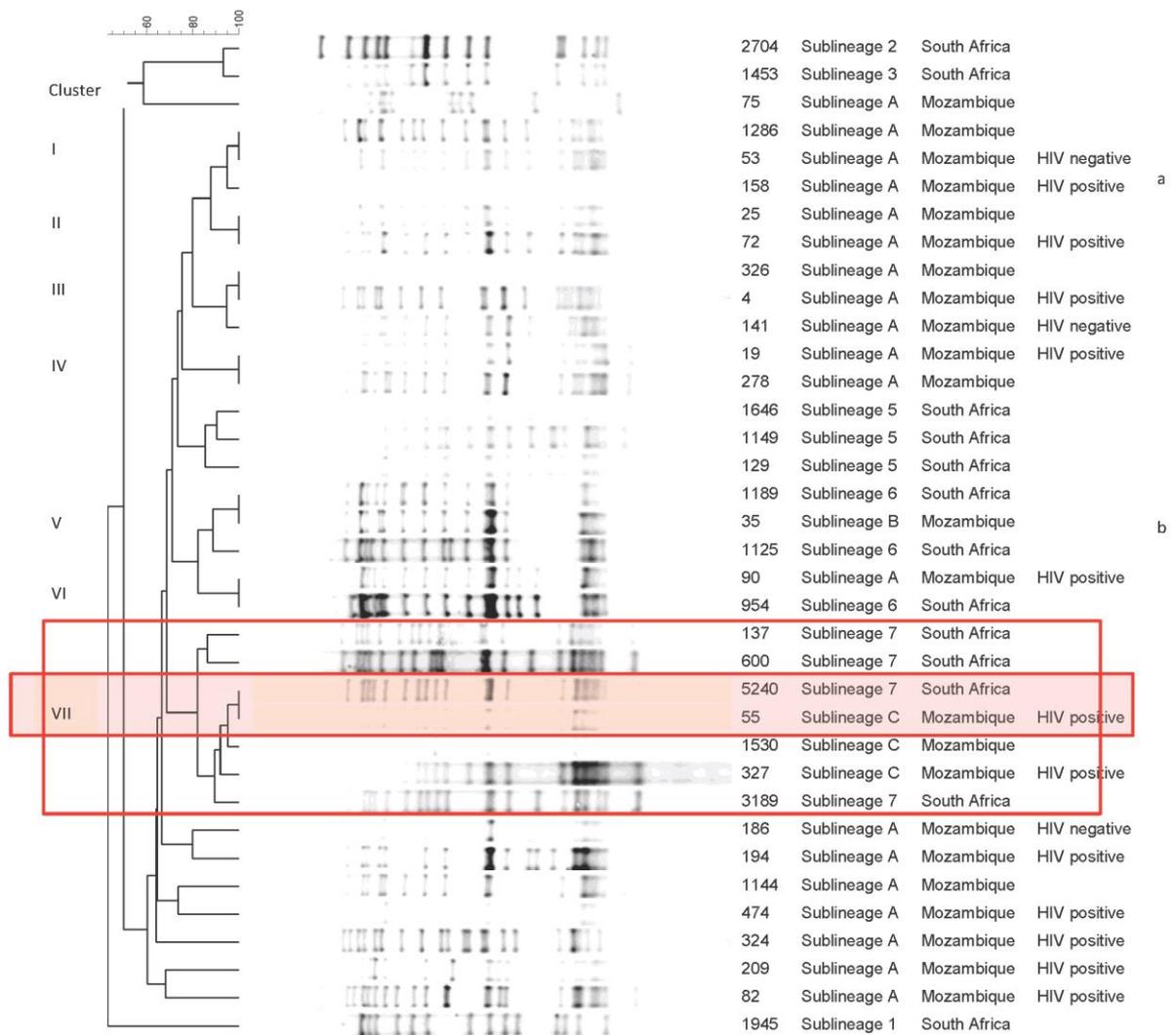


Figure 6. IS6110 RFLP dendrogram of Beijing genotype strains from Mozambique and South Africa.

The dendrogram includes 36 *M. tuberculosis* Beijing genotype strains, 23 from Mozambique and 13 from South Africa. Red rectangle indicates sublineage 7 and sublineage C isolates from South Africa and Mozambique respectively; Highlighted area shows the clustered isolates from both sublineages. a) Drug resistant isolate; b) SIT 190 isolate

MIRU-VNTR analysis was done on 30 Beijing strains from Mozambique and the obtained results were compared with 54 isolates from South Africa previously described (122), each one with one unique pattern. The isolates from Mozambique formed seven clusters (2 or 4 isolates per cluster). Although the Mozambican sublineage C isolates analyzed (55, 327 and 1530) did not cluster by 24 loci MIRU-VNTR to any of the South African “sublineage 7” isolates, they had similar patterns.

Mozambique and South Africa are neighbouring countries and have a history of cross border migration. The fact that four of the Beijing genotype isolates had the RD150 deletion, were clustered or were similar by RFLP with South African “sublineage 7” isolates (168), and also by MIRU-VNTR were close to the South African “sublineage 7” isolates, and considering the high endemicity of this sublineage in South Africa and the low prevalence in Mozambique (4/33) suggests that this sublineage could have been recently introduced in Mozambique from South Africa.

The findings on the low clonality of the strains by MIRU-VNTR and RFLP demonstrate that the population structure of the Beijing genotype in Mozambique consists of more than one sublineage, indicating that these strains were introduced to the country on separate occasions.

