Genetic relatedness of *Mycobacterium tuberculosis* strains obtained from Kalafong Hospital in Pretoria using Spoligotyping and MIRU-VNTR typing

by

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Declaration

I, the undersigned, declare that the dissertation hereby submitted to the University of Pretoria for the degree MSc (Medical Microbiology) and the work contained therein is my own original work and has not previously, in its entirety or in part, been submitted to any university for a degree.

Signed…….this……..day of ……2012

Dedication

I would like to say thank you to my mum, you known you're the best person in my life, thank you a million times Mummy for everything. My siblings, guys you're my rock and my reality check could not have taken my first step if it was not for you believing in me. To my late Dad you still are my Hero. I also would like to thank my colleagues in the post graduate office for the warm office environment that they provided for me to be able to think and work effectively. To all my friends both in the department and off the campus premises thank you for the good times we had. To everyone that supported me in my studies that were not mentioned here thank you a lot, could not have made it without your input and support. Lastly, I would like to humbly say thank you to God for being so faithful to me and for the reassurance every single day that I should be still and trust he is GOD.

When I hear somebody sigh "Life is hard," I am always tempted to ask, "Compared to what?" Sydney J, Harris

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LIST OF ABBREVIATIONS

A	Adenine
AFB	Acid fast bacilli
AMI	Amikacin
ATCC	American Type Culture Collection
BCG Vaccine	Bacillus Calmette-Guérin Vaccine
C	Cytosine
CAP	Capreomycin
CCL2	(C-C motif) Ligand 2
CD4+ cells	Clusters of differentiation 4 cells
CD8+ cells	Clusters of differentiation 8 cells
CM	Common mycobacteria
DNA	Deoxyribonucleic acid
DOTS	Directly Observed Treatment, Short-Course
DR	Direct repeats
DVR	Direct variable repeats
ECL	Enhanced chemiluminescence
EMB	Ethambutol
FDA	Food and Drug Administration
G	Guanosine
GLC	Gas liquid chromatography
HEX	6-carboxy-2′, 4, 4′, 5′, 7, 7′-hexachlorofluorescein dye
HGDI	Hunter-Gaston Discriminatory Index
HIV	Human immunodeficiency virus
IFN-γ	Interferon-gamma
IL-12	Interleukin-12
IL-18	Interleukin-18
IL-1α	Interleukin-1-alpha
IL-1-β	Interleukin-1-beta
INH	Isoniazid
IS	Insertion sequence
IS*3*	Insertion sequence *3*
IS*1081*	Insertion sequence *1081*
IS*6110*	Insertion sequence *6110*
KAN	Kanamycin
KZN	KwaZulu-Natal
LAM	Lipoarabinomannan
LM	Lipomannan
MDR-TB	Multidrug-resistant tuberculosis

MIRU	Mycobacterial interspersed repetitive units
MIRU-VNTR	Mycobacterial interspersed repetitive units-variable number tandem repeats
MOTTS	*Mycobacterium*-other-than-tuberculosis
NAAT	Nucleic acid amplification test
NED	4,7-trichloro-5-carboxyfluorescein
OADC	Oleic albumin dextrose catalase
PAS	Para-aminosalicylic acid
PCR	Polymerase chain reaction
PGRS	Polymorphic GC-rich repetitive sequence typing
PZA	Pyrazinamide
RIF	Rifampicin
SCC	Short Course Chemotherapy
SIT	Spoligo-international type
SITVIT2	Spoligo-international type and MIRU-VNTR international type 2
SpolDB1	Spoligotype Database 1
SpolDB2	Spoligotype Database 2
SpolDB3	Spoligotype Database 3
SpolDB4	Spoligotype Database 4
ST	Shared types
STR	Streptomycin
T	Thymine
TAE	Tris-acetate-EDTA
TB	Tuberculosis
T-cells	Thymus cells
TLR2	Toll-like receptor 2
TLR9	Toll-like receptor 9
TNF-α	Tumor necrosis factor-alpha
UPGMA	Unweighted pair group method with arithmetic mean
WHO	World Health Organization
XDR-TB	Extensively drug-resistant tuberculosis
ZN	Ziehl-Neelsen

LIST OF PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

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Genetic relatedness of *Mycobacterium tuberculosis* using Spoligotyping and MIRU–VNTR typing

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SUMMARY

Every year close to 9 million people contract tuberculosis (TB) and approximately 2 million die from the disease. The highest number of TB cases is in Asia while Africa has the highest incidence rates due to high rates of HIV and malnutrition that weakens the immune systems and speeds up the spread of the disease. The management of TB has faced many challenges in the past but the two most important threats to global TB control are the HIV epidemic and the increasing prevalence of drug resistance.

The occurrence and transmission of *Mycobacterium tuberculosis* (*M. tuberculosis*) strain families vary by countries or by regions within the same country. Correct identification of *M. tuberculosis* strain families in a given geographical area is therefore, important for epidemiological investigation. Molecular typing of *M. tuberculosis* isolates has facilitated the understanding of the epidemiology of TB, its control and prevention.

The insertion sequence *6110* restriction fragment length polymorphism (IS*6110* RFLP) has been considered the 'gold standard' in *M. tuberculosis* genotyping due to its high discriminatory power. However, due to limitations, such as the requirement of large quantities of DNA, several polymerase chain reaction (PCR) based genotyping methods have been developed. These methods include spoligotyping and mycobacterial interspersed

repetitive units-variable number of tandem repeats (MIRU-VNTR). Spoligotyping is widely used because of its low cost, high reproducibility, simplicity and ease of interpretation due to its binary results format, while the MIRU-VNTR assay is robust, reliable and easier to perform compared to IS*6110* RFLP typing.

In South Africa, *M. tuberculosis* genotyping assays have been applied in only a few provinces, such as the Western Cape and KwaZulu-Natal. The purpose of this study was to determine the prevalence of the *M. tuberculosis* strains circulating in the Kalafong Hospital in Pretoria, Gauteng province by using spoligotyping and MIRU-VNTR typing methods.

Spoligotyping identified 39 distinct spoligotypes of which 36% (14/39) were unreported in the SITVIT2 database. There were three strain families that were found to be represented by most of the isolates in the study ('ill-defined' T, Beijing and LAM). These strain families fall within the major families of the *M. tuberculosis* strains (Brudey *et al.*, 2006). The T1 subfamily, which is a member of the 'ill-defined' T family had the highest number of isolates (19). In the 12 loci based MIRU-VNTR typing analysis, 87 distinct patterns were obtained of which 79 were unique patterns and the remaining eight were represented by 21 clustered isolates. The 12 MIRU loci included were 02, 04, 10, 16, 20, 23, 24, 26, 27, 31, 39 and 40. The MIRU locus 10 was found to be the most discriminatory among the 12 loci with an allelic diversity of 0.743. The combination of spoligotyping and MIRU-VNTR typing data resulted in a 0.998 discriminative power. Combining the two methods proved to result in a higher discriminatory power than using the methods individually.

Using these typing methods, the study has identified the most prevalent circulating *M. tuberculosis* strain families, subfamilies and variants in patients seeking medical attention at the Kalafong Hospital. The study has shown that the use of two molecular genotyping methods improves the discriminatory power of the techniques. Hence, these genotyping methods can be used as an alternative for the IS*6110* RFLP typing method to analyse *M. tuberculosis* strains from clinical settings.

CHAPTER 1

INTRODUCTION

Tuberculosis (TB), remains a globally significant public health problem and is reported to be the second most common cause of death after human immunodeficiency virus (HIV) infection (Dye *et al*., 1999; Corbett *et al*., 2003). The World Health Organization (WHO) declared TB a global emergency in 1993 (WHO, 2009). Irrespective of this, new cases of TB were estimated to be 9.24 million in 2006 (WHO, 2006) and 9.27 million in 2007 globally (WHO, 2009). In 2009, the number of cases increased to 9.4 million (WHO, 2010). The countries contributing the most to the increase of TB cases are the Asian countries; India, China, Indonesia with 55%, followed by Africa; Nigeria and South Africa with 30% (WHO, 2010). These countries make up the first five of the 22 high burden countries in the world with South Africa being in the third position (WHO, 2010). Furthermore, it is estimated that 22.5 million of people living in sub-Saharan Africa are HIV positive, which drives the rapid increase of TB in this region (UNAIDS, 2010). Among the 15 countries with the highest estimated TB incidences, 13 are in Africa (WHO, 2009). These 13 African countries contribute up to 79% of the HIV related TB cases (WHO, 2009) and South Africa contributes 31% of these cases (WHO, 2009).

Several factors have led to the increase and the spread of TB, including the development of anti-TB drug resistance by *Mycobacterium tuberculosis* (*M. tuberculosis*) strains, resulting in the increase of multi-drug resistant (MDR) and extensively-drug resistant (XDR) *M. tuberculosis* strains (Joshi *et al*., 2006; Menzies *et al*., 2007). The development of resistance can be attributed to non-compliance of patients, such as not completing treatment regimens or it could be due to incorrect anti-TB drug prescription and the erratic supply of drugs by medical personnel (Sharma and Mohan, 2003). Multi-drug resistant tuberculosis is *M. tuberculosis* strains resistant to at least isoniazid (INH) and rifampicin (RIF), the two fundamental components of any regimen for the treatment of drug-susceptible TB (WHO, 2009). According to the WHO report (2009), among the 10.4 million episodes of TB, about 4.9% or 511 000 patients were diagnosed with MDR-TB globally (WHO, 2009). Two hundred and eighty-nine thousand cases were new MDR-TB cases, while 221 000 were

MDR-TB cases that had received previous TB treatment (WHO, 2009). The emergence of XDR-TB was also observed (CDC, 2006a). Extensively-drug resistant TB can be defined as *M. tuberculosis* strains that are in addition to INH and RIF resistance, also resistant to any of the fluoroquinolones and at least to one of the three injectable second-line anti-TB drugs (amikacin, kanamycin or capreomycin) (CDC, 2006b). These resistant *M. tuberculosis* strains make TB treatment more challenging and less successful when compared to the treatment of patients with non-resistant *M. tuberculosis* strains (CDC, 2006b).

Various molecular typing methods have been developed and are used to determine the genetic relatedness between *M. tuberculosis* strain families (Corless *et al*., 2001; Van Soolingen *et al*., 2001; Moström *et al*., 2002). The differentiation methods of these *M. tuberculosis* strain families include, the insertion sequence *6110* restriction fragment length polymorphism (IS*6110* RFLP), spacer oligonucleotide typing (spoligotyping) and mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing methods (Kamerbeek *et al*., 1997; Supply *et al*., 2000; Van Soolingen, 2001). These genotyping methods were found to be useful in the clinical management of TB patients, TB control programmes and epidemiological studies (Van Soolingen, 2001). The IS*6110* RFLP genotyping method is the most commonly used and the globally accepted method, which is currently considered as the 'gold standard' for *M. tuberculosis* strain family differentiation (Kremer *et al*., 1999; Nakamura *et al*., 2004; Houben and Glynn, 2009). Although this typing method has shown acceptable performance in *M. tuberculosis* strain family discrimination, it is laborious, expensive and requires uniform data processing systems and deoxyribonucleic acid (DNA) of a high quality (Scott *et al*., 2005; Houben and Glynn, 2009). To improve on these limitations, spoligotyping and MIRU-VNTR typing methods were developed (Kamerbeek *et al*., 1997; Allix *et al*., 2004; Supply *et al*., 2006), which are polymerase chain reaction (PCR) based (Goyal *et al*., 1999; Supply *et al*., 2000; Supply *et al*., 2001; Van Soolingen, 2001; Kremer *et al*., 2005). These PCR based typing methods are cost-effective, robust, reliable and easy to perform when compared to IS*6110* RFLP typing (Van Soolingen *et al*., 2001; Allix *et al*., 2004).

Genotyping of *M. tuberculosis* strains has shown that knowledge regarding the circulating *M. tuberculosis* strains is crucial for TB control programmes (Sun *et al*., 2004). The Beijing strain family has shown a high dominance worldwide because it has an intrinsic advantage over other *M. tuberculosis* strain families in terms of virulence (Van Soolingen *et al*., 1995;

Tsolaki *et al*., 2005). It is also hypothesized that the Beijing strain family has spread and co-evolved with humans long before the vaccine programmes and antibiotic treatment regimens were introduced (Van Soolingen *et al*., 2001; Van Crevel *et al*., 2009).

Even though South Africa was ranked third among the 22 high TB burden countries, there is little information available regarding the circulating *M. tuberculosis* strains in the different provinces. Previous studies mainly focused on the Western Cape and KwaZulu-Natal provinces (Warren *et al*., 2002; Streicher *et al*., 2004; Victor *et al*., 2004; Cohen *et al*., 2011). It has been reported that different *M. tuberculosis* strains occur at different frequencies in different areas (Van Soolingen, 2001; Filliol *et al*., 2002) and the strains that were found predominating, such as Beijing, F11 and F28 in those provinces are not an indicator of strains prevailing in other provinces of the country.

The aim of this study was to determine the predominant *M. tuberculosis* strain families in the Kalafong hospital in the Pretoria region, Gauteng province. Spoligotyping and MIRU-VNTR genotyping methods were performed on isolates obtained from sputum and gastric aspirate specimens. The specimens were submitted from Kalafong hospital to the Diagnostic labaratory of the Department of Medical Microbiology, UP/NHLS. These specimens were tested as TB positive by microscopy, culture in MiddleBrook 7H9 medium and identified with the GenoType MTBC® line probe assay (Hain Lifescience, Nehren, Germany).

The objectives of this study were:

- To collect a minimum of 67 *Mycobacterium tuberculosis* isolates from sputum and gastric aspirate specimens submitted from Kalafong Hospital to the Diagnostic laboratory of the Department of Medical Microbiology, UP/NHLS
- To ensure purity of *M. tuberculosis* using blood and 7H11 agar and further subculture on Middle-brook 7H9 liquid medium
- To genotype *M. tuberculosis* isolates using spoligotyping
- To genotype *M. tuberculosis* isolates using MIRU-VNTR typing
- To determine the genetic relatedness of *M. tuberculosis* by constructing dendrograms
- To analyse data

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Tuberculosis (TB) causes high morbidity and mortality, especially in countries, such as Asia and Africa (WHO, 2009; WHO, 2010). Tuberculosis prevails and is on the increase despite the introduction of the live attenuated Bacillus Calmette-Guérin (BCG) vaccine in the 1920s, which is still used in the *Mycobacterium tuberculosis* (*M. tuberculosis*) vaccination programme and which covers 85% of the global population (Kaufmann and Schaible, 2005; Apt and Kondratieva, 2008). It was initially assumed that patients experiencing relapse of TB infection was due to the same *M. tuberculosis* strain that caused the first infection (Gruft *et al*., 1984). This assumption was due to studies that were based on phenotypic diagnostic assays, such as drug susceptibility profiles and phage typing of *M. tuberculosis* isolates, which do not differentiate between *M. tuberculosis* strains (Crawford *et al*., 1981; Gruft *et al*., 1984; Mathema *et al*., 2006). However, several studies have shown that the reoccurence of tuberculosis was caused by *M. tuberculosis* strains other than those involved in the first TB episodes (De Boer and Van Soolingen, 2000; Richardson *et al*., 2002; Warren *et al*., 2004). Since, the introduction of molecular typing methods that can discriminate on the genetic level of bacilli, it has been possible to determine whether the reoccurrence of a TB infection was due to an initial *M. tuberculosis* strain or due to re-infection with an exogenous *M. tuberculosis* strain (Gagneux and Small, 2007).

The discovery of multiple genetic markers for the *M. tuberculosis* complex has led to the introduction of deoxyribonucleic acid (DNA) genotyping assays also called the DNA fingerprinting of *M. tuberculosis* strains in the early 1990s (Kremer *et al*., 1999; Van Soolingen *et al*., 2001; Moström *et al*., 2002; Djelouadji *et al*., 2008). Genotyping seems to be a more sensitive tool to detect epidemiological links between TB cases compared to conventional contact tracing (Veen, 1992; Solsona *et al*., 2001; Inigo *et al*., 2003). However, only a few genotyping methods, such as the IS*6110* RFLP typing, spoligotyping and MIRU-VNTR typing appear to offer enough discriminatory power and reproducibility for *M. tuberculosis* epidemiological studies (Kremer *et al*., 1999; Kremer *et al*., 2005). These genotyping assays have been used worldwide in studying the TB epidemiology (Van

Soolingen *et al*., 1999; National TB Controllers Association/CDC Advisory Group on Tuberculosis Genotyping, 2004). These molecular assays have shown that the global epidemiology of TB is made up of thousands of different genotypes of *M. tuberculosis* strains (Van Soolingen *et al*., 1999; Warren *et al*., 1999; Brudey *et al*., 2006). These *M. tuberculosis* strains occur at different frequencies in different areas (districts, cities, countries and continents) (Van Soolingen, 2001; Filliol *et al*., 2002). The dynamics of the TB epidemic in a given area and time frame may be an indicator of the different strains circulating in that region (Filliol *et al*., 2002). The global *M. tuberculosis* strain diversity resulted in the establishment of several databases, such as the Spoligotype Database1, Spoligotype Database2, Spoligotype Database3 and Spoligotype Database4 (Sola *et al*., 1999; Sola *et al*., 2001; Filliol *et al*., 2002; Brudey *et al*., 2006). The Spoligotype Database4 was updated to the spoligo-international type and MIRU-VNTR international type2 (SITVIT2) (http://www.pasteur-guadeloupe.fr/tb/bd_myco.html). The spoligotype database shows the genetic diversity of the direct repeat locus of *M. tuberculosis*. The information of the diversity of *M. tuberculosis* is used in the analysis of *M. tuberculosis*' population structure and understanding of global *M. tuberculosis* transmission (Hirsh *et al*., 2004; Brudey *et al*., 2006).

In South Africa, the Western Cape and KwaZulu-Natal (KZN) are the most studied provinces in terms of TB epidemiology (Victor *et al*., 2004; Pillay and Sturm, 2007). The *M. tuberculosis* F11 (Western Cape province), Beijing and F15/LAM4/KZN (KZN province) strain families were found to be the dominant strains in these provinces (Victor *et al*., 2004; Pillay and Sturm, 2007). There is insufficient data regarding other provinces in terms of *M. tuberculosis* strain family prevalence. The purpose of this study was to genotype 100 *M. tuberculosis* isolates using spoligotyping and MIRU-VNTR typing to determine the most prevalent circulating *M. tuberculosis* strain families in Pretoria. The *M. tuberculosis* isolates were obtained from sputum and gastric aspirates specimens that were collected during April 2009 to August 2009 from TB patients attending the Kalafong Hospital in Pretoria, Gauteng, South Africa.

2.2 History of tuberculosis

Gutierrez and colleagues (2005) hypothesised that an early progenitor of *M. tuberculosis* was already present in East Africa approximately three million years ago and may have infected hominids during that time. All modern members of the *M. tuberculosis* complex, such as *M. tuberculosis*, *M. africanum*, *M. canettii* and *M. bovis* may have originated from a common African ancestor about 35 000 to 15 000 years ago (Brosch *et al*., 2001b; Gutierrez *et al*., 2005).

In 1790, Benjamin Marten hypothesised that tuberculosis is infectious in nature (Doetsch, 1978). This hypothesis was proven correct by the French military surgeon Jean-Antoine Villemin who demonstrated the infectious nature of tuberculosis in 1865 by inoculating a rabbit with a purulent liquid from a tuberculous cavity (Major, 1945). The rabbit presented with extensive tuberculosis when euthanized and autopsied three months later (Major, 1945). In 1882, Hermann Heinrich Robert Koch further confirmed the infectious nature of the bacilli in his presentation in Berlin resulting in Koch's postulates (Daniel, 2005).

2.3 Classification of *M. tuberculosis*

The *Mycobacterium* genus belongs to the *Mycobacteriaceae* family and is classified as indicated in Table 2.1 (Pfyffer, 2007; Euzéby, 2012). The cladistics method classifies the genus *Mycobacterium* into *M. tuberculosis* complex and the non-tuberculosis mycobacteria for diagnostic and treatment purposes. This classification is based on shared derived characters and homologous features (Pfyffer, 2007). The *Mycobacterium* genus includes more than 50 species (Van Soolingen *et al*., 1997; Pfyffer, 2007). The *M. tuberculosis* complex consists of *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. canettii* and *M. pinnipedii* species, which are the common causes of infection in humans and animals (Pfyffer, 2007).

