Karolinska Institutet

Thesis for licentiate degree 2011

MOLECULAR CHARACTERIZATION OF *M. TUBERCULOSIS* ISOLATES IN MOZAMBIQUE

Sofia Omar Viegas

Thesis for licentiate degree
2011

MOLECULAR CHARACTERIZATION OF *MYCOBACTERIUM TUBERCULOSIS* ISOLATES IN MOZAMBIQUE

Sofia Omar Viegas

Karolinska Institutet

From Department of Microbiology, Tumor and Cell Biology
Karolinska Institutet, Stockholm, Sweden; Department of Preparedness, Swedish Institute for Communicable Disease Control, Solna, Sweden and Faculty of Veterinary, Eduardo Mondlane University, Maputo, Mozambique

MOLECULAR CHARACTERIZATION OF *MYCOBACTERIUM TUBERCULOSIS* ISOLATES IN MOZAMBIQUE

Sofia Omar Viegas

Karolinska
Institutet

Stockholm 2011

The previously published paper was reproduced with permission from the publisher.

Published by Karolinska Institutet. Printed by [name of printer]

© Sofia Omar Viegas, 2011
ISBN 978-91-7457-329-9

Printed by

www.reproprint.se
Gårdsvägen 4, 169 70 Solna

"The future belongs to those who believe in the beauty of their dreams"

Eleanor Roosevelt (1884-1962)

ABSTRACT

Tuberculosis (TB) is one of the major health problems in Mozambique. It is estimated that 27,000 deaths caused by TB occur each year with an estimated incidence and prevalence rate of 431 and 504 per 100,000 population respectively. Mozambique ranks 19th on the list of 22 high-burden TB countries in the world. A steady increase in the prevalence rate of HIV/AIDS and the emergence of drug-resistant bacilli makes the situation even more precarious. Moreover, Mozambique shares geographical borders with six other countries where TB is also endemic, i.e., South Africa, Swaziland, Zimbabwe, Zambia, Malawi and Tanzania. Different genotypes of *Mycobacterium tuberculosis* complex (MTC) predominate in different geographical regions of the world and have differences in virulence, clinical presentation as well as transmission potential.

This study described the molecular epidemiology of MTC in Mozambique, identified the predominant genotypes responsible for TB transmission and prevalence, and investigated the association between predominant spoligotypes and HIV sero-status. Furthermore, the study investigated the prevalence and transmission of the Beijing genotype in Mozambique.

For the epidemiological characterization, 445 *M. tuberculosis* isolates from seven different provinces of Mozambique were characterized by spoligotyping and resulting profiles were compared with the international spoligotyping database SITVIT2. 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%); an evolutionary recent but yet ill-defined T clade, (n=52 or 11.6%) and the globally-emerging Beijing clone, (n=31 or 7%); nearly equally attributed both to ancestral and evolutionary modern *M. tuberculosis* lineages with an exceptionally high biodiversity documented for the EAI, LAM and T lineages. Furthermore, the presence of predominant lineages in neighboring countries indicates TB transmission by migration from one country to another, particularly to South Africa.

To investigate the prevalence and transmission of the Beijing genotype in Mozambique, a total of 543 *M. tuberculosis* isolates, from different regions of Mozambique were spoligotyped. Of these, 33 were of the Beijing lineage.

By combined use of spoligotyping and Region of Difference (RD) deletions, we found a predominant group of 25 isolates having deletions of RD105 and RD181. Another group of three isolates lacked RD150, a signature of the reference strain "sublineage 7" recently emerging in South Africa.

A comparison with South African Beijing strains, by Restriction Fragment Length Polymorphism (RFLP) and MIRU-VNTR, suggests multiple introductions of different sublineages leading to an emerging epidemic associated with HIV.

Moreover, the majority of the Beijing strains were found in the South Region of Mozambique, particularly in the capital, Maputo city (17%). Additionally, it was found that the Beijing strains were associated with HIV positive serostatus (p= 0.049) but not with drug resistance.

LIST OF PUBLICATIONS

I. **Sofia O Viegas**, Adelina Machado, Ramona Groenheit, Solomon Ghebremichael, Alexandra Pennhag, Paula S Gudo, Zaina Cuna, Paolo Miotto, Véronique Hill, Tatiana Marrufo, Daniela M Cirillo, Nalin Rastogi, Gunilla Källenius, Tuija Koivula. Molecular diversity of *Mycobacterium tuberculosis* isolates from patients with pulmonary tuberculosis in Mozambique. BMC Microbiology 2010; **10**:195 doi: 1471-2180/10/195.

II. **Sofia O Viegas**, Adelina Machado, Ramona Groenheit, Solomon Ghebremichael, Alexandra Pennhag, Egidio Langa, Zaina Cuna, Paolo Miotto, Daniela M Cirillo, Nalin Rastogi, Rob M Warren, Paul D van Helden, Gunilla Källenius, Tuija Koivula. Beijing *Mycobacterium tuberculosis* genotype is emerging in Mozambique in association with HIV co-infection. Manuscript.

CONTENTS

1 INTRODUCTION 1
 1.1 TUBERCULOSIS AND HIV IN MOZAMBIQUE 2
 1.2 TUBERCULOSIS AND HIV CO-INFECTION 4
 1.3 THE *MYCOBACTERIUM TUBERCULOSIS* COMPLEX (MTC) .. 4
  1.3.1 *Mycobacterium tuberculosis* 4
  1.3.2 *Mycobacterium bovis* and *Mycobacterium bovis* BCG 5
  1.3.3 *Mycobacterium africanum* 6
  1.3.4 *Mycobacterium canettii* 6
  1.3.5 *Mycobacterium microti* 6
  1.3.6 *Mycobacterium pinnipedii* 6
  1.3.7 *Mycobacterium caprae* 7
 1.4 LABORATORY DIAGNOSIS OF TUBERCULOSIS 7
  1.4.1 Identification of the MTC 7
  1.4.2 Strain identification of the MTC 8
  1.4.3 Drug resistant TB 10
2 THE PRESENT INVESTIGATION 12
 2.1 PURPOSE OF THE STUDY 12
  2.1.1 Objectives 12
 2.2 MATERIALS AND METHODS 12
  2.2.1 Study area and patients 12
  2.2.2 Sample processing 12
  2.2.3 HIV testing 12
  2.2.4 Chromosomal DNA isolation 13
  2.2.5 Spoligotyping 13
  2.2.6 RFLP 13
  2.2.7 Regions of Difference (RD) analysis 14
  2.2.8 MIRU-VNTR 14
 2.3 RESULTS AND DISCUSSION 14
  2.3.1 Paper I 14
  2.3.2 Paper II 17
 2.4 CONCLUDING REMARKS AND FUTURE PERSPECTIVES .. 20
3 ACKNOWLEDGEMENTS 21
4 REFERENCES 23

LIST OF ABBREVIATIONS

AIDS	Acquired Immuno Deficiency Syndrome
BCG	Bacillus Calmette-Guérin
BTB	Bovine tuberculosis
DNA	Deoxyribonucleic Acid
DOTS	Directly Observed Treatment Short Course
DR	Direct Repeat
DRS	Drug Resistance Surveillance
DVR	Direct Variable Repeat
EAI	East African Indian
HIV	Human Immunodeficiency Virus
IS	Insertion Sequence
LAM	Latin American Mediterranean
LJ	Lowenstein-Jensen
MDR	Multidrug Resistant
MIRU	Mycobacterial Interspersed Repetitive Units
MTC	*Mycobacterium tuberculosis* complex
NAA	Nucleic Acid Amplification
NTM	Nontuberculous mycobacteria
PCR	Polymerase Chain Reaction
PGG	Principal Genetic groups
PZA	Pyrazinamidase
RFLP	Restriction Fragment Length Polymorphism
RD	Region of Difference
SIT	Spoligotyping Shared Type
Spoligotyping	Spacer Oligonucleotide Typing
SITVIT	*Mycobacterium tuberculosis* molecular markers database
SIT	Spoligotype International Type
ST	Shared Type
TB	Tuberculosis
TCH	Thiophene-2-Carboxylic Acid Hydrazide
VNTR	Variable Number of Tandem Repeats
WHO	World Heath Organization
XDR	Extensively Drug-Resistant

1 INTRODUCTION

For millennia, the history of medicine has reported tuberculosis (TB) as responsible for high morbidity and mortality, generating social and economic imbalances. Over the years, the development of diagnostic methods (acid fast staining and chest X-ray), the development of a vaccine (BCG, Bacillus Calmette-Guérin) and the discovery of antitubercular compounds for the treatment of the disease, have led to more effective measures for control of the disease. The existence of good national programs to fight TB, well-structured and organized health services, decrease the risk of infection. In addition, technological advances allow faster and accurate diagnoses, resulting in reduction of infectivity of the disease.