There are certain limitations of the present study. The study is based on a sample of isolates from a drug resistance survey, and may not reflect the true population structure. The low number of viable specimens (543 *M. tuberculosis* isolates from a bank of 1124), and a low number of Beijing genotype strains, and the fact that HIV status was not determined for all patients and that not all isolates were genotyped by RFLP and MIRU-VNTR are further limitations of the study. For this reason further longitudinal studies are recommended, both to test the hypothesis that the Beijing genotype is emerging in Mozambique, and to further investigate the potential role of HIV infection in this setting.

2.4.3 Paper III

Considering existing doubts on the ability of spoligotyping alone to reveal exact phylogenetic relationships between *M. tuberculosis* complex strains (128,168), particularly the classification of evolutionary recent TbD1–/PGG2/3 group (171); Six different subsets of *M. tuberculosis* complex isolates encompassing 7793 strains were studied. The purpose of this paper was to: (i) classify these strains based on 12 locus MIRU-VNTR typing data; (ii) to draw the evolutionary history of various *M. tuberculosis* complex members (species, subspecies, groups) leading to the diversity of newly described phylogenetic lineages/groups; (iii) to see how the geographical distribution of these lineages reinforces the history of human settlement in the world, and finally, (iv) to evaluate the MLVA based classification of *M. tuberculosis* complex genotypic lineages as a means to provide with an accurate and robust phylogeographic interpretation of its worldwide diversity. MLVA is useful to establish transmission routes and sources of infections for various microorganisms including *M. tuberculosis* complex.

Phylogenetic inferences were drawn from 12-loci MIRU based MSTs constructed on all the 7009 *M. tuberculosis* complex patterns taken from the SITVIT2 database (for which both spoligotyping and 12-loci MIRU-VNTR data were available; figure not shown since the resulting tree was over-crowded). From this tree, seven major central nodes (or lineages) were identified, represented by their respective prototypes: Indo-Oceanic/ MIRU International Types - MIRU International Types (MIT) 57, East Asian and African Indian/MIT17, Euro American / MIT116, West African-I/MIT934, West African-II/MIT664, *M. bovis*/MIT49, *M. canettii* / MIT60. Further MST subdivision identified an additional 34 sublineage MIT prototypes. The phylogenetic relationships among the 37 newly defined MIRU-VNTR lineages were inferred using a classification algorithm based on a Bayesian approach.

A MIRU-based MST tree drawn on 176 strains of the MIRU-VNTR_{plus} database (Figure 7) showed that the three phylogenetic groups – West African I, West African II and *M. bovis* are phylogenetically close. Considering the fact that the oldest lineages are most distant from the Euro American lineage, the tree suggests that West African I and West African II lineage strains appeared before *M. bovis*.

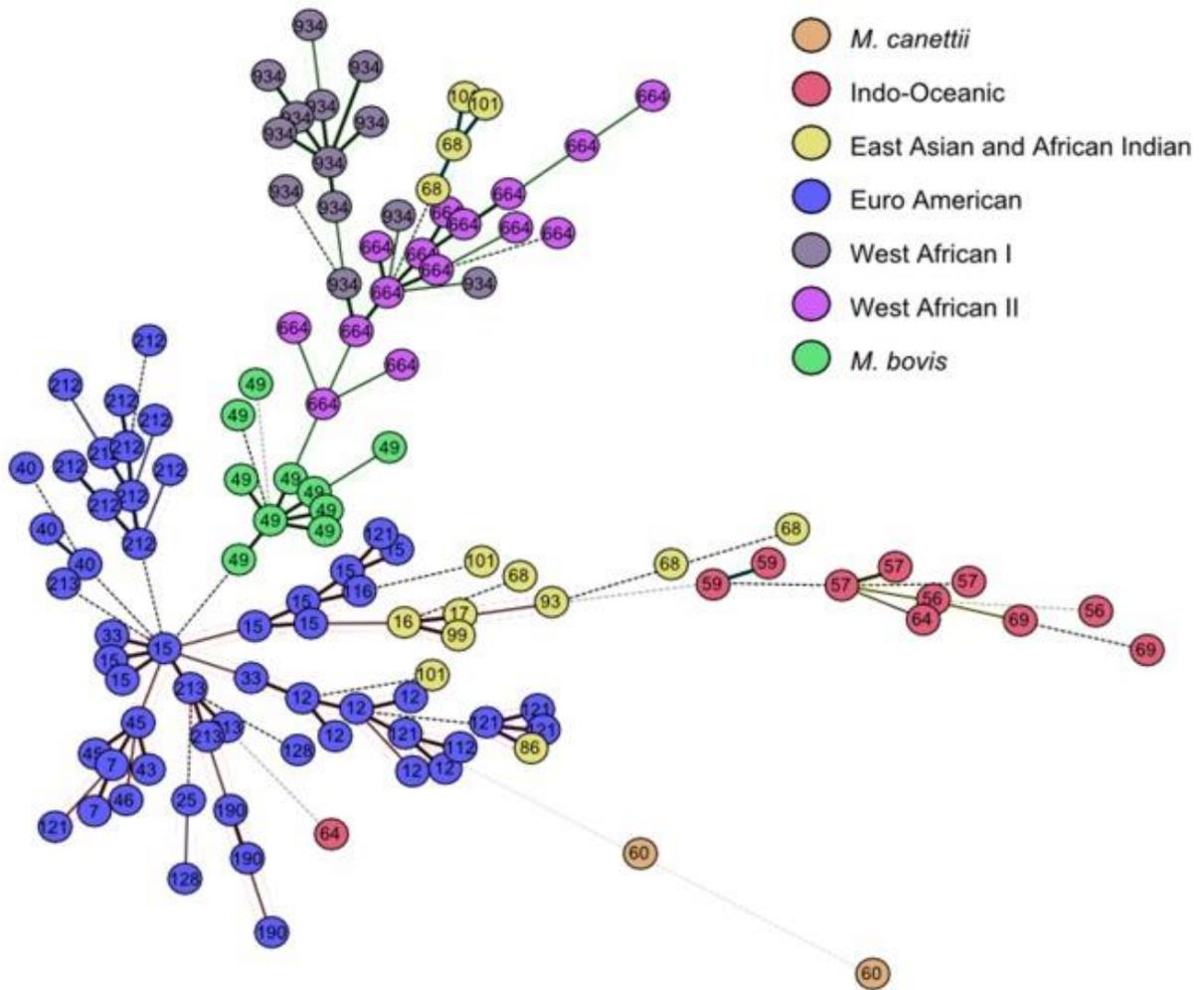


Figure 7. MST tree done with 12 MIRU-VNTR loci of 176 strains from MIRU-VNTR $plus$

The MIRU-based classification superimposes quite well with that of Brudey for sublineages belonging to PGG1, nonetheless discrepancies do exist for PGG2/3 lineages. The classical spoligotyping method which uses 43 spacers out of 104 reported spacers in tubercle bacilli (172), may not systematically reflect the succession and exact order of spacers on the genome. We therefore thought it desirable to have a: (i) finer view of the DR locus using extended spoligotyping (57,173), (ii) to detect IS6110 insertions in the DR locus using methodology described earlier (169), (iii) use IS6110AD-typing to investigate the role of IS6110 insertional event(s) causing deletions in the *M. tuberculosis* complex genome elsewhere than the DR locus. All these three techniques were used on a same set of 100 *M. tuberculosis* complex isolates blindly sampled from an initial set of 445 clinical isolates studied in Mozambique (154). The results obtained for selected isolates are summarized in Figure 8.

Regarding the demonstration of the *IS6110* in the DR locus (Figure 8A), hybridization of a spacer by the primer sets (biot)DRa-IS3 or (biot)DRb-IS6 is positive evidence for *IS6110* insertion in the DR preceding the spacer in question in 5'→3' direction, while with primer sets (biot)DRa-IS6 or (biot)DRb-IS3, it is an evidence for insertion in the direction 3'→5'. Nonetheless, asymmetrical insertion of *IS6110* in the DR can prevent the binding of one of the two primers and affect the amplification of the upstream or downstream spacer. Hence, we amplified the spacers both on the right and left of the DR repeats to evidence *IS6110* insertions; indeed these four pairs of primers are expected to produce an amplicon containing only a single spacer as shown in Figure 8A. The results obtained for the 86 extended spacers are summarized for a strain in Figure 8B: 1st line corresponds to use of classical spoligotyping primers DRa-Drb, while the 4 other lines correspond respectively to primer sets: (biot)DRa-IS3, (biot)DRb-IS6, (biot)DRa-IS6 and (biot)DRb-IS3, and are helpful to highlight the presence of *IS6110* element(s) in the DR locus. As shown in Figure 8B, the presence of *IS6110* often results in revelation of 1 or 2 adjacent spacers leading to 2 possible assumptions: (i) either there are several *IS6110* inserted into contiguous DR, or (ii) part of the amplicon carried by the *IS6110* had length variations (since transposable elements are known sometimes to carry pieces of genomic sequences; (174).

Several RDs are reportedly located next to *IS6110*, e.g., RD152, RD207, RD5, RD11, RD14, MiD2 (156,175,176). To determine whether the *IS6110* was involved in genetic recombination that may cause adjacent deletions (177), we applied *IS6110* AD-typing to selected strains from Mozambique and *M. tuberculosis* H37Rv. The results obtained underlined deletions adjacent to *IS6110* insertions. One may postulate that the high *IS6110* copy number in the H37Rv genome (16 copies) conferred a high mutation rate to the DR locus, since the latter is known to be an *IS6110* preferential locus. However, mechanisms other than *IS6110* insertion have been suggested to cause the loss of spacers in the DR locus – which is a member of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) – such as homologous recombination between DR (178) or *IS6110* (179), and slippage during DNA replication (180). In a recent study, different spoligotypes observed among epidemiologically related strains were attributed to the loss of spacer blocks due to recombination between DRs, an event favored by the formation of a secondary structure involving two *IS6110* in opposite orientation (181), an explanation that argues in favor of more complex and interlinked way of *M. tuberculosis* complex evolution involving 2 or more mechanisms simultaneously. In conclusion, insertion sequences undoubtedly induce adjacent deletions (177), and no matter the mechanism, the fact that *IS6110* are observed next to deleted spacers on the DR locus underlines their active involvement in DR evolution by loss of spacers. In conclusion, the discrepancies observed between spoligotype and MIRU based classification schemes in the cases cited above underline that MIRU-based classification tends to group *M. tuberculosis* complex isolates that are phylogenetically close or almost similar albeit they might appear distant if only judged based on their spoligotyping patterns.

The global geographical distribution of the newly defined MIRU-VNTR lineages is summarized in Figure 9. The map drawn illustrates the information available in the SITVIT2 database for the 6800 *M. tuberculosis* complex isolates recognized as *M. tuberculosis sensu stricto*. The figure shows pie charts with two circles – the inner circle shows the three most predominant newly-described lineages, i.e., Indo Oceanic, East Asian and African Indian (EAAI), Euro American, whereas the outer circle shows the sublineages belonging to uniquely the most predominant of the three lineages (please refer to the color scheme shown in the legend to Figure 9).

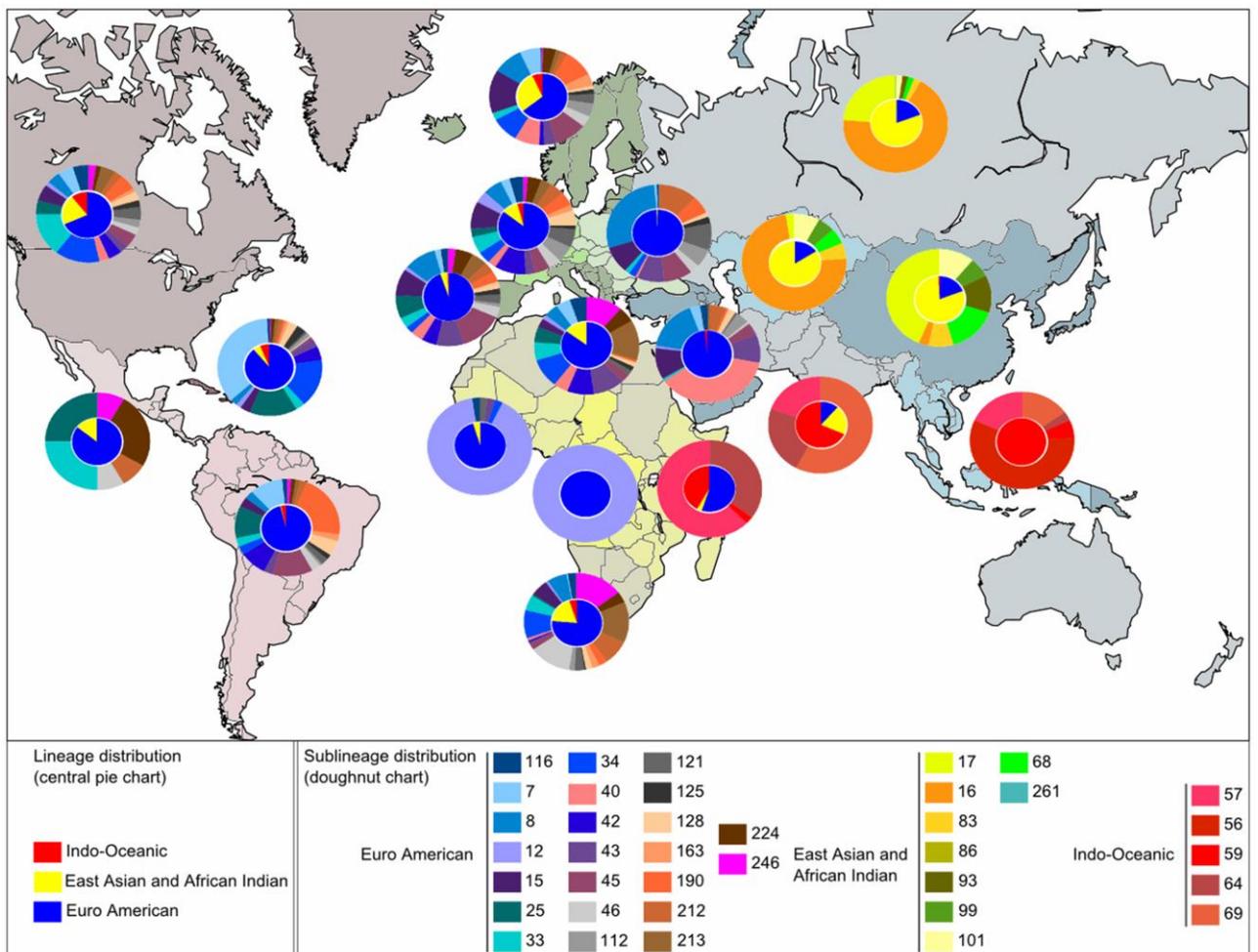


Figure 9. Global geographical distribution of the newly defined MIRU-VNTR lineages.

To evaluate the ability of 12-loci versus 24-loci MIRU-VNTRs to discriminate *M. tuberculosis* complex sublineages, we constructed two MST phylogenetic trees with 95 strains of Mozambique (Figure 10), which essentially contained two main lineages – Indo-Oceanic (42.1%) and Euro American (54.7%). Irrespective of the typing format used (12-loci, Figure 10A vs. 24-loci, Figure 10B), none of the trees showed a strong link between these two main lineages. Almost the totality of Euro American strains (84.6%) belonged to the LAM phylogenetic sub-group B (essentially sublineages Euro American-163 and Euro American-128). Regardless of the typing format used, the trees showed the same two big clusters (even though the tree made with 24-loci had much more ramifications). We therefore conclude that 12-loci format is sufficient to discriminate the present MIRU-VNTR based *M. tuberculosis* complex lineages.