Table 2.1: Classification of *Mycobacterium tuberculosis* (Pfyffer, 2007; Euzéby, 2012)

RANK	NAME
Domain	*Bacteria*
Phylum	*Actinobacteria*
Class	*Actinobacteria*
Subclass	*Actinobacteridae*
Order	*Actinomycetales*
Suborder	*Corynebacterineae*
Family	*Mycobacteriaceae*
Genus	*Mycobacterium*
Species	*Mycobacterium tuberculosis*

The non-tuberculosis mycobacteria also have species that are of medical importance, such as *M. avium, M. gordonae, M. intracellulare, M. kansasii, M. leprae, M. malmoense* and *M. marium* (Pfyffer, 2007). Among the non-tuberculous mycobacterial species, *M. avium* and *M. intracellulare* form the *M. avium* complex, which is also referred to as the *M. avium-intracellulare* complex (Field *et al*., 2004).

The non-tuberculous mycobacteria are ubiquitous and can be found in fresh and sea water, soil, dust and several animals species, such as pigs and dogs and bird droppings (Field *et al*., 2004). Infections caused by the *M. avium-intracellulare* complex are commonly due to the inhalation or ingestion of environmental *M. avium* and *M. intracellulare* strains (Akisamit, 2002). The infections are frequently associated with immunocompromised people, such as human immunodeficiency virus (HIV) positive patients (Akisamit, 2002).

2.4 Characteristics and morphology of *M. tuberculosis*

Mycobacterium tuberculosis bacteria are obligate aerobes and found in the well-aerated upper lobes of the lungs where these bacteria exist as facultative intracellular pathogens of macrophages (Cole, 2002). *Mycobacterium tuberculosis* bacilli are non-sporulated rods that do not contain capsules nor produce any toxins. The *M. tuberculosis* bacterium is structurally more related to Gram-positive than Gram-negative bacteria as it has peptidoglycan in the cell walls and stain very weakly Gram-positive (Todar, 2005). The *M. tuberculosis* bacterium is also referred to as an acid-fast bacterium because of the presence of the thick mycolic acid

structure with the ability to retain the primary stain during the decolourisation step with acid alcohol during the Ziehl-Neelsen staining procedure (Iseman, 2000).

The cell wall of *M. tuberculosis* is composed of peptidoglycan and more than 60% of lipids (Todar, 2005). The lipids consist of mycolic acid, cord factor and wax-D (Brennan, 2003; Alderwick *et al*., 2007). The *M. tuberculosis* cell wall is divided into two layers; the lower and upper layer (Brennan, 2003). The lower layer consists of peptidoglycan, which is covalently linked to arabinogalactan and mycolic acid, resulting in the mycolyl arabinogalactan-peptidoglycan complex (Van Soolingen *et al*., 1997; Brennan, 2003). The upper layer is composed of free lipids linked to fatty acids (Brennan, 2003). Interspersed in the cell wall are proteins, lipoarabinomannan (LAM), phosphatidylinositol mannosides (PIMs), the phthiocerol containing lipids and lipomannan (LM) (Brennan, 2003).

Microscopically, *M. tuberculosis* cells appear as either straight or curved non-motile rods that are arranged in cords with the cells ranging between 2 to 4 µm long and 0.2 to 0.5 µm in width (Todar, 2005). On solid media the *M. tuberculosis* bacterial colonies appear rough with or without pigmentation (Cole, 2002). Pigmented colonies are yellow, orange (rarely pink) due to the carotenoid pigments (Pfyffer, 2007). The growth of *M. tuberculosis* species is slow with generation times of 12 to 24 hours (hr) (Iseman, 2000) compared to other bacteria, like *Escherichia coli* (*E. coli*) with a generation time of 20 minutes (Iseman, 2000).

The complete genome sequence of the *M. tuberculosis* strain H37Rv indicated that it consists of 4 411 529 base pairs (bp) with a 65.6% G+C content and densely packed coding regions within a chromosome (Cole *et al*., 1998; Nicol and Wilkinson, 2008). The *M. tuberculosis* genome is known to be highly conserved but still has some genetic polymorphic regions (Van Soolingen, 2001). The polymorphic regions of the mycobacterial genome are caused by the periodic repeats of monomeric sequences called repeat sequences (Van Soolingen, 2001). There are two types of repeat sequences, interspersed and tandem repeats (Van Soolingen, 2001; Mathema *et al*., 2006). The interspersed region consists of insertion sequences (IS) and direct repeats (DR) (Van Soolingen *et al*., 2000).

2.5 Pathogenesis and virulence factors of *M. tuberculosis*

Infection commence when droplets containing *M. tuberculosis* bacilli from an infected person are inhaled into the upper respiratory tract of a susceptible person, either through the mouth or the nose (Cole and Cook, 1998; Behr *et al*., 1999; Nicas *et al*., 2005; Konstantinos, 2010). Mycobacteria in the droplets bypass the defences of the upper airway (nasal mucosa and ciliated epithelium cells) and penetrate deep into the lung alveoli where the bacteria are absorbed by the alveolar macrophages and live as intracellular pathogens (Figure 2.1) (Houben *et al*., 2006).

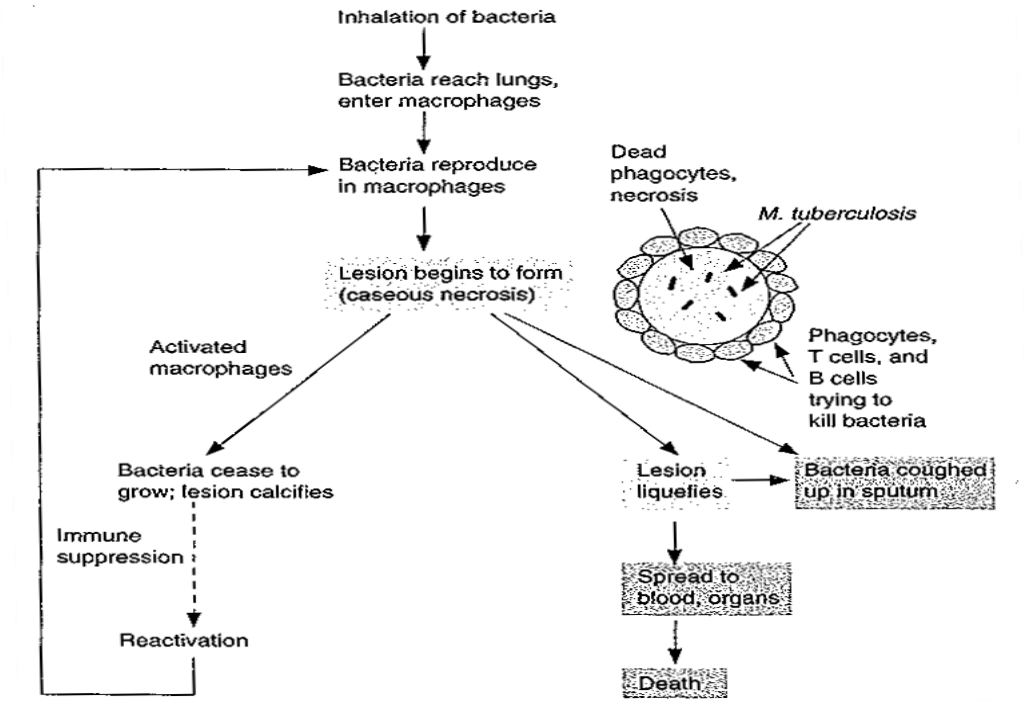

Figure 2.1: The infection process of *M. tuberculosis* and the development of tuberculosis disease (Slayer and Whitt, 2002)

Inside the macrophage, the phagolysosome forms and the *M. tuberculosis* survive and replicate by using several defence pathways (Vergne *et al*., 2004; Kumar *et al*., 2007). There are several *M. tuberculosis* virulence factors (structural components and secretions) that are considered to play a role in the *M. tuberculosis* survival, causing infection and disease manifestation. The virulence factors are classified as cell secretions and envelope functions, cell surface components and enzymes involved in general cellular metabolism (Smith, 2003).

In cell secretion and envelope function, there are a number of components that take part in the *M. tuberculosis* pathogenesis, such as the catalase-peroxidase and superoxide dismutase enzymes, which are produced by the bacilli that degrade reactive oxygen intermediates and that are important for *M. tuberculosis* survival during infection (Braunstein and Belisle, 2000). Cell surface components, such as LAM is a major component, which functions by down-modulating host responses to *M. tuberculosis* infection (Knutson *et al*., 1998; Vergne *et al*., 2003). The LAM blocks phagosomal maturation in the host cell and inhibits T-cell proliferation, protecting the bacilli from the potentially lethal host response mechanisms (Chan *et al*., 1991; Knutson *et al*., 1998; Vergne *et al*., 2003). In order to survive, the *M. tuberculosis* also produces enzymes, such as isocitrate lyase and lipase, which are used in lipid and fatty acid metabolism to enable growth in the infected host.

2.5.1 Human immune response to *M. tuberculosis* infection

In *M. tuberculosis* infection, the bacilli first interact with resident macrophages and result in the formation of a primary focus (Schlesinger, 1993). The infected macrophages produce cytokines and accessory molecules, which are involved in the recruitment of T-cells and serves as a signal for infection (Stewart *et al*., 2003; Russell, 2007). Three weeks after the infection, the recruited T-cells move from the bloodstream to the focus of the infection (Kaufmann, 2002). The T-cells interact with infected living macrophages and the clusters of differentiation 4 (CD4+) T-cells recognise the mycobacterial proteins on the macrophages and start producing Interferon-gamma (IFN-γ), which triggers the direct antimycobacterial activity in the macrophages (Kaufmann, 2002). The triggered macrophages produce tumor necrosis factor-alpha (TNF-α) and Interleukin-12 (IL-12) as a feedback and further inducers of IFN-γ production from the CD4+ T-cells resulting in an adaptive protective response (Kaufmann, 2001). The macrophages activated by the IFN-γ produce bacteriostatic and bactericidal molecules, such as oxygen and nitrogen radicals that attack the intracellular mycobacteria (MacMicking *et al*., 1997; Scanga *et al*., 2001). The activated macrophages produce lipocalins, iron-chelating agents that restrict the ability of mycobacteria to compete for iron in the host cells, which is fatal for the bacteria (Schaible *et al*., 1999; Collins and Kaufmann, 2001).

The clusters of differentiation 8 (CD8+) T-cells produce granulysin and perforin (Stenger *et al*., 1998). The perforin mediate the lysis of the infected host cells; therefore, the intracellular bacteria are released, which allow the activated infiltrating macrophages to take up and kill the bacilli (Stenger *et al*., 1998). The CD8+ T-cells can also kill the intracellular bacteria by producing the antimicrobial peptide, granulysin (Stenger *et al*., 1998).

2.6 Clinical manifestation of *M. tuberculosis* infection

After the primary infection, the infected person's immune system can control and clear the infection or the immune system can be unable to control infection and the person develops the disease. The bacilli can also become latent, at this stage the person is non-infectious to others but is a reservoir for the bacilli (Russell, 2007). In case of a compromised immune system, most patients with primary pulmonary TB or reactivation, first presents with a chronic productive cough, fever, weight loss, haemoptysis, thoracic pain and dyspnoea (Frieden *et al*., 2003). Atypical features, consisting of lower lobe involvement with a trend towards diffuse infection rather than cavitation, are seen frequently (Frieden *et al*., 2003).

The person infected with *M. tuberculosis* cannot always contain the initial infection, there can be reactivation of the latent infection due to a weak immune system either because of immunosuppressive drugs, HIV infection, malnutrition and aging (Dheda *et al*., 2005). The granuloma centre becomes liquefied and becomes a rich medium where the bacteria multiplies and escape from the granuloma (Dheda *et al*., 2005). The bacteria spread within the lungs causing active TB resulting in an infectious person or spreading to extra-pulmonary organs via the lymphatic system and the blood resulting in extra-pulmonary TB (Dheda *et al*., 2005).

There is a complex biological interaction between *M. tuberculosis* and HIV in the co-infected host, this aggravates both pathogens (Mariani *et al*., 2001; Rosas-Taraco *et al*., 2006). Human immunodeficiency virus promotes progression of *M. tuberculosis* latent infection to disease while *M. tuberculosis* in turn enhances HIV replication, accelerating the natural evolution of HIV infection through a principle, which is not yet clear (Goletti *et al*., 1996; Mariani *et al*., 2001; Rosas-Taraco *et al*., 2006). Human immunodeficiency virus infection does not only increase the risk for latent *M. tuberculosis* to be reactivated but it increases the risk of the

rapid development of TB soon after infection or re-infection (Small *et al*., 1993; Van Rie *et al*., 1999). Extra-pulmonary TB which includes: bone marrow, hepatic, splenic, cerebral, vertebral, meningeal, spinal and kidney involvements have been described in HIV infected patients (Friedland, 2009). In HIV-positive patients, the clinical pattern of TB correlates with the host's immune status (WHO, 2004). If TB occurs in the early stages of HIV infection, when the patient's immunity is only partially compromised, the patient's symptoms are characteristic of post-primary TB, the patient's chest radiography shows lung destruction, cavitation and upper-lobe disease and sputum smears are positive for acid-fast bacilli (AFB) (Raviglione *et al*., 1997). In the late stage of HIV, patients present with a primary TB-like pattern with diffuse interstitial or miliary infiltrates with little or no cavitations and intrathoracic lymphadenopathy (Raviglione *et al*., 1997).

2.6.1 Treatment of *M. tuberculosis* infections

The first chemotherapy of tuberculosis (TB) began in the 1940s when *para*-aminosalicylic acid (PAS) and the first antibiotic against TB, which was streptomycin (STR) were discovered (Iseman, 2002; Chalmer and Clarke, 2004). In 1950, the two anti-TB drugs' efficacy was tested as monotherapy and combined therapy (Iseman, 2002; Chalmer and Clarke, 2004). The combined therapy was found to be more effective as compared to monotherapy because there was no emergence of anti-TB drug resistance (Rieder, 1993). In 1952, the third anti-TB drug was discovered, isoniazid (INH) and was added to the combination therapy (Slayden and Barry, 2000). Isoniazid improved the efficiency of the TB treatment resulting in anti-TB drugs being administered for 18 to 24 months (Iseman, 2002). In 1960, ethambutol (EMB) was discovered and substituted PAS and the treatment course was reduced to 18 months (O'Brien, 1994). In the 1970s, rifampicin (RIF) was included into the combination therapy, which shortened the treatment to nine months (Hong Kong Chest Service, BMR Council, 1979). Pyrazinamide (PZA) was introduced in 1980 into the TB drug regimen and the treatment duration was reduced to six months. The drug was found to be active against semi-dormant non-growing bacilli, therefore preventing TB reactivation and development of drug resistance (Zhang and Mitchison, 2003).

Anti-TB drugs are classified as first and second-line drugs (Jassal and Bishai, 2009). There are five first-line drugs: INH, RIF, PZA, EMB and STR (Todar, 2005). The development of

resistance to the two first-line anti-TB drugs (INH and RIF) used for treatment resulted in multi-drug resistant TB (MDR-TB), therefore requiring the implementation of second-line anti-TB drugs (Jassal and Bishai, 2009). These drugs are less effective and not well-tolerated by patients when compared to first-line drugs (Mukherjee *et al*., 2004). The second-line anti-TB drugs consist of aminoglycosides [(kanamycin (KAN), amikacin (AMI)], polypeptides [capreomycin, (CAP)], *para*-aminosalicylic acid (PAS), cycloserine, thioamides (ethionamide, prothionamide) and fluoroquinolones (moxifloxacin, gatifloxacin, levofloxacin, ciprofloxacin and ofloxacin) (Todar, 2005). If these second-line drugs are misused or mismanaged, extensively drug-resistant TB (XDR-TB) develops (CDC, 2006). The XDR-TB, being MDR as well as being resistant to at least one of the fluoroquinolones and to any of the injectable second-line drugs, has therefore limited treatment options available (Guidelines for the programmatic management of drug-resistant tuberculosis, 2011).

2.7 Diagnostic detection and identification of *M. tuberculosis*

Accurate tuberculosis case detection is known to be the rate-limiting step in tuberculosis control (Perkins and Kritski, 2002). About two-thirds of sputum smear-positive cases remain undetected worldwide due to the misdiagnosis of tuberculosis in children under the age of 10 years, the elderly and HIV positive patients; due to the poor quality of sputum produced (Gupta *et al*., 2004). Most efforts in the control of tuberculosis have focused on the cure of tuberculosis rather than on the detection of the disease (Gupta *et al*., 2004). Laboratory diagnosis of *M. tuberculosis* is an important step in detection and control of tuberculosis, but it does not receive enough consideration specifically in developing countries where acid-fast bacilli (AFB) sputum microscopy is mostly the only tool that is used to diagnose tuberculosis (Hall and Robert, 2006). Various phenotyping and molecular assays are currently available to detect, identify and characterise active pulmonary or extra-pulmonary TB (Somoskövi *et al*., 2001).

2.7.1 Phenotypic methods in the diagnosis of *M. tuberculosis* infection

Several phenotypic methods are available to detect *M. tuberculosis* from clinical specimens. However, the most common methods are acid-fast bacilli smear microscopy and culture (Heymann, 2006). There are three techniques of staining that are used; the Ziehl-Neelsen

(ZN), Kinyoun and Auramine for microscopy (Heymann, 2006). However, culture is the 'gold standard' for the diagnosis of tuberculosis (Getahun *et al*., 2007). Different solid and liquid media can be used in the isolation of mycobacteria but the most commonly used media are complex egg-based media with malachite green to prevent contamination with other bacteria (Heymann, 2006). The Löwenstein-Jensen, Stonebrink and Ogawa media are examples of complex egg-based culture media (Heymann, 2006). Synthetic agar-based culture media, such as MiddleBrook 7H10 and 7H11 are prepared from powder base agar and MiddleBrook Oleic Albumin Dextrose Catalase (OADC) enrichment supplement (Mathur, 2002). The main disadvantage of both the egg and the agar-based solid media culture technique is that the bacterial growth is slow and takes about six to nine weeks to be confirmed as *M. tuberculosis* (Davies *et al*., 1999). Due to this disadvantage, the solid culture media are often replaced by commercially available liquid culture media based systems: the VersaTREK, BacT/Alert 3D, BACTEC TB 460 and BACTEC MGIT 960 systems (Watterson and Drobniewski, 2000). These systems take about 10 days to confirm the presence of *M. tuberculosis* (Mathur, 2002; Palomino *et al*., 2008). All the liquid culture media systems use MiddleBrook 7H9 liquid media (Mathur, 2002). The drawback of liquid media and these systems is that both are expensive (Heymann, 2006).

2.7.2 Molecular assays for the identification and drug susceptibility testing of *M. tuberculosis*

Molecular assays are based on the amplification of nucleic acids of specific regions, such as the 16S rRNA gene of the *M. tuberculosis* genome, these allow direct detection of the bacteria in specimens and culture (Alcaide and Coll, 2011). Molecular methods can also be used to determine antibiotic susceptibility based on the identification of specific mutations in the mycobacterial genes that confer resistance to antibiotics (Ramaswamy and Musser, 1998). Molecular assays have been found to be rapid, highly sensitive and specific (Dinnes *et al*., 2007). However, disadvantages are that, the assays are expensive, require expertise and may not differentiate active infection, since DNA from dead bacteria may also be detected and amplified (Alcaide and Coll, 2011).

There are several molecular assays that have been introduced, the first two commercially available assays approved by the US Food and Drug Administration (FDA) were the

Amplified *Mycobacterium tuberculosis* Direct test (MTD test) (Gen-Probe, San Diego, CA, USA) and the Cobas Amplicor *M. tuberculosis* assay (Roche Diagnostics, Mannheim, Germany) (Woods, 2001). There are also other molecular methods, which are used in the identification of *M. tuberculosis* and drug susceptibility testing, such as real-time PCR assays (Beqaj *et al*., 2007), microarrays (Tobler *et al*., 2006) and the GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA), which is an automated one-step system for the direct detection of TB and RIF-resistance. Commercial DNA strip assays, which include the INNO-LiPA Mycobacteria (Innogenetics NV, Ghent, Belgium), GenoType *Mycobacterium*, GenoType MTBC® (Hain Lifescience, Nehren, Germany), INNO-LiPARif.TB (Innogenetics NV, Ghent, Belgium) and GenoType MTBDR*Plus* (Hain Lifescience, Nehren, Germany) are used for identification of *Mycobacterium* species and antibiotic resistance determination (Miller *et al*., 2000; Hillemann *et al*., 2007)

2.8 Genetic relatedness of *M. tuberculosis* strains

Molecular epidemiology studies use DNA fingerprinting (genotyping), which is based on polymorphisms in the *M. tuberculosis* genome to characterise the bacteria into different strain families (Narayanan, 2004; Lillebaek, 2005; Mathema *et al*., 2006). The genotyping methods allow a high degree of discrimination between different *M. tuberculosis* strains (Van Soolingen, 2001; Narayanan, 2004; Lillebaek, 2005; Mathema *et al*., 2006).

There are several genotyping assays that have been developed including: IS*6110* restriction fragment length polymorphism (IS*6110* RFLP) (Van Soolingen *et al*., 1995; Van Soolingen, 2001), spoligotyping (Kamerbeek *et al*., 1997), mycobacterial interspersed repetitive units typing variable-number tandem repeat typing (MIRU-VNTR) (Supply *et al*., 2000, Supply *et al*., 2001) and polymorphic GC-rich repetitive sequence typing (PGRS) (Chaves *et al*., 1996). The three main molecular assays used for the genotyping of *M. tuberculosis* worldwide are currently: i) IS*6110* RFLP using the IS*6110* element; ii) spoligotyping using the direct repeat sequences and iii) MIRU-VNTR using the mycobacterial interspersed repetitive elements (Van Embden *et al*. 1993; Kamerbeek *et al*. 1997; Supply *et al*., 2000) as summurised in Figure 2.2.

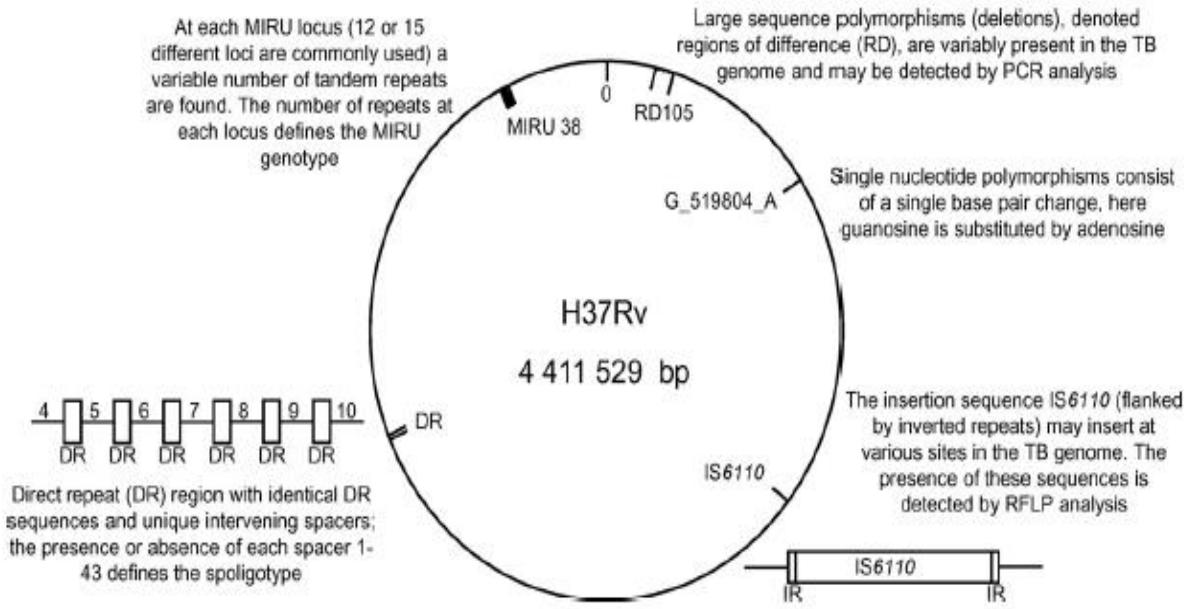

Figure 2.2: Schematic representation of the *M. tuberculosis* genome, indicating the genetic basis of genotyping techniques. The circular chromosome of the reference strain H37Rv is shown together with examples of the major genetic elements used for strain genotyping (Nicol and Wilkinson, 2008)

Among the mostly used genotyping assays, IS*6110* RFLP is the 'gold standard' (Van Embden *et al*., 1993). The spoligotyping and MIRU-VNTR typing assays have a lower discriminatory power than IS*6110* RFLP, while a combination of these typing assays results in a discriminatory power close to that of the IS*6110* RFLP typing assay (Blackwood *et al*., 2004).

2.8.1 IS*6110* RFLP typing of *M. tuberculosis* strains

The IS elements are small, mobile repetitive elements present on the chromosome of *M. tuberculosis* with variable copy numbers (Figure 2.3) (Van Soolingen, 2001; Warren *et al*., 2002; Mathema *et al*., 2006). More than 14 different kinds of IS elements have been identified in the *M. tuberculosis* complex (Mathema *et al*., 2006). These IS elements are usually less than 2.5 kb in size (Barnes and Cave, 2003). The most common IS elements used in determining the molecular epidemiology of *M. tuberculosis* strains is IS*6110,* which is unique for the *M. tuberculosis* complex and can range from 0 to 25 copies in different

M. tuberculosis strains (Khosravi and Seghatoleslami, 2009). The IS*6110* element copies differs between *M. tuberculosis* strains in numbers and location and can be used for *M. tuberculosis* strain differentiation (Van Embden *et al*., 1993; Khosravi and Seghatoleslami, 2009). The IS*6110* are non-randomly distributed throughout the chromosome, indicating the presence of insertion "hot spots" (Van Soolingen *et al*., 1991; Van Embden, *et al*., 1993; Van Soolingen *et al*., 1995; Van Soolingen, 2001). Insertion sequences only carry the genetic information related to their transposition and regulation, hence, their true function is not yet known (Barnes and Cave, 2003).

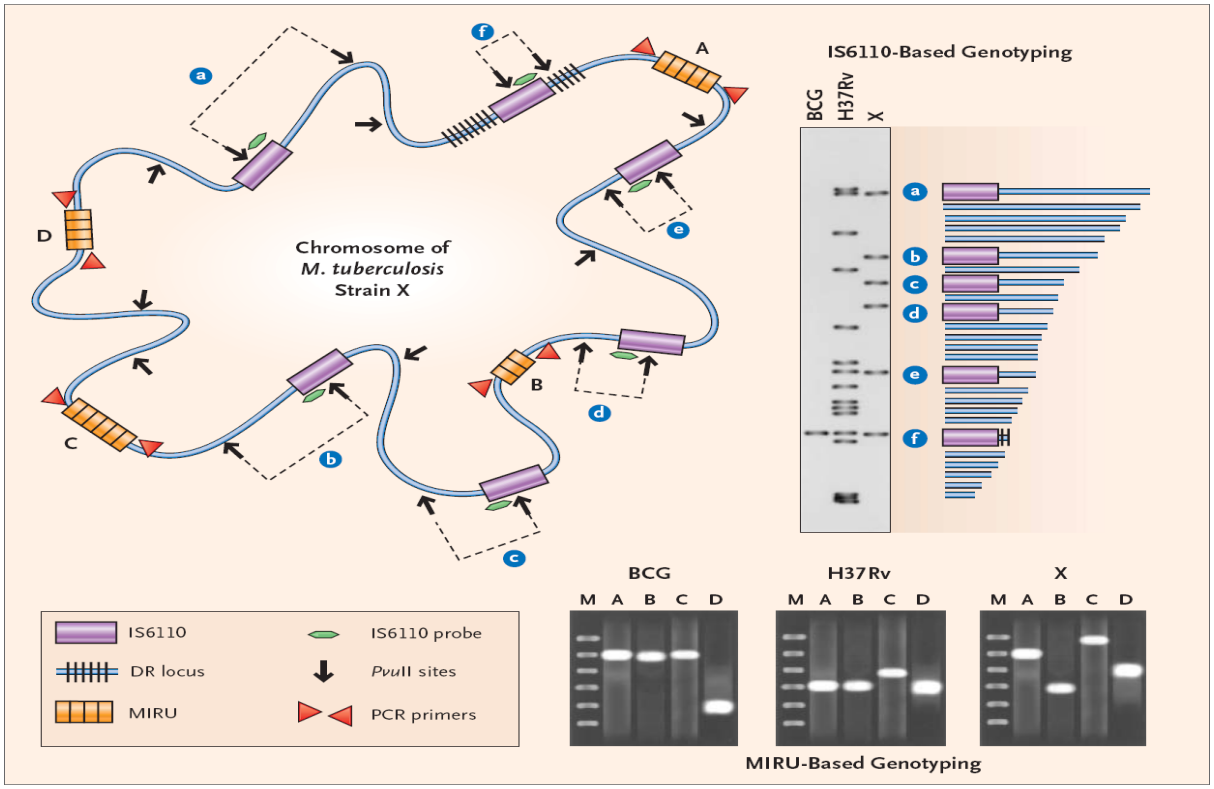

Figure 2.3: The chromosome of a *M. tuberculosis* strain showing the insertion sequence *6110* throughout the genome (Barnes and Cave, 2003)

Since, 1993 the restriction fragment length polymorphism (RFLP) typing using IS*6110* as a DNA probe was adopted as a 'gold standard' to which other molecular typing techniques are presently evaluated, this technique has been standardised and is widely used (Van Embden, 1993; Van Soolingen, 2001). The IS*6110* RFLP typing assay uses DNA probes to visualise the restriction fragments with repetitive DNA sequences complementary to the specific probe (Van Soolingen, 2001). The probe mostly used in RFLP typing detects IS*6110*, which is

located on the chromosome of the *M. tuberculosis* (Van Soolingen *et al*., 1995; Van Soolingen, 2001; Warren *et al*., 2002). In brief, the method is performed by first extracting, the chromosomal DNA, which is digested with restriction enzyme *Pvu*II (cleaves IS*6110* at a single asymmetric site) (Hermans *et al*., 1990). The digested DNA is separated on an agarose gel and the DNA fragments are transferred to a nylon membrane (Van Soolingen, 2001). The DNA fragments on the nylon membrane are hybridised with the IS*6110* DNA probe which consists of a 245 bp sequence. The IS*6110* DNA probe is labeled with peroxidase, enabling enhanced chemiluminesence (ECL) detection of the IS*6110*-containing restriction fragment (Hermans *et al*., 1990; Van Soolingen, 1994; Burgos and Pym, 2002).

However, population-based molecular epidemiological studies have shown that most *M. tuberculosis* strains have between 8 to 18 copies of the IS*6110* insertion element, a number that is adequate to allow discrimination between the majority of *M. tuberculosis* strains (Van Soolingen, 2001). Unfortunately, a proportion of *M. tuberculosis* strains contain no, or only a few copies of the IS*6110* element exist and these *M. tuberculosis* strains differ significantly by geographical area (Van Soolingen *et al*., 1993; Van Soolingen *et al*., 2001).

Mycobacterium tuberculosis strain typing based on a low copy number of the IS*6110* is not sufficiently discriminatory; therefore, other secondary typing methods, such as spoligotyping and MIRU-VNTR typing could help discriminate between the strains (Van Soolingen *et al*., 1993; Yang *et al*., 2000; Van Soolingen *et al*., 2001; Sola *et al*., 2003; Mathema *et al*., 2006). On average, half of the *M. tuberculosis* complex strains have a band-shift in the IS*6110* pattern within a three to four year period (De Boer *et al*., 1999; Van Soolingen, 2001). This is fast enough so that unrelated *M. tuberculosis* strains can be identified and slow enough that strains from related cases are indistinguishable (De Boer *et al*., 1999; Van Soolingen, 2001).

2.8.2 Spacer oligonucleotide typing of *M. tuberculosis* strains

The *M. tuberculosis* complex strains contain a distinct chromosomal region consisting of multiple 36 bp direct repeats (DRs) interspersed by 43 unique spacer DNA sequences that are 37 bp to 41 bp in length (Figure 2.4) (Barnes and Cave, 2003). This chromosomal region shows considerable strain to strain polymorphism (Goyal *et al*., 1997; Kamerbeek *et al*., 1997). The polymorphism in the DRs is hypothesised to be caused by homologous

recombination between DRs, rearrangements caused by the IS*6110* elements and the successive deletion of a single or multiple direct variable repeats (DVR) from the DR region (Groenen *et al*., 1993; Van Embden *et al*., 2000).

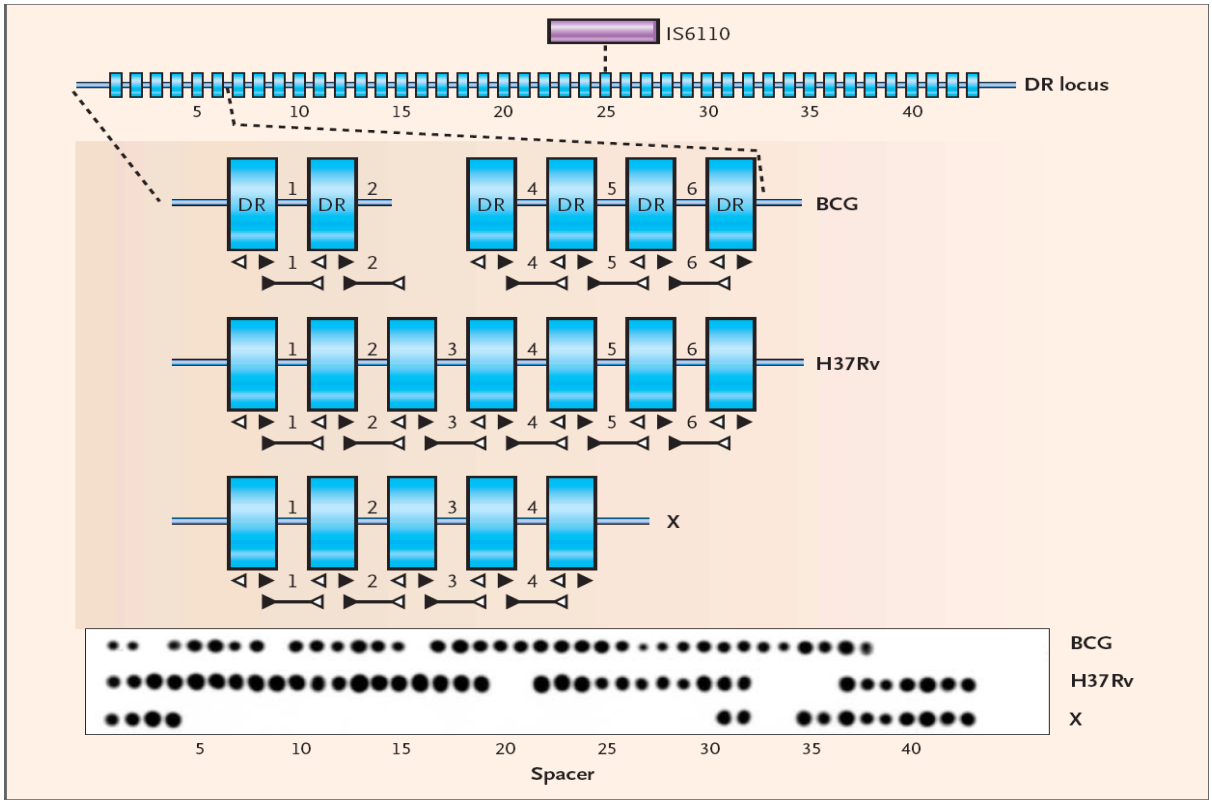

Figure 2.4: The direct-repeat (DR) locus, chromosomal region of a *M. tuberculosis* strain that contains 10 to 50 copies of a 36 bp direct repeat separated by spacer DNA with various sequences, each of which is 37 to 41 base pairs (Barnes and Cave, 2003)

These events (homologous recombination between DRs, rearrangements by the IS*6110* elements and the successive deletion of a single or multiple direct variable repeats) are believed to be unidirectional and to occur over time making the DR region an informative locus for studying the evolution and epidemiology of the *M. tuberculosis* complex (Groenen *et al.*, 1993; Warren *et al*., 2004; Filliol *et al*., 2006). To obtain information regarding the structure of the DR region in *M. tuberculosis* strains and in different members of the *M. tuberculosis* complex, the spacer oligonucleotide typing (spoligotyping) method was developed as a genotyping tool (Kamerbeek *et al*., 1997; Barnes and Cave, 2003).

Spoligotyping is a polymerase chain reaction (PCR) based method (Hermans *et al*., 1992). Spoligotyping identifies the presence or absence of one or more of the 43 spacer DNA sequences between the variable direct repeats in the amplified genome DR region of *M. tuberculosis* strains, through the use of reverse hybridisation and an array of DNA probes, covalently bound on a nylon membrane in parallel lines (Goyal *et al*., 1997; Kamerbeek *et al*., 1997). Spoligotyping is done by amplifying simultaneously the spacers using one set of primers; DRa forward (5'-GGTTTTGGGTCTGACGAC-3'biotinylated) and DRb (5'-CCGAGAGGGGACGGAAAC-3') reverse (Goyal *et al*., 1997; Kamerbeek *et al*., 1997). The *M. bovis* BCG vaccine strain P3 and *M. tuberculosis* H37Rv strains are used as positive controls and deionised water as a negative control (Kamerbeek *et al*., 1997). The presence or absence of spacers in a given biotinylated strain is determined by reverse hybridisation with a set of 43 oligonucleotides from spacer sequences of *M. tuberculosis* H37Rv and *M. bovis* BCG vaccine strain P3 (Goyal *et al*., 1997; Kamerbeek *et al*., 1997; Mathema *et al.*, 2006). The detection of the hybrids is done by chemiluminescence (Kamerbeek *et al.*, 1997).

Spoligotyping targets a single locus, which represents less than 0.1% of the *M. tuberculosis* genome compared to IS*6110*-based RFLP analysis, which examines the distribution of IS*6110* throughout the entire genome (Van Soolingen *et al*., 1999). Spoligotyping uses relatively smaller amounts of genomic DNA (10 ng) compared to IS*6110* RFLP, which needs about 2 µg (Kamerbeek *et al*., 1997; Christianson *et al*., 2010). Strains having identical spoligotyping patterns, yet distinct IS*6110* fingerprint profiles are often encountered (Van Embden *et al*., 2000). One example is the W-Beijing strain family, which are a large phylogenetically related group of *M. tuberculosis* strains that comprise hundreds of similar yet distinct IS*6110* variations, but these strains have an identical spoligotyping pattern, lacking spacers 1 to 34 (Bifani *et al*., 2002; Kremer *et al*., 2004).

The results of spoligotyping are highly reproducible and data generated can be easily interpreted and computerised because the results are in binary format (present/absent), enabling intra-laboratory comparisons (Kamerbeek *et al*., 1997; Nicol and Wilkinson, 2008). Although spoligotyping is simple and highly reproducible, its discriminatory power is lower compared to IS*6110*-based RFLP analysis, except for strains with low copy numbers (less than six copies of IS*6110*) (Van Soolingen *et al*., 1999; Van Soolingen *et al*., 2001).

To compare strains that are circulating, dominanting and to trace the origin of strains globally, different international spoligotype databases were created (Filliol *et al*., 2002; Brudey *et al*., 2006). These databases reveal the global structure of the *M. tuberculosis* complex population (Sola *et al*., 2001). The spoligotyping databases are SpolDB1, SpolDB2, SpolDB3, SpolDB4 and SITVIT2. The SpolDB1 was built by comparing the Caribbean spoligotypes and those of other geographical regions (Sola *et al*., 1999). This resulted in a database of 610 spoligotypes (218 from the Caribbean and 392 from other counties) (Sola *et al*., 1999). Later there was an update of SpolDB1 to SpolDB2, which had 3 319 spoligotype patterns from 47 countries with 259 shared types and 540 "orphan patterns" (Sola *et al*., 2001). The SpolDB2 was built from the systematic analysis of published spoligotypes mostly from Europe and the USA (Sola *et al*., 2001). In 2002, SpolDB3 was published as an update of the SpolDB2 with the global distribution of the *M. tuberculosis* complex (Filliol *et al*., 2002). This database was built to improve the SpolDB2 because it poorly represented the worldwide diversity of the *M. tuberculosis* genome as two-thirds of the isolates were from Europe and the United States (Sola *et al*., 1999; Soini *et al*., 2000; Sola *et al*., 2001). The SpolDB3 has 13 008 spoligotype patterns from more than 90 countries grouped into 813 shared types and has 11 708 of isolates and 1 300 orphan patterns (Filliol *et al*., 2002). The SpolDB3 shows a high genetic diversity index of 97.4%, while that of SpolDB2 was 93% as well as an increased number of clustered isolates (shared types), which increased from 84% (2 779 of 3 319) to 90% (11 708 of 13 008) (Filliol *et al*., 2002). The spoligotyping database was further broadened into SpolDB4, which was built through data-mining by statistical and mixed expert-based/bioinformatical approaches and which includes 1 939 shared-types (STs) and 3 370 orphans from a total of 39 295 spoligotype patterns from 122 countries (Brudey *et al*., 2006).

The SpolDB4 was also updated to the SITVIT2 database (Brudey *et al*., 2006). The SITVIT2 database defines 62 genetic lineages/sub-lineages which includes signatures for different *M. tuberculosis* complex members like *M. bovis*, *M. microti*, *M. pinnipedii* as well as *M. africanum* (Brudey *et al*., 2006). This database includes rules defining major lineages/sub-lineages for *M. tuberculosis stricto sensu*: that includes the Beijing clade, the East African-Indian (EAI) clade and nine sublineages, the Central Asian (CAS) clade and two sublineages, the Haarlem (H) clade and three sublineages, the ancestral "Manu" lineage and three sublineages, the S clade, the 'ill-defined' T clade and five sublineages and the IS*6110*-low banding X clade and three sublineages (Brudey *et al*., 2006).

These databases, have played a major role in the analysis of global TB epidemiology (Van Embden and Van Soolingen, 2000).

2.8.3 Mycobacterial interspersed repetitive units-variable number tandem repeat typing of *M. tuberculosis* strains

The genome of *M. tuberculosis* strains contain many mycobacterial interspersed repeat units (MIRU) (Supply *et al*., 2000). Some *M. tuberculosis* bacilli contain identical repeat units, while other bacilli contain repeats that vary slightly in sequence and length (40 to 100 bp) (Frothingham and Meeker-O'Connell, 1998; Mazars *et al*., 2001; Mathema *et al*., 2006). These MIRU elements are found as tandem repeats and are dispersed in the intergenic region of the genome of *M. tuberculosis* (Frothingham and Meeker-O'Connell, 1998; Mazars *et al*., 2001; Mathema *et al*., 2006). The variable number tandem repeats (VNTR) are consecutive base pair repeats situated in the non-coding region of the *M. tuberculosis* genome (Supply *et al*., 2000). Differences in the number of MIRU are used to distinguish between *M. tuberculosis* strains (Mathema *et al*., 2006). Twelve to 24 of these MIRU loci are found in *M. tuberculosis* and are used for the genotyping of *M. tuberculosis* strains (Mathema *et al*., 2006). These polymorphic loci are reported in a 12-digit format that corresponds with the number of repeats at each chromosomal locus (Supply *et al*., 2000; Mazars *et al*., 2001; Allix-Béguec *et al*., 2008). The discriminatory power of MIRU-VNTR analysis is proportional to the number of loci analysed (Mathema *et al*., 2006). The discriminatory power increases when more than 12 MIRU loci are used, such as 15 or 24 loci (Supply *et al*., 2000).

The MIRU-VNTR typing is a PCR-based assay and is performed using primers specific to flank the regions of each locus. The number of the targeted MIRU-VNTR marker copies reflect the discriminatory power of the marker (Supply *et al*., 2001; Mathema *et al*., 2006). The determination of the amplicon sizes can be done using capillary (1, 24 or 28) analysis, gel electrophoresis or nondenaturing high performance liquid chromatography after PCR amplification to detect the presence of the amplicon (Mathema *et al*., 2006). Unlike IS*6110*-based genotyping, the MIRU-VNTR typing method can be automated and used to evaluate large numbers of *M. tuberculosis* strains (Supply *et al*., 2001; Mathema *et al*., 2006). A fully automated system using PCR primers labelled with one of four dyes (FAM, NED, VIC and HEX) and an automated sequencer has been developed (Supply *et al*., 2001; Allix-Béguec *et*

al., 2008). The high-resolution MIRU-VNTR genotyping system allows amplification of four different loci simultaneously using a multiplex PCR assay (Supply *et al*., 2001; Allix-Béguec *et al*., 2008). The results of MIRU-VNTR genotyping are expressed as numerical codes (almost all MIRU loci have up to 9 repeats) and can be catalogued on a computer database (Supply *et al*., 2001). A website has been created and is maintained so that a worldwide database of MIRU patterns can be compiled (Supply *et al*., 2001; Allix-Béguec *et al*., 2008).

When more than 12 loci are used, or MIRU analysis is combined with spoligotyping, the MIRU-VNTR discriminatory power approximates that of IS*6110* RFLP typing analysis (Frothingham and Meeker-O'Connell, 1998; Supply *et al*., 2001, Cowan *et al*., 2002). The MIRU-VNTR genotyping method is more discriminatory than either IS*6110* RFLP or spoligotyping for strains with copy numbers of less than six of the IS*6110* elements (Frothingham and Meeker-O'Connell, 1998; Cowan *et al*., 2002). A comparative study of genotyping methods aimed at evaluating novel PCR-based typing techniques found MIRU-VNTR analysis to have the highest discriminatory power among amplification-based genotyping approaches (Kremer *et al*., 2005).

The combination of the three methods, IS*6110* RFLP typing, spoligotyping and MIRU-VNTR, in *M. tuberculosis* strain differentiation has shown high specificity (Cowan *et al*., 2002). These genotyping assays have been used worldwide and have provided significant knowledge contributing to tuberculosis control and prevention. These assays have enhanced the understanding of the transmission of TB (Daley and Kawamura, 2003).

2.9 Epidemiology of *M. tuberculosis* strain families

The different polymorphic or hypervariable genetic markers are characterised and used to discriminate or sub-speciate clinical isolates of *M. tuberculosis* (Kamerbeek *et al*., 1997). The *M. tuberculosis* strains are divided into seven major spoligotyping-based families (Table 2.2) (Brudey *et a*l., 2006).

Table 2.2: The seven major *M. tuberculosis* spoligotyping based families and subfamilies according to the SpolDB4 database (Brudey *et al*., 2006)

Families	Subfamilies
East African-Indian (EAI)	EAI1, EAI2-Nonthaburi. EAI3, EAI4, EAI5, EAI6-Bangladesh/1, EIA7-Bangladesh/2 & EIA8-Madagascar
Haarlem (H)	Haarlem1, Haarlem2, Haarlem3 & Haarlem 4
Central and Middle Eastern Asia (CAS)	CAS1-Delhi, CAS1-Kilimanjaro & CAS2
European Family X	X1, X2 & X3
Default family T	T1-Russia/2,T2-Uganda, T3 -Ethiopia, T3-Oscar, T4-Central Europe/1, T5-Russia/1, T5-Madrid/2,"Tuscany"
W-Beijing	None
Latino-American and Mediterranean	LAM1, LAM2, LAM3, LAM4, LAM5, LAM6, LAM7-Turkey, LAM8, LAM9, LAM10-Camreoun, LAM11-ZWE, LAM12-Madrid1

These seven strain families are further subdivided into subfamilies (Table 2.2) (Filliol *et al*., 2002; Brudey *et al*., 2006). The most prevalent strain family is the Beijing strain family, which was described in Beijing, China, in 1995 and accounted for 92% of the *M. tuberculosis* strains in China (Glynn *et al*., 2002). The spoligotype signature of the strain is the absence of spacers 1 to 34, while the IS*6110* RFLP signature is the inverted IS*6110* copy within the DR region (Brudey *et al*., 2006). However, the Chinese Beijing strain family has also been detected in other places, such as Estonia (Europe), Asia and Houston, Texas [United States of America (USA)] (Figure 2.5) (Van Soolingen *et al*., 1995; Qian *et al*., 1999; Krüüner *et al*., 2001). The New York City multi-drug resistant strain known as "W," which was found in the early 1990s in New York among 350 patients who presented with multi-drug resistant TB (Kurepina *et al*., 1998; Bifani *et al*., 2002) was recognised as a variant of the Beijing strain family (Bifani *et al*., 2002). The Beijing strain family, as well as the "W" variant, have an insertion of the IS*6110* element in the genomic *dna*A-*dna*N locus (Kurepina *et al*., 1998; Bifani *et al*., 2002) and have an inverted IS*6110* copy within the DR region (Bifani *et al*., 2002). The Beijing strain family and the "W" variant have a characteristic spoligotype with

the presence of spacers 35 to 43 and the absence of spacers 1 to 34 in the direct repeat region (Van Soolingen *et al*., 1995; Van Crevel *et al*., 2001).

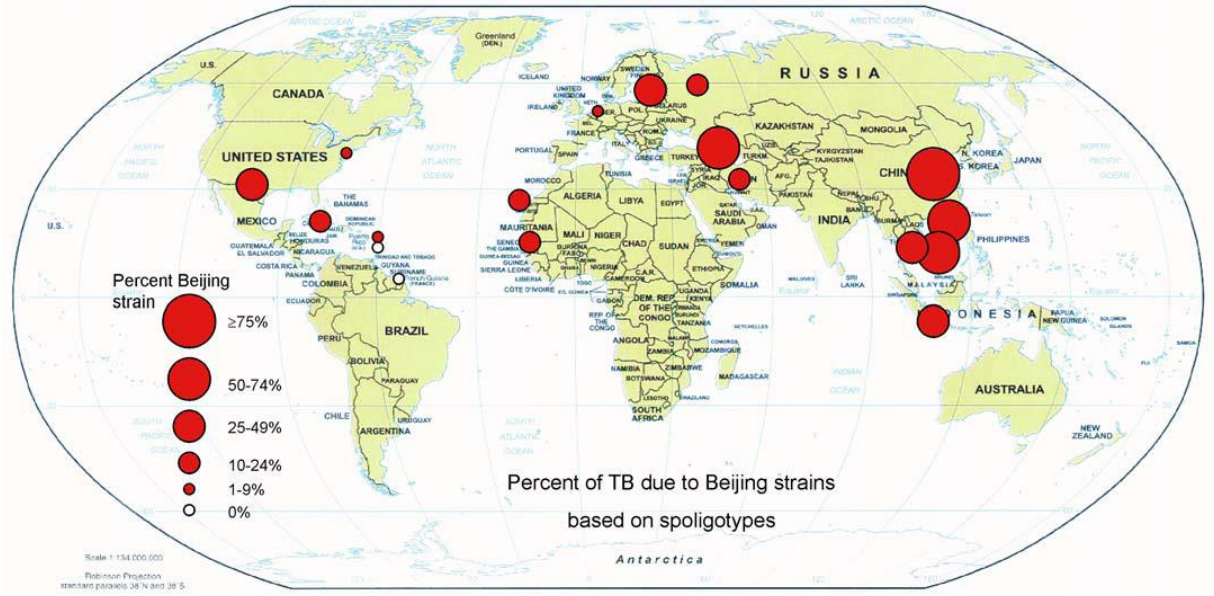

Figure 2.5: Global distribution and prevalence of the Beijing *M. tuberculosis* strains based on spoligotyping data (Glynn *et al*., 2002)

The analysis of spoligotype distribution on SpolDB3 done by Filliol and colleagues (2003) has shown that there is a variation in the distribution of the different strain families of *M. tuberculosis* within continents, such as Africa, Europe, North America, South America, Middle East and Central Asia, Far East Asia and Oceania (island of the tropical Pacific Ocean). The Beijing strain family was found to dominate in the Far East Asia region and North America, while the Haarlem strain family was found in Central America, Europe, Africa and South America (Filliol *et al*., 2003; Brudey *et al*., 2006). The T1 subfamily, which is a member of the 'ill-defined' T family was found predominantly in Africa, Central America, Europe and South America, while the *M. tuberculosis* East African-Indian (EAI) strain family was found in Guinea-Bissau and dominantly found in South East Asia, India and East Africa (Kremer *et al*., 1999; Brudey *et al*., 2006). The Central-Asian (CAS) strain family was isolated from Middle Eastern and Central-Asian patients (Filliol *et al*., 2003). The Latino-American and Mediterranean (LAM) family is mostly found in Africa, Central America, Europe and South America while the European family X is mostly found in Europe (Brudey *et al*., 2006).

The EAI strain family has a low copy number of the IS*6110* elements and the spacers 29 to 32 and 34 are absent (Soini *et al*., 2000; Sola *et al*., 2001). New variants of the EAI strain family, which includes: EAI2-Nonthaburi, EAI6-Bangladesh/1, EAI7-Bangladesh/2 and EAI8-Madagascar have been included in the SpolDB4 (Brudey *et al*., 2006). According to Douglas and colleagues (2003), the EAI2 subfamily was assigned as the "Manila family". The EAI2-Nonthaburi variant from Thailand was further linked to the "Manila family". The EAI3 and EAI4 subfamilies were shown to have originated from India and Vietnam respectively, therefore, it was suggested that these subfamilies can be called EAI3-IND and EAI4-VNM variants (Brudey *et al*., 2006). In Bangladesh the EAI family was found and called EAI-Bangladesh/1 and EAI-Bangladesh/2 (Brudey *et al*., 2006). In the Eastern part of the South Asian region, EAI6-BGD1 was found and was shown to be prevalent in the neighbouring Myanmar (Brudey *et al*., 2006).

The Haarlem (H) strain family is characterised by the presence of a double band at 1.4 kb on the IS*6110* elements, the absence of spacer 31 in spoligotyping, which is caused by the presence of a second copy of IS*6110* in the DR (Filliol *et al*., 2000). This second IS*6110* is inserted asymmetrically within the DR locus, therefore, hindering the detection of spacer 31 (Filliol *et al*., 2000; Legnard *et al*., 2001). Based on MIRU-VNTR analysis, the H strain family frequently has 33233 characteristic patterns (Kremer *et al*., 1999). There are three main spoligotype signatures that define the H strain family (H1 to H3) according to the SpolDB3 (Filliol *et al*., 2003). In SpolDB4 a fourth spoligotype is added resulting in the H4 subfamily (Brudey *et al*., 2006). The H4 subfamily is characterised by the absence of spacers 29 to 31 and 33 to 36 in the DR region of the genome (Brudey *et al*., 2006). More than 60% of the *M. tuberculosis* strains belonging to the H4 subfamily is found in Armenia, Austria, Finland, Georgia, Iran and Russia (Brudey *et al*., 2006).

The Central-Asian (CAS) strain family has a specific spoligotyping signature, which is represented by the absence of spacers 4 to 27 or 23 to 34 (McHugh *et al*., 2005). The CAS strain family is mostly found in India and the Indian subcontinent (Singh *et al*., 2004). The strain family has several subfamilies and numerous variants, such as CAS1-Kilimanjaro, which is found in Tanzania and CAS1-Dar from Dar-es-Salaam (McHugh *et al*., 2005).

The Latin-American strain family has twelve subfamilies (LAM1 to LAM12) according to SpolDB3 (Filliol *et al*., 2003). According to the SpolDB4 there is a new variant called

LAM11-ZWE from Zimbabwe, which is likely identical to the "Meru" family found in Tanzania and the LAM12-Madrid1 (García de Viedma *et al*., 2005). The LAM7 subfamily was renamed LAM7-Turkey because it was predominantly found in Asia Minor (Zozio *et al*., 2005). The LAM-10 subfamily was renamed LAM10-Cameroon (Niobe-Eyangoh *et al*. 2003).

The *M. tuberculosis* S strain family is prevalent in Sicily and Sardinia and identical to the F28 family that is found in South Africa (Sola *et al*., 2001; Warren *et al*., 2002). The origin of this family is not yet known (Sola *et al*., 2001). The 'ill-defined' T strain family is divided into five subfamilies (T1 to T5) and "Tuscany" (Brudey *et al*., 2006). The T strain family names include geographical specificity: T1-Russia/2, T2-Uganda, T3-Osaka, T3-Ethiopia, T4-Central Europe/1, T5- Russia/1, T5-Madrid/2 (Brudey *et al*., 2006). The T1-Russia/2 and T5-Russia/1 subfamilies are from clinical isolates obtained from Russia, former Russian Soviet Republics and from Northern and Eastern European countries, such as Estonia, Finland, Georgia, Latvia, Poland and Russia (Brudey *et al*., 2006). The "Tuscany" subfamily was found to have a single spacer difference from the T5-Russia/1 variant and is found in Italy (Lari *et al*., 2005). The T2-Uganda variant was mainly found in East Africa with seven shared types (STs) that are linked to East African countries (Brudey *et al*., 2006). The T3-Osaka variant was first identified in Finland and is now frequently found in the Okayama district and other parts of Japan (Ohata and Tada, 2004). The T4-Central-Europe/1 variant was identified based on its similarities with some strain families that are found in South and North America, such as the T4_CEU1 variant represented by ST94, ST430, ST1258 (Brudey *et al*., 2006). The T5-Madrid/2 variant was found in Spanish related settings (Garcia de Viedma *et al*., 2005).

The X strain family was first found in Guadeloupe (Sola *et al*., 1997) and in French Polynesia (Torrea *et al*., 1995). The epidemic variants of this genotype family were found in South Africa in the rural districts of the Western Cape province, from drug-resistant *M. tuberculosis* isolates collected from January 2001 to February 2002 (Streicher *et al*., 2004). This strain family is defined by the low number of IS*6110* copies and the absence of spacer 18 using spoligotyping (Sebban *et al*., 2002). The X strain family is found to be dominant in the United Kingdom (UK), the USA and in the former British colonies (Sebban *et al*., 2002; Garcia de Viedma *et al*., 2005). The X strain family is divided into three subfamilies (X1 to X3) (Sebban *et al*., 2002). A genotype family from India was called the "Manu" strain

family, which is divided into Manu1, with a deletion of spacer 34, Manu2 with a deletion of spacers 33 to 34 and Manu3 with a deletion of spacers 34 to 36 (Singh *et al*., 2004).

In South Africa, the most studied setting is the Western Cape province and according to the literature the most prevalent strain family is the *M. tuberculosis* strain family 11 (F11) (Warren *et al*., 1999; Warren *et al*., 2002; Victor *et al*., 2004). The F11 strain family is characterised by the presence of 11 to 19 IS*6110* RFLP bands (Victor *et al*., 2004). The F11 family does not have spoligotype spacers 9 to 11, 21 to 24 and 33 to 36 and this serves as unique markers for the identification of the family (Warren *et al*., 1999; Warren *et al*., 2002). The F11 strain family is not only found in South Africa but in other parts of the world, such as the Netherlands, Chile, Honduras, Cuba, France, Italy, Austria, UK, Spain, Brazil, Argentina, French Guiana, Sweden, Venezuela and the USA (Victor *et al*., 2004).

2.9.1 Control programmes for tuberculosis infection

In order to achieve complete elimination of both actively dividing and dormant bacilli with first-line anti-TB drugs, the short-course chemotherapy (SCC) with two phases was introduced: initial phase and continuation phase (Mitchison, 2005). The initial phase involved the use of three or more anti-TB drugs (INH, RIF, PZA and EMB or STR) (WHO, 1997). These anti-TB drugs are given for two months and rapidly kill actively dividing bacteria, which results in smear negative sputum results (Elzinga *et al*., 2004). The continuation phase uses fewer anti-TB drugs (INH and RIF) and is given for four to seven months to kill the remaining or dormant bacilli, therefore preventing recurrence (The Tuberculosis Trials Consortium, 2002). Unfortunately, the SCC for controlling TB was not always properly followed and this resulted in its failure (Espinal *et al*., 2000). Reacting to this situation, the WHO in 1993 (WHO, 1994) recommended a multifaceted strategy known as directly observed treatment, short-course (DOTS), which incorporates standardised supervised SCC and was aimed at detecting at least 70% of all new infectious cases and to cure at least 85% of the cases detected (WHO, 1994; WHO, 2002).

The DOTS strategy focuses on five main points of action (WHO, 2002). These points includes the government's commitment in the control of TB, the detection of TB using sputum-smear microscopy, direct observation short-course chemotherapy treatment, a reliable supply of anti-TB drugs and a standard recording and reporting system to monitor cases and

treatment outcome (Elzinga *et al*., 2004). Furthermore, the Stop-TB 2006-2015 was published by the Stop TB strategy to enhance DOTS. The Stop-TB 2006-2015 includes ten actions to achieve the global plan in fighting TB (WHO, 2006). These plans were divided into three parts; Part I sets out the Partnership's strategic directions for 2006-2015, based on recent achievements and the current situation. Part II summarises planned regional activities, costs and impact for all regions with a high burden of TB, based on an ambitious but realistic scenario. The Stop-TB 2006-2015 considers what would be needed to accelerate progress towards halving the TB prevalence and death rates in Africa and Eastern Europe (WHO, 2006). Part III summarises the strategic plans of the Partnership (WHO, 2006).

Directly observed treatment, short course is effective in patients with drug susceptible TB, while it has shown poor outcome with patients who are infected with MDR-TB (Espinal *et al*., 2000). In 1999, the WHO introduced the DOTSplus programme for treatment of patients with MDR-TB. The programme included the use of susceptible first-line and second-line anti-TB drugs. Challenges for the success of the DOTSplus programme include: i) capacity to perform drug-susceptibility testing; ii) the availability of second-line drugs, which are expensive; iii) difficulty to administer second-line drugs and iv) poor tolerance of patients to these drugs (Farmer, 2001). The Green Light Committee (GLC) was created by the World Health Organisation in 2000 (WHO, 2000) to make sure that second-line drugs are affordable and accessible to every one who needs them.

2.9.2 Summary

Regardless of efforts to control TB disease, the disease is still the leading cause of death worldwide after the human immunodeficiency virus (HIV) (WHO, 2010). The control of TB disease is complicated by TB and HIV coinfection and the development of resistance to some of the anti-TB drugs (Mathema *et al*., 2006). In an attempt to control and better understand the disease, there were several diagnostic tools developed, among them were genotyping assays (Van Embden and Van Soolingen, 2000).

Genotyping assays are useful in studying TB epidemiology, since it enables researchers to explore the genetic diversity within the *M. tuberculosis* genome (Moström *et al*., 2002; Hershberg *et al*., 2008; Wirth *et al*., 2008). The discovery of the genetic diversity in

M. tuberculosis has resulted in an entry point for studies evaluating the clinical implication of such diversity. Genotyping methods have also been used extensively to understand the transmission and prevalence of different *M. tuberculosis* strains (Van Soolingen, 2001; Moström *et al*., 2002; Djelouadji *et al*., 2008). Multiple genotyping methods have been developed to differentiate clinical isolates of *M. tuberculosis* strains (Kamerbeek *et al*., 1997; Supply *et al*., 2000; Supply *et al*., 2001). The IS*6110* RFLP typing method is considered to be the 'gold standard' for *M. tuberculosis* genotyping due to its high discriminatory power (Van Embden *et al*., 1993; Van Soolingen, 2001; Stavrum *et al*., 2009). However, the IS*6110* RFLP genotyping assay is a laborious and lengthy method (Sun *et al*., 2004). Therefore, several PCR-based genotyping methods have been used, particularly spoligotyping and MIRU-VNTR to replace the IS*6110* RFLP assay, since these assays are faster compared to the IS*6110* RFLP assay (Supply *et al*., 2000; Mazars *et al*., 2001; Supply *et al*., 2001; Allix *et al*., 2004; Supply *et al*., 2006).

Spoligotyping and MIRU-VNTR genotyping methods have facilitated the development of international databases (SITVIT2 and MIRU-VNTR*plus*) (Filliol *et al*., 2002; Brudey *et al*., 2006). The spoligotyping method has also introduced a degree of consistency to the naming of the major and minor families of *M. tuberculosis* (Sola *et al*., 2001). Databases assist in the analysis of *M. tuberculosis* population structure and to assess the complexity of global TB transmission (Filliol *et al*., 2002; Brudey *et al*., 2006). In this study both the spoligotyping and MIRU-VNTR typing methods were used to determine the genetic relatedness of the circulating *M. tuberculosis* strains in patients who attended the Kalafong Hospital because the combined discriminatory power of these typing methods is close to that of the 'gold standard,' the IS*6110* RFLP assay (Cowan *et al*., 2002).

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CHAPTER 3

SPOLIGOTYPING AND MIRU-VNTR TYPING OF *MYCOBACTERIUM TUBERCULOSIS* ISOLATES OBTAINED FROM A PROVINCIAL HOSPITAL IN PRETORIA, GAUTENG PROVINCE, SOUTH AFRICA

The editorial style of the International Journal of Medical Microbiology was followed in this chapter

3.1 Abstract

Mycobacterium tuberculosis (*M. tuberculosis*), the causative agent of tuberculosis (TB), is one of the most successful bacterial pathogens worldwide. Molecular typing of *M. tuberculosis* isolates has improved the understanding of the epidemiology of TB. Various deoxyribonucleic acid (DNA) typing methods, such as spoligotyping and mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) typing have been developed and have shown to be faster and easier to perform compared to the internationally standardised IS*6110* restriction fragment length polymorphism typing method (IS*6110* RFLP). Limited information is currently available regarding, which dominant *M. tuberculosis* strains are circulating in most of the provinces in South Africa. The aim of this study was to determine the dominant circulating strains of *M. tuberculosis* in the Pretoria region, Gauteng province, South Africa. A total of 100 *M. tuberculosis* isolates were analysed using spoligotyping and MIRU-VNTR typing. Spoligotyping showed 39 different spoligopatterns of which 36% (14/39) were unreported in the SITVIT2 database. The T1 subfamily (19%) and Beijing strain family (18%) were the most dominant spoligopatterns, followed by the LAM3 subfamily (13%). The *M. tuberculosis* isolates were further genotyped using the 12 loci based MIRU-VNTR typing method. Eighty-seven distinct MIRU-VNTR patterns were obtained of which 79 were unique patterns and the remaining eight represented by 21 clustered isolates. Among the 12 MIRU loci; loci 10, 26 and 40 were the most discriminatory. Combining the spoligotyping and MIRU-VNTR typing results showed a Hunter-Gaston discriminatory index of 0.998, indicating that these genotyping methods are useful tools to determine the epidemiology of these *M. tuberculosis* isolates. This study indicated that the *M. tuberculosis* strains circulating in the Pretoria region were diverse and strain families dominating in this region were in agreement with results obtained in similar studies conducted in the KwaZulu-Natal and Western Cape provinces of South Africa.

Key words: *Mycobacterium tuberculosis*, molecular epidemiology, spoligotyping, MIRU-VNTR

3.2 Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), remains one of the leading causes of death worldwide with an estimated 9.4 million new TB cases in 2009 (WHO, 2010). Most of the patients with TB, live in Asia (55%) and Africa (30%) (WHO, 2010). In 2009, South Africa was the third highest country worldwide in terms of TB burden with an incidence of 59% (WHO, 2010). Genotyping methods have been widely applied to analyze the transmission dynamics of *M. tuberculosis* strains (Van Soolingen, 2001; Moström *et al*., 2002; Djelouadji *et al*., 2008). The most commonly used genotyping methods for *M. tuberculosis* are IS*6110* RFLP typing, spoligotyping and MIRU-VNTR typing (Van Soolingen, 2001; Moström *et al*., 2002). These genotyping methods use different genomic markers (Barnes and Cave, 2003). The IS*6110* RFLP typing method is based on the transposable IS*6110* element (Van Embden *et al*., 1993). The spoligotyping method functions by detecting the variation of spacers in the direct-repeat (DR) regions of the *M. tuberculosis* genome, while the MIRU-VNTR typing method is based on the detection of tandem repeats in the *M. tuberculosis* genome (Cowan *et al*., 2002; Hawkey *et al*., 2003). The IS*6110* RFLP typing method is regarded as the 'gold standard' for genotyping of *M. tuberculosis* due to its high discriminatory power (Van Embden *et al*., 1993; Van Soolingen, 2001; Stavrum *et al*., 2009). However, this typing method is expensive, labour intensive and time consuming (Sun *et al*., 2004). These limitations have made spoligotyping and MIRU-VNTR typing [deoxyribonucleic acid (DNA) amplification methods] acceptable alternative typing methods for *M. tuberculosis* strains (Supply *et al*., 2006). These typing methods are easier to perform and faster than IS*6110* RFLP typing (Van Soolingen, 2001).

Genotyping of *M. tuberculosis* isolates combined with the demographic data can provide useful information regarding the understanding of the epidemiology of TB (Barnes and Cave, 2003). Genotyping methods can be used to prove suspected TB transmission from the epidemiological information by determining the diversity of strains circulating in the suspected area (Barnes and Cave, 2003). Genotyping methods can cluster related cases together or can show distinct genotypes, which could be due to several factors, such as reactivation, primary infection and reinfection (Alland *et al*., 1994). Genotyping of *M. tuberculosis* strains is important in determining the strain family diversity of *M. tuberculosis* strains within a population (Kato-Maeda *et al*., 2001). Despite the high TB burden in South Africa, there is currently limited data available pertaining to the genetic

relatedness of *M. tuberculosis* strains circulating in most provinces including the Gauteng province. The aim of this study was to determine the prevalence of circulating genotypes of *M. tuberculosis* obtained from patients attending a provincial hospital in Pretoria, South Africa.

3.3 Materials and Methods

3.3.1 Study site and bacterial isolate collection

A provincial hospital situated west of Pretoria was used as the study site. The hospital sent the specimens from different hospital wards and casualty to the Diagnostic laboratory of the Department of Medical Microbiology, UP/NHLS, for routine diagnostic analysis. A total of 100 consecutive *M. tuberculosis* isolates were obtained from sputum (38) and gastric aspirate (62) specimens, which were collected from the Diagnostic laboratory of the Department of Medical Microbiology, UP/NHLS, from April 2009 to August 2009. Contamination of the *M. tuberculosis* isolates was excluded by culturing the *M. tuberculosis* isolates on sheep blood agar plates (Diagnostic laboratory, Department of Medical Microbiology, UP/NHLS) for 24 hr at 37ºC. The *M. tuberculosis* isolates were also cultured on MiddleBrook 7H11 agar plates (Diagnostic Media Products, South Africa) at 37ºC for three weeks, in an aerobic environment, to detect the presence of mycobacteria other than *M. tuberculosis* (MOTTS). The pure *M. tuberculosis* isolates were cultured in MiddleBrook 7H9 medium (Diagnostic Media Products, South Africa) using the BACTEC MGIT 960 system (Becton Dickinson, Sparks, Md) according to the manufacturer's protocol.

3.3.2 *Mycobacterium tuberculosis* DNA extraction

A pasteur pipette was used to collect 100 µl of the MiddleBrook 7H9 medium (Diagnostic Media Products, South Africa) after three weeks of culturing. The *M. tuberculosis* DNA extraction was done using the Amplicor Respiratory Specimen Preparation kit according to the manufacturer's instructions (Roche Diagnostics, USA). The final elution volume of 200 µl, which consisted of neutralisation reagent, lysine reagent and extracted DNA were stored at -20ºC until required for further analysis.

3.3.3 Spoligotyping of *M. tuberculosis* isolates

Spoligotyping was performed according to the modified method of Kamerbeek and colleagues (1997), using a commercial kit (Ocimum BioSolution, India). In brief, the method is based on the amplification of the DNA direct repeat (DR) locus in the *M. tuberculosis* genome with primers DRa (5'-GGT TTT GGG TCT GAC GAC -3') (biotinylated 5' end) and DRb (5'-CCG AGA GGG GAC GGA AAC-3') (Kamerbeek *et al*., 1997). The reaction mixture consisted of 12.5 µl Qiagen mastermix (Qiagen, Germany), 2 µl of each primer (forward primer biotinylated DRa and reverse primer DRb) as described by Kamerbeek and colleagues (1997), 3.5 µl deionised water (Qiagen, Germany) and 5 µl DNA template prepared to a final reaction volume of 25 µl. The following cycle programme was used and run on an Eppendorf MasterCycler (Hamburg, Germany): intial denaturation at 96ºC for 15 min, 30 cycles of denaturation at 96ºC for 1 min, annealing at 55ºC for 1 min, extension at 72ºC for 30 sec and a final extension step at 72ºC for 10 min with a 4ºC holding time. The PCR products were hybridised to the 43 spacer oligonucleotides of *M. tuberculosis* H37Rv and *M. bovis* BCG P3 that were covalently linked to the spoligo-membrane (Ocimum BioSolution, India). *Mycobacterium tuberculosis* and *M. bovis* BCG P3 reference strains were included in each test as positive controls and RNase free ultra pure water (Roche Diagnostics, USA) was used as negative control. The hybrids were detected using an enhanced chemiluminescence (ECL) detection liquid (Amersham, Sweden) after 45 min to 60 min incubation with a streptavidin-peroxidase conjugate (Qiagen, Germany) at 42ºC and exposure to a X-ray film (Ocimum BioSolution, India). The results were entered into a Microsoft excel sheet in binary format representing the 43 spacers (1 is hybridisation and 0 is no hybridisation). The binary format was converted into an octal code. This was done by dividing the binary format into 14 sets of three digits (1 to 42) plus one additional digit (spacer 43). The three-digit set was converted to octal code (000=0, 001=1, 010=2, 011=3, 100=4, 101=5, 110=6, 111=7) and with the last digit remaining either 1 or 0, this resulted in a 15-digit octal code (Dale *et al*., 2001). The octal code was entered into the international spoligotyping database SITVIT2 to determine the spoligo-international type (SIT) and the *M. tuberculosis* strain names (Brudey *et al*., 2006). Those spoligopatterns not found in the SITVIT2 database were analysed using "Spotclust", which assigns families based on SpolDB3.0 (Vitol *et al*., 2006).

3.3.4 Mycobacterial interspersed repetitive units-variable number of tandem repeats typing of the *M. tuberculosis* isolates

The MIRU-VNTR typing method was performed according to the method by Le-Fleche and colleagues (2002). This method is based on the PCR amplification of 12 loci (Appendix A: Table 3) found on the *M. tuberculosis* genome (Supply *et al*., 2001). An individual locus was amplified using a reaction mixture, which consisted of 10 µl Qiagen mastermix (Qiagen, Germany), 0.5 µl of each primer (forward primer and reverse primer) according to Le-Fleche and colleagues (2002), 7 µl deionised water (Qiagen, Germany) and 2 µl DNA template were prepared to a final reaction volume of 20 µl. The following cycle programme was utilised and run on an Eppendorf MasterCycler (Hamburg, Germany): initial denaturation at 94ºC for 1 sec, 40 cycles of denaturation at 94ºC for 5 min, annealing at 62ºC for 1 min, extention at 72ºC for 1 min 50 sec and a final extension step at 72ºC for 10 min with a 4ºC holding time. The PCR amplicons were electrophoresed on a 2% (m/v) agarose gel (Whitehead Scientific, South Africa) containing 5 µl ethidium bromide (10 mg/ml) (Promega, Madison, USA) at 85 V/cm (Eilte-300 Power supply, Wealtec Corp, Kennesaw, GA) for 2 hr in 1 X TAE [40 mM Tris Base (Sigma Chemical, USA), 20 mM glacial acid (Merck, Germany) and 0.5 mM EDTA (Promega, USA)] running buffer (pH 8.0 - 8.5). A 50 bp DNA ladder (Fermentas, Thermo Scientific, USA) was used as a molecular size marker. All agarose gels were visualised using UV illumination (TFM-26 Ultra Transilluminator, UVP, Upland, CA). The image was captured using a digital gel documentation system (DigiDoc-It imaging system, UVP, Upland). The size of the PCR fragments for each locus was estimated by visual comparison with the molecular marker and the MIRU allele frequency table to determine the number of repeats (Supply, 2005). The numbers of repeats for each of the 12 loci per isolate was entered in a Microsoft excel sheet to create a 12-digit allelic profile. The results were analysed using the MIRU-VNTR*plus* database.

3.3.5 Data analysis

Dendrogrammes were constructed for spoligotyping, MIRU-VNTR typing and the combined results of the two typing methods. The distance matrix was constructed with the categorical coefficient and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm was used to construct the dendrogrammes. The clustering rate of *M. tuberculosis* strains based on spoligotyping, MIRU-VNTR typing and the combined typing methods was

defined as: $(n_c-c)/n$, where n_c is the total number of clustered *M. tuberculosis* isolates, c is the number of clusters and n is the total number of *M. tuberculosis* isolates in the study. The Hunter–Gaston Discriminatory Index (HGDI) (Hunter and Gaston, 1988) was used as a numerical index to calculate the discriminatory power of each typing method alone as well as in combination. The HGDI is based on the probability that two unrelated strains sampled from the population will be placed into different typing groups. The HGDI was calculated by using the following formula:

$$\mathbf{HGDI= 1-[1/N(N-1)\sum^{s} n_j(n_j-1)]}$$

where N is the total number of strains in the typing scheme, s is the total number of different patterns and n_j is the number of strains belonging to the jth pattern. Allelic diversity of each loci in MIRU-VNTR typing was classified as "highly discriminant" (HGDI>0.6), "moderately discriminant"(0.3≤HGDI≤0.6) and "poorly discriminant" (HGDI<0.3) (Sola *et al*., 2003).

3.4 Results

3.4.1 Study population and preliminary results of the patients used in the study

Based on the demographic information, there were 46% female and 54% male patients. In total, 91% of the *M. tuberculosis* isolates were from patients who were 18 years and older, while 7% was less than 18 years and two patients were of unknown age. Gastric aspirates represented 62%, while sputum specimens represented 38% of the specimens analysed in this study. Drug susceptibility results were available for only 95 of the 100 *M. tuberculosis* isolates, with 98% (93/95) of the *M. tuberculosis* isolates susceptible to both INH and RIF, while one isolate was resistant to INH and the other isolate was resistant to RIF.

3.4.2 Spoligotyping results of the *M. tuberculosis* isolates

Spoligotyping gave 39 distinct spoligotypes after analysis of the 100 *M. tuberculosis* clinical isolates (Figure 3.1). A total of 73% (73/100) of the isolates clustered within 12 of these distinct spoligotypes with each cluster containing between two to 18 *M. tuberculosis* isolates. The remaining 27% (27/100) isolates showed unique spoligotypes (spoligotypes that occurred only once). According to the SITVIT2 database, 86% of the *M. tuberculosis* isolates

belonged to four families, nine subfamilies and two variants, with only 14% of the *M. tuberculosis* isolates that were regarded as orphans. The T1 (ST719, ST53, ST1294) subfamily (19%), Beijing (ST1) strain family (18%) and the LAM3 (ST33, ST130, ST1293) subfamily (13%) were the three most dominating spoligopatterns. The S (ST34, ST71, ST884) strain family (7%), CAS_KILI (ST21) variant (6%), T2-T3 (ST73) subfamily (4%), X3 (ST92) strain family (3%), H1 (ST47, ST62) subfamily (3%), LAM11-ZWE (ST59, ST807) variants (3%), U (ST124, ST721) strain family (3%), the LAM4 (ST60, ST811) subfamily (2%), X1-LAM9 (ST1614) subfamily (2%), Beijing-like (ST269) strain family (1%), LAM1 (ST20) subfamily (1%) and T3 (ST37) subfamily (1%) were the least represented spoligopatterns (Figure 3.1). The 14% orphan strains belonged to the Euro-American subfamily According to "Spotclust" (Vitol *et al*., 2006).

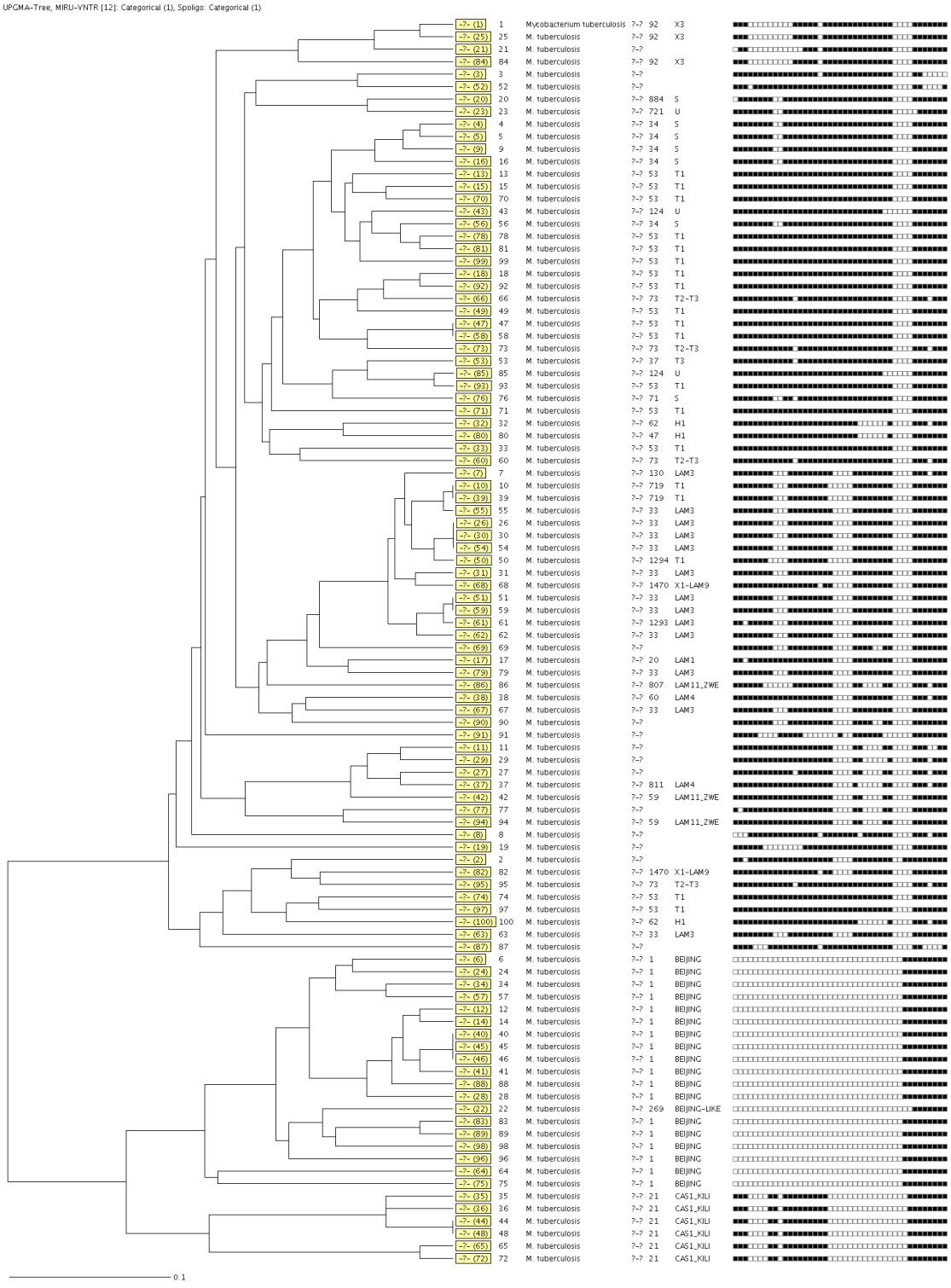

Figure 3.1: **Dendrogramme presentation of the spoligotypes detected among the 100 *M. tuberculosis* isolates collected from a provincial hospital in the Pretoria region, South Africa using the MIRU-VNTR*plus* database and the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm**

3.4.3 MIRU-VNTR results of the *M. tuberculosis* isolates

The MIRU-VNTR typing analysis showed a total of 87 distinct MIRU patterns (Figure 3.2). Among the 87 distinct MIRU patterns, eight clusters and 79 unique patterns were obtained. Overall, 21% (21/100) of the *M. tuberculosis* isolates were included in these eight clusters with each cluster containing between two to four isolates. Allele polymorphism analysis of the 12 MIRU loci used in this study showed that MIRU loci 10, 26 and 40 had the highest allelic diversity (ranging from 0.678 to 0.743) and were, therefore, highly discriminant. The MIRU loci, 16, 23, 27, 31 and 39 were moderately discriminant (ranging from 0.394 to 0.53), while MIRU loci 2, 4, 20 and 24 showed poor discrimination (ranging from 0.0398 to 0.246) (Table 3.1).

Table 3.1: Summary of the allelic diversity of 100 *Mycobacterium tuberculosis* isolates obtained from a provincial hospital in the Pretoria region, South Africa

MIRU loci	Number of isolates with indicated MIRU allele										Allelic Diversity
	0	1	2	3	4	5	6	7	8	9	
MIRU2		3	90		5	2					0.188
MIRU4			86		13	1					0.246
MIRU40			34	41	21	3	1				0.678
MIRU10			4	14	39	29	5		4	5	0.743
MIRU16		13	10	70	7						0.483
MIRU20		3	97								0.0588
MIRU23				10		60	29	1			0.551
MIRU24	1	98	1								0.0398
MIRU26		6	1	3	6	52	6	15	10	1	0.692
MIRU27		13	15	72							0.447
MIRU31			5	65	8	21	1				0.53
MIRU39		1	74	25							0.394

The allelic diversity of the loci was classified as highly discriminant (Hunter-Gaston index >0.6), moderately discriminant (0.3≤HGDI≤0.6) and poorly discriminant (HGDI<0.3) (Sola *et al*., 2003)

3.4.4 Combined results of the spoligotyping and MIRU-VNTR typing methods

Combining the spoligotyping (octal code) and MIRU-VNTR (12-digit allelic profile) typing results using the MIRU-VNTR*plus* database, resulted in 92 distinct genotypes among which six clusters containing between two to three isolates (representing 14 isolates in total) could be identified. The non-clustered genotypes consisted of 86 unique profiles. The summary of the discriminative power of each typing method alone and of the two typing methods combined are shown in Table 3.2 and were calculated according to the HGDI. The combined typing methods showed the highest discriminatory power of 0.998, followed by the MIRU-VNR typing method with 0.996 and lastly the spoligotyping method with 0.926.

Table 3.2: Discriminatory power of spoligotyping and MIRU-VNTR typing, alone and in combination according to the HGDI

Methodologies	Distinct patterns n	Clusters n	Clustered isolates n	Clustering rate (%)	HGDI
Spoligotyping	39	12	73	61%	0.926
MIRU-VNTR typing	87	8	21	13%	0.996
Spoligotyping and MIRU-VNTR typing	92	6	14	8%	0.998

HGDI: Hunter-Gaston Discriminatory index

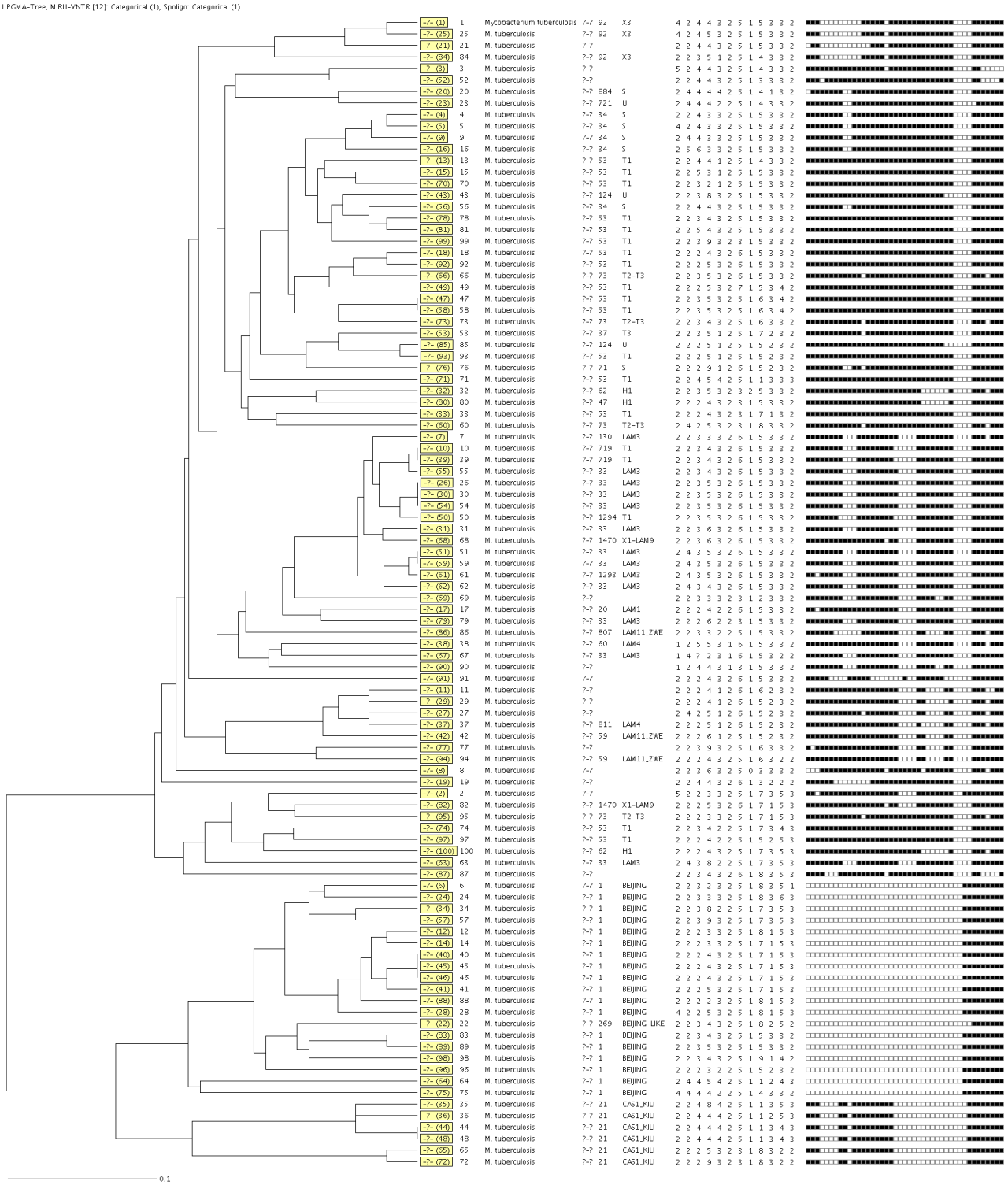

Figure 3.2: **Combined results of spoligotyping and MIRU-VNTR typing depicted in a dendrogramme for the 100 *M. tuberculosis* clinical isolates analysed from a provincial hospital using the MIRU-VNTR*plus* database and the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm**

3.5 Discussion

The results from this study showed that TB in this provincial hospital is caused by multiple *M. tuberculosis* strain families. Among the 100 *M. tuberculosis* isolates included in this study, four strain families, nine subfamilies and two variants were obtained using spoligotyping. Spoligotyping has shown that TB in the provincial hospital is mostly caused by three *M. tuberculosis* strain families: the 'ill-defined' T strain family represented by the T1 subfamily, the Beijing strain family and the LAM strain family represented by the LAM3 subfamily. These families are members of the seven major *M. tuberculosis* spoligotyping based families (Brudey *et al*., 2006) and represented 50% of the *M. tuberculosis* isolates in the study. This study corroborated the results from similar genetic relatedness studies performed in other provinces of South Africa, such as KwaZulu-Natal and the Western Cape where the 'ill-defined' T, Beijing and LAM families were also reported as the most prevalent *M. tuberculosis* strain families (Victor *et al*., 2004; Nicol *et al*., 2005; Cohen *et al*., 2011).

In this study the dominance of the T1 subfamily, which represented 19% of the *M. tuberculosis* isolates was in agreement with the findings of a study by Stavrum and colleagues (2009) from 252 *M. tuberculosis* isolates, collected from 2001 to 2002. The research assessed the distribution and diversity of multi-drug resistant tuberculosis (MDR-TB) genotypes in eight (Eastern Cape, Limpopo, North West, Free State, Mpumalanga, Gauteng, KwaZulu-Natal and Western Cape) of the nine provinces of South Africa and determined if there is an association between MDR-TB and a particular genotype. Stavrum and colleagues (2009) found the T1 subfamily to be dominant in Gauteng and the Free State provinces. A drawback of the Stavrum *et al*. (2009) study was the small sample size used to represent the provinces ranging from 23 *M. tuberculosis* isolates from North West to 41 *M. tuberculosis* isolates from KwaZulu-Natal while the Gauteng province was presented by 35 *M. tuberculosis* isolates. In addition, for the Gauteng province, it was not clear where the isolates were obtained.

According to SpolDB4 database, the 'ill-defined' T strain family is among the most dominating family of strains found in Africa (Brudey *et al*., 2006). All the T1 subfamily isolates obtained in this study were susceptible to rifampicin (RIF) and isoniazid (INH), except one isolate, which was found to be rifampicin mono-resistant. This kind of resistance

(RIF mono-resistance) is rare and considered a surrogate marker for MDR-TB, which is a major threat to the control of tuberculosis (Gillespie, 2002).

The Beijing strain family representing 18% of the *M. tuberculosis* isolates was the second most dominant family found in the study population. This is similar to previous studies conducted in the Western Cape, where the Beijing strain family was found to be among the dominanting strain families (Streicher *et al*., 2004; Victor *et al*., 2004; Nicol *et al*., 2005; Cowley *et al*., 2008). The Beijing strain family is considered not to be common in Africa, except for the Cape Town area (Van Helden *et al*., 2002). It is believed to have been introduced in Cape Town around the 17th and 18th century following the sea trade route from East Asia to Europe, whereby the Dutch colonist at the Cape of Good Hope imported slaves from Indonesia, Madagascar, Mozambique and India (Van Helden *et al*., 2002). The Beijing strain family has been found among the dominating *M. tuberculosis* strains in studies conducted in other provinces of South Africa (Stavrum *et al*., 2009).

All the Beijing strain family isolates in this study were susceptible to INH and RIF. This differs from other studies conducted in other provinces of South Africa as well as worldwide where this strain family is associated with anti-TB drug resistance (Van Rie *et al*., 1999; Krüüner *et al*., 2001; Toungoussova *et al*., 2002; Sun *et al*., 2007). According to a study conducted in the Western Cape province on 438 and 652 MDR-TB isolates collected from January 2001 to December 2002 and January 2005 to December 2006 respectively, from patients attending 65 health care clinics in the province, it was observed that the Beijing strain family contributed to 36.5% of the drug resistant *M. tuberculosis* isolates (Johnson *et al*., 2010). In a study done by Mlambo and colleagues (2008) among 699 *M. tuberculosis* isolates submitted to the National Health Laboratory Service (NHLS) TB Referral Laboratory, Johannesburg from seven provinces (Western Cape, Eastern Cape, Northern Cape, Free State, North West, Gauteng and Limpopo) of South Africa from June 2005 to December 2006, the Beijing strain family accounted for 34% of the XDR-TB cases. The 34% of the XDR-TB was found among 41 *M. tuberculosis* isolates, which were found to be XDR-TB in four (Northern Cape, North West, Gauteng and Limpopo) of the seven provinces (Mlambo *et al*., 2008). The Beijing strain family is associated with drug resistance in other parts of the world, such as India (Almeida *et al*., 2005), Russia (Toungoussova *et al*., 2004), Korea (Park *et al*., 2005), Vietman (Anh *et al*., 2000), Japan (Iwamoto *et al*., 2008) and Germany (Hillemann *et al*., 2005).

The LAM family represented by the LAM3 subfamily was the third most dominating group (13%) of the *M. tuberculosis* isolates in the study. The LAM3 subfamily is also named the F11 strain family due to the distinctive C-T SNP in the *rrs* 491 gene (Victor *et al*., 2001). The F11/LAM3 strain was found in the high incidence communities of the Western Cape in a study conducted from July 1992 to December 1998 (Victor *et al*., 2004). The dominance of the LAM3 subfamily in the current study was not a surprise as this subfamily was found among the strain subfamilies that contributed to the TB epidemic in South Africa (Victor *et al*., 2004). The strain subfamily is believed to be globally distributed causing about 15% of TB cases worldwide (Gibson *et al*., 2008).

Among the minor strain families and variants the S (ST34) strain family, LAM11-ZWE (ST59) variant and LAM4 (ST60) subfamily were of importance. The S strain family, which is known to be dominating in Sicily and Sardinia (Sola *et al*., 2001) contributed 7% of the *M. tuberculosis* isolates in this study. The S strain family is similar to the F28 strain family that was found in the Western Cape province in patients visiting primary health care facilities in the province between mid-1992 to 1998 (Warren *et al*., 2002). The variant, LAM11-ZWE was represented by three isolates in this study. The variant was first identified in high numbers in Harare, Zimbabwe (Easterbrook *et al*., 2004; Chihota *et al*., 2007). The presence of this variant in the provincial hospital can be explained by the fact that Zimbabwe shares a border with South Africa and currently there is a high migration rate of Zimbabweans into South Africa, especially to the Gauteng province in search for jobs and a better life.

The LAM4 subfamily, contributed to 2% of the *M. tuberculosis* isolates in this study. One of the LAM4 subfamily spoligopatterns was found to be similar to the F15/LAM4/KZN spoligopattern (Pillay and Sturm, 2007). The F15/LAM4/KZN strain family was found among 966 *M. tuberculosis* isolates analysed from 1994 to 2002 from patients in KwaZulu-Natal where this strain family was consistently associated with MDR-TB (Pillay and Sturm, 2007). The LAM4 with F15/KZN spoligopattern showed no association with drug resistance in this study. Since the number of LAM4 isolates in this study population was small, a study of longer duration with higher numbers of LAM4 strain family isolates with the F15/KZN spoligopattern is required to confirm this finding.

The spoligotyping method was proved useful in classifying *M. tuberculosis* isolates into different families and was used as an initial screening method, which required an additional

method for optimal discriminatory power. In this study, the MIRU-VNTR typing method was used to increase the resolution and discrimination of spoligotyping. The number of clusters decreased from 12 by spoligotyping to eight clusters by MIRU-VNTR typing. According to the MIRU-VNTR typing method, the Beijing strain family was further differentiated into 17 distinct patterns and the T1 subfamily, which had three spoligotyping patterns also showed 17 distinct patterns. Despite the high discriminatory power of the MIRU-VNTR typing method observed in this study, there was still only a few *M. tuberculosis* isolates that were clustered using this typing method. Some of these clusters were discriminated by the spoligotyping method. Therefore, the results obtained in this study showed the importance of combining more than one typing method, such as spoligotyping and MIRU-VNTR typing to obtain a higher discriminatory power for epidemiological studies. The combination of the spoligotyping and MIRU-VNTR typing methods resulted in a HGDI of 0.998. According to Cowan and colleagues (2002), the combined discriminatory power of spoligotyping and MIRU-VNTR typing is similar to that of the 'gold standard' (IS*6110* RFLP). The combined HGDI in this study was higher than that of spoligotyping (0.926) and MIRU-VNTR typing (0.996).

The MIRU-VNTR loci have variable numbers of alleles (Supply *et al*., 2006). In this study the loci, which were found to be most polymorphic were 10 (seven allelic copies), 26 (nine allelic copies) and 40 (five allelic copies), respectively. The results were in agreement with other studies that showed MIRU 10 to be the most polymorphic locus having mostly seven allelic copies (Cowan *et al*., 2002; Sola *et al*., 2003; Sun *et al*., 2004; Smittipat *et al*., 2005). According to studies by Valcheva and colleagues (2008) and Sola and colleagues (2003) the MIRU locus 40 was the most discriminatory locus with six and eight allelic copies, respectively. The MIRU 26 was also found to be highly discriminatory by Sola *et al*., 2003 but moderate by Valcheva and colleagues (2008). The MIRU loci 27 (three allelic copies), 31 (five allelic copies) and 39 (three allelic copies), which were found to be moderately discriminatory in this study, were reported as poorly discriminatory by Valcheva and colleagues (2008) with three allelic copies for MIRU 27 and 31 and two allelic copies for MIRU 39. The MIRU 16, which was moderate in the present study was also found to be moderate according to Sola and colleagues (2003) and Valcheva and colleagues (2008) with four allelic copies in all the studies. The three poorly discriminatory (MIRU 2, MIRU 20, MIRU 24) loci in this study were also found to be poorly discriminatory by other molecular

studies (Mazars *et al*., 2001; Valcheva *et al*., 2008). This proves that the polymorphism of each MIRU locus vary with the geographical origin of the *M. tuberculosis* isolates.

According to this study almost an equal number of male (31%) and female (29%) participants were TB smear positive, unlike in the WHO report (2009) where more males (1.65 million) were found to be TB smear positive than females (0.9 million) with a ratio of 1:8 worldwide. In this study 61% of *M. tuberculosis* isolates were smear positive, which suggest that most of these *M. tuberculosis* strains are highly infectious; however, based on the results, there was no TB transmission noted in this hospital, since there was no association between hospital wards and different *M. tuberculosis* strains obtained.

This study provided baseline information on the diversity of *M. tuberculosis* strains circulating in a provincial hospital in Gauteng province. The study laid a foundation for *M. tuberculosis* genotyping in this hospital. Several *M. tuberculosis* strain families, subfamilies and variants are circulating in this provincial hospital in Pretoria among TB patients attending this clinical setting. The information can be used to monitor transmission of the *M. tuberculosis* strains in the hospital periodically as well as to follow these *M. tuberculosis* strains in the community.

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CHAPTER 4

CONCLUSION

4.1 Concluding Remarks

Molecular epidemiology is now a major field of research in infectious diseases, particularly tuberculosis (TB) (Tazi *et al*., 2002). Epidemiological studies have shown that the distribution of *M. tuberculosis* strains vary in different geographical regions (Filliol *et al*., 2002). There are numerous genetic markers developed for use in epidemiological studies but the most commonly used is the insertion sequence (IS) *6110* element, which is used in the restriction fragment length polymorphism (RFLP) method. Since 1993, genotyping of *M. tuberculosis* relied heavily on IS*6110* RFLP typing as the 'gold standard' because it has a high discriminatory power in the genotyping of *M. tuberculosis* strains (Kremer *et al*., 1999; Houben and Glynn, 2009). The discriminatory power of the method is directly proportional to the number of IS*6110* copies present (Cowan *et al*., 2002). The higher the IS*6110* copy number, the greater the possibility that two or more identical RFLP patterns will be grouped together to form a cluster and are considered to be epidemiologically related (Das *et al*., 1995). However, related *M. tuberculosis* strains with less than six IS*6110* copy numbers may be indicated as epidemiologically unrelated when the IS*6110* RFLP typing method is used (McHugh and Gillespie, 1998).

Due to this disadvantage, polymerase chain reaction (PCR) based methods were introduced (Supply *et al*., 2000; Van Soolingen, 2001). The most commonly used PCR based genotyping methods are spoligotyping and mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) typing (Mazars *et al*., 2001). The spoligotyping and MIRU-VNTR typing methods applied in this study were able to determine the *Mycobacterium tuberculosis* (*M. tuberculosis*) strain families that caused TB among 100 patients that seeked medical attention at the Kalafong Hospital in Pretoria. The spoligotyping method was found useful as a screening method with a discriminatory index of 0.926 to classify *M. tuberculosis* isolates into different families. Eight strain families were obtained in the present study (CAS, Beijing, LAM, H, 'ill-defined' T, X, S and U) using spoligotyping. The most dominating strain families found among the eight strain families were the 'ill-

defined' T strain family, Beijing strain family and LAM strain family according to the descending order of families. These strain families were also identified in similar studies conducted in other provinces of South Africa, such as KwaZulu-Natal and the Western Cape provinces (Victor *et al*., 2004; Nicol *et al*., 2005; Cohen *et al*., 2011) and other parts of the world, such as Asia, Africa, Europe and South America (Brudey *et al*., 2006). The spoligotyping method was easy to perform and allowed the comparison of the fingerprints obtained in the study with those found internationally through the use of the SITVIT2 database. The SITVIT2 database is an update of the SpolDB4, which is an open access and internet available international database (Brudey *et al*., 2006).

In this study the single primer pair MIRU-VNTR typing method was used as a secondary genotyping method to further discriminate the *M. tuberculosis* strains obtained by spoligotyping. The MIRU-VNTR typing method was informative and was able to subdivide the *M. tuberculosis* isolates that were clustered together by spoligotyping into distinct genotypes. The MIRU-VNTR typing showed a discriminatory power of 0.966 with three MIRU loci (10, 26, 40) having the highest discriminatory power, five MIRU loci (16, 23, 27, 31 and 39) showed a moderate discriminatory power and four MIRU loci (2, 4, 20, 24) showed a poor discriminatory power. To obtain the genetic relatedness of the strains, the results were entered into the MIRU-VNTR*plus* database. The MIRU-VNTR*plus* database was built using 186 reference strains representing primary *M. tuberculosis* complex strains (Allix-Béguec *et al*., 2008).

The MIRU-VNTR typing method is an important genotyping method in determining the heterogeneity of *M. tuberculosis* strains within a patient (Garcia de Viedma *et al*., 2004; Martin *et al*., 2007). The heterogeneity in the patient can be due to diversification of *M. tuberculosis* strains following single infection events or infection with more than one *M. tuberculosis* strain resulting in mixed infections (Garcia de Viedma *et al*., 2004; Martin *et al*., 2007). Infection with heterogeneous *M. tuberculosis* strains is clinically important especially when one of the strains is associated with resistance to any of the TB drugs, since it will have an effect on the treatment outcome of the patient (Garcia de Viedma *et al*., 2004; Martin *et al*., 2007). No heterogeneity was found in the *M. tuberculosis* isolates obtained from the patients from Kalafong Hospital that were included in this study.

Mycobacterium tuberculosis was initially thought to have no genetic variability but it was later found that limited horizontal gene transfer does occur in this bacterium therefore, there is presence of non-clonal populations (Alland *et al*., 2003; Achtman, 2008). Based on the research findings in this study, a high diversity with a total of 92 distinct patterns was obtained when the spoligotyping and MIRU-VNTR typing methods were combined. These results were important because it has been shown that genetic variability among clinical isolates may have a significant outcome in *M. tuberculosis* infections with diverse TB clinical presentations, such as acute primary TB (localised or disseminated), latent disease and reactivation (O'Brien *et al*., 1994; Rhoades and Orme 1997; Firmani and Riley, 2002; Barczak *et al*., 2005; Malik and Godfrey-Faussset, 2005; Nicol and Wilkinson, 2008). The genetic variation among the *M. tuberculosis* isolates can alter future diagnostics and vaccines and may have an impact on the management of patients. It is, therefore, important that genotypic analysis on *M. tuberculosis* strains be performed throughout the world, especially in high TB burdened countries in order to establish the diversity of the *M. tuberculosis* strains.

4.2 Future research

The present study was able to determine the dominating *M. tuberculosis* strain families in the Kalafong Hospital in the Pretoria region. Future research should focus on determining the dominating and circulating *M. tuberculosis* strain families in the Pretoria region by including several hospitals and clinics in the area. It can be useful to determine if the same *M. tuberculosis* strains are also prevalent in the greater Pretoria region. The present study showed that circulating *M. tuberculosis* strains in this setting are genetically diverse. Community based genotyping studies on *M. tuberculosis* should be conducted in all the communities that seek medical care from the Kalafong Hospital. This could help in understanding the cause of the high strain diversity at the hospital, whether it is caused by factors, such as reactivation or by migrants seeking medical care at the hospital. More genotyping studies are needed to assess the association between *M. tuberculosis* strain families and pulmonary and extrapulmonary TB, since it has been hypothesised that *M. tuberculosis* strain families differ in their ability to disseminate and cause extrapulmonary TB (Kong *et al*., 2007; Caws *et al*., 2008). These findings could help in determining the virulent *M. tuberculosis* strains in this region because the ability of a strain to disseminate is regarded as a virulence marker.

Although the single primer pair 12 MIRU-VNTR typing showed a high discriminatory power in this setting, the method was found to be labour intensive and time consuming. In order to overcome this disadvantage, the multiplex PCR based MIRU-VNTR typing method also known as the automated fluorescence-based MIRU-VNTR typing method, though expensive, is recommended for studies on *M. tuberculosis* genotyping (Supply *et al*., 2001). According to the literature, this genotyping method either based on a 12, 15 or 24 set of MIRU-VNTR loci is less labour intensive and can be completed in a shorter period of time because the analysis is done using a fluorescence-based DNA analyser (Supply *et al*., 2001). The MIRU-VNTR typing method based on 24 sets of loci when combined with spoligotyping, the discriminatory power can exceed the discriminatory power of the IS*6110* RFLP typing method, the 'gold standard' (Allix-Béguec *et al*., 2008; Maes *et al*., 2008). Therefore, the use of the 24 MIRU-VNTR loci automated multiplex-PCR is recommended.

More genotyping studies are needed not only in the Pretoria region but in the Gauteng province as a whole, since little is known of the *M. tuberculosis* strain families circulating in this province. The information will be of clinical importance because it can assist clinicians to differentiate between TB relapse and reinfection in a patient presenting with recurrence, since these cannot be done on clinical presentation. Knowledge of the cause of the high TB burden in all the provinces will have an impact on the country's TB control programme's strategies and interventions. Tuberculosis relapses in a patient shows that the patient was inadequately treated, therefore, it might be an indicator of failure of one or more of the directly observed treatment, short-course (DOTS) five main points of action or the DOTSplus programme. In cases of reinfection, genotyping can identify unsuspected epidemiological links and sites of transmission. This information can help TB control programme coordinators to evaluate the programme activities and act appropriately.

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APPENDIX A

1. *Mycobacterium tuberculosis* DNA extraction method

A pasteur pipette was used to collect 100 µl of the MiddleBrook 7H9 broth culture medium (Diagnostic Media Products, South Africa). The deoxyribonucleic acid (DNA) extraction was performed using the Amplicor Respiratory Specimen Preparation kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. In brief; 500 µl wash solution and 100 µl *M. tuberculosis* broth culture was added in a labelled 1.8 ml Eppendorf tube (Lasec, South Africa). The H37Rv (ATCC 25177) broth culture was added in 500 µl of wash solution as a positive control. The mixtures were vortexed (Labnet International, USA) for 5 s and centrifuged (Spectrafuge, Labnet International, USA) (12 500 *x g*) for 10 min. The supernatant was aspirated and 100 µl lysis buffer was added to the cell pellet and vortexed for 5 s. The cell suspension and positive control (H37Rv; ATCC 25177) were incubated in a 60°C +/- 2°C dry heating block (AccuBlock Digital Bath, Labnet International, USA) for 45 min. The tubes were pulse-centrifuged (Spectrafuge, Labnet International, USA) for 5 s to remove condensate that might have accumulated in the cap. Neutralisation reagent (100 µl) was added and the tubes were vortexed for 5 s. The DNA sample was stored at -20°C until further analysis.

1.1 Determination of the extracted DNA concentration using the Nanodrop spectrophotometer

The concentration of the extracted DNA was determined by pipetting 2 µl of sample onto the Nanodrop spectrophotometer sensor (Thermo Scientific, USA). The Nanodrop uses the Beer-Lambert equation (C=A/CE*b) to determine the concentration of the nucleic acid (DNA). The C is the nucleic acid concentration in ng/µl, A is the absorbance in AU, E is the wavelength dependent extinction coefficient in ng-cm/µl and b is the path length in cm. In DNA concentration determination, the absorbance peak is at 260 nm and the ratio of 260/280 should be 1.8. The concentration of the DNA was found to range between 122.4 ng/µl and 499.7 ng/µl.

2. Spoligotyping method

The spoligotyping method is a polymerase chain reaction (PCR) based method. The method involves the amplification of the direct repeat region (DR) of the extracted genomic DNA. The PCR amplified spacers found in the DR region are hybridised with the probes derived from 43 spacers covalently bounded to the membrane (Ocimum BioSolution, India). The hybrids are detected by enhanced chemiluminescence (ECL).

2.1 Polymerase chain reaction for the spoligotyping method

The spoligotyping of the *M. tuberculosis* isolates was done using the DNA extracted as described in Section 1. The spoligotyping was done according to the modified method of Kamerbeek *et al*., 1997. The direct region (DR) of the *M. tuberculosis* DNA was amplified using the biotin-labeled forward primer (5'-GGT TTT GGG TCT GAC GAC-3') T_m=59.9ºC and reverse primer (5'-CCG AGA GGG GAC GGA AAC-3') T_m=64.46ºC described by Kamerbeek *et al*., 1997 and manufactured by Inqaba Biotec, South Africa. The PCR reagents were prepared as indicated in Table 1. The amplication cycling profile was programmed as shown in Table 2.

Table 1: Polymerase chain reaction reagents and volumes for the amplification of the 43 spacers for spoligotyping

PCR reagents	Volumes (µl)
Qiagen Mastermix (Qiagen, Germany)	12.5
Forward primer (biotinylated 5'end) (20 µg/µl)	2
Reverse primer (20 µg/µl)	2
DNA template	5
Deionised water (Qiagen, Germany)	3.5
Total	25

Table 2: Polymerase chain reaction profile for the amplification of the 43 spacers used in spoligotyping

PCR cycle parameter	Temperature	Time	Number of cycles
Initial denaturation	96ºC	15 min	1
Denaturation	96ºC	1 min	30
Annealing	55ºC	1 min	
Extension	72ºC	30 sec	
Final extension	72ºC	10 min	1
Hold	4ºC	-	1

2.2 Spoligotyping buffer solution preparation for the detection of the 43 spacers

1. The 20x Saline Sodium Phosphate EDTA (SSPE) buffer, was prepared by adding 0.2 M sodium hydrogen phosphate ($Na_2HPO_4*2H_2O$ (35.6 g/l) (Merck, Germany), 3.6 M sodium chloride ($NaCl_2$) (210.24 g/l) (Merck, Germany) and 20 mM Ethylenediaminetetraacetic acid (EDTA) (7.4 g/l) (Promega, USA).

2. The mixture was autoclaved and stored at room temperature (T°C) as stock solution.

3. The 10% sodium dodecyl sulfate (SDS) CALBIOCHEM (Merck, Germany) solution was prepared by adding 10 g SDS in 100 ml of deionised water.

4. The 2x SSPE was prepared by diluting the 20x SSPE stock solution with deionised water in a ratio 1:10.

5. The 2x SSPE/0.1% SDS was prepared by adding 100 ml of the 20x SSPE stock solution and 10 ml of 10% SDS to 890 ml of deionised water.

6. The 2x SSPE/0.5% SDS was prepared by adding 100 ml 20x SSPE and 50 ml 10% SDS to 850 ml of deionised water.

2.3 Hybridisation and detection of the 43 spacers of the *M. tuberculosis* isolates

The hybridisation of the PCR products was done by adding 20 µl of the amplicons to 150 µl of 2x SSPE/0.1% SDS (Merck, South Africa) in 60ºC. The diluted PCR products were heat-denatured at 99ºC for 10 min in an Eppendorf MasterCycler (Hamburg, Germany) and immediately cooled on ice. The membrane (Ocimum BioSolution, India) was washed for 5 min at 60ºC in 250 ml 2x SSPE/0.1% SDS (Merck, South Africa) and placed into the miniblotter (Ocimum BioSolution, India) supported by the cushion (Ocimum BioSolution, India). The membrane was placed in such a way that the slots were perpendicular to the line pattern of the applied oligonucleotides (PCR products). The residual fluid was removed from the slots of the miniblotter by aspiration. A volume of 170 µl of denatured PCR products was transferred into the 43 membrane slots and hybridised at 60ºC in a waterbath (SANJEEV Scientific UDYOGI, India) for 60 min. The sample was aspirated from the miniblotter after 60 min and the membrane was placed into a washing buffer. The membrane was washed twice in 250 ml 2x SSPE/0.5% SDS at 60ºC for 10 min. The washed membrane was incubated in a mixture of 7.5 µl (500 U/ml) streptavidin-peroxidase conjugate (Ocimum BioSolution, India) and 20 ml of 2x SSPE/0.5% SDS at 42ºC for 45 min to 60 min in a rolling bottle (Lasec, South Africa). The membrane was washed twice in 250 ml of 2x SSPE/0.5% SDS (Ocimum BioSolution, India) at 42ºC for 10 min in a shaking incubator (Labcon, US) followed by rinsing twice in 250 ml 2x SSPE at room temperature (25ºC) for 5 min. The detection of the hybrids was done by incubating the membrane for 1 min in 30 ml of chemiluminiscence (ECL) detection liquid (solution 1 and solution 2) (Amersham, Sweden). The membrane was exposed to the light sensitive X-ray film (Ocimum BioSolution, India) for 20 min. The signal was developed by placing the X-ray film into the developing solution (AGFA Health Care, South Africa) for 10 min, rinsed in water, placed in the fixing solution (Ocimum BioSolution, India) for 10 min and left to air dry. The results were entered into a Microsoft Excel sheet as octal codes (Appendix B).

3. MIRU-VNTR typing method

The MIRU-VNTR typing was done according to the described method of Le-Fleche and colleagues (2002). The extracted *M. tuberculosis* DNA was amplified using the 12 primer set described by Supply *et al.* (2001) (Table 3). The PCR reactions were prepared as indicated in

Table 4. The amplification was done according to the cycling profile as described in Table 5. The amplicons were analysed by agarose gel electrophoresis (Section 3.2).

Table 3: Twelve MIRU loci primer sequences for the MIRU-VNTR typing method (Supply *et al.*, 2001)

MIRU locus	MIRU-VNTR length bp	*Primer sequence (5'↔3')	Melting Temperature (T_m/°C)
4	77	GCGCGAGAGCCCGAACTGC	68.79
		GCGCAGCAGAAACGTCAGC	64.48
26	51	TAGGTCTACCGTCGAAATCTGTGAC	64.58
		CATAGGCGACCAGGCGAATAG	64.52
40	54	GGGTTGCTGGATGACAACGTGT	64.54
		GGGTGATCTCGGCGAAATCAGATA	64.57
10	53	GTTCTTGACCAACTGCAGTCGTCC	66.28
		GCCACCTTGGTGATCAGCTACCT	66.33
16	53	TCGGTGATCGGGTCCAGTCCAAGTA	67.86
		CCCGTCGTGCAGCCCTGGTAC	70.38
31	53	ACTGATTGGCTTCATACGGCTTTA	61.15
		GTGCCGACGTGGTCTTGAT	62.32
2	53	TGGACTTGCAGCAATGGACCAACT	64.57
		TACTCGGACGCCGGCTCAAAAT	64.54
23	53	CTGTCGATGGCCGCAACAAAACG	66.33
		AGCTCAACGGGTTCGCCCTTTTGTC	67.86
39	53	CGCATCGACAAACTGGAGCCAAAC	66.28
		CGGAAACGTCTACGCCCCACACAT	67.98
20	77	TCGGAGAGATGCCCTTCGAGTTAG	66.28
		GGAGACCGCGACCAGGTACTTGTA	67.98
24	54	CGACCAAGATGTGCAGGAATACAT	62.86
		GGGCGAGTTGAGCTCACAGAA	64.52
27	53	TCGAAAGCCTCTGCGTGCCAGTAA	66.28
		GCGATGTGAGCGTGCCACTCAA	66.4

*Primers manufactured by Inqaba Biotec, South Africa

Table 4: Polymerase chain reaction reagents for the amplification of the 12 MIRU loci (Le-Fleche *et al*., 2002)

PCR reagents	Volumes (µl)
Qiagen Mastermix (Qiagen, Germany)	10
Forward primer (20 µg/µl)	0.5
Reverse primer (20 µg/µl)	0.5
DNA template	2
Deionised water (Qiagen, Germany)	7
Total	20

Table 5: Mycobacterial interspersed repetitive units-variable number tandem repeat (MIRU-VNTR) typing PCR conditions for the amplification of each of the 12 loci (Le-Fleche *et al*., 2002)

PCR Cycle parameters	Temperature	Time	Number of Cycles
Initial denaturation	94ºC	1 sec	1
Denaturation	94ºC	5 min	40
Annealing	62ºC	1 min	
Extension	72ºC	1 min 50 sec	
Final extension	72ºC	10 min	1
Hold	4ºC	-	1

3.1 Preparation of a buffer for MIRU-VNTR typing amplicon analysis

The 50 X TAE buffer was prepared by adding 242 g of Tris Base (Sigma Chemical, USA) to deionised water. A volume of 57.1 ml of glacial acid (Merck, Germany) and 100 ml of 0.5 M EDTA (pH 8.0) (Promega, USA) were added to the solution. The 0.5 M EDTA was prepared by dissolving 186.12 g of EDTA in a liter of deionised water (pH 8.0). The volume of the 50 X TAE was adjusted to a final volume of 1000 ml and stored at room temperature (25ºC) as stock solution until further use.

The 1 X TAE working buffer solution was prepared by adding 10 ml of the stock solution into 990 ml of deionised water. The 2% (m/v) agarose gel was prepared by adding 2 g of agarose powder (Whitehead Scientific, South Africa) in 100 ml of the 1 X TAE working buffer

solution. The agarose powder was dissolved in the TAE working buffer solution by heating in microwave oven. The mixture was cooled in the Hybridiser HB-1D (Lasec, South Africa) for 30 min. After cooling, 5 µl ethidium bromide was added (10 mg/ml) (Promega, Madison, USA). The gel was poured into a casting tray (Bio-RAD, USA) with a comb to form the wells of the gel. The gel was placed into the electrophoresis tank (Bio-RAD, USA) with a 1 X TAE buffer after it solidified.

3.2 Analysis of the amplicons by gel electrophoresis

The PCR amplicons were electrophoresed on a 2.0% (m/v) agarose gel (Whitehead Scientic, South Africa) at 85 V/cm (Eilte-300 Power supply, Wealtec Corp., Kennesaw, GA) for 2 hr in 1 X TAE (pH 8.0 - 8.5) [40 mM Tris Base (Sigma Chemical, USA), 20 mM glacial acid (Merck, Germany), 0.5 M EDTA adjusted with Sodium hydroxide pellets to pH 8.0 (Promega, USA)]. A 50 base pair DNA ladder (Fermentas, Thermo Scientific, USA) was used as molecular size marker. The amplicons were visualised under UV illumination (TFM-26 Ultra Transilluminator, UVP, Upland, CA). The image was captured using a digital gel documentation system (DigiDoc-It imaging system, UVP, Upland). The size of the PCR fragments for each locus was estimated by visual comparison with the molecular marker and the MIRU allele frequency table to determine the number of repeats (Supply, 2005). The number of repeats of each of the 12 loci per isolate was entered into a Microsoft Excel sheet to create a 12-digit allelic profile. The results were analysed using the MIRU-VNTR*plus* database, (http://miru-vntrplus.org).

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APPENDIX B

Table 1: Summary of the clinical information and the initial diagnostic test results of the patients involved in the study

Isolate number	Age	Gender	Susceptibility profile	Microscopy results	Spp type	Location
1	32	M	INH (S), RIF (S)	Sc	SP	Medical out patients
2	32	M	INH (S), RIF (S)	2+	SP	Casualty
3	39	M	INH (S), RIF (S)	Neg	SP	Casualty
4	44	M	INH (S), RIF (S)	1+	SP	Family Medicine Secondary Clinic
5	29	M	INH (S), RIF (S)	Neg	SP	Family Medicine Secondary Clinic
6	36	M	INH (R), RIF (S)	3+	SP	Family Medicine Secondary Clinic
7	15	M	INH (S), RIF (S)	Neg	SP	Family Medicine Secondary Clinic
8	40	M	INH (S), RIF (S)	Neg	SP	Ward 16
9	26	F	-	3+	SP	Family Medicine Secondary Clinic
10	45	M	-	3+	GA	Ward 22
11	33	M	INH (S), RIF (S)	3+	GA	Ward 22
12	34	F	INH (S), RIF (S)	2+	GA	Ward 13
13	18	F	INH (S), RIF (R)	2+	SP	Casualty
14	N/S	M	INH (S), RIF (S)	3+	GA	Ward 22
15	45	M	INH (S), RIF (S)	3+	SP	Ward 22
16	29	F	INH (S), RIF (S)	3+	SP	Ward 19
17	64	M	INH (S), RIF (S)	Neg	SP	Ward 9
18	45	M	INH (S), RIF (S)	Neg	GA	Ward 16
19	35	F	-	Neg	SP	Casualty
20	28	F	INH (S), RIF (S)	3+	SP	Ward 20
21	27	F	-	Neg	SP	Medical Out Patients Department
22	31	F	INH (S), RIF (S)	2+	GA	Ward 20
23	36	F	INH (S), RIF (S)	3+	GA	Ward 19
24	31	M	-	3+	GA	Ward 22
25	30	F	INH (S), RIF (S)	3+	GA	Ward 19
26	23	M	INH (S), RIF (S)	3+	GA	Ward 22
27	42	M	INH (S), RIF (S)	Sc	SP	
28	56	F	-	3+	GA	Ward 20
29	33	F	INH (S), RIF (S)	3+	GA	Casualty
30	26	F	INH (S), RIF (S)	1+	GA	Ward 19
31	28	M	INH (S), RIF (S)	Sc	GA	Ward 22
32	63	F	-	3+	GA	Ward 19
33	49	F	INH (S), RIF (S)	Neg	GA	Ward 20
34	38	M	INH (S), RIF (S)	3+	GA	Ward 22
35	65	M	INH (S), RIF (S)	2+	GA	Ward 22
36	35	M	INH (S), RIF (S)	2+	GA	Ward 22
37	13d	M	INH (S), RIF (S)	Neg	GA	Ward 6B
38	29	F	INH (S), RIF (S)	Neg	GA	Ward 20
39	39	F	-	Neg	GA	Ward 20

Spp-specimen Sp-sputum GA-Gastric aspirate

Table 1: Summary of the clinical information and the initial diagnostic test results of the patients involved in the study (Continued)

Isolate number	Age	Gender	Susceptibility profile	Microscopy results	Spp type	Location
40	16	F	INH (S), RIF (S)	3+	GA	Ward 20
41	29	M	INH (S), RIF (S)	3+	GA	Ward 20
42	31	F	INH (S), RIF (S)	2+	GA	Ward 19
43	52	M	INH (S), RIF (S)	1+	GA	Family Medicine Secondary Clinic
44	68	F	-	3+	GA	Ward 19
45	30	F	INH (S), RIF (S)	3+	GA	Ward 20
46	28	M	INH (S), RIF (S)	3+	GA	Ward 22
47	34	F	INH (S), RIF (S)	Neg	SP	ward 26
48	49	M	INH (S), RIF (S)	Neg	SP	Out Patients Department
49	24	M	INH (S), RIF (S)	1+	GA	Casualty
50	31	F	INH (S), RIF (S)	Neg	SP	Immunology
51	43	M	INH (S), RIF (S)	Neg	GA	Casualty
52	47	M	INH (S), RIF (S)	2+	SP	Family Medicine Secondary Clinic
53	46	F	INH (S), RIF (S)	1+	GA	Ward 19
54	35	F	INH (S), RIF (S)	2+	SP	Ward 20
55	34	F	INH (S), RIF (S)	3+	SP	Ward 19
56	49	M	INH (S), RIF (S)	3+	GA	Ward 22
57	30	M	INH (S) RIF (S)	Sc	GA	Ward 22
58	29	M	INH (S), RIF (S)	2+	SP	Out Patients Department
59	32	M	INH (S), RIF (S)	2+	GA	Ward 22
60	54	M	INH(S), RIF (S)	Neg	GA	Ward 22
61	33	M	INH (S), RIF (S)	3+	GA	Ward 22
62	48	M	INH (S), RIF (S)	3+	SP	Casualty
63	30	M	INH (S), RIF (S)	Neg	GA	Ward 22
64	49	F	INH (S), RIF (S)	3+	GA	Ward 20
65	49	M	INH (S), RIF (S)	Sc	GA	Ward 22
66	6m	F	INH (S), RIF (S)	Neg	GA	Ward 6A
67	35	M	INH (S), RIF (S)	3+	GA	Ward 22
68	45	M	INH (S), RIF (S)	3+	SP	Family Medicine Secondary Clinic
69	10m	M	INH (S), RIF (S)	Neg	GA	Ward 6A
70	40	F	INH (S), RIF (S)	Neg	GA	Ward 19
71	37	M	INH (S), RIF (S)	Neg	SP	Family Medicine Secondary Clinic
72	26	F	INH (S), RIF (S)	Neg	GA	Ward 20
73	32	M	INH (S), RIF (S)	Neg	SP	Casualty
74	29	F	INH (S), RIF (S)	2+	GA	Ward 20
75	67	M	INH (S), RIF (S)	1+	GA	Ward 21
76	8m	F	INH (S), RIF (S)	Neg	GA	Drip Room
77	49	M	INH (S), RIF (S)	Sc	GA	Ward 22
78	49	F	INH (S), RIF (S)	3+	SP	Family Medicine Secondary Clinic
79	29	F	INH (S), RIF(S)	2+	SP	Casualty
80	42	M	INH (S), RIF (S)	3+	SP	Casualty

Spp-specimen Sp-sputum GA-Gastric aspirate

Table 1: Summary of the clinical information and the initial diagnostic test results of the patients involved in the study (Continued)

Isolate number	Age	Gender	Susceptibility profile	Microscopy results	Spp type	Location
81	22	F	INH (S), RIF (S)	2+	GA	Ward 19
82	41	F	INH (S), RIF (S)	1+	GA	Ward 19
83	33	M	INH (S), RIF (S)	Neg	SP	Ward 22
84	29	F	INH (S), RIF (S)	1+	GA	Ward 19
85	36	F	INH (S), RIF (S)	2+	GA	Ward 19
86	35	F	INH (S), RIF (S)	Neg	GA	Ward 22
87	46	M	INH (S), RIF (S)	Neg	GA	Ward 22
88	58	F	INH (S), RIF (S)	Neg	SP	Immunology Paediatric Out Patients Department
89	36	F	INH (S), RIF (S)	Sc	SP	Ward 20
90	30	F	-	Neg	GA	Ward 19
91	77	M	INH (S), RIF (S)	Neg	GA	Ward 22
92	45	M	INH (S), RIF (S)	2+	SP	Family Medicine Secondary Clinic
93	3y8m	F	INH (S), RIF (S)	Neg	GA	Ward 6A
94	25	F	INH (S), RIF (S)	2+	GA	Ward 20
95	N/S	M	INH (S), RIF (S)	3+	SP	Ward 22
96	35	M	INH (S), RIF (S)	3+	GA	Casualty
97	35	M	INH (S), RIF (S)	2+	SP	Casualty
98	34	M	INH (S), RIF (S)	3+	GA	Ward 22
99	34	M	INH (S), RIF (S)	3+	GA	Ward 22
100	40	F	INH (S), RIF (S)	2+	SP	Ward 19

Spp-specimen Sp-sputum GA-Gastric aspirate

Table 2: Spoligotyping and the MIRU-VNTR typing results of the 100 patients attending the Kalafong hospital, Pretoria

ID	MIRU02	MIRU04	MIRU40	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	Octal Code
1	4	2	4	4	3	2	5	1	5	3	3	2	700076777760771
2	5	2	2	3	3	2	5	1	7	3	5	3	777177417777751
3	5	2	4	4	3	2	5	1	4	3	3	2	777776777760600
4	2	2	4	3	3	2	5	1	5	3	3	2	776377777760771
5	4	2	4	3	3	2	5	1	5	3	3	2	776377777760771
6	2	2	3	2	3	2	5	1	8	3	5	1	3771
7	2	2	3	3	3	2	6	1	5	3	3	2	776177607760731
8	2	2	3	6	3	2	5	0	3	3	3	2	77776775760731
9	2	4	4	3	3	2	5	1	5	3	3	2	776377777760731
10	2	2	3	4	3	2	6	1	5	3	3	2	776177407760771
11	2	2	2	4	1	2	6	1	6	2	3	2	777777606060711
12	2	2	2	3	3	2	5	1	8	1	5	3	3771
13	2	2	4	4	1	2	5	1	4	3	3	2	777777777760771
14	2	2	2	3	3	2	5	1	7	1	5	3	3771
15	2	2	5	3	1	2	5	1	5	3	3	2	777777777760771
16	2	5	6	3	3	2	5	1	5	3	3	2	776377777760771
17	2	2	2	4	2	2	6	1	5	3	3	2	677777607760771
18	2	2	2	4	3	2	6	1	5	3	3	2	777777777760771
19	2	2	4	4	3	2	6	1	3	2	2	2	770017777760771
20	2	4	4	4	4	2	5	1	4	1	3	2	376377777760771
21	2	2	4	4	3	2	5	1	5	3	3	2	376377777760771
22	2	2	3	4	3	2	5	1	8	2	5	2	3771
23	2	4	4	4	2	2	5	1	4	3	3	2	776377777760371
24	2	2	3	3	3	2	5	1	8	3	6	3	3771
25	4	2	4	5	3	2	5	1	5	3	3	2	700076777760771

Table 2: Spoligotyping and the MIRU-VNTR typing results of the 100 patients attending the Kalafong hospital, Pretoria

ID	MIRU02	MIRU04	MIRU40	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	Octal Code
26	2	2	3	5	3	2	6	1	5	3	3	2	776177607760771
27	2	4	2	5	1	2	6	1	5	2	3	2	77773760606731
28	4	2	2	5	3	2	5	1	8	1	5	3	3771
29	2	2	2	4	1	2	6	1	5	2	3	2	777777606020731
30	2	2	3	5	3	2	6	1	5	3	3	2	776177607760771
31	2	2	3	6	3	2	6	1	5	3	3	2	776177607760771
32	2	2	3	5	3	2	3	2	5	3	3	2	777777774020731
33	2	2	2	4	3	2	3	1	7	1	3	2	777777777760771
34	2	2	3	8	2	2	5	1	7	3	5	3	3771
35	2	2	4	8	4	2	5	1	1	3	5	3	703377400001771
36	2	2	4	4	4	2	5	1	1	2	5	3	703377400001771
37	2	2	2	5	1	2	6	1	5	2	3	2	777777604060731
38	1	2	5	5	3	1	6	1	5	3	3	2	777777607760731
39	2	2	3	4	3	2	6	1	5	3	3	2	776177407760771
40	2	2	2	4	3	2	5	1	7	1	5	3	3771
41	2	2	2	5	3	2	5	1	7	1	5	3	3771
42	2	2	2	6	1	2	5	1	5	2	3	2	777777606060771
43	2	2	3	8	3	2	5	1	5	3	3	2	777777777700771
44	2	2	4	4	4	2	5	1	1	3	4	3	703377400001771
45	2	2	2	4	3	2	5	1	7	1	5	3	3771
46	2	2	2	4	3	2	5	1	7	1	5	3	3771
47	2	2	3	5	3	2	5	1	6	3	4	2	777777777760771
48	2	2	4	4	4	2	5	1	1	3	4	3	703377400001771
49	2	2	2	5	3	2	7	1	5	3	4	2	777777777760771
50	2	2	3	5	3	2	6	1	5	3	3	2	774177407760771

Table 2: Spoligotyping and the MIRU-VNTR typing results of the 100 patients attending the Kalafong hospital, Pretoria

ID	MIRU02	MIRU04	MIRU40	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	Octal Code
51	2	4	3	5	3	2	6	1	5	3	3	2	776177607760771
52	2	2	4	4	3	2	5	1	3	3	3	2	737777777760601
53	2	2	3	5	1	2	5	1	7	2	3	2	777737777760771
54	2	2	3	5	3	2	6	1	5	3	3	2	776177607760771
55	2	2	3	4	3	2	6	1	5	3	3	2	776177607760771
56	2	2	4	4	3	2	5	1	5	3	3	2	776377777760771
57	2	2	3	9	3	2	5	1	7	3	5	3	3771
58	2	2	3	5	3	2	5	1	6	3	4	2	777777777760771
59	2	4	3	5	3	2	6	1	5	3	3	2	776177607760771
60	2	4	2	5	3	2	3	1	8	3	3	2	777737777760731
61	2	4	3	5	3	2	6	1	5	3	3	2	676177607760771
62	2	4	3	4	3	2	6	1	5	3	3	2	776177607760771
63	2	4	3	8	2	2	5	1	7	3	5	3	776177607760771
64	2	4	4	5	4	2	5	1	1	2	4	3	3771
65	2	2	2	5	3	2	3	1	8	3	2	2	703377400001771
66	2	2	3	5	3	2	6	1	5	3	3	2	777737777760731
67	1	4	3	2	3	1	6	1	5	3	2	2	776177607760771
68	2	2	3	6	3	2	6	1	5	3	3	2	777776607760771
69	2	2	3	3	3	2	3	1	2	3	3	2	777776607760771
70	2	2	3	2	1	2	5	1	5	3	3	2	777777777760771
71	2	2	4	5	4	2	5	1	1	3	3	3	777777777760771
72	2	2	2	9	3	2	3	1	8	3	2	2	703377400001771
73	2	2	3	4	3	2	5	1	6	3	3	2	777737777760731
74	2	2	3	4	2	2	5	1	7	3	4	3	777777777760771

Table 2: Spoligotyping and the MIRU-VNTR typing results of the 100 patients attending the Kalafong hospital, Pretoria

ID	MIRU02	MIRU04	MIRU40	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	Octal Code
75	4	4	4	4	2	2	5	1	4	3	3	2	3771
76	2	2	2	9	1	2	6	1	5	2	3	2	776337777760771
77	2	2	3	9	3	2	5	1	6	3	3	2	577777606060771
78	2	2	3	4	3	2	5	1	5	3	3	2	777777777760771
79	2	2	2	6	2	2	3	1	5	3	3	2	776177607760771
80	2	2	2	4	3	2	3	1	5	3	3	2	777777774020771
81	2	2	5	4	3	2	5	1	5	3	3	2	777777777760771
82	2	2	2	5	3	2	6	1	7	1	5	3	777776607760771
83	2	2	3	4	3	2	5	1	5	3	3	2	3771
84	2	2	3	5	1	2	5	1	4	3	3	2	700076777760771
85	2	2	2	5	1	2	5	1	5	2	3	2	777776607760771
86	2	2	3	3	2	2	5	1	5	3	3	2	777776607760771
87	2	2	3	4	3	2	6	1	8	3	5	3	743776777760601
88	2	2	2	2	3	2	5	1	8	1	5	3	3771
89	2	2	3	5	3	2	5	1	5	3	3	2	3771
90	1	2	4	4	3	1	3	1	5	3	3	2	777776607760771
91	2	2	2	4	3	2	6	1	5	3	3	2	777777777760771
92	2	2	2	5	3	2	6	1	5	3	3	2	777777777760771
93	2	2	2	5	1	2	5	1	5	2	3	2	777777606060771
94	2	2	2	4	3	2	5	1	6	3	2	2	777737777760731
95	2	2	2	3	3	2	5	1	7	1	5	3	3771
96	2	2	2	3	2	2	5	1	5	2	3	2	777777777760771
97	2	2	2	4	2	2	5	1	5	2	5	3	777777774020731
98	2	2	3	4	3	2	5	1	9	1	4	2	3771
99	2	2	3	9	3	2	3	1	5	3	3	2	776177607760771
100	2	2	2	4	3	2	5	1	7	3	5	3	777777607760731