Today, TB remains a major global health problem particularly in resource-poor countries where 95% of all TB cases occur. Twenty-two high burden countries were identified as having more than 80% of the new cases of TB worldwide. In the 1980s, the severity and consequences of the human immunodeficiency virus/Acquired Immuno Deficiency Syndrome (HIV/AIDS) epidemic were becoming increasingly apparent in sub-Saharan Africa (De Cock *et al.*, 1992). TB was identified as the major cause of death among HIV-positive Africans (Lucas *et al.*, 1993), and there was growing recognition that HIV was leading to a fundamental change in the epidemiology of TB throughout the continent (De Cock *et al.*, 1992)

Beside the HIV pandemic, the global problem of TB has worsened due to increased drug resistance, and the now also emerging multidrug resistance (MDR) and extensively drug-resistance (XDR) (CDC, 2006).

Molecular epidemiologic studies of TB have focused largely on utilizing molecular techniques to address short and long-term epidemiologic questions, such as in outbreak investigations and in assessing the global dissemination of strains, respectively (Moro *et al.*, 2002; Franzatti *et al.*, 2010; Houben and Glynn, 2009). For instance, molecular epidemiologic studies have added much-needed accuracy and precision in describing transmission dynamics (Wu *et al.*, 2010; Stephen *et al.*, 2002), and they have facilitated investigation of previously unresolved issues, such as estimates of recent-versus-reactive disease and the extent of exogenous reinfection (Caminero *et al.*, 2001; Bandera *et al*, 2001; van Rie *et al.*, 1999; Andrews *et al.*, 2008). In addition, there is mounting evidence to suggest that specific strains of *Mycobacterium tuberculosis* belonging to discrete phylogenetic clusters (lineages) may differ in virulence, pathogenesis, and epidemiologic characteristics (Gagneux *et al.*, 2006), all of which may significantly impact TB control and vaccine development strategies.

The present study characterized *M. tuberculosis* complex (MTC) isolates from patients with pulmonary TB from Mozambique to identify the predominant lineages that cause TB in the country and deeper investigated the globally emerging Beijing genotype.

1.1 TUBERCULOSIS AND HIV IN MOZAMBIQUE

Mozambique, with a population of around 20 million inhabitants, is located in Southern Africa (Figure 1) and divided per 11 provinces and 128 districts. The country suffered almost five centuries of Portuguese colonization, a massive migration of skilled workers after 1975 (the independence) and a terrible civil war that ended in 1990 where half of public health facilities and schools were destroyed. These historical, social and economic factors had strong impact in the present extreme poverty and health inadequacy (Owen, 1997; Tick *et al*., 2007). TB thrives in times of social and environmental upheaval. Poverty, malnutrition, poor general health, social disruption, natural disasters are factors placing individuals at a higher TB risk. The observed increase in TB in sub-Saharan Africa may have resulted from several of these factors.

This year TB was decreed a National Emergency in Mozambique. This disease represents one of the principal causes of morbidity and mortality 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 TB National Control Program (Ministério da Saúde, 2007). Since 1993, Mozambique stands in the list of the 22 TB high burden countries, currently ranking in the 19th position, where the TB incidence and prevalence rate is 431 and 504 per 100,000 population respectively (WHO, 2009).

Mozambique has pioneered the introduction of the Directly Observed Treatment Short Course (DOTS) strategy in the late 80's and actually, Institutional DOTS has been implemented in all the country. However, funds to TB National Control Program are place serious constraints on their function and the program relies heavily on donor support. As a result, in 2010 there was a partial shortage of 1st line drugs.

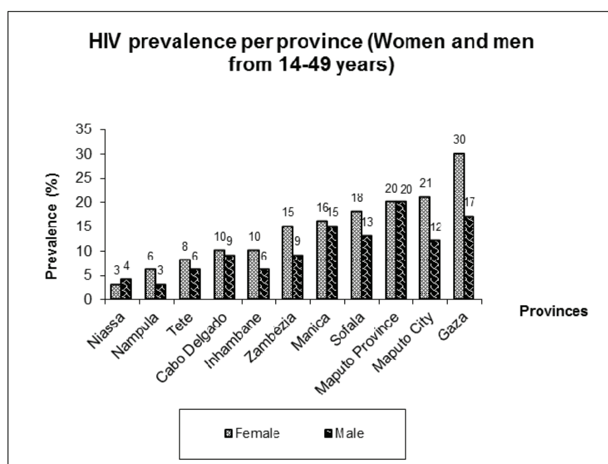
Presently Mozambique has 430 district laboratories performing smear microscopy for TB diagnosis and three Reference Laboratories (two of them fully operational, performing cultures and drug susceptibility testing of MTC, and the other still under refurbishment), which is still an insufficient number to cover the all Country.

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 36.9% and 46.3% of total notified cases (46,174), respectively (Ministério da Saúde, 2010).

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 (Foreit *et al*., 2001) 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 prevalence (Mworozi, 1993). 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%) (INS, 2010).

Figure 1. Mozambique Geographic Location

The HIV prevalence is higher in urban areas (12.3% for both sexes) and in the South Region (12.3%). Province of Gaza presents the highest prevalence in the country (29.9%) and Niassa the lowest (3.3%) (Figure 2).



INSIDA, 2009

Figure 2. HIV prevalence per province in Mozambique (Women and men from 14-49 years). Light color- Women; Dark color- Men

1.2 TUBERCULOSIS AND HIV CO-INFECTION

HIV infection exerts immense influence on the natural course of TB disease. 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 (Selwyn *et al.*, 1992). The HIV and TB epidemics fuel each other and the relationship between HIV and *M. tuberculosis* infection in co-infected individuals has been shown to be synergistic; latent *M. tuberculosis* infection is activated by HIV-induced immunodeficiency and dormant HIV is triggered by TB-induced immune activation (Toossi *et al.*, 2001; Diedrich and Flynn, 2011). With the introduction of highly active antiretroviral therapy for HIV, the risk of progression to TB among those co-infected with *M. tuberculosis*, while higher than among HIV-negative cases, is considerably lower (Badri *et al.*; 2002; Girardi *et al.*, 2005). The role for $CD4^{+}$ T cells in protecting against disease progression is underscored by the marked susceptibility to TB in patients with advanced HIV-induced $CD4^{+}$ T-cell depletion (Di Perri *et al*. 1989; Daley *et al.*, 1992). The natural course of HIV disease may also be influenced by *M. tuberculosis* infection by deregulation of the cytokine and chemokine balance (Lawn *et al.,* 2001). *M. tuberculosis* infection results in macrophage activation, which can house resident HIV virions, resulting in active expression of HIV antigens rather than the prolonged latency without antigenic expression of HIV proteins (Toossi *et al.*, 1993). Thus, HIV infection tends to accelerate the progression of TB, while in turn the host immune response to *M. tuberculosis* can enhance HIV replication and may accelerate the natural course of HIV/AIDS (Toossi *et al.*, 1993).

1.3 THE *MYCOBACTERIUM TUBERCULOSIS* COMPLEX (MTC)

TB is caused by bacteria from the MTC, which consists of highly related slow growing, acid-fast, non-motile bacilli belonging to the genus *Mycobacterium* that differs substantially from other bacteria due to the exceptionally thick cell wall and high genomic guanine-cytosine content. The MTC 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.

Although the mycobacterial species in MTC are highly similar to each other on DNA level, MTC members differ widely in terms of host tropism, phenotype and pathogenicity (Brosch *et al.*, 2002; Huard *et al.*, 2003; Smith *et al.*, 2006). 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 MTC.

1.3.1 *Mycobacterium tuberculosis*

M. tuberculosis is the principal agent of the disease in humans, first described by Robert Koch in 1882. There has been much debate regarding the origin of the MTC strains as it was previously thought that *M. tuberculosis* had evolved from *M. bovis* by specific adaptation of an animal pathogen to human host (Stead *et al.*, 1995; Brisse *et*

al., 2006; Smith *et al*., 2006). However, genomic analysis has shown that *M. bovis* has a smaller genome, suggesting that it is evolutionary younger (Brosch *et al*, 2002).
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 (PZA) (Hoffner *et al*., 1993; Niemann *et al.*, 2002). Genotypically, by spoligotyping *M. tuberculosis* has been classified into different phylogenetic lineages (Brudey *et al.*, 2006).

1.3.2 *Mycobacterium bovis* and *Mycobacterium bovis* BCG

M. bovis is the leading cause of TB in cattle that occasionally affects other species of mammals. This disease is a significant zoonosis spread to humans, typically by the inhalation of aerosols or the ingestion of unpasteurized milk or contaminated meat.
Information on human disease due to *M. bovis* in developed and developing countries is scarce. From a review of a number of zoonotic tuberculosis studies, published between 1954 and 1970 and carried out in various countries around the world, it was estimated that the proportion of human cases due to *M. bovis* acccounted for 3.1% of all forms of tuberculosis: 2.1% of pulmonary forms and 9.4% of extrapulmonary forms (Gervois *et al.*, 1972)

In developed countries, eradication programs have reduced or eliminated TB in cattle, and human disease is now rare; however, reservoirs in wildlife can make complete eradication difficult.

Bovine TB (BTB) is an economical and public health threat in developing countries, including Africa (Ayele *et al.*, 2004). In many African settings, domestic animals are an integral part of human social life and in those cases the risk factors for *M. bovis* infection in both animals and humans are close contact, food hygiene practices and HIV/AIDS infection (Cosivi *et al.*, 1998). Control policies have not been enforced due to cost implications, lack of capacity and infrastructure limitations (Cosivi *et al.*, 1998; Ayele *et al.*, 2004).

Most *M. bovis* infections are extra-pulmonary TB cases with rare cases of pulmonary TB. The clinical signs of the disease in humans are indistinguishable from those occurring due to infection with *M. tuberculosis*. *M. bovis* can be distinguished from *M. tuberculosis* on the basis of epidemiology, phenotype and some genetic markers. *M. bovis* does not produce niacin, does not reduce nitrate and is sensitive to TCH but resistant to PZA (Niemann *et al.*, 2002).

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. 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, it may cause disease in humans.

1.3.3 *Mycobacterium africanum*

Since its first description in 1968 (Bonard *et al.*, 2000), *M. africanum* has been found in several regions of Africa, where it represents up to 60% of clinical isolates obtained from patients with pulmonary TB (Haas *et al.*, 1997, Viana-Niero *et al.*, 2001).
Recent surveys show highly variable prevalences of *M. africanum* in different African regions. For example, *M. africanum* was found in approximately 5% of patients with TB in the Ivory Coast (Bonard *et al.*, 2000) and in at least 60% of patients in Guinea-Bissau (Källenius *et al.*, 1999; Groenheit *et al.*, 2011). In contrast to *M. tuberculosis* and *M. bovis*, *M. africanum* strains show a higher variability of phenotypic attributes, comprising characteristics common to both *M. tuberculosis* and *M. bovis*. This phenotypic heterogeneity of *M. africanum* complicates its unequivocal identification and may lead to misclassification of clinical strains. According to their biochemical characteristics, two major subgroups of *M. africanum* have been described, corresponding to their geographic origin in western (subtype I) or eastern (subtype II) Africa (Collins *et al.*, 1982). Recently, *M. africanum* subtype II has been shown to correspond to a particular sublineage of *M. tuberculosis* (Mostowy *et al.*, 2004; Gagneux *et al.*, 2006).

1.3.4 *Mycobacterium canettii*

Mycobacterium canettii, a novel rare variant of MTC with a smooth colony morphology was first isolated from a Somali-born patient in 1969 by Canetti (van Soolingen *et al.*, 1997). Daffe (Daffe *et al.*, 1991) demonstrated that this particular strain differed from the commonly rough strains by having large amounts of lipooligosaccharides. 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 (van Soolingen *et al.*, 1997).

1.3.5 *Mycobacterium microti*

Mycobacterium microti typically causes disease in voles, wood mice, and shrews, although it was also detected in a limited number of other mammalian species. The causative agent was named *M. tuberculosis subsp. muris*, and later this species was designated *M. microti* and classified as a member of the MTC .
It was first reported in humans in 1998 in immunocompromised patients (van Soolingen *et al.*, 1998), although human to human transmission of *M. microti* infection seems to be rare (Xavier *et al.*, 2007).
M. microti differs from other MTC strains in its S-shaped cell morphology, extremely slow growth in vitro, and distinct host-specific pathogenicity for laboratory animals. Based on biochemical properties, this bacterium is difficult to distinguish from *M. tuberculosis*, *M. africanum*, or *M. bovis*, but *M. microti* strains display characteristic IS*6110* banding patterns and spoligotypes, distinct from types previously observed in other MTC strains (van Soolingen *et al*., 1998).

1.3.6 *Mycobacterium pinnipedii*

In 1993, it was reported for the first time that isolates from seals captured on the coast of Argentina had a characteristic Insertion Sequence (IS) *6110* Restriction Fragment Length Polymorphism (RFLP) pattern (Cousins *et al.*, 1993). This seal bacillus was

later designated *M. pinnipedii* and appeared to have a unique position in the MTC (Cousins *et al.*, 2003). Later on, reports had described *M. pinnipedii* infections in various marine mammals (Forshaw and Phelps 1991; Thompson *et al.*, 1993; Hunter *et al.*, 1998).
Transmission of *M. pinnipedii* to humans has been reported in individuals who are in close contact with marine mammals (Thompson *et al.*, 1993; Kiers *et al.*, 2008).
M. pinnipedii isolates present a distinct spoligotype pattern when compared to other members of the MTC (Cousins *et al.*, 2003).

1.3.7 *Mycobacterium caprae*

Mycobacterium caprae was first isolated from goats in Spain (Aranaz *et al.*, 1999), but has since been found in other animals, such as cattle (Prodinger *et al.*, 2002; Erler *et al.*, 2004; Boniotti *et al.*, 2009), pigs (Pavlik *et al.*, 2002), red deer (Pavlik *et al.*, 2002), and wild boars (Erler *et al.*, 2004). Its isolation from humans has also been described (Erler *et al.*, 2004, Kubica *et al.*, 2003); often, a contact with livestock has been suggested as a likely means of transmission (Prodinger *et al.*, 2002). To our knowledge, this pathogen has never been isolated outside continental Europe, except from a European patient in Australia (Sintchenko *et al.*, 2006) and a cow in Algeria (Sahraoui *et al.*, 2009).
For a long time this species was considered as *M. bovis*, because the biochemical test results were 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* (Aranaz *et al.*, 2003; Aranaz *et al.*, 1999).

1.4 LABORATORY DIAGNOSIS OF TUBERCULOSIS

1.4.1 Identification of the MTC

1.4.1.1 Microscopy

Smear examination is a primary tool 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 economically developed countries. However, it has low sensitivity as 5,000 to 10,000 acid fast bacilli per mL of sputum must be present in order to be detected. In addition it cannot distinguish MTC from other mycobacteria.
Smears are positive in fewer than 50% of culture positive sputum specimens in new TB patients. This rate is even lower (down to 30%) in patients who are co-infected with HIV.
Two procedures, the Ziehl-Neelsen stain and auramine O fluorescence acid fast stain, are the most widely used methods in detection.

1.4.1.2 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. Radiometric liquid culture (Bactec), the most rapid culture technique widely utilized, requires an average of 13 days to become positive (Abe *et al.*, 1992). The most sensitive and rapid culture and staining techniques available are not currently utilized by all laboratories as a result of limited funding, reduced number

of trained and qualified personnel, and training difficulties. On the other hand, proper biosafety protocols and equipment are required for culture isolation.

1.4.1.3 Phenotypic identification of the MTC

Accurate species identification of the MTC members is essential, particularly in countries with high HIV prevalence, where species other than *M. tuberculosis* have been characterized in human TB, and *M. bovis* remains an enormous 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 MTC 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 MTC species (Frothingham *et al*., 1999). All these tests, although simple and inexpensive to perform, require experienced personal to interpret the results and do not clearly differentiate between species (Springer *et al.*, 1996; Djelouadji *et al.*, 2008).

1.4.1.4 Genotypic identification of the MTC

In recent years, the identification of non-tuberculous mycobacteria (NTM) has become a challenge for clinical laboratories since there are currently more than 90 accepted species, coupled with an increased recognition of the significant role of the organisms in a range of clinical presentations (Griffith *et al.*, 2002).
Molecular biology techniques have been successfully used for identification of MTC, with the advantage being that they are more rapid and accurate than conventional methods (Somoskovi *et al.*, 2002).
The introduction of radioisotope-labelled DNA probes and acridinium ester-labeled DNA probes (AcuProbes; Gen.Probe) greatly facilitated the identification of commonly isolated mycobacteria. Subsequently, commercially available and in-house developed nucleic acid amplification (NAA) tests were successfully used for early identification of MTC grown in liquid cultures (Katila *et al.*, 2000).
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 MTC.

1.4.2 Strain identification of the MTC

Genotyping methods are based on the analysis of chromosomal DNA of MTC isolates.
A large number of different molecular methods have been developed to measure the genetic relationship between different MTC 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. 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 too costly and time consuming only parts of the genome are being examined (Moström *et al.*, 2002). 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.

1.4.2.1 Spoligotyping

Spoligotyping is a simple, rapid, reproducible and cost effective method for simultaneous detection and differentiating the MTC 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 (Hermans *et al.*, 1991).
Spoligotyping can be applied directly to cultured cells and to clinical samples (Kamerbeek *et al.*, 1997). The absence of five spacers (numbers 38-43) enables the *M. bovis* species to be determined. 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 *M. tuberculosis* as well as IS*6110* does (i.e., strains with different IS*6110* RFLP patterns may have the same spoligotype).

Polymorphism in the DR locus tend to group strains into larger groups than does IS*6110* analysis and have been used to link strains to specific geographic areas (Brudey *et al.*, 2006). A major criticism of spoligotyping is that it measures a small number of polymorphisms at a single genetic locus.
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 (Brudey *et al.*, 2006). This database defines 62 genetic lineages/sub-lineages (Brudey *et al.*, 2006). These include specific signatures for various MTC members such as *M. bovis, M. caprae, M. microti, M. canettii, M. pinnipedii,* and *M. africanum*, as well as rules defining major lineages/sub-lineages for *M. tuberculosis* sensu stricto; these include the Beijing clade, the Central Asian (CAS) clade and 2 sublineages, the East African-Indian (EAI) clade and 9 sublineages, the Haarlem (H) clade and 3 sublineages, the Latin American-Mediterranean (LAM) clade and 12 sublineages, the ancestral "Manu" lineage and 3 sublineages, the S clade, the IS*6110*–low-banding X clade and 3 sublineages, and an ill-defined T clade with 5 sublineages (as well as further well-characterized phylogeographical specificity for 8 additional spoligotype signatures).

1.4.2.2 IS6110-RFLP

RFLP is the first and most widely applied and standardized molecular typing method. This method is based on the detection of the IS*6110.* The IS*6110* is present in different copy numbers and integrated at different chromosomal sites in MTC isolates. The fragments based on the IS*6110* are highly polymorphic but stable enough for epidemiological studies (van Soolingen *et al.*, 1991). Strains with fewer copies of IS*6110* are more homogenous and the fingerprints are not as reliable concerning epidemiological links as of those containing multiple copies (van Soolingen *et al.*, 1993).
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 IS*6110* that lies upstream of *PvuII* site. The RFLP patterns are entered into a computerized database and analyzed with an image analysis system.

IS*6110* fingerprinting has proven useful for investigating nosocomial transmission, investigating outbreaks, confirming instances of laboratory cross contamination, differentiating relapse caused by endogenous reactivation from re-infection by an exogenous strain and studying TB transmission in large populations.

1.4.2.3 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) (Supply *et al.*, 2000). In its original format, the PCR primers are each run in separate reactions and the sizes of the products are analyzed by gel electrophoresis
Currently, the most widely used version of MIRU-VNTR analysis includes 12 tandem repeat loci (Supply *et al.*, 2000; Supply *et al.*, 2001). A set of 24 MIRU-VNTR loci was standardized to increase the discrimination power (Supply *et al.*, 2006).
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 IS*6110* RFLP genotyping for isolates with high-copy-number IS*6110* insertions but more discriminating than IS*6110* RFLP genotyping for isolates with low-copy-number IS*6110*. When more than 12 loci are used, or MIRU analysis is combined with spoligotyping, the discriminatory power approximates that of IS*6110* RFLP analysis. A comparative study of genotyping methods aimed at evaluating novel PCR-based typing techniques found VNTR analysis to have the greatest discriminatory power among amplification-based approaches (Kremer *et al.*, 2005). MIRU-VNTR genotyping has been used in a number of molecular epidemiologic studies, as well as to elucidate the phylogenetic relationships of clinical isolates (Warren *et al.*, 2004; Kremer *et al.*, 2005). VNTR analysis has also been used to evaluate *M. bovis* transmission (Roring *et al.*, 2004)

1.4.2.4 Genomic deletion analysis

Regions of difference (RD) are used to differentiate between species in the MTC. It is a rapid, simple and reliable PCR-based MTC typing method that makes the use of MTC chromosomal region-of-difference deletion loci. Several specific primers are used to amplify specific loci which together formed a MTC PCR typing panel. The final pattern of amplification products of all reactions, given by failure or success, segregates the tested strains from NTM isolates and by MTC subspecies identity. The panel not only provides an advanced approach to determine the subspecies of MTC isolates but also differentiate them from clinically important NTM species (Huard *et al.*, 2003).

1.4.3 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 the lengthy therapy of at least 6 months makes patient compliance

very difficult, which frequently creates drug resistant strains of *M. tuberculosis.* Other factors that contribute to the development of resistance are poor quality of drugs and wrong treatment regimens prescribed.

Two forms of resistance are known; acquired resistance when the organism becomes resistant after treatment within the same host and primary resistance when a resistant organism is transmitted to a different host.

MDR-TB is caused by strains of *M. tuberculosis* resistant to at least isoniazid and rifampicin. A strain is called XDR when a MDR-TB strain develops further resistance to any member of the quinolones and at least to one injectable drug (kanamycin, capreomycin or amikacin) (Shenoi and Friedland, 2009).

While drug-sensitive TB can be effectively treated, treatment of MDR-TB can exceed 2 years, thus increasing the cost and side effects significantly. In the absence of any new treatment and the growing epidemic of HIV infection, which weakens the host immune system and allows easier transmission of TB and the drug-resistant form, there is increasing concern about the control of the disease (Nunn *et al.*, 2005).

2 THE PRESENT INVESTIGATION

2.1 PURPOSE OF THE STUDY

2.1.1 Objectives

BTB is an important zoonotic disease, particularly in developing countries, and mainly in countries with high rates of HIV/AIDS, where little is known about the impact of BTB on public health.
In this study *M. tuberculosis* isolates from a population-based study in Mozambique were examined, with particular emphasis on BTB.

2.1.1.1 General objective

- To characterize MTC isolates and estimate the relative incidence of BTB in humans in Mozambique.

2.1.1.2 Specific objectives

- By molecular genetic methods characterize MTC isolates into sub-families and clones

- To relate those findings with the findings in husbandry

- To relate the obtained result with international databases and with the results of other studies accomplished in neighboring country

2.2 MATERIALS AND METHODS

2.2.1 Study area and patients

This study included *M. tuberculosis* isolates collected during a one year (2007-2008) Nation Wide Drug Resistance Surveillance (DRS) study performed by the National TB Control Program of Mozambique in 40 randomly selected districts around the country.

2.2.2 Sample processing

Clinical specimens were processed at the individual district laboratories for smear microscopy, and the sputum samples were referred to the National Reference Laboratory for culture and drug susceptibility testing. During the DRS, 1124 positive cultures were analyzed.

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 Swedish Institute for Communicable Disease Control, in Stockholm.

2.2.3 HIV testing

All the patients with TB were advised and tested voluntarily for HIV/AIDS. The patient had a right to refuse HIV testing. For patients who consented to undergo testing, blood was collected via veni-puncture and tested at the local Sanitary Unit.

HIV testing was performed according to the recommendations given by the Ministry of Health, Mozambique at the Sanitary Unit of enrolment. 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.

2.2.4 Chromosomal DNA isolation

Briefly, mycobacteria were harvested, heat killed at 80ºC for 20 minutes and then subjected to repeated freeze thawing. Bacteria were resuspended in TE (Tris; EDTA) buffer and lysed for two hours at 37ºC. Incubation were made at 65ºC 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 redissolved in TE buffer.

2.2.5 Spoligotyping

Spoligotyping, previously described in the introduction, was performed to assign all isolates into lineages and sub-lineages. 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 DNAwere used with each experiment.

Spoligotyping results were analyzed with a Bionumerics Software version 5.01 (Applied Maths, Kortrijk, Belgium). Dendrograms were generated and the obtained signatures were compared with the SITVIT2 database.

2.2.6 RFLP

IS*6110* RFLP genotyping, previously described in the introduction, was performed using the insertion sequence IS*6110* as a probe and *PvuII* as the restriction enzyme (van Embden *et al.*, 1993; van Soolingen *et al.*, 1995). Visual bands were analyzed using the BioNumerics software version 5.10 (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 IS*6110* copies of each isolate, fingerprint patterns were compared by the un-weighted pair-group method of arithmetic averaging using the Jaccard coefficient. Dendrograms were constructed to show the degree of relatedness among strains according to a previously described algorithm (van Soolingen *et al.*, 1991) and similarity matrixes were generated to visualize the relatedness between the banding patterns of all isolates.

2.2.7 Regions of Difference (RD) analysis

In paper I, we investigated five Manu pattern isolates for the presence of genomic deletion of RD105 (deleted in the Beijing lineage). The DNA was analyzed by PCR using primers previously described (Hanekom *et al.*, 2007). PCR was carried out under the following conditions:10mM Tris–HCl (pH 8.8), 1.5mM MgCl2, 50mM KCl, 0.1% Triton X-100, 0.5mM primers, 0.2mM deoxynucleoside triphosphates, 1U of Taq polymerase (Dynazyme) and 10ng DNA per 50 ml 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.

In paper II, the identification of the genomic deletions RD105, RD142, RD150 and RD181 in the Beijing isolates was performed using the protocol described above.

2.2.8 MIRU-VNTR

Standardized 24-locus MIRU-VNTR typing (Supply *et al.*, 2006) 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 4.0 (Applied Biosystems) according to the manufacturers' instructions.

2.3 RESULTS AND DISCUSSION

2.3.1 Paper I

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

In total we studied 445 isolates from new pulmonary TB cases from 7 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 EAI, an evolutionary recent but yet ill-defined T clade, and the globally-emerging Beijing clone (Figure 3). The predominance of the LAM family is not surprising as it is believed that this lineage is globally disseminated, causing about 15% of TB worldwide (Gibson *et al.*, 2008). Indeed, out of the 12 sublineages reported so far worldwide for the LAM clade (Brudey *et al.*, 2006), a total of 8 sublineages were present in our 1 year recruitment.

The major phylogenetic lineages were nearly equally attributed both to ancestral and evolutionary modern *M. tuberculosis* lineages with a high spoligotype diversity documented for EAI, LAM and T lineages. 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. Nowadays, 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 % (Foster, 1996).

Paper I also attempted to describe the worldwide distribution of predominant Spoligotype 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) (Brudey *et al.*, 2006).

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 (Warren *et al.*, 2004).

No *M. africanum* isolates were detected. *M. africanum* is highly prevalent in West African countries, with its epicentre in Guinea Bissau (Koivula *et al.*, 2004; de Jong *et al.*, 2009; Groenheit *et al.*, 2011) but is rarely seen in East and Southern Africa (Chihota *et al.*, 2007; Asiimwe *et al.*, 2008). The *M. tuberculosis* genotype T2-Uganda (previously designated *M. africanum* subtype II) was shown to be mainly responsible for the TB epidemic in Kampala, Uganda (Asiimwe, 2008), although not so common in other East African countries as Kenya (Githui *et al.*, 2004) and the Mozambican neighbour Tanzania (Kibiki *et al.*, 2007). In this study, no strains of the *M. tuberculosis* genotype T2-Uganda (Asiimwe *et al.*, 2008) were found.

The total absence of *M. bovis* in this one year study is noteworthy. Although BTB is an important disease of cattle and other domestic animals in Mozambique, no *M. bovis*, the causative agent of BTB, was found. One reason could be that we have studied only sputum isolates. *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 BTB in cattle as this information was not accessible at that time. Today having that information available it would be interesting to investigate BTB in humans in areas with high prevalence of BTB in cattle to unveil the importance this zoonosis to public health in Mozambique.

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.

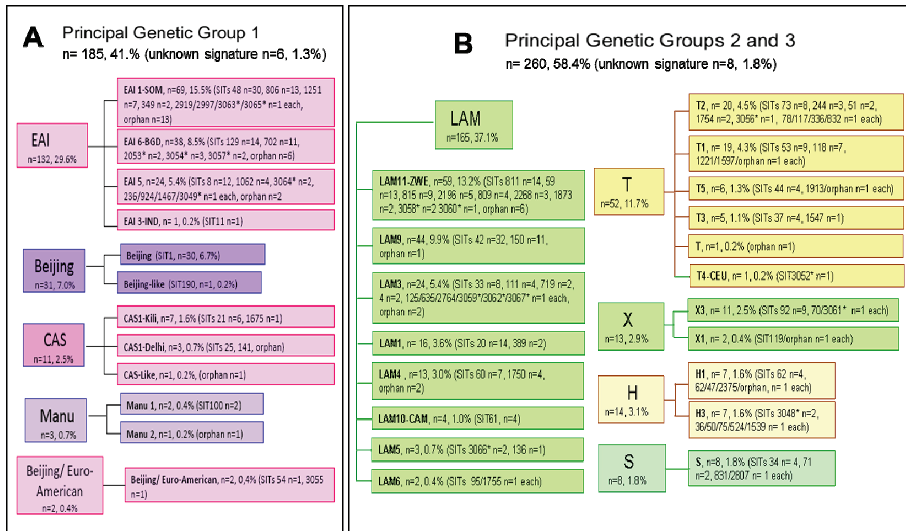


Figure 3. The principal genetic groups (PGG) in Mozambique

The figure illustrates the 4 most predominant clades in our study comprised both PGG1 and PGG2/3 lineages: LAM (PGG 2/3); ancestral EAI (PGG1); T clade (PGG 2/3); and the globally-emerging Beijing clone (PGG1).

2.3.2 Paper II

Paper II presents the in depth characterisation of the Beijing family, one of the predominant lineages found in Mozambique (Viegas *et al*, 2010). By spoligotyping out of 543 *M. tuberculosis* isolates, 33 were identified as Beijing lineage strains. Of these, 32 had all the characteristic Beijing spacers 35-43, corresponding to the shared type SIT1 as defined in SITVIT2 and one strain (code 35) lacked spacer 40, corresponding to SIT190 (Table 1).

We further analysed 29 Beijing strains for RD genomic deletions. All Beijing strains in this study had the RD105 deletion (Table 1). The large deletion of RD105 is considered to be a marker for Beijing strains (Tsolaki *et al.*, 2005; Kong *et al.*, 2006; Hanekom *et al.*, 2007), although deletion of the RD105 was recently found also in ancestral strains with non-Beijing spoligoprofiles (Flores *et al.*, 2007). Additional deletions of RD142, RD150 and RD181 may further divide this family into different sublineages (Tsolaki *et al.*, 2005). Moreover, no isolate showed deletion of the RD142 region, in accordance with the low frequency of this deletion event reported elsewhere (Tsolaki *et al.*, 2005; Hanekom *et al.*, 2007).
By a combined use of RD deletions and spoligotyping the 29 Beijing strains could be divided into 3 arbitrary genetic sublineages (Table 1). The sublineage 3, including 3 SIT1 isolates, with deletions of RD 105, 150 and 181, a characteristic signature of the reference strain "sublineage 7" recently emerging in South Africa (Hanekom *et al.*, 2007). These three isolates were from young patients (33, 20 and 19 years of age) from different provinces of the country.

Table 1. Polymorphisms of *M. tuberculosis* isolates of Beijing genotype

		Spoligotype description		Region of difference (RD)[a]			
Genotypic sublineage	Number of isolates (n=29)	SIT[b]	Binary format [c]	105	142	150	181
1	25	1	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	−	+	+	−
2	1	190	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■□■■■	−	+	+	−
3	3	1	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	−	+	−	−

[a] presence (+) or absence (−) of the specific genomic region

[b] spoligotype international type, designations were assigned according to the definition in the SITVIT2 database.

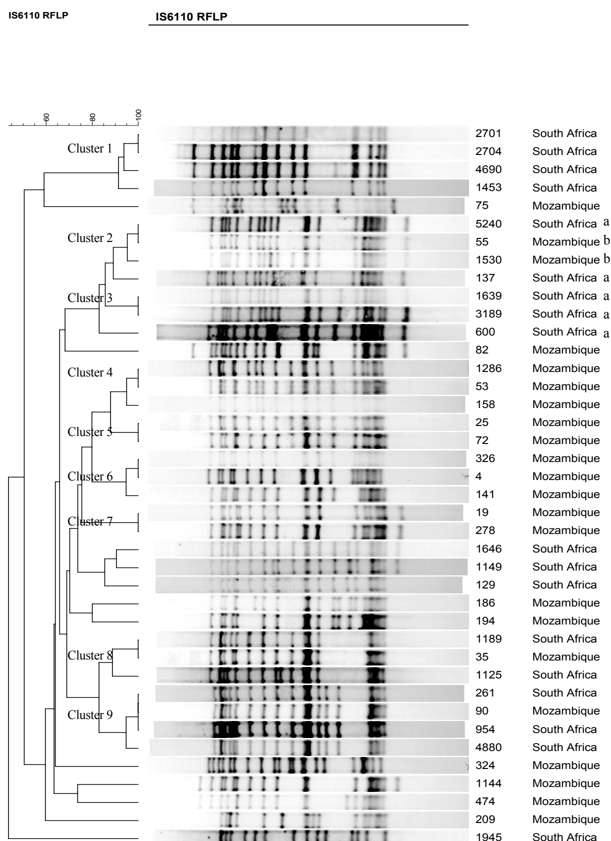
[c] The black and white boxes indicate the presence and absence, respectively, of the specific spacer at positions 1–43 in the DR locus.

IS*6110* RFLP and MIRU-VNTR were performed in Mozambican and South African reference strains for identification of similarities. When comparing the Beijing isolates from both countries by IS*6110* RFLP, the sublineage 3 isolates (55, 327 and 1530) from this study were very similar to the South African "sublineage 7" (Figure 4), although, the RFLP was not performed on the third sublineage 3 isolate. By MIRU-

VNTR analysis, the sublineage 3 isolates were also similar to the two South African "sublineage 7" isolates (Figure 5). These similarities may indicate that this sublineage has been recently introduced from South Africa.

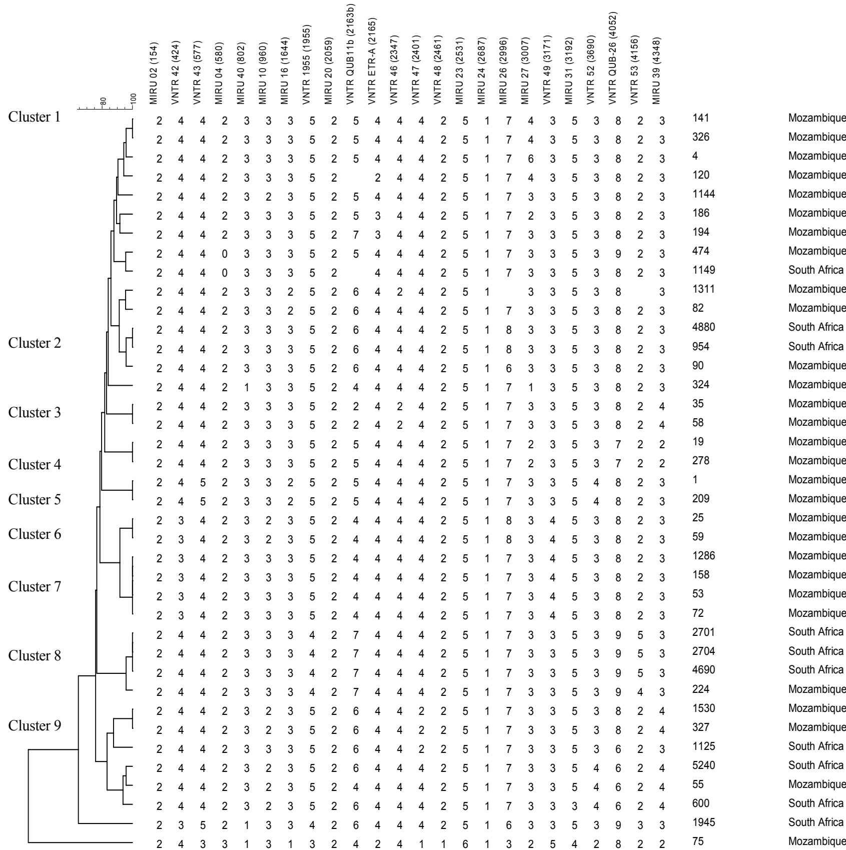
The cross border migration between Mozambique and South Africa is notorious. A comparison of the MIRU types for the Beijing strains from Mozambique and previously published MIRU type data from South Africa and East Asia (Middelkoop *et al.*, 2009) show that 23 of the Mozambican Beijing MIRU types were shared between these geographical settings forming 12 clusters (data not shown). This may suggest that the shared Beijing MIRU types represent founder strains that were introduced into Mozambique from South Africa and/or East Asia.

The Mozambican strains by RFLP and MIRU-VNTR formed 4 clusters (clusters 4, 5, 6 and 7) and 7 clusters (clusters 1, 3, 4, 5, 6, 7 and 9) respectively with 2 to 4 isolates each, demonstrating that the population structure of the Beijing genotype in Mozambique consists of more than one sublineage, indicating that these strains were introduced in the country on separate occasions.



[a] South Africa Beijing sublineage 7
[b] Mozambican sublineage 3

Figure 4. IS*6110* dendrogram of *M. tuberculosis* Beijing strains from Mozambique and South Africa



[a] South Africa Beijing sublineage 7
[b] Mozambican sublineage 3

Figure 5. 24 MIRU-VNTR dendrogram of *M. tuberculosis* Beijing strains from Mozambique and South Africa

Univariable and multivariable logistic regression models were estimated for Beijing lineage as outcome and sex, age, HIV status and province (Maputo city or other) included as covariates. Interestingly, the majority of the Beijing strains were found in the South region of Mozambique, with predominance in the capital Maputo City (17%; $P<0.001$). Beijing strains were significantly predominant among HIV positive individuals ($p=0.049$) in Mozambique, a worrying fact as Beijing strains have recently been reported to be associated with HIV positive serostatus in South Africa (Middelkoop *et al.*, 2009), a connection which is further supported here. Although Beijing strains have been associated with MDR tuberculosis (Drobniewski *et al.*, 2005), only one from Mozambican strain was drug resistant.

2.4 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This study represents the first report on the genetic diversity of *M. tuberculosis* in Mozambique, a TB high burden country with high prevalence of HIV/AIDS and surrounded by countries where those pandemics represent an enormous public health problem. This study demonstrated the importance of molecular typing in assessing the global dissemination of strains and in understanding the transmission dynamics, and emphasizes the importance of monitoring the transmission between countries, particularly with countries with strong relationship, migration history and geographic proximity, as a tool in the development of National strategies for TB control.

In paper I we found that TB in Mozambique is mainly caused by *M. tuberculosis* strains, no *M. bovis* was found, and that the predominant lineages were also common in neighboring countries, particularly in South Africa. Regarding the interesting finding on the predominance of the Beijing lineage we further investigated this global emerging strain (Glynn *et al.*, 2002), reported to be associated with HIV infection (Caws *et al.*, 2008), enhanced virulence (Mathema *et al.*, 2006) and MDR (Glynn *et al.*, 2002), and compared our results with selected Beijing strains from South Africa, indicating a recently introduction of the South African Sublineage 7 in Mozambique. Furthermore, in this study, no association with drug resistance was found, however a significant association between Beijing strains and HIV positive serostatus was found.

In Mozambique, domestic animals are an integral part of human social life and risk factors for *M. bovis* infection are close contact, food hygiene practices and HIV/AIDS infection (Cosivi *et al.*, 1998). In the country, control policies have not been enforced due to cost implications, lack of capacity and infrastructure limitations.

The fact that no human *M. bovis* infection was found does not answer the concern if it really represents a public health problem in Mozambique. Our sampling only included sputum isolates. *M. bovis* is thought to spread through unpasteurized milk, and hence would mainly cause abdominal or disseminated TB. Moreover at the time we did not have information of which areas had higher prevalence of BTB in cattle. For this reason we recommend a further study, including isolates from patients with extra pulmonary TB, in areas with high prevalence of BTB in cattle, with the aim to ascertain the public health importance of this zoonosis.

With the ongoing strengthening and capacity building at the laboratory reference level in Mozambique, we plan in the near future to introduce molecular genetic methods at reference level, particularly for migrant patients, with emphasis on mine workers from South Africa in order to control the transmission between countries, as well as for HIV positive individuals for monitoring possible epidemics related to opportunistic strains and drug resistance.

3 ACKNOWLEDGEMENTS

Many people have contributed to this work either directly or indirectly, colleagues, friends and family. My work would not have been the same without any of you and I am therefore grateful for all your help and support. Especially, I would like to express my sincere gratitude to the following persons:

Tuija Koivula, my main supervisor, for your guidance through all the process of research, generosity, for helping me in the right direction. Thank you for all your positive support and encouragement during the past years.

Gunilla Källenius, my co-supervisor, for inspiring me during this work and also for being most helpful in every possible way during the years. Thanks for never giving up on me, for all support and continuous interest.

Adelina Machado, for encouraging me during the past years and for your support during all the process. *Kanimanbo!*

Sven Hoffner, for welcoming me to his lab. During the time spent in Sweden I was given the opportunity to work independently and take responsibilities.

Ricardo Thompson, for giving me the opportunity to participate in this project. I really appreciate the trust you put in me.

Ilesh Jani, my recent director, for his support and encouragement during the past year. Thank you for believing in me and giving me the challenge of coordinating the TB Reference Laboratories in my country.

Ramona, Solomon and Alexandra, for your friendship and generosity. Thank you for introducing and teaching me the molecular techniques used during this training.

The co-authors for their valuable help: Paula S Gudo, Zaina Cuna, Paolo Miotto, Véronique Hill, Tatiana Marrufo, Daniela M Cirillo, Nalin Rastogi, Rob Warren and Egidio Langa.

To all great colleagues and strong team from the TB National Reference Lab, Khalid, Nureisha, Carla, Ezembro, Cátia, Cristolde, Salomão, Mercedes, João Manuel, Dimande and Leonel, thank you for receiving me as a member of the TB family.

Luís Neves and all friends from the Center of Biotechnology for kindly offering me laboratory access.

Past and present members of the TB group at SMI, Senia, Benon, Freddie, Lisbeth, Emma, Melles, Maria, Anna, Pontus, Jim, Jolanta and Andrzej for your kindness.

All Sida-SAREC project 12 team, for helping and supporting.

Noémia, *minha amiga companheira, obrigada pelo apoio durante os longos periodos que passei fora de casa e tu foste o meu ombro amigo.*

My journey fellows, Paula, Belisário, Meraldina, Nádia, Lucilio e Magaia *obrigada pela vossa companhia e pelo vosso apoio.*

Anisha Prabhu, thanks for your kindness and for critically revising my thesis.

Special thanks to Djion e Mena, my parents in law, for encouraging me to do what I believe.

My brother Dário and my sisters Edna, Aissa e Alanis. *Obrigada meus manos pelo vosso amor genuíno e por estarem sempre presentes para todo e qualquer momento da minha vida.*

My father Ricardo, for his endless love, priceless advice and supporting me in every possible way. *Obrigada meu pai por seres o meu exemplo de vida.*

My husband Ébano*, amor da minha vida, obrigada pelo teu apoio incondicional e pela compreenção durante o tempo que passei fora de casa*.

My son Zidane*, minha benção e a minha razão de viver! Obrigada meu filhote, por cada sorriso, cada abraço, cada passo teu deu-me forças para terminar esta tese*.

Most of all, my mother Ratiba, who although far away has always been my inspiration, and to whom I dedicate this thesis.

4 REFERENCES

Abe C. et al. **1992**; J Clin Microbiol 30:878-81.
Andrews J. R. et al. Exogenous reinfection as a cause of multidrug-resistant and extensively drug-resistant tuberculosis in rural South Africa. J Infect Dis **2008**; 1;198:1582-9.
Aranaz A. et al. *Mycobacterium tuberculosis* subsp. *caprae* subsp. nov.: a taxonomic study of a new member of the *Mycobacterium tuberculosis* complex isolated from goats in Spain. Int J Syst Bacteriol **1999**; 49 Pt 3:1263-73.
Aranaz A. et al. Elevation of *Mycobacterium tuberculosis* subsp. *caprae* to species rank as *Mycobacterium caprae* comb. nov., sp. nov. Int J Syst Evol Microbiol **2003**; 53 Pt 6:1785-9.
Ayele W. Y. et al. Bovine tuberculosis: an old disease but a new threat to Africa. Int J Tuberc Lung Dis **2004**; 8: 924–37.
Asiimwe B. B. et al. *Mycobacterium tuberculosis* spoligotypes and drug susceptibility pattern of isolates from tuberculosis patients in peri-urban Kampala, Uganda. BMC Infect Dis **2008**; 8:101.
Badri M. et al. Effect of highly active antiretroviral therapy on incidence of tuberculosis in South Africa: a cohort study. Lancet **2002**; 359:2059–2064
Bandera A. et al. Molecular epidemiology study of exogenous reinfection in an area with a low incidence of tuberculosis. J Clin Microbiol **2001**; 39(6):2213-8.
Bonard D. et al. What is the meaning of repeated isolation of *Mycobacterium africanum?* Int J Tuberc Lung Dis **2000**; 4(12):1176-80.
Boniotti M. B. et al. Molecular typing of *Mycobacterium bovis* strains isolated in Italy from 2000 to 2006 and evaluation of variable-number tandem repeats for geographically optimized genotyping. J Clin Microbiol **2009**; 47(3):636-44.
Brisse S. et al. "A re-evaluation of M. prototuberculosis": continuing the debate. PLoS Pathog **2006**; 2(9):e95.
Brosch R. S. et al. A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proc. Natl. Acad. Sci. USA **2002**; 99:3684–3689.
Brudey K. et al. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol **2006**; 6:23.
Caminero J. A. et al. Epidemiological evidence of the spread of a *Mycobacterium tuberculosis* strain of the Beijing genotype on Gran Canaria Island. Am J Respir Crit Care Med **2001**;164(7):1165-70.
Centers for Disease Control and Prevention (CDC). Emergence of *Mycobacterium tuberculosis* with extensive resistance to second-line drugs--worldwide, 2000-2004. MMWR Morb Mortal Wkly Rep **2006**; 24;55(11):301-5.
Chihota V. et al. Predominance of a single genotype of *Mycobacterium tuberculosis* in regions of Southern Africa. Int J Tuberc Lung Dis **2007**; 11(3):311-318.
Caws M. et al. The influence of host and bacterial genotype on the development of disseminated disease with Mycobacterium tuberculosis. PLoS Pathog **2008**; 4: e1000034.
Collins C. H. et al. Subdivision of *Mycobacterium tuberculosis* into five variants for epidemiological purposes: methods and nomenclature. J Hyg (Lond) **1982**; 89(2):235-42.
Cousins D. V. et al. Tuberculosis in wild seals and characterisation of the seal bacillus. Aust Vet J **1993**; 70(3):92-7.

Cousins D. V. et al. Tuberculosis in seals caused by a novel member of the *Mycobacterium tuberculosis* complex: *Mycobacterium pinnipedii* sp. nov. Int J Syst Evol Microbiol **2003**; 53(Pt 5):1305-14.
Cosivi O. et al. Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries. Emerg Infect Dis **1998**; 4(1):59-70.
Daffe M. et al. Novel type-specific lipooligosaccharides from *Mycobacterium tuberculosis*. Biochemistry **1991**; 15; 30(2):378-88.
Daley C. L. et al. An outbreak of tuberculosis with accelerated progression among persons infected with the human immunodeficiency virus. An analysis using restriction-fragment-length polymorphisms. N. Engl. J. Med. **1992**; 326:231–235.
de Cock K. M. et al. Tuberculosis and HIV infection in sub-Saharan Africa. JAMA **1992**; 23-30; 268(12):1581-7. Review.
de Jong B. C. et al. Use of spoligotyping and large sequence polymorphisms to study the population structure of the *Mycobacterium tuberculosis* complex in a cohort study of consecutive smear-positive tuberculosis cases in The Gambia. J Clin Microbiol **2009**; 47(4):994-1001.
Di Perri G. M. et al. Nosocomial epidemic of active tuberculosis among HIV-infected patients. Lancet **1989**; ii:1502–1504.
Diedrich C. R and Flynn J. L. HIV-1/*Mycobacterium tuberculosis* coinfection immunology: how does HIV-1 exacerbate tuberculosis? Infect Immun **2011**; 79(4):1407-17.
Djelouadji Z. et al. A single-step sequencing method for the identification of *Mycobacterium tuberculosis* complex species. PLoS Negl Trop Dis **2008**; 18; 2(6):e253.
Drobniewski F. et al. Drug-resistant tuberculosis, clinical virulence, and the dominance of the Beijing strain family in Russia. JAMA **2005**; 293: 2726-2731.
Erler W. et al. Molecular fingerprinting of *Mycobacterium bovis* subsp. caprae isolates from central Europe. J Clin Microbiol **2004**; 42(5):2234-8.
Flores L. et al. Large sequence polymorphisms classify *Mycobacterium tuberculosis* strains with ancestral spoligotyping patterns. J Clin Microbiol **2007**; 45: 3393-3395.
Foreit K. F. et al. Population movements and the spread of HIV/AIDS in Mozambique. J Health Hum Serv Adm **2001**; 24(3):279-94.
Forshaw D. and Phelps G. R. Tuberculosis in a captive colony of pinnipeds. J Wildl Dis **1991**; 27(2):288-95.
Foster S. The implications of HIV/AIDS for South African mines. AIDS Anal Afr **1996**; 7(3):5.
Franzetti F. et al. Genotyping analyses of tuberculosis transmission among immigrant residents in Italy. Clin Microbiol Infect **2010**; 16(8):1149-54.
Frothingham R. et al. Phenotypic and genotypic characterization of *Mycobacterium africanum* isolates from West Africa. Clin Microbiol **1999**; 37(6):1921-6.
Gagneux S. et al. The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. Science **2006**; 312:1944–1946.
Gervois M. et al. Epidemiology of the human infection due to *Mycobacterium bovis*. Arch Monaldi**1972**; 27(3):294-317.
Gibson A. L. et al. Application of sensitive and specific molecular methods to uncover global dissemination of the major RDRio Sublineage of the Latin American-Mediterranean *Mycobacterium tuberculosis* spoligotype family. J Clin Microbiol. **2008** Apr; 46(4):1259-67. Epub 2008 Jan 30.

Girardi E. et al. Incidence of tuberculosis among HIV-infected patients receiving highly active antiretroviral therapy in Europe and North America. Clin. Infect. Dis **2005**; 41:1772–1782.
Githui W. A. et al. Identification of MDR-TB Beijing/W and other *Mycobacterium tuberculosis* genotypes in Nairobi, Kenya. Int J Tuberc Lung Dis **2004**; 8(3):352-360.
Glynn J. R. et al. *Mycobacterium tuberculosis* Beijing genotype, northern Malawi. *Emerg Infect Dis* **2005**; 11(1):150-153
Griffith D. E. et al. Diagnosing nontuberculous mycobacterial lung disease. A process in evolution. Infect Dis Clin North Am **2002**; 16(1):235-49. Review.
Groenheit R. et al. The Guinea-Bissau Family of *Mycobacterium tuberculosis* Complex Revisited. G.PLoS One **2011**; 20; 6(4):e18601.
Hanekom M. et al. A recently evolved sublineage of the *Mycobacterium tuberculosis* Beijing strain family is associated with an increased ability to spread and cause disease. J Clin Microbiol **2007**; 45: 1483-1490.
Haas W. H. et al. Comparison of DNA fingerprint patterns of isolates of *Mycobacterium africanum* from east and west Africa. J Clin Microbiol **1997**; 35(3):663-6.
Hermans P. W. et al. Insertion element IS987 from *Mycobacterium bovis* BCG is located in a hot-spot integration region for insertion elements in *Mycobacterium tuberculosis* complex strains. Infect Immun **1991**; 59(8):2695-705.
Hoffner S. E. et al. Biochemical heterogeneity of *Mycobacterium tuberculosis* complex isolates in Guinea-Bissau. J Clin Microbiol **1993**; 31(8):2215-7.
Houben R. M. and **Glynn J. R.** A systematic review and meta-analysis of molecular epidemiological studies of tuberculosis: development of a new tool to aid interpretation. Trop Med Int Health **2009**; 14(8):892-909.
Huard R. C. et al. PCR-based method to differentiate the subspecies of the *Mycobacterium tuberculosis* complex on the basis of genomic deletions. J Clin Microbiol **2003;** 41(4):1637-50.
Hunter J. E. et al. First report of potentially zoonotic tuberculosis in fur seals in New Zealand. N Z Med J **1998**; 111(1063):130-1.
Instituto Nacional de Saúde (INS), Instituto Nacional de Estatística (INE), e ICF Macro. Inquérito Nacional de Prevalência, Riscos Comportamentais e Informação sobre o HIV e SIDA em Moçambique **2009**. 2010 pp 1-333.
Kamerbeek J. et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol. **1997**; 35(4):907-14.
Katila M. L. et al. Accelerated detection and identification of mycobacteria with MGIT 960 and COBAS AMPLICOR systems. J Clin Microbiol **2000**; 38(3):960-4.
Kibiki G. S. et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J. Clin. Microbiol **1997**; 35, pp. 907–914.
Kiers A. et al. Transmission of *Mycobacterium pinnipedii* to humans in a zoo with marine mammals. Int J Tuberc Lung Dis **2008**; 12(12):1469-73.
Kubica T. et al. *Mycobacterium bovis* subsp. *caprae* caused one-third of human *M. bovis*-associated tuberculosis cases reported in Germany between 1999 and 2001. J Clin Microbiol **2003**; 41(7):3070-7.
Koivula T. et al. Genetic characterization of the Guinea-Bissau family of *Mycobacterium tuberculosis* complex strains. Microbes Infect **2004**; 6(3):272-278.

Kong Y. et al. Population-based study of deletions in five different genomic regions of *Mycobacterium tuberculosis* and possible clinical relevance of the deletions. J Clin Microbiol **2006**; 44: 3940-3946.
Kremer K. et al. Discriminatory power and reproducibility of novel DNA typing methods for *Mycobacterium tuberculosis* complex strains. J Clin Microbiol **2005** Nov; 43(11):5628-38.
Källenius G. et al. Evolution and clonal traits of *Mycobacterium tuberculosis* complex in Guinea-Bissau. J Clin Microbiol **1999**; Dec; 37(12):3872-8.
Lawn S. D. et al. Contribution of immune activation to the pathogenesis and transmission of human immunodeficiency virus type 1 infection. Clin Microbiol Rev **2001**; 14(4):753-77. Review.
Lucas S. B. et al. The mortality and pathology of HIV infection in a west African city. AIDS **1993**; 7(12):1569-79.
Mathema B. et al. Molecular epidemiology of tuberculosis: current insights. Clin Microbiol Rev **2006**; 19(4):658-85. Review.
Middelkoop K. et al. Molecular epidemiology of *Mycobacterium tuberculosis* in a South African community with high HIV prevalence. J Infect Dis **2009**; 200: 1207-1211.
Ministério da Saúde Programa Nacional de Controle da Tuberculose. Plano Estratégico Nacional Para o Controle da Tuberculose em Moçambique, 2008-2012. **2007**; pp 1-74
Ministério da Saúde Programa Nacional de Controle da Tuberculose. Relatório das Actividades Desenvolvidas Durante o Ano **2010**; pp 1-13.
Moström P. et al. Methods used in the molecular epidemiology of tuberculosis. Clin Microbiol Infect **2002** Nov; 8(11):694-704. Review.
Moro M. L. et al. Two-year population-based molecular epidemiological study of tuberculosis transmission in the metropolitan area of Milan, Italy. Eur J Clin Microbiol Infect Dis **2002**; 21(2):114-22.
Mostowy S. et al. Genomic analysis distinguishes Mycobacterium africanum. J Clin Microbiol **2004** Aug;42(8):3594-9.
Mworozi E. A. AIDS and civil war: a devil's alliance. Dislocation caused by civil strife in Africa provides fertile ground for the spread of HIV. AIDS Anal Afr **1993**; 3(6):8-10.
Niemann S. et al. Biochemical and genetic evidence for the transfer of *Mycobacterium tuberculosis* subsp. caprae Aranaz et al. 1999 to the species Mycobacterium bovis Karlson and Lessel 1970 (approved lists 1980) as *Mycobacterium bovis* subsp. *caprae* comb. nov. Int J Syst Evol Microbiol **2002**; 52(Pt 2):433-6.
Nunn P. et al. M. Tuberculosis control in the era of HIV. Nat Rev Immunol **2005**; 5(10):819-26. Review.
Owen M. The bottom line: Mozambique. Plan Parent Chall **1997**; (1-2):45-7.
Pavlik I. et al. Molecular epidemiology of bovine tuberculosis in the Czech Republic and Slovakia in the period 1965–2001 studied by spoligotyping. Vet Med (Praha) **2002**; 47:181–94.
Prodinger W. M. et al. Infection of red deer, cattle, and humans with *Mycobacterium bovis* subsp. *caprae* in western Austria. J Clin Microbiol **2002**; 40(6):2270-2.
Roring S. et al. Evaluation of variable number tandem repeat (VNTR) loci in molecular typing of *Mycobacterium bovis* isolates from Ireland. Vet Microbiol **2004**; 101(1):65-73.

Sahraoui N. et al. Molecular characterization of *Mycobacterium bovis* strains isolated from cattle slaughtered at two abattoirs in Algeria.BMC Vet Res **2009**; 27; 5:4.
Selwyn, P. A. et al. High risk of active tuberculosis in HIV-infected drug users with cutaneous anergy. JAMA **1992**; 268:504–509.
Shenoi S. and Friedland G. Extensively drug-resistant tuberculosis: a new face to an old pathogen. Annu Rev Med **2009**; 60:307-20. Review.
Sintchenko V. et al. A case of urinary tuberculosis due to *Mycobacterium bovis* subspecies *caprae*. Pathology **2006**; 38(4):376-8.
Smith N. H. et al. Bottlenecks and broomsticks: the molecular evolution of *Mycobacterium bovis*. Nat Rev Microbiol **2006**; 4:670-681.
Somoskovi A. et al. Laboratory diagnosis of nontuberculous mycobacteria. Clin Chest Med **2002**; 23(3):585-97. Review.
Springer B. et al. Two-laboratory collaborative study on identification of mycobacteria: molecular versus phenotypic methods. J Clin Microbiol **1996**; 34(2):296-303.
Stead W. W. et al. When did *Mycobacterium tuberculosis* infection first occur in the New World? An important question with public health implications. Am J Respir Crit Care Med **1995**; 151(4):1267-8.
Stephen H. G. et al. Evolution of drug resistance in *M. tuberculosis*. Clinical and molecular perspective of antimicrobial agents and chemotherapy. **2002**. DOI: 10.1128/AAC.46.2.267–274. pp. 267–274
Supply P. et al. Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Mol Microbiol **2000**; 36:762-71.
Supply P. et al. Automated high-throughput genotyping for study of global epidemiology of *Mycobacterium tuberculosis* based on mycobacterial interspersed repetitive units. J Clin Microbiol **2001**; 39(10):3563-71.
Supply P. et al. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol **2006**; 44(12):4498-510.
Thompson P. J. et al. Seals, seal trainers, and mycobacterial infection. Am Rev Respir Dis **1993**; 147(1):164-7.
Tick E. et al. Mozambique: building hope, seeking help. Explore (NY) **2007**; 3(5):511-3.
Toossi Z. J. G. et al. Enhanced susceptibility of blood monocytes from patients with pulmonary tuberculosis to productive infection with human immunodeficiency virus type 1. J. Exp. Med **1993**; 177:1511–1516
Tsolaki A. G. et al. Genomic deletions classify the Beijing/W strains as a distinct genetic lineage of *Mycobacterium tuberculosis*. J Clin Microbiol **2005**; 43: 3185-3191.
van Rie A. et al. Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment. N Engl J Med **1999**; 341(16):1174-9.
van Soolingen D. et al. Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. J Clin Microbiol **1991**; 29(11):2578-86.
van Soolingen D. et al. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of Mycobacterium tuberculosis. J Clin Microbiol **1993**; 31(8):1987-95.
van Soolingen D. et al. A novel pathogenic taxon of the *Mycobacterium* tuberculosis complex, Canetti: characterization of an exceptional isolate from Africa. Int J Syst Bacteriol **1997**; 47(4):1236-45.

van Soolingen D. et al. Diagnosis of *Mycobacterium microti* infections among humans by using novel genetic markers. J Clin Microbio **1998**; 36(7):1840-5.
van Soolingen D. et al. *M. tuberculosis* genotypic diversity and drug susceptibility pattern in HIV-infected and non-HIV-infected patients in northern Tanzania. BMC Microbiol **2007**; 7:51.
Viana-Niero C. et al. Genetic diversity of *Mycobacterium africanum* clinical isolates based on IS*6110*-restriction fragment length polymorphism analysis, spoligotyping, and variable number of tandem DNA repeats. J Clin Microbiol **2001**; 39(1):57-65.
Viegas S. O. et al. Molecular diversity of *Mycobacterium tuberculosis* isolates from patients with pulmonary tuberculosis in Mozambique. BMC Microbiol **2010**; 10: 195.
Warren R. M. et al. Patients with active tuberculosis often have different strains in the same sputum specimen. Am J Respir Crit Care Med **2004**; 169(5):610-614.
World Health Organization. Global tuberculosis control - epidemiology, strategy, financing. WHO Report **2009**. pp 39. WHO/HTM/TB/2009.411
Wu P. et al. The Transmission Dynamics of Tuberculosis in a Recently Developed Chinese City. PLoS One. **2010**; 5(5): e10468.
Xavier E. F. et al. Human and animal infections with *Mycobacterium microti*, Scotland. Emerg Infect Dis **2007**; 13(12):1924-7.