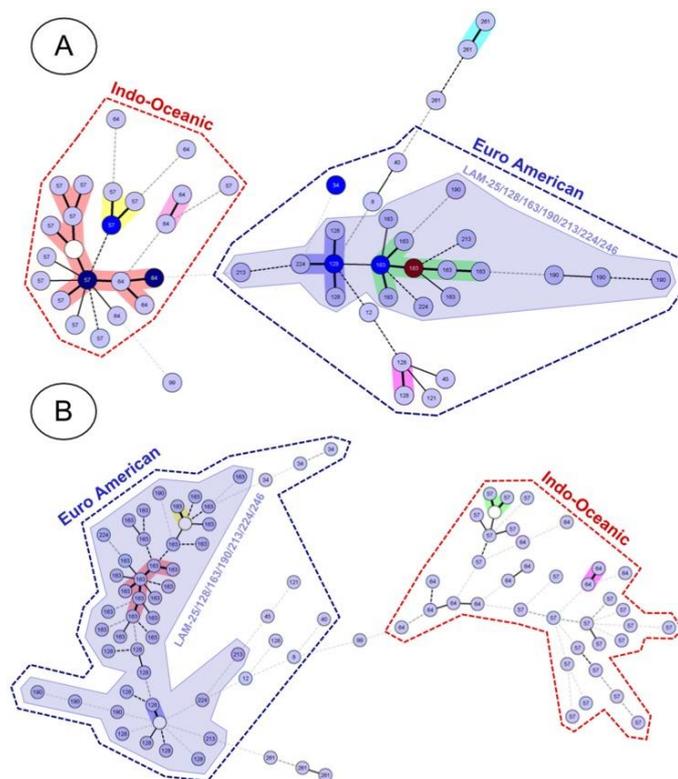


Figure 10. Two MST phylogenetic trees done with 95 Mozambican strains based on 12-loci MIRU-VNTRs (A), and 24-loci MIRU-VNTRs (B).

The results from the present investigation allowed the construction of an updated phylogenetic and phylogeographic snapshot of worldwide *M. tuberculosis* complex diversity studied both at the regional, sub-regional, and country level according to the United Nations specifications. We also looked for *IS6110* insertional events that are known to modify the results of the spoligotyping in specific circumstances, and showed that a fair portion of convergence leading to the currently observed bias in phylogenetic classification of strains may be traced back to the presence of *IS6110*. These results shed new light on the evolutionary history of the pathogen in relation to the history of peopling and human migration.

2.4.4 Paper IV

In paper IV, we explored the public health risk for bovine TB in Maputo, capital of Mozambique, by characterizing the isolates from tuberculous lymphadenitis (TBLN) cases, during one year, in the Pathology Service of Maputo Central Hospital. In this study we presented for the first time the genetic lineages of *M. tuberculosis* complex from extrapulmonary TB cases from Maputo, Mozambique.

A total of 110 patients, suspected of having TBLN, were recruited to participate in the study (Figure 11). From those patients, 45 isolates were analyzed by genotyping methods, one isolate per patient.

Cervical lymphadenitis was the main cause of TBLN in Maputo. Of the lymph node samples 39 (86.7%) were collected from the cervical region, two (4.4%) from axillary site and one (2.2%) from inguinal region. Other sites were breast, chest and thigh (1 case, 2.2% from each site).

Among the 45 patients, 30 (66.7%) were HIV positive (19 males and 11 females), nine (20.0%) were HIV negative and six (13.3%) were not tested for HIV. Of the cervical TBLN cases, 26 (66.7%) were HIV positive patients. No statistical association was found between HIV serology and cervical TBLN. Several studies have shown correlation between HIV infection and TBLN (182–184). The synergies between TB and HIV infection (185,186) have resulted in an increase in the incidence of TBLN and have further complicated TB control. In this study, the high prevalence of HIV positive patients (66.7%) among TBLN cases, might suggest a rising trend of HIV infection associated with TBLN in Maputo.

Two patients, EBOV 13-23 (27 years, Lineage T1, SIT53) and EBOV 13-29 (28 years, Lineage Beijing, SIT1), both males, HIV positive and not previously treated for TB, were diagnosed with MDR-TB (Figure 11).

Among all, 49 patients had a positive mycobacterial culture, giving a culture positivity rate of 44.5%. Of them, 48 isolates were identified as *M. tuberculosis* complex and one as NTM (32 years, male, HIV positive). From the 59 culture negative patients there were an additional 15 (25.4%) cases that were ZN positive on cytology (morphological evidence of mycobacterial infection). In the remaining 44 patients, based on cytology, there was a specific diagnosis other than mycobacterial infection.

Among the 48 culture confirmed TB cases, 45 isolates were analyzed by spoligotyping and MIRU-VNTR. For the remaining three isolates and for the NTM, DNA was not available, because there was no growth during the re-culture procedure (Figure 11).

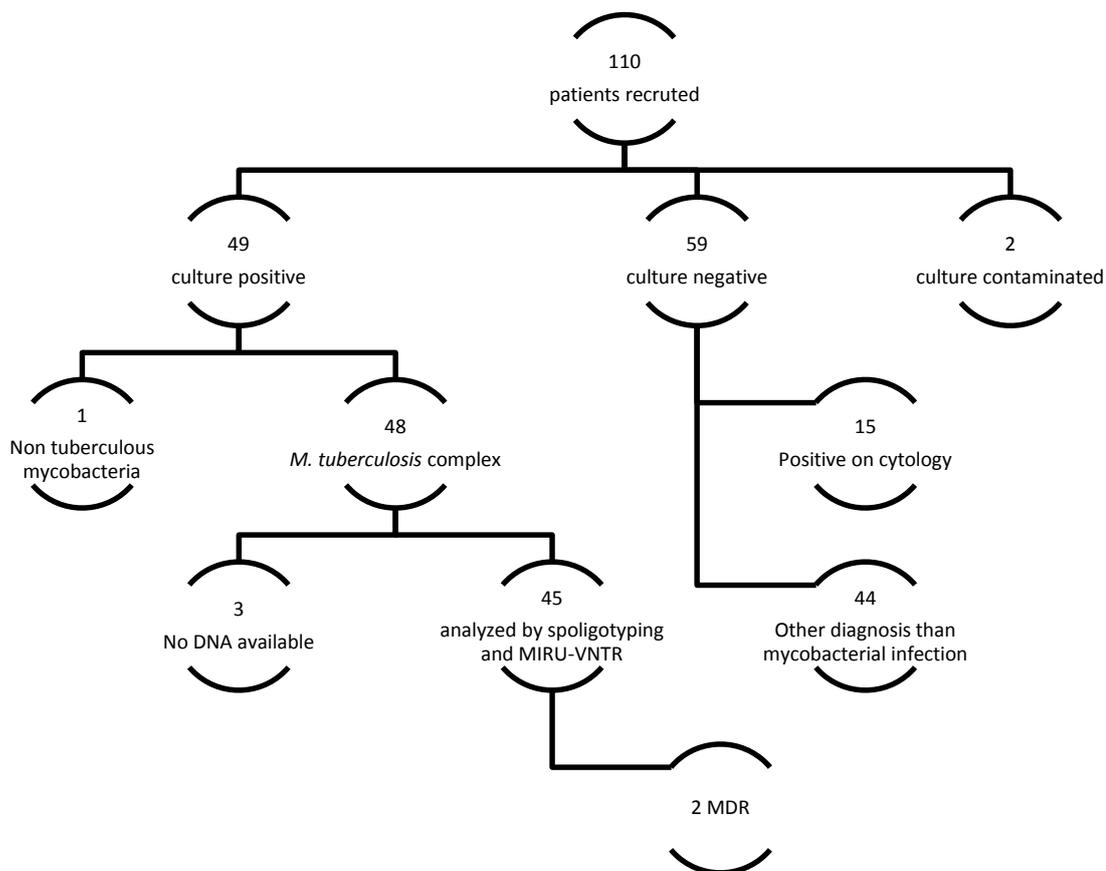


Figure 11. Chart showing patient’s recruitment, culture, identification and resistance results

Mixed *M. tuberculosis* infections is a potential obstacle for TB treatment and control, occurs when an individual is simultaneously infected with more than one strain of *M. tuberculosis* complex. In high TB prevalence settings, mixed infections are frequent, implying high reinfection rates and the absence of efficient protective immunity conferred by the initial infection (134). In patient EBOV 13-19 (male, 59 years, HIV positive), a spoligotype SIT2117 (Manu 2; all spacers present except spacers 9, 10, 33 and 43) was observed. This type of pattern may correspond to a mixed infection, due to concomitant Beijing and Euro-American lineage strains (the latter comprising H, LAM, X, and T lineages per spoligotyping defined clades). A mixed infection was defined as the occurrence of strains with different 24-loci MIRU-VNTR patterns at two or more loci in the same sample. We further investigated this isolate; by spoligotyping we could observe different intensities of the spacers in the spoligotyping pattern (Figure 12) and by MIRU-VNTR we could observe double alleles in three different locus (MIRU26, Mtub21, and Mtub30), confirming a mixed infection pattern. Since it was confirmed as a mixed infection, the isolate EBOV 13-19 was excluded from further genotypic analysis.

In paper I, we have shown mixed infection with a Beijing and non-Beijing strain in two out of five Manu strains from pulmonary TB cases from Mozambique (154). In South Africa, a study conducted in Cape Town, showed that 57% of patients infected with a Beijing strain were also infected with a non-Beijing strain (134). Other countries have also reported mixed infection within different strains from the *M. tuberculosis* complex, i.e. Botswana (187), China (188), Taiwan (189).



Figure 12. Spoligotyping pattern of the strain EBOV 13-19. The picture shows different intensities of the spacers

Spoligotyping was performed on 45 isolates. Of them, all were defined as *M. tuberculosis* and no *M. bovis* was found. Among the 44 isolates (excluding the mixed infection isolate), 23 spoligopatterns were obtained. Three patterns corresponded to orphan strains that were unique in the SITVIT2 database, as opposed to 20 patterns from 41 patients that corresponded to shared-types (SITs), i.e. an identical pattern shared by two or more patients worldwide (within this study, or matching another strain in the SITVIT2 database), as shown in Table 3.

For each isolate, their binary/octal description, their lineages and SITs are summarized in Table 3. Four SITs (containing seven isolates) were newly created either within the present study or after a match with an orphan in the database. Nine patterns were in clusters, containing 30 isolates (2 to 6 isolates per cluster), amounting to an overall clustering rate of 68.2% (30/44).

As shown in Table 3 the most common spoligotypes found in this study were SIT48 (East African-Indian_Somalia; EAI1_SOM) with six isolates; SIT1 (Beijing lineage) and SIT42 (LAM 9) with five isolates each and SIT53 (T1) with four isolates. The most common lineage was the EAI (n=19; 43.2%).

livestock samples, where one *M. tuberculosis* was isolated in camels, suggesting transmission between livestock and humans in this pastoral area. This last study emphasizes the importance of an appropriate study area, where risk factors, including close contact between humans and livestock, and consumption of raw milk and meat by the communities, are present. That can be one of the reasons for the absence of *M. bovis* in the present study.

By spoligotyping, the main lineages of TBLN were the EAI, Beijing; LAM and T1; and the major SITs, were SIT48, SIT1, SIT42 and SIT53. These genotypes are also predominant in pulmonary cases in Mozambique (154), indicating that there are no differences in the population of strains in pulmonary and extrapulmonary cases. Similarities between pulmonary and extrapulmonary cases were observed in other countries, i.e. Ethiopia (195), Thailand (196), Madagascar (197) and Brasil (198).

In African countries, little is known about the common lineages responsible for extrapulmonary TB. A study conducted in the neighboring South Africa, in children, showed similarities with our findings, stressing the proximity between the two countries. In the South African study, 21.2% of the *M. tuberculosis* isolates belonged to the LAM lineage, and 20% to the Beijing lineages (199). In Uganda, a recent study conducted in lymphadenitis cases, T2 was the most common lineage (200). In another study performed in Ethiopia, spoligotyping revealed that the most common spoligotypes in extrapulmonary TB were SIT54, SIT53, and SIT149 (201); SIT54, Lineage T1 was also found to be common in TBLN cases from the present study.

The ancestral EAI lineage, most predominant in this study (42.2%), and one of the prevalent lineages in pulmonary TB cases in Mozambique, with a predominance of 29.7% (154); is considered to be endemic in the Southern region of India (202,203). The migration link between India, particularly South India, and Mozambique arisen since the second half of the 19th century, when Indian traders practiced the trade routes of the Indian Ocean, for transnational connections. The high prevalence of EAI lineages in Maputo might represent an indication of TB transmission between the two countries.

The Beijing lineage was also found to be one of the most common lineages in TBLN cases from this study. In South Africa, it was the second most common spoligotype found in extrapulmonary cases (199) and in Thailand, Beijing lineage was reported to be the most predominant in extrapulmonary TB cases, with 56% (204) and 57.9% (205). Further research is needed to evaluate whether Beijing lineage has any particular association with extrapulmonary TB.

We have also shown an association between the Beijing lineage and HIV infection (206), although in this study, perhaps because of the sample size, it was not possible to find any relationship between a particular lineage and HIV infection. Furthermore, analysis of the spoligotyping lineages did not show any association with a particular clinical expression of the disease (data not shown).

Based on MIRU-VNTR analysis, we could observe a wide diversity of patterns, within strain lineages, showing that TB lymphadenitis in Maputo is not caused by a particular strain but a wide variety of strains, an indication that risk factors for developing TBLN are rather associated with host than *M. tuberculosis* strain.

In this study, no *M. bovis* was found. In low income countries there are no effective animal TB control programs and surveillance, and the epidemiological and public health aspects of infection due to bovine TB are scarce (58,59,61). This situation is aggravated by the presence of additional risk factors such as human behavior and the high prevalence of HIV infections (58,59,62). In African countries, a median of 2.8% (range 0%–37.7%) of all humans cases of

TB are estimated to be caused by *M. bovis* (61). Variances on prevalence are observed in different sites; those differences might be influenced by sampling, study area and diagnostic methods. Prevalence varies from; 17% and 4.4% in Ethiopia (207,208), 16% in Tanzania (209), 7% in Uganda (210), 3% in Ghana (211) and 15.38 % in Nigeria (212).

The genotyping findings from this study and the findings in pulmonary cases (154) indicate that the overall contribution of *M. bovis* to human TB in Maputo is minor. However, the present study has certain limitations. The small number of positive cases on culture might have reduced the chances of finding *M. bovis* as well as the statistical power and have affected the conclusions regarding the significance of the different variables and *M. tuberculosis* lineages. Furthermore, patients from this study are from urban or peri-urban areas of Maputo where livestock and consumption of unpasteurized milk is minor, thus, exposure to *M. bovis* is less.

The occurrence of zoonotic TB is greatly dependent on the presence of bovine TB in cattle. *M. bovis* in cattle is very frequent in certain areas of Mozambique; a recent publication has demonstrated a high prevalence of bovine TB in cattle of 39.6% (95% CI 36.8–42.5) in one particular district of Mozambique, district of Govuro (213). Another study carried out in 2008 in the same region reported a bovine TB prevalence rate of 61.9% (95% CI: 55.8–67.8) (214).

In study V, isolates from the Govuro district were evaluated.

2.4.5 Study V – Preliminary results

M. bovis in cattle is very frequent in the district of Govuro, located in the province of Inhambane, Mozambique. In order to identify the public health importance of bovine TB we have characterized mycobacteria isolates from that particular district.

Preliminary results are based on 24 samples from cases of pulmonary and TBLN suspected patients from the Health Center of Govuro and from the community. Basic demographic information was collected and spoligotyping and MIRU/VNTR were performed on positive cultures in order to identify cases of TB due to *M. bovis*.

Among all, 17 were from pulmonary and seven from TBLN suspected cases. Of all, nine were culture positive. Of those, six were confirmed as *M. tuberculosis* complex and three as NTM. Spoligotyping and MIRU-VNTR were performed in all *M. tuberculosis* complex isolates and one was confirmed as *M. bovis*, SB0961. The patient was a female, 40 years, HIV positive with TBLN. Interestingly, the same spoligopattern was found in 61% of 178 *M. bovis* isolates from cattle in eight provinces of Mozambique (215).

The remaining isolates were identified as *M. tuberculosis* sensu stricto. The NTM isolates are still to be identified.

3 CONCLUDING REMARKS

Paper I represents the first baseline study of the *M. tuberculosis* population structure in Mozambique, a useful guide for future epidemiological studies in the country and extending the picture of global TB distribution. Predominant lineages from Mozambique were also found in neighbouring countries and no *M. bovis* was found in pulmonary cases.

A particular lineage, the Beijing, was found to be more common in the South 27/282 (9.6%) compared to the North 4/163 (2.5%). We further investigated the Beijing isolates with the aim to investigate the prevalence and possible transmission of Beijing strains in Mozambique and compared our findings with the ones from South Africa (paper II). An association was found between HIV positive serostatus and Beijing lineage ($p=0.023$) and similarities with the South African “sublineage 7”, suggesting that this sublineage could have been recently introduced in Mozambique from South Africa, in association with HIV infection.

Based on paper I and paper II findings, my recommendation is to in the near future introduce molecular genetic methods at reference level in Mozambique, prioritizing migrant patients, with emphasis on mine workers from South Africa in order to identify outbreaks and control the transmission between countries, as well as for HIV positive individuals for monitoring possible epidemics related to opportunistic strains and drug resistance.

In paper III, we evaluated the MLVA based classification of *M. tuberculosis* genotypic lineages as a means to provide with an accurate and robust phylogeographic interpretation of its worldwide diversity. An updated phylogenetic and phylogeographic snapshot of worldwide *M. tuberculosis* complex diversity was studied both at the regional, sub-regional, and country level according to the United Nations specifications. This novel classification scheme seems to be a good alternative to support future phylogenetic and epidemiologic studies.

In paper IV, we explored the public health risk for bovine TB in Maputo, by characterizing the isolates from TBLN cases. In this study we presented the genetic lineages of *M. tuberculosis* complex from extrapulmonary TB cases from Maputo, Mozambique. No *M. bovis* was found.

The occurrence of zoonotic TB is greatly dependent on the presence of bovine TB in cattle. A study is being conducted presently in the district of Govuro, a region where the prevalence of bovine TB in cattle is high, and we have identified the first case of bovine TB in Mozambique in that particular area.

Further investigation is needed on cases of abdominal TB and other forms of TB in Govuro and in other pastoral areas, where the prevalence of bovine TB in cattle is known to be high in order to have a better answer about the public health importance of this zoonotic disease in Mozambique.

The National TB Control Program in Mozambique should improve on the diagnoses of *M. bovis* as well as encourage collaboration between public health and veterinary public health. A ‘one health’ approach can have a positive impact towards the design and implementation of appropriate diagnosis, surveillance and prevention of mycobacterial infection, especially in areas in which different mycobacteria species are known to be infecting humans and livestock.

4 REFLECTIONS CONCERNING LEARNING OUTCOMES

In October 2007, I was registered as a licentiate student at the Department of Microbiology, Tumour and Cell Biology / Karolinska Institutet during my first visit to the Swedish Institute for Infectious Disease Control (presently the Public Health Agency of Sweden). On the 17th of June 2011, I defended the licentiate thesis with success at the Karolinska Institutet and in August 2012, I was successfully registered as a PhD student at the Department of Clinical Sciences and Education / Karolinska Institutet.

During my PhD studies, I could improve my understanding and gain laboratory skills in molecular typing methods, acquired during my stay in Stellenbosch University, in South Africa and several visits to the Public Health Agency of Sweden. I could attend several postgraduate courses and improve my understanding in different fields, among others, on Ethics, Biostatistics, Biosafety, Public Health, basic epidemiology and basic immunology. I could also participate in international meetings, several journal clubs and other scientific meetings and improve my communication skills and understanding science in different contexts. I could also learn how to write science, during these years I had the opportunity to write and interact with the journal's editor in three of the papers from this thesis.

The sample collection and field work was performed in Mozambique. The sample processing and part of the molecular testing was performed at the National TB Reference Laboratory and at the Center of Biotechnology of Eduardo Mondlane University. This training allowed installation of capacity and training of the laboratory personnel from the National TB Reference laboratory in performing spoligotyping.

The PhD training also had a big impact from the professional point of view. In 2010 I was appointed to coordinate the activities of the TB Reference Laboratories in Mozambique and in 2014 as the head of the Department of Laboratory Network and Referral Services. Those experiences allowed me to learn how to lead a team, interact with different stakeholders and somehow participate in the decision making process of TB diagnosis in my country.

In general, during the past years, I could gain knowledge and systematic understanding of TB, and TB molecular epidemiology, be able to carry out scientific analysis and syntheses, gain intellectual independence and scientific conscientiousness, gain in-depth insight into scientific possibilities and limitations and their role in society.

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