Molecular typing of drug-susceptible-, mono-resistant-, multi-drug resistant- (MDR) and extensively-drug resistant- (XDR) *Mycobacterium tuberculosis strains* in Johannesburg.

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Dedication

This work is dedicated to my lovely wife *Debra Ester Gondwe,* my children; Mphatso Peter Gondwe, Memory Gondwe, and Lombani Gondwe, and to my father Christopher Kamudumuli Gondwe.

Your support was awesome

Thank you very much

Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own, original work and has not previously in its entirety or in part been submitted at any university for a degree.

Signature.....

Date.....

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Summary

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB), remains a challenging disease, killing more people today than any other bacterial infection. The problem has further been compounded by the emergence of multi-drug resistant (MDR) and extensively drug-resistant (XDR) TB. Through standardized DNA fingerprinting methods, insight is gained into the disease dynamics thereby enabling investigations of transmission patterns and strain properties such as drug resistance.

The aims of this study were to determine the genetic diversity of drug susceptible and drug resistant MTB strains in Johannesburg and to potentially identify a predominant clone circulating in the area. Johannesburg is the most populated, densely inhabited industrialized city in South Africa and it is likely that the introduction and spread of mycobacterial strains is not the same as seen in other major African cities.

Isolates (n=500) were collected from the NHLS Mycobacteriology (TB) Culture Referral Laboratory. The isolates originated from patients residing in the seven regions (Regions A, B, C, D, E, F and G) of Johannesburg suspected for drug-resistant TB who had submitted specimens for routine culture and susceptibility testing according to the national TB control programme of Republic of South Africa. These isolates were typed using spoligotyping and IS*6110* RFLP.

Spoligotyping results showed a wide genetic diversity of strains circulating in the Johannesburg area with eight shared types [Beijing (SIT 1), S (SIT 34), LAM4 (SIT 60), T1 (SIT 53), LAM3 (SIT 33), H3 (SIT 50), EAI1_SOM (SIT 48) and X3 (SIT 92)] from 340 isolates being the most prevalent. This indicates that TB incidences in Johannesburg are not due to clonality but rather due to diversity of strains, with Beijing SIT 1 isolates being the most predominant at (n = 135) 27%. There is a considerably high relationship between the eight predominant families and drug-resistance with a P value of <0.001. The Beijing SIT 1 and LAM 4 SIT 60 (F15/LAM4/KZN) genotypes were found to contribute significantly to drug-resistant incidences in Johannesburg. Of the 135 isolates of Beijing SIT 1, 52% were associated with drug-resistance while 93% of the LAM 4 SIT 60 (F15/LAM4/KZN) isolates were associated with drug-resistance, with 54% of the later being XDR. Through data comparison, it was noticed that Beijing SIT 1, LAM4 SIT 60 and LAM3 (SIT 33) genotypes are more prevalent in South Africa than any other African country. Furthermore, the study did not identify other families such as LAM10-CAM (SIT 61) and T2-U which dominate in other African countries such as Cameroon, Nigeria and Uganda. There is geographical specificity of the isolates which would be expected to have an impact on TB epidemiology.

A subset of isolates from spoligotyping clusters were analyzed by IS*6110* RFLP technique. Thirty distinct RFLP patterns were revealed from 74 MTB isolates and results showed highly variable IS*6110* fingerprinting patterns. The common fingerprinting patterns found in this study were the F29, F15, F28 and F11. Of the thirty obtained RFLP fingerprint patterns, 15 (20%) were represented by a single isolate from which 3 (4%) had zero copy number. The clustering percentage

of the isolates was considerably high at 80% (59/74), which may suggest cases representative of recent transmission, while the 15 unique isolates may indicate epidemiological independence or recurrent infection. Comparison of the results for the two methods showed that 56 isolates had identical IS*6110* RFLP patterns as well as spoligotypes. This study has demonstrated the usefulness of spoligotyping technique in genotyping the MTB isolates containing a low copy number of IS*6110* between zero and six. Potential ongoing transmission of TB, as evidenced through a high strain cluster rate by both methods, necessitates the evaluation of TB monitoring policies in the area and urges the implementation of appropriate infection control measures in order to minimize the spread of the disease.

Publications and Presentations

The following work is in the process to be published through International Journal for Lung and Tuberculosis Disease.

- Molecular typing of drug susceptible and drug-resistant *Mycobacterium tuberculosis* in Johannesburg, South Africa

Part of this work has been presented at the following scientific meeting:

- Poster presentation at Faculty of Health Sciences Research day, University of the Witwatersrand, Johannesburg, South Africa. 2012.

Table of contents

List of figures

Chapter One: General Introduction

Chapter two: Spoligotyping of *Mycobacterium tuberculosis* strains

Chapter three: DNA fingerprinting of *mycobacterium tuberculosis* through RFLP IS*6110*

List of Tables

Chapter One: General Introduction

Chapter Two: Spoligotyping of *Mycobacterium tuberculosis* strains

List of Abbreviations

AFB	Acid Fast Bacilli
AIDS	Acquired Immunodeficiency Syndrome
AMK	Amikacin
BCG	Bacille Calmette – Guerin
bp	base pair
CAS	Central Asian
CAS – KILLI	Central Asian Kilimanjaro
CNS	Central Nervous System
CPC	Capreomycin
CTAB	Cetyl Trimethyl Ammonium Bromide
DH2O	Distilled Water
DNA	Deoxyribonucleiric Acid
DOT	Direct Observable Treatment
DR	Direct Repeat
DRs	Direct Repeats
DST	Drug Sensitivity Testing
EAI	East – African – Indian
EAI – SOM	East African Indian-Somalia
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid

EMB	Ethambutol
EPTB	Extra Pulmonary Tuberculosis
FQ	Fluoroquinolone
HCL	Hydrochloric Acid
H	Haarlem
HIV	Human Immunodeficiency Virus
hrs	hours
IMWM	Internal Molecular Weight Marker
INH	Isoniazid
IR	Inverted Repeats
IS	Insertion Sequence
KM	Kanamycin
KZN	KwaZulu-Natal Province
LAM	Latin American Mediterranean
LAM – ZWE	Latin American Zimbabwe
LJ	Löwenstain Jensen
LTBI	Latent Tuberculosis Infection
LTB	Latent Tuberculosis
MTB	*Mycobacterium tuberculosis*
MDR-TB	Multi drug-resistant tuberculosis
MDR	Multi drug-resistant
MGIT	Mycobacterium Growth Indicator Tube
MIRU	Mycobacterium Interspaced Repetitive Unit
MTBDR*plus*	*Mycobacterium tuberculosis* drug-resistant plus

MTBDR*sl*	*Mycobacterium tuberculosis* drug-resistant second line
MTC	*Mycobacterium tuberculosis* complex
NaCL	Sodium Chloride
NaOH	Sodium Hydroxide
NHLS	National Health Laboratory Services
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PT	Pulmonary Tuberculosis
PZA	Pyrazinamide
RFLP	Restriction Fragment Length Polymorphism
RIF	Rifampicin
RTI	Recent Transmission Index
SDS	Sodium Dodecyl Sulfate
SIT	Shared International Type
SN	Study Number
SOM	Somalia
SpolDB4	Spoligotype Data Base 4
Spoligotyping	Spacer oligonucleotide typing
SSC	Standard Saline Concentrate
SSPE	Sodium Chloride Sodium Phosphate Ethylenediaminetetraacetic acid
TBE	Tris base Boric acid Ethylenediaminetetraacetic acid
TB	Tuberculosis
Tris	Tris base (Hydroxymethyl) aminomethane
VNTR	Variable Number Tandem Repeat

WCP	Western Cape Province
WHO	World Health Organization
XDR-TB	Extensively Drug – Resistant Tuberculosis
XDR	Extensively Drug – Resistant

Chapter one: Introduction

1.0 General Introduction

1.1 The history of tuberculosis

Tuberculosis (TB) is a common, and in many cases lethal, infectious disease caused by various species of the *Mycobacterium tuberculosis* complex (MTBC), usually *Mycobacterium tuberculosis* **(**MTB**)** (Smith 2003b; Glickman et al. 2001; Vasconcellos et al. 2010). The origin of MTB has been the subject of much investigation and it is thought that the bacteria in the genus Mycobacterium, like other actinomycetes, were initially found in soil and that some species evolved to live in mammals (Smith, 2003). TB has plagued humankind throughout history and human prehistory. Modern techniques of molecular genetics and genomic sequencing of several strains of MTB allow a more rigorous estimation of the time of origin of MTB to be more than 150 million years ago (Daniel, 2006). This is seen through evidence of spinal disease found in Egyptian mammals of several thousand years BC as well as in the reference found in ancient Babylonian and Chinese writings (Ahmad, 2011a; Daniel, 2006). There are other well documented cases of TB disease such as bone TB, including Pott's disease in Peruvian mummies, and the availability of abundant archaeological evidence in Columbia from the Andean Region which shows that the disease occurred throughout the hemisphere prior to the arrival of the first European explorers, while modern strains of MTB appear to have originated from a common ancestor approximately 15, 000 - 20,000 years ago (Daniel, 2006).

The history of TB was changed dramatically on 20 March 1882, when Hermann Heinrich Robert Koch made his justly famous presentation, "Die Aetiologie der Tuberculose", to the Berlin Physiological Society in which he elucidated the aetiological agent of TB by identifying the tubercle bacillus (Daniel, 2006). Unfortunately, TB is still one of the most prevalent infections of humankind and a formidable public health challenge that shows little sign of abating (Ahmad, 2011a).

WHO has prioritized to improve TB diagnosis, treatment, and vaccination, as well as development of new tools to control and fight the epidemic as it aims to eliminate TB by 2050 (WHO report, 2010). Understanding disease dynamics is one way to achieve controlling the disease epidemic. The discovery of polymorphic DNA in MTB has led molecular typing to become a variable tool for the study of the disease dynamics. Phenotypic methods such as Phage typing has been used to type MTB, unfortunately, this method has a low discriminatory power due to its failure to discriminate between two unrelated strains of MTB as well as the inability to detect laboratory cross contamination is a major setback. This triggered the development of more sophisticated typing methods to study the disease dynamics and its epidemiology. In the early 1980s, under the influence of advances in molecular biology, DNA-based typing methods were developed with improved discriminatory power over phenotypic methods (Schürch & Van Soolingen 2012; Lopez-Alvarez et al. 2010).

The effective strain genotyping techniques like IS*6110* DNA fingerprinting, the PCR based spoligotyping technique, and most recently mycobacterial interspersed repetitive unit-variable of number of tandem repeats (MIRU-VNTR) analysis have been established to analyze diversity and population structure of clinical MTBC and MTB strains (Homolka et al. 2008). Molecular typing classifies the MTB strains into lineages which provides insight into the genetic diversity of the strains being investigated and helps identify the predominant genetic groups in a population (Shabbeer et al. 2012). Recent reports indicate that genetic heterogeneity of MTB strain is higher than previously anticipated and may influence host pathogen interaction, immunogenicity, transmissibility, development of drug resistance, and the performance of diagnostic tests. This depicts the importance of genotyping the MTB strains in a community (Homolka et al. 2008). Further discussion of molecular background and techniques is continued in chapter one section 1.8, chapter two and chapter three.

1.2 *Mycobacterium tuberculosis*

1.2.1 Classification of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis falls in the taxonomical group (phylum) of Actinobacteria under the genus of *Mycobacterium* (Figure 1.1)

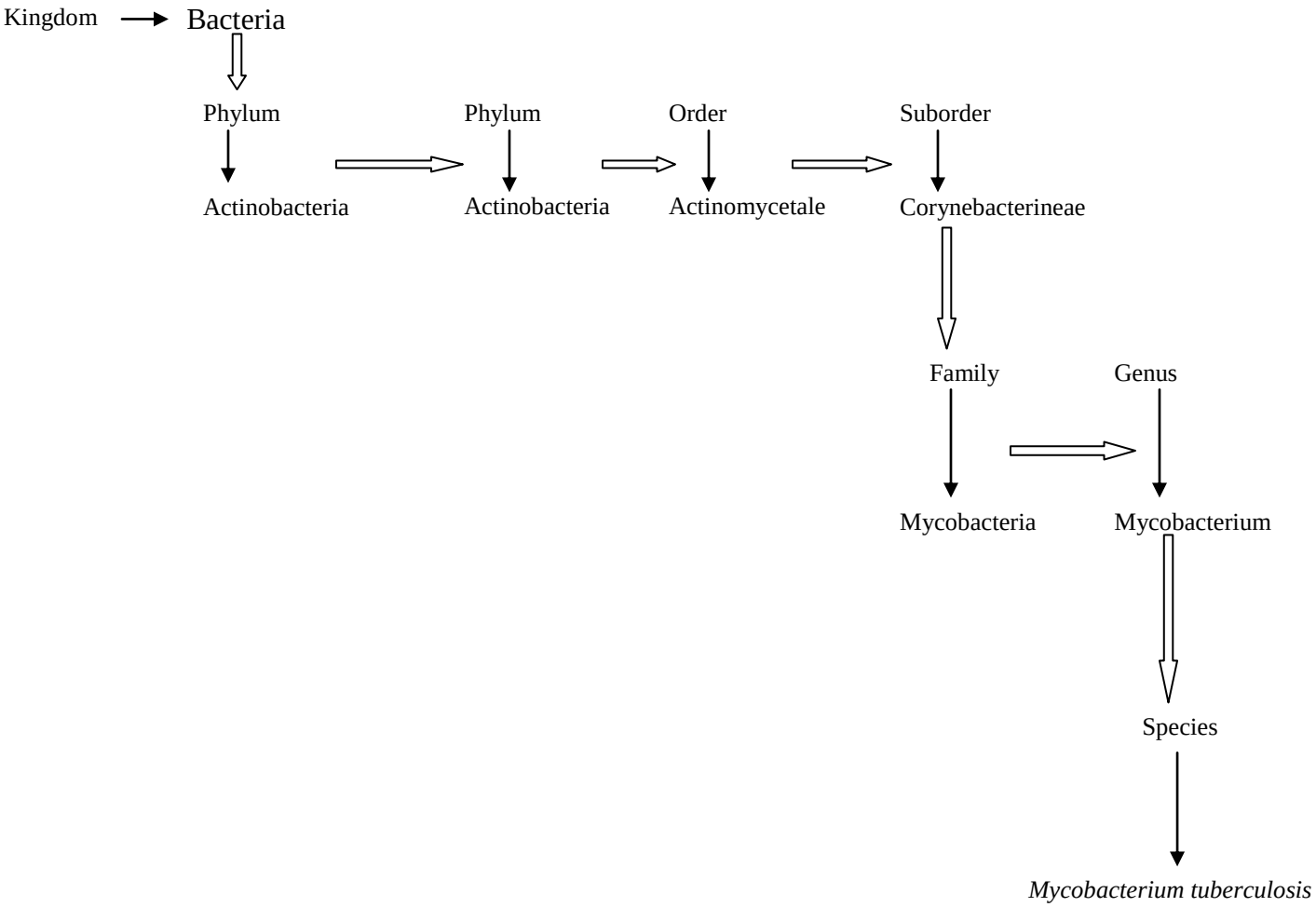


Figure 1.1: Phylogenetic tree of Mycobacterium tuberculosis.

1.2.2 *Mycobacterium Tuberculosis* Complex

There are eight MTBC species which can cause tuberculosis disease. The eight species are; *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis* BCG, *Mycobacterium microti*, *Mycobacterium canetti, Mycobacterium tuberculosis caprae, dassie bacillus* and the recently discovered *Mycobacterium tuberculosis pinipedii*. (Lawn & Zumla 2011; Vasconcellos et al. 2010; Mostowy et al. 2002).

The group of bacteria in MTBC are characterized by 99% similarity at the nucleotide level and identical 16S rRNA sequences but differ widely in terms of their host tropism, phenotypes, certain physiological features, biochemical features and pathogenicity (Chernick, 2004). Therefore, it is believed that the members of the MTBC are the clonal progeny of a single successful ancestor, resulting from a recent evolutionary bottleneck that occurred 20,000 to 35,000 years ago and the MTBC members, all agents responsible for TB, are considered to be the most successful human pathogen (Gutierrez et al. 2005). The MTB is the most common member within the complex to cause human tuberculosis, and thus the focus of this paper.

1.2.3 Cell Biology of *Mycobacterium tuberculosis*

MTB is an aerobic, non-motile, non-encapsulated, non-spore forming and distinctive rod shaped bacillus (Figure 1.2). It grows most successfully in tissues with high oxygen content, such as the lungs. Compared with the cell walls of the other bacteria, the lipid-rich cell wall is relatively impermeably to basic dyes unless combined with phenol. Thus MTB is neither gram positive nor gram negative but is instead described as acid-fast, since once stained it resists decolourisation with acidified organic solvents.

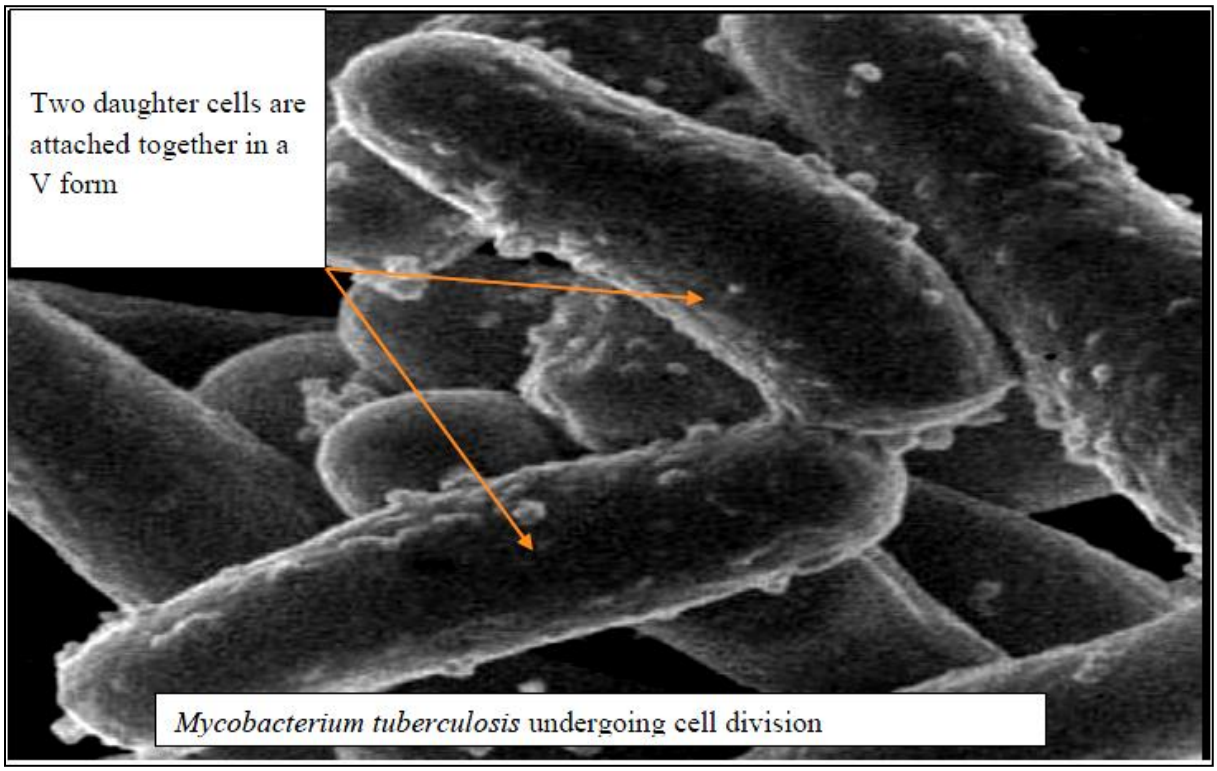


Figure 1.2: *Mycobacterium tuberculosis* is a non-motile, non-encapsulated, distinctively rod shaped bacillus. Adapted from Dahl, 2004.

In addition to the lipids, the cell wall also contains a remarkable mixture of polypeptides, lipoglycans and glycolipids in its waxy envelope layers (Figure 1.3) (Ventura et al. 2007). MTB is a slow growing organism with replication occurring every 15 to 20 hours. This slow replication rate together with the ability to persist in a latent state results in the need for long duration of both drug therapy for TB and preventive therapy in people with MTB infection (Lawn & Zumla, 2011; Glickman et al. 2001).

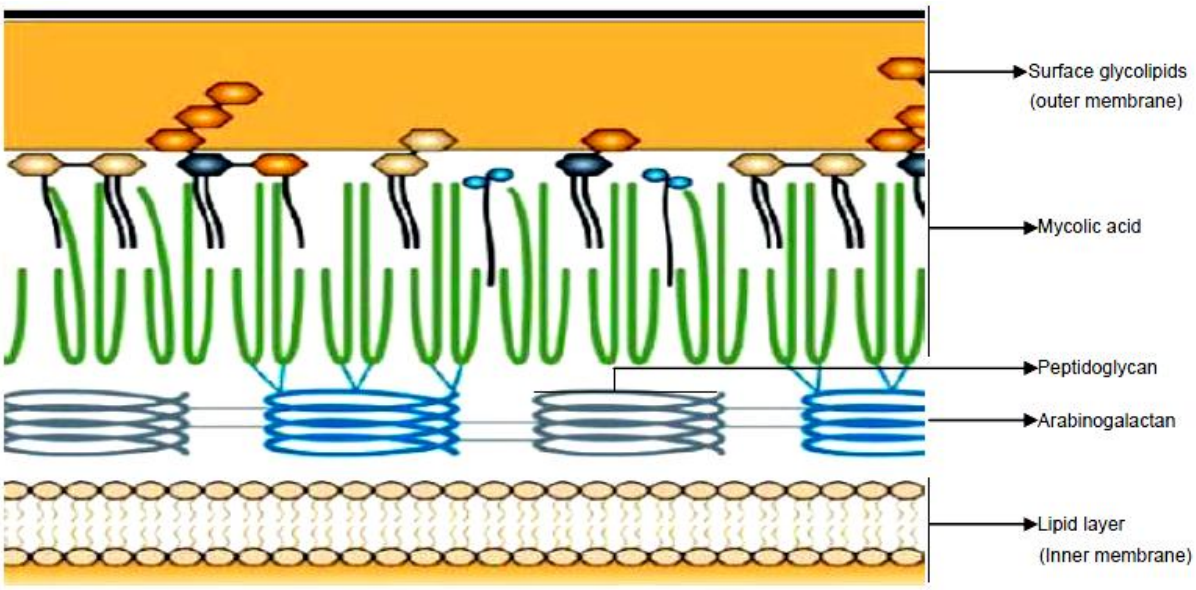


Figure 1.3: Schematic diagram of the *Mycobacterium tuberculosis* cell wall. Adapted from Abdallah et al. 2007

1.3 Transmission of *Mycobacterium tuberculosis* and contributing factors

1.3.1 Transmission of *Mycobacterium tuberculosis*

TB is a communicable disease and patients with pulmonary or laryngeal TB are the most important source of infection. Infection is initiated by inhalation of droplet nuclei, which are particles of 1-5μm in diameter containing MTB. These droplets are expectorated by patients with active pulmonary TB, typically when the patient coughs, sneezes, speaks, or sings and since the droplet nuclei are very small, they can remain in the air for several minutes to hours (Standards, 2000; Ahmad, 2011a). The MTB containing droplet nuclei traverse the mouth, the nasal passage, the upper respiratory tract and the bronchi to reach the alveoli of the lungs. The risk of infection is dependent on several factors such as the infectiousness of the source case, the closeness of the contact, the bacillary load inhaled, and the immune status of the potential host (Ahmad, 2011a).

1.3.2 Contributing factors for TB transmission

The current TB epidemic is being sustained and fuelled by two important factors namely the Human Immunodeficiency Virus (HIV) epidemic and its association with TB disease and the increasing rates of drug resistant TB. Other contributing factors include population expansion, poor case detection and cure rates in impoverished countries, active transmission in overcrowded hospitals, prisons, and other public places, migration of individuals from high-incidence countries due to wars or famine, drug abuse, social decay, and homelessness (Ahmad, 2011a).

1.3.2.1 TB and HIV

Human Immunodeficiency Virus (HIV) and TB are interacting epidemic diseases (Kantipong et al. 2012). Starting in the 1980s, the HIV epidemic led to a major upsurge in TB cases and TB mortality in many countries (WHO global TB report, 2011). HIV infected people are at an increased risk of acquiring MTB infection and to an increased risk of progressing to active TB disease. (Kantipong et al. 2012). HIV and TB coinfected individuals are approximately 21 to 34 times more likely to develop TB disease when compared with those who are HIV negative (WHO global report, 2011). Globally, just over one in ten of the almost nine million people who develop TB each year are HIV positive, which equates to 1.1 million new TB cases among people living with HIV in 2010. In the African region, this accounted for 82% of the new TB cases that were living with HIV in 2010 and 79% of new TB cases that were living with HIV in 2012. An estimated 900 000 (39%) of the 2.3 million people who developed TB in 2010 were HIV-positive from which 0.4% million deaths occurred (WHO global report, 2011; WHO global report, 2012).

1.4 Pathogenesis of *Mycobacterium tuberculosis*

Infection with MTB starts with the phagocytosis of the bacilli that have reached the alveoli, antigen-presenting cells such as alveolar macrophages and dendritic cells (Ahmad, 2011a). Although the majority of these bacilli are destroyed, a small number may replicate within the alveolar macrophages (Glickman, et al. 2001). Once the macrophages die, the bacilli can spread via the lymphatic system or bloodstream to more distant sites, such as the brain and bone and this

is termed miliary TB (Rastogi et al. 2001; Ray et al. 2013). The dissemination of TB from the lungs to other parts of the body leads to the development of extrapulmonary TB (EPTB) such as bone TB and central nervous system (CNS) TB (Ray et al. 2013; Thwaites et al. 2009). The ability of the bacilli to grow inside phagosomes and phagolysosomes, as well as to inhibit the fusion of bacteria-containing phagosomes with lysosomes upon macrophage infection, is the main strategy to evade the immune system and disseminate the organism (Rastogi et al. 2001). MTB has no known endotoxins or exotoxins and therefore; there is no immediate host response to infection. In the vast majority of the infected individuals, an effective cell-mediated immune response develops 2-8 weeks after infection that stops further multiplication of the tubercle bacilli. The establishment of an infection in the lungs depends on both the bacterial virulence and the inherent microbicidal ability of the alveolar macrophage (Standards, 2000; Ahmad, 2011a).

1.4.1 Latent TB Infection

In persons with an intact cell-mediated immunity, granulomas will form due to collections of activated T lymphocytes, macrophages and other immune cells (Figure 1.4). The formed granulomas will wall off the growing necrotic tissue, hence limiting further replication and spread of the tubercle bacilli. While inside granulomas, the bacilli may either die or survive and assume a non-replicating state, which is referred to as latent tuberculosis infection (LTBI). The bacilli can stay in this non-replicating state for decades until the immune system wanes. Antibodies against MTB are formed during this time but do not appear to be protective. The evidence of infection at this stage may be defined by immunological sensitization such as Tubercle Skin Test (TST) and interferon-gamma release assays (IGRA) (Standards, 2000; Ahmad, 2011a).

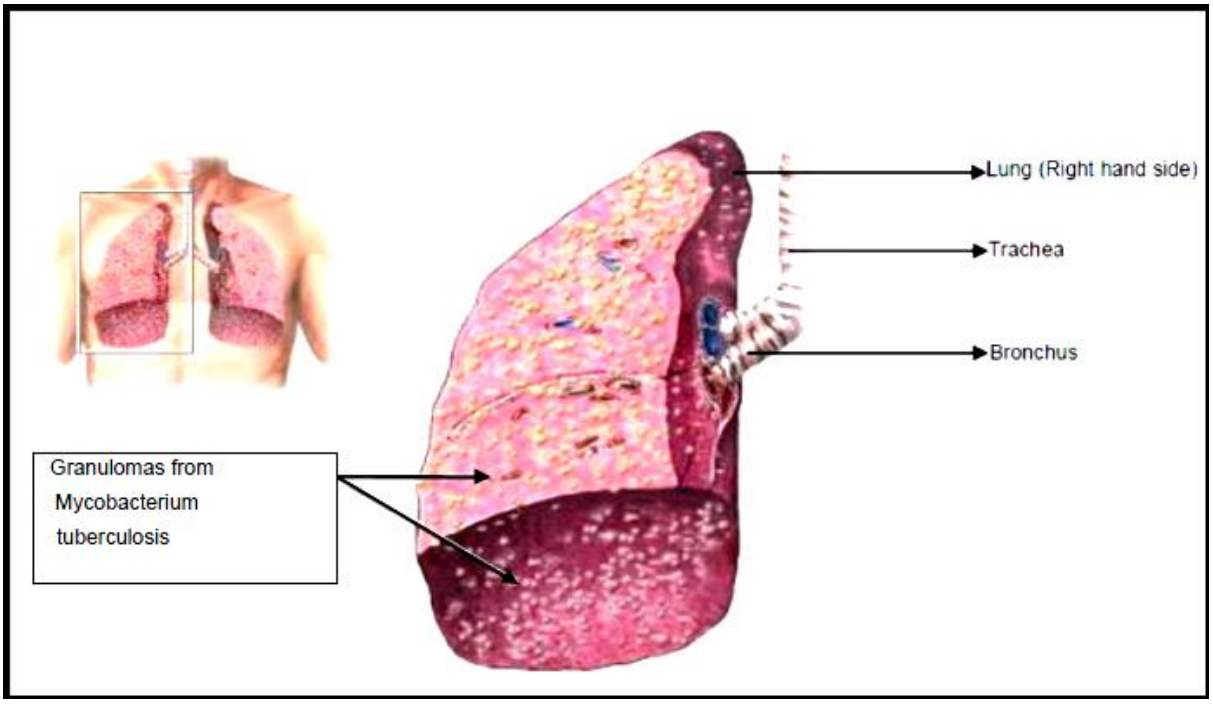
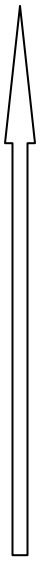


Figure 1.4: Formation of granulomas from *Mycobacterium tuberculosis*. Adapted from Vyas 2012.

Several features such as HIV infection, malnutrition, drug use, cancer, diabetes, chronic renal insufficiency and immunosuppressive drug therapy may alter the course of latent TB (LTB) (Table 1.1) and influence the development of active disease (Parrish et al. 1998). It is estimated that approximately 10% of individuals with LTB infection (who are not further compromised by the above-mentioned comorbid diseases) that are not given preventive therapy, will develop active tuberculosis but in those with comorbid diseases it is higher than 10% (Standards, 2000).

Table 1.1: Proposed framework of potential outcomes of the host-pathogen relationship following exposure to MTB infection. Source: Lawn, et al. 2011

	Terminology	T cell priming	Mycobacterial numbers	Symptoms	Impact of HIV infection	Impact of ART
1 Infection eliminated by innate immune system	Innate Immune	–	–	–		
2 Infection eliminated by acquired immune response	Acquired immune	+	–	–		
3 Infection occurs but remains under immune control	Quiescent infection	+	+	–		
4 Active bacterial replication but remains subclinical	Active infection	+	+++	–		
5 Uncontrolled bacterial replication and clinical TB disease	TB disease	+	+++++	++		

1.5. Disease presentation and diagnosis

1.5.1 Disease presentation

Clinical manifestations of TB are quite variable and depend on a number of factors. Prior to the HIV epidemic, approximately 85% of reported TB cases were limited to the lungs, with the remaining 15% involving either exclusively only non-pulmonary or both pulmonary and nonpulmonary sites. This situation has now changed mainly in HIV infected individuals who may present with TB disease localized in pulmonary or extrapulmonary sites or both (Standards, 2000). Signs and symptoms of pulmonary TB include a cough for at least two weeks, fever, chills, night sweats, unexplained weight loss, haemoptysis and fatigue. In contrast signs and symptoms of EPTB depend on the site of disease (Steingart et al. 2013).

1.5.2 Diagnosis of Tuberculosis

1.5.2.1 TB Microscopy

Efficient TB control is based on an early diagnosis followed by the rapid identification of drug resistance, to ensure adequate treatment of patients to break the chain of transmission and avoid the spread of resistant strains (Lacoma et al. 2012a). In spite of modern advances, microscopy remains a cornerstone of TB control because it identifies smear microscopy positive (most infectious) cases and is rapid and affordable, although has a limited specificity (Drobniewski et al. 2003). In most countries, smear microscopy through Ziehl-Neelsen (ZN) acid-fast stain continues to be the primary method for diagnosing TB and will remain to be favored mostly in

low income and high TB prevalence countries as it provides most of the essential laboratory-epidemiological indicators needed for the evaluation of the National TB Programme (NTP) Page-shipp et al. 2010; Drobniewski et al. 2003). While cheap and relatively easy to perform, the method is insensitive and performs poorly in HIV infected patients. It requires ≥ 5000 bacilli/ml of sputum to achieve a positive result. In addition, the procedure requires the observation of 100 to 300 fields in order to obtain accurate results. It detects 75% of all people who have active pulmonary TB (PTB) (Dawson, 2000; Page-shipp et al. 2010; Drobniewski et al. 2003). Fluorescence microscopy with fluorochrome dyes such as auramine O or auramine-rhodamine is considered to be a more sensitive test than ZN smear for the diagnosis of TB. However, the procedure is more expensive than the conventional method as it requires unstable and expensive fluorescent staining reagents including expensive equipment (Patino et al. 2008).

1.5.2.2 TB Diagnosis through solid and liquid culture

Mycobacterial culture is generally considered the best available diagnostic test for the microbiological TB diagnosis and is the first step in detecting drug resistance. Culture is a more sensitive test than smear microscopy for the detection of MTB since it can detect as few as 10 to 100 viable bacteria/ml. Culture is recognized as being critically important in detecting TB, mainly in HIV-positive individuals who often have low bacillary loads in sputum. However, the procedure is not routinely performed in resource poor countries (Steingart et al. 2013) where coinfection of HIV/TB is high. It is also a relatively complex and slow procedure. It requires specialized laboratories and highly skilled staff (Parsons et al. 2011; Steingart et al. 2013).

Solid culture (Löwenstein Jensen [LJ] medium, Middlebrook solid agar), typically takes four to eight weeks for results whereas liquid cultures (Middlebrook liquid broth, BackTAlert [BioMerieux] medium and Mycobacteria Growth Indicator Tube® [Becton Dickson, Sparks, USA] [MGIT]) though more rapid than solid culture, still requires an average of seven days for the results (Dionne et al. 2007; Bardarov et al. 2003). These phenotypic methods may also be used to detect drug resistant MTB. In addition, liquid culture is hampered with higher rates of contamination (Rodrigues et al. 2009).

Lack of culture diagnostic services in high-burden countries, has seen programmes often employ diagnostic algorithms, which require that TB suspects with a negative smear are first treated with antibiotics which are ineffective against TB. Only after this treatment has failed (or in critically ill patients) is TB treatment started (Borgdorff et al. 2002). This may lead to development of drug resistance and increased mortality rate.

1.5.2.3 TB Diagnosis through molecular techniques

Although conventional microbiology methods still constitute the principal tool for the diagnosis of TB, direct microscopy testing has low sensitivity and culture requires long culturing time. This often leads to considerable delays, compromising patient care and outcomes. Nucleic acid amplification tests (NAAT) for rapid TB diagnosis are increasingly becoming available and being utilized to provide a more rapid TB diagnosis (Lawn & Zumla, 2012; Sala et al. 2008).

1.5.2.3.1 GeneXpert® MTB/RIF assay (Cepheid Inc., CA, USA)

The GeneXpert® is a multifunctional, automated diagnostic platform. Through real-time Polymerase Chain Reaction (PCR) the diagnosis of TB and simultaneous assessment of rifampicin (RIF) resistance is made (Lawn & Zumla, 2012; Carlton, 2011). The test procedure may be used directly on raw sputum samples or sputum pellets. The assay limit of detection is 5 genome copies of purified DNA per reaction or 131 colony forming units (CFU) per ml of MTB spiked sputum. Through the use of molecular beacon technology RIF resistance TB is detected. These nucleic acid probes recognize and report the presence or absence of the 'wild type' (RIF-susceptible) sequence of the 81 base pair *rpoB* gene of MTB (Steingart et al. 2013).

The GeneXpert detects MTB in >98% of smear positive, culture positive specimens (Scott et al. 2011). Although the GeneXpert can detect resistance to RIF, which is regarded as a marker of MDR, it cannot diagnose MDR-TB (which is defined as MTB resistant to isoniazid (INH) and RIF, with or without resistance to other first-line drugs). Culture and drug susceptibility testing (DST) to confirm RIF resistance and testing for resistance to isoniazid and other first and second-line drugs is recommended once rifampicin resistant TB has been detected (Steingart et al. 2013). The use of the GeneXpert in the diagnosis of EPTB has recently been endorsed by the WHO (WHO recommendations, 2013).

1.5.2.3.2 GenoType MTBDR plus and MTBDRsl (Hain Lifesciences)

The standard methods for detecting extensively drug-resistant MTB (XDR – defined as MTB resistant to at least INH and RIF as well as to any fluoroquinolone, and at least to one of the three injectable drugs; amikacin (AMK), capreomycin (CPM), and kanamycin (KM) (Lacoma et al. 2012) are not only difficult but time consuming as mentioned earlier. Modern broth-based culture systems, such as MGIT 960 (Becton Dickinson, Cockeysville, MD) or BacT/ALERT 3D (bioMerieux, Durham, NC), are somewhat quicker but are expensive and need sophisticated equipments. Molecular tests such as MTBDR*plus* and MTBDR*sl* probe assays (Hain Lifescience GmbH, Nehren Germany) do provide results within a day or two and thus enable for early and appropriate treatment of the drug-resistant cases which will reduce morbidity and mortality of MDR-TB cases and also interrupt transmission of drug-resistant TB strains (Gupta et al. 2013).

The GenoType MTBDR*plus* detect mutations in the *inhA*, *katG*, and *rpoB* genes that confer INH and RIF resistance. It is a DNA strip assay which uses PCR and hybridization to detect mutations (Anek-vorapong et al. 2010). Multiple reports have shown that the performance of the Genotype MTBDR*plus* assay in terms of sensitivity and specificity closely resembles that of conventional culture-based identification and susceptibility testing and its potential applications in laboratory diagnosis of drug-resistant TB have been widely validated (Zhang et al. 2011; Barnard et al. 2008; Huang et al. 2009).

The Genotype MTBDR*sl* assay uses multiplex PCR and reverse hybridization to identify MTB and relevant mutations in the *gyrA*, *rrs* and *embB* genes, which confer resistance respectively to fluoroquinolone (FQ), CPM, AMK, KM and Ethambutol (EMB) (Ehlers, 2012). This assay was developed for the rapid detection of MTB resistance against second-line drugs. Studies have shown that the assay has a high accuracy for FQ, amikacin and capreomycin resistance detection in clinical isolates despite its failure to detect all mutations in these genes, which is why results require confirmation by culture based DST methods (Kiet et al. 2010; Felkel et al. 2013).

1.6. Treatment of tuberculosis and development of drug-resistance

1.6.1 Treatment of Tuberculosis

In the first half of the 20th century, the disease was considered incurable after sulfonamides and Penicillin antibiotics showed no useful activity against MTB and the lipid-rich cell wall was believed to make chemotherapy impossible (Gillespie, 2002). The effective treatment of TB started in 1946 with the introduction of streptomycin (SM) (Mitchison & Davies, 2012) as monotherapy which rapidly dispersed the first hopes of a conquest of TB. Although patients on therapy improved compared to those not on therapy, relapse occurred in many patients and the organisms were found to be resistant to SM. The combined prescription of INH, pyrazinamide (PZA), RIF, EMB, and other drugs brought cure rates of over 95% and shortened treatment time from 2 years to 6 months. This gave the medical community the basic tools for TB control (Gillespie, 2002).

TB can be treated with either first-line drugs which are used to treat susceptible TB or second-line drugs which are used to treat resistant TB. The most common second line drugs are CPM, KM, AMK, Ethionamide (ETHIO), para-aminosalicylic acid (PAS), Ofloxacin (Ofxn), Ciprofloxacin (Cpfxn), Cycloserine (Csn), Levofloxacin (Lflxn), and Clofazimine (Cfmn). The downfall with the second-line drugs is that they are inherently more toxic and less effective than the first-line drugs and the use of second-line drugs require a prolonged period (9 months) as opposed to the six months with first-line drugs (Raja et al., 2010).

A combination of multiple TB drugs is necessary. MTB grows in different locations in the human body: pulmonary cavities, pleural cavity pus and solid caseous material within granulomas. Each of the antituberculosis drugs has a major role in dealing with one of these populations. INH is active mainly against the organisms growing aerobically in pulmonary cavities. It is critical early in therapy as its bactericidal activity rapidly reduces the sputum viable count. PZA is only active at a low pH, making it ideally suitable for killing the organisms inside the phagosome. This explains the finding that PZA appears to have no benefit after the second month of therapy (Raja, 2010; Zhang et al. 2003; Mitchison & Davies 2012). RIF is important in killing organisms that are metabolizing slowly, killing the “persisters”, and sterilizing the patient’s sputum (Gillespie 2002).

1.6.2. Development of drug-resistance

Despite the availability of chemotherapy against the tubercle bacillus and the implementation of DOT, drug-resistant TB is still emerging (Johnson et al. 2003). The prolonged treatment regimen could be the additional contributing factor (Mitchison & Davies, 2012). With the rather unusual biological characteristics of this pathogen, the disease shows a distinctive natural history and a very slow response to existing chemotherapeutic agents (Chang et al. 2011).

MDR-TB/XDR-TB seems to be a man made phenomenon that has emerged because of improper TB treatment. MDR-TB has emerged since INH and RIF were used in the 1970s. Increasing the use of FQ in TB treatment in the 1990s promoted the development of pre-XDR-TB and XDR-TB. In comparison with drug-susceptible TB, MDR-TB and XDR-TB are more difficult to treat (Chang & Yew, 2013).

Risk factors for MDR- and XDR-TB seems to be non-adherence to treatment, development of active TB disease after having taken TB medicine in the past, residing in areas where drug-resistant TB is common, being exposed to someone with drug-resistant TB disease and co-infection with HIV (Marwar et al. 2009a).

1.6.2.1 Molecular mechanisms of drug-resistance

Major advances in molecular biology tools and the availability of new information generated after deciphering the complete genome sequence of MTB has increased our knowledge of understanding of the mechanisms of resistance to the main anti-tuberculosis drugs. These have shown that specific gene mutations are associated with drug resistance (Almeida Da Silva & Palomino 2011) as shown in Table 1.2

Gaining insight into how MTB develops drug resistance is essential to control the drug resistance epidemic. Mutations in the genome of MTB that can confer resistance to anti-TB drugs occur spontaneously with an estimated frequency of 3.5×10^{-6} for INH and 3.1×10^{-8} for RIF. Because the chromosomal loci responsible for resistance to various drugs are not linked, the risk of a double spontaneous mutation is extremely low: 9×10^{-14} for both INH and RIF. MDR-TB will thus occur mainly in circumstances where sequential drug resistance follows sustained treatment failure (Raja et al. 2010).

Table 1.2: Molecular mechanisms of drug resistance in *Mycobacterium tuberculosis*

Drug (year of discovery)	Gene(s) involved in resistance	Gene function	Role	Mechanism of action	Most frequently mutated codes associated with resistance
Isoniazid (1952)	katG inhA ahpC kasA ndh	Catalase-peroxidase Enoyl ACP reductase Alkyl hydroperoxide reductase β-ketoacyl-ACP Synthase NADH dehydrogenase	Pro-drug Conversion Drug target	Inhibition of Mycolic acid biosynthesis and other multiple effects	katG 315
Rifampicin (1966)	ropB	β subunit of RNA polymerase	Drug target	Inhibition of RNA synthesis	rpoB 526,531
Pyrazinamide (1952)	pncA	Nicotinamidase/pyrazinamidase	Pro-drug conversion	Depletion of membrane energy	
Ethambutol (1961)	embB	Arabinosyl transferase	Drug target	Inhibition of arabinogalactan synthesis	306
Streptomycin (1944)	rpsL rrs gidB	S12 ribosomal protein 16S rRNA rRNA methyltransferase (G527 in 530 loop)	Drug target Drug target Drug target	Inhibition of protein synthesis	43 & 88 bp530&915
Amikacin/Kanamycin (1957)	rrs	16S rRNA	Drug target	Inhibition of protein synthesis	bp1400
Capreomycin (1960)	tlyA	2'-O-methyltransferase	Drug target	Inhibition of protein synthesis	
Fluoroquinolone (1963)	gyrA gyrB	DNA gyrase subunit A DNA gyrase subunit B	Drug target Pro-drug conversion	Inhibition of DNA gyrase	90,91,94
Ethionamide (1956)	etaA/ethA inhA	Flavin monooxygenase Enoyl ACP reductase	Drug target	Inhibition of Mycolic acid synthesis	inhA21,94,44
PAS (1946)	thyA	Thymidylate synthase	Drug activation?	Inhibition of folic acid and iron metabolism?	

Sources: Almeida Da Silva & Palomino, 2011; Mathema et al. 2006.

1.7. Epidemiology of *Mycobacterium tuberculosis*

1.7.1 TB epidemiology

Even though the causative organism was discovered more than 100 years ago and that highly effective drugs and a vaccine are available, TB remains a worldwide public health problem (Marwar et al. 2009b). The disease contributes considerably to illness and death around the world, exacting a heavy toll on the world's most vulnerable citizens (Ahmad, 2011a).

It is estimated that one-third of the world's population has been infected with MTB (Pang et al., 2012) resulting in more than eight million people contracting the disease annually. Of these, more than 25% die each year (Jagielski et al., 2010). TB continues to be one of the most challenging communicable diseases for developing countries where the situation is worse particularly in Sub-Saharan Africa (Amin, 2006). In 2003, an estimated nine million new cases of TB resulted in 1.7 million deaths. Twenty seven percent of these cases and 31% of these deaths arose in Africa, home to only 11% of the world's population (Corbett et al. 2006). According to the WHO, approximately 95% of the eight million new cases each year occur in middle-and low-income countries where 98% of TB death occur with the highest prevalence and estimated annual risk of TB infection in sub-Saharan Africa and Southeast Asia (1.5% to 2.5% and 1% to 2% respectively) (WHO, 2008).

In 2011, the largest number of new TB cases occurred in Asia, with 60% of new cases globally. However, Sub-Saharan Africa carried the greatest proportion of new cases per population with over 260 cases per 100 000 population (WHO, 2012) (Figure 1.5). Almost 80% of TB cases among people living with HIV reside in Africa (WHO, 2012), indicating that the incidence of TB from sub-Saharan Africa is fuelled by the HIV epidemic (O'Grady et al. 2011). Worldwide, 3.7% of new cases and 20% of previously treated cases were estimated to have MDR-TB. India, China, the Russian Federation and South Africa have almost 60% of the world's cases of MDR-TB (WHO, 2012).

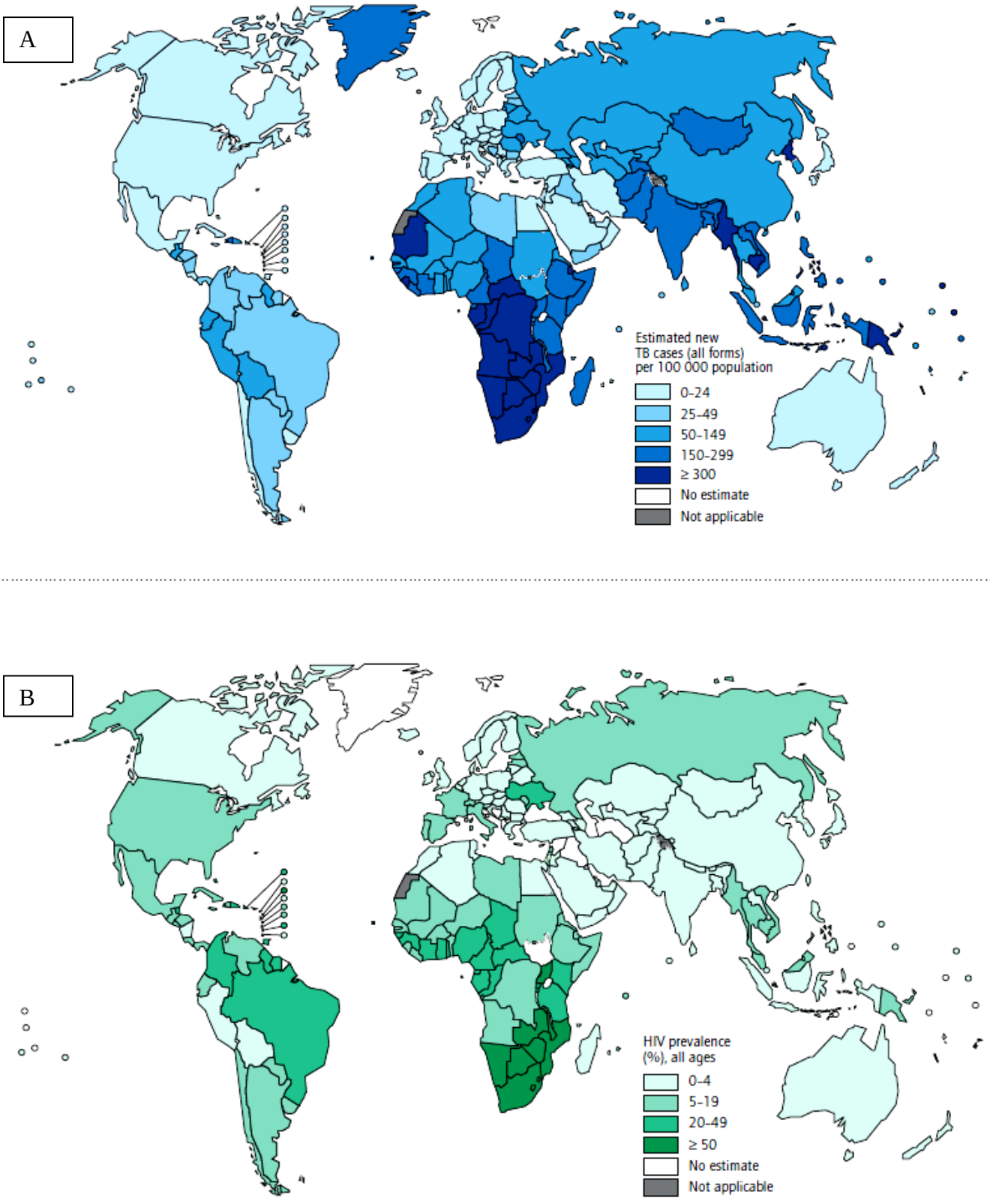


Figure 1.5: A - Estimated of TB incidence rates, 2011 and B - Estimated HIV prevalence in new TB cases, 2011 Source: Global Tuberculosis Report, 2012.

1.7.2 Epidemiology of TB in South Africa

South Africa remains the country with the greatest burden of HIV positive individuals and the second highest estimated TB incidence per capita worldwide (Houlihan et al. 2010). South Africa alone has 28% of the world's population of HIV and TB coinfected individuals (Louw et al. 2012). Approximately, 73% of the new TB cases in South Africa are co-infected with HIV which contributes to 84% of TB deaths in the country (Daftary & Padayatchi, 2012). The prevalence of HIV and TB is high throughout South Africa with the most escalating situation from the KwaZulu-Natal (KZN) province. Research conducted at Edendale Hospital in the KZN province, has observed that up to 50% of patients admitted to the hospital and 60% of women presenting for antenatal care are confirmed to be HIV positive (Dong et al. 2007). The province has the highest number of HIV-positive individuals (one million people) with even higher TB notification rates of 1094 cases per 100 000 population. The high HIV/TB prevalence figures together with TB drug-resistant (MDR-TB and XDR-TB) has gained national and international attention (Houlihan et al. 2010). TB incidences in South Africa have kept rising since 2002 as presented in Figure 1.6.

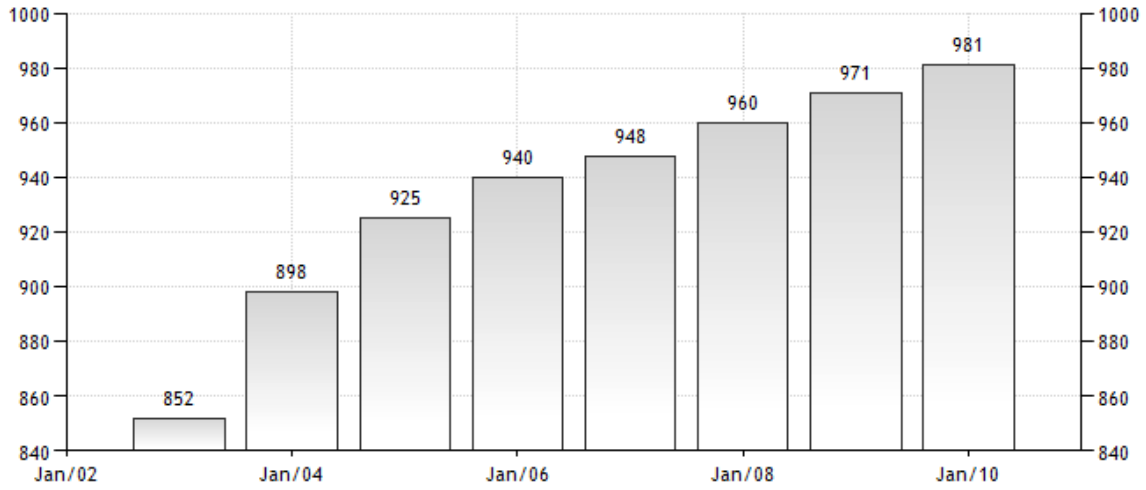


Figure 1.6: Incidence of Tuberculosis (per 100 000 people) in South Africa
Source: http://www.tradingeconomics.com/south-africa/incidence-of-tuberculosis-per-100-000-people-wb-data.html

1.8 Molecular Epidemiology of TB

1.8.1 General overview

The resurgence of TB in the world, but more importantly in developing countries has renewed interest in understanding the epidemiology and pathogenesis of the disease (Kehinde, 2012). The TB research community entered the genomic era in 1998 with the publication of the complete annotated genome of the MTB laboratory strain H37Rv. While previous methods, such as colony morphology, comparative growth rates, susceptibility to selected antibiotics, and phage typing, were useful, they did not provide sufficient discrimination of MTB strains, thus limiting their utility in TB epidemiology (Mathema et al. 2006a; Burgos & Pym, 2002).

The advancement of molecular techniques that allow identification and tracking of strains within MTBC has revolutionized TB research. The contribution of molecular epidemiological methods in the prevention and control of TB is made possible through their ability to differentiate between infecting strains, assessment of the overall diversity of circulating MTB strains, pinpointing differences by regions and subpopulations, and measurement of prevalence of endemic strains (Kehinde 2012). The clustered strains defined as two or more isolates from different patients but having a matching genotype may indicate recent transmission while non clustering isolates may indicate reactivation of TB disease. The gained information is vital in making TB policy (Rodwell et al. 2012).

Over the past 15 years approximately 900 pertinent publications have substantiated the value of the genotyping approach for TB control. Several European countries have profited from nationwide prospective fingerprinting. After providing genotyping results to public health officials, these were able to document epidemiological links for substantially more TB patients. On a global scale, strain families and particular strains have been identified, characterized and traced in their spread (Prodinger, 2007). Unfortunately, only in the developed countries, where more resources are available, have the results been used to guide tuberculosis-control efforts (Barnes & Cave, 2003).

The essence of conducting molecular epidemiological studies has been demonstrated in other studies done elsewhere in the world. A study done in San Francisco, New York and Amsterdam, demonstrated that the rate of recent infection was much higher than the estimated 10% predicted by traditional epidemiological studies. In San Francisco, the study showed that almost one-third of new cases of TB were as a result of recent infection, while in New York and Amsterdam clustering was estimated to be 40% and 47%, respectively (Burgos & Pym, 2002). In another study conducted in Hong Kong it was shown that 88% of the isolates from patients with relapses matched those with the pre-treatment counterparts indicating a high frequency of occurrence of infections caused by endogenous reactivation of MTB (Narayanan, 2004).

1.8.2 Genotyping Methodologies

There are a wide variety of genotyping markers for MTB (Figure 1.8). However, only a few of these, namely Spacer oligonucleotide typing (Spoligotyping), Mycobacterial Interspersed Repetitive-Unit Variable Number Tandem Repeat (MIRU-VNTR), and Insertion Sequence (IS) *6110* Restriction Fragment Length Polymorphism (IS*6110* RFLP), exhibit enough discrimination, have been internationally standardized and are currently the most commonly used in the globe (Hanekom et al. 2008). Spoligotyping and IS*6110* RFLP are discussed in detail in chapters two and three respectively.

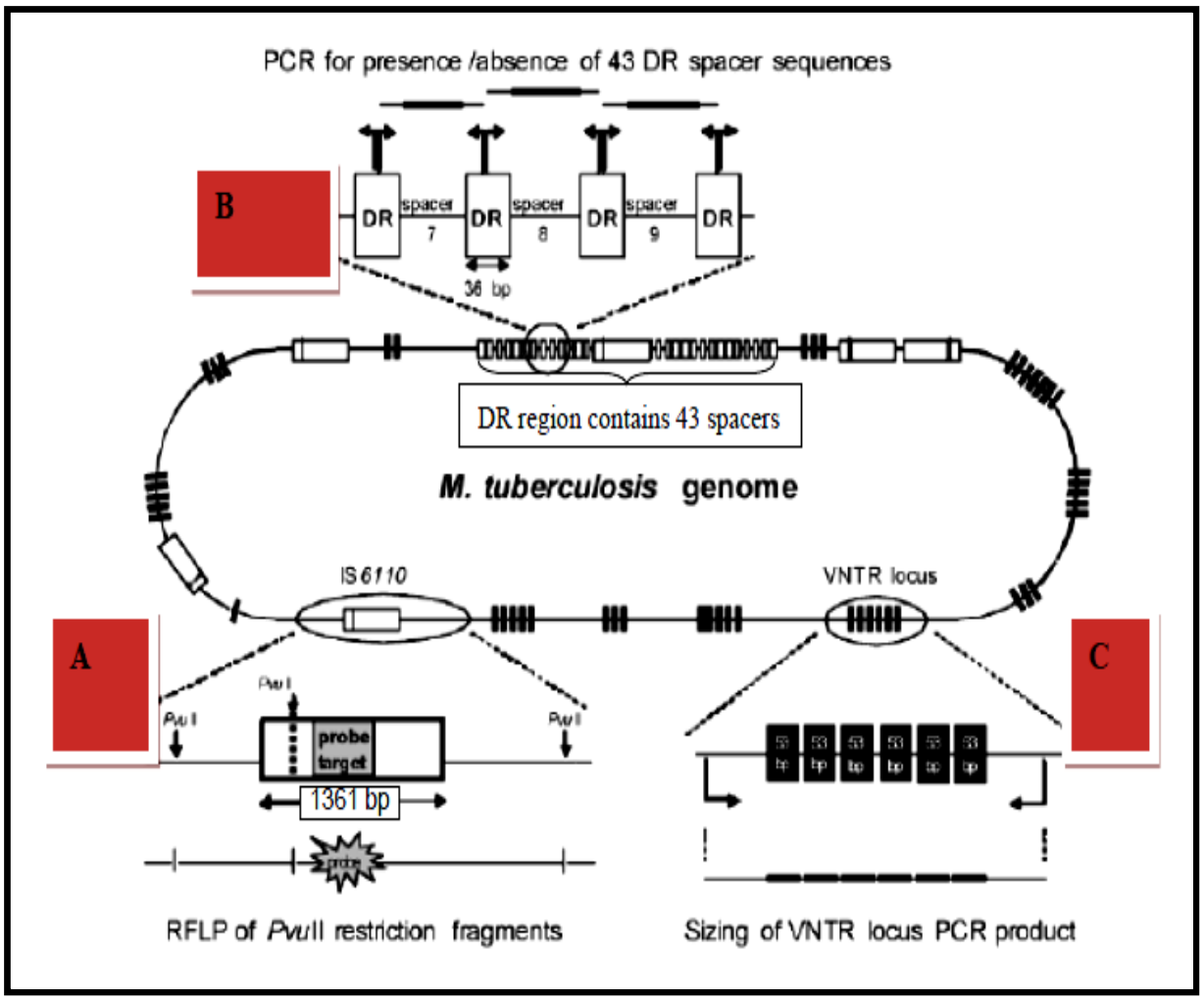


Figure 1.7: the genome of a virtual Mycobacterium tuberculosis complex isolate with the elements targeted by common genotyping methods indicated: (a) there are six copies of insertion element IS6110 in this genome, which will yield a strain-specific six-band RFLP pattern after hybridisation with an IS6110-specific probe. (b) The single DR locus harbours 36 bp direct repeats (boxes) and unique spacer sequences in between. Those are amplified by PCR at once and the strain-specific presence of 43 such spacers is tested in a next step by reversed line blotting. (c) Twelve VNTR loci (out of many more eligible for testing) are individually amplified by twelve sets of primers and the VNTR copy number is determined. The combination of the copy numbers from each locus is specific for a strain. VNTRs are between 40 and 100 bp in size, a size of 53 if often encountered MIRU-VNTRs.

Adapted from: (Prodinger, 2007).

In summary, spoligotyping is a PCR – based technique targeting the well-conserved 36-bp direct repeat (DR) regions in MTB which are interspersed with nonrepetitive spacer sequences of 34 to 41 bp in length. Its detection of polymorphism in the direct repeat region is based on identifying the presence or absence of 43 spacer DNA sequences between the variable direct repeats (VDRs) (Asho et al. 2008; Boer et al. 2004). The polymorphisms detected by spoligotyping are used to identify some members (i.e. *M. bovis*) of MTBC and distinguish MTB into distinct families and sub families that are quiet useful for identifying the source and spread of TB, TB control, and examining host-pathogen relationships. The major families of MTB include Beijing, CAS, T, LAM, X, H, S, EAI and MANU. These major families are further divided into sub families. Some of the sub families are; typical and atypical for the Beijing family, CAS-Kili, CAS-Delhi for the CAS family, T1, T2, T3 etc for the T family, LAM1, LAM2, LAM3, LAM4 etc for the LAM family, X1, X2 etc for the X family, H1, H2, H3 etc for the H family and EAI1 – SOM for the EAI family. These major families and sub families have been extensively studied through molecular epidemiological studies and have assisted to shed light on TB dynamics (Ozcaglar et al. 2011; Demay et al. 2012).

The IS*6110* RFLP technique is currently considered the gold-standard method for MTB typing. It is based on the occurrence of number and location of the IS*6110* element which is unique to MTB. Different strains carry different copy numbers of the IS*6110* elements and this polymorphism is highly discriminatory and reproducible to genotype the isolates (Asho et al. 2008; Van Embden et al. 1993; Yang et al. 1995).

MIRU-VNTR is one of the PCR based methods which is used to genotype MTB (Alonso-Rodríguez et al. 2008). This technique has been introduced recently, and is currently the most used method, based on determining the length variation at 12 independent minisatellite-like loci scattered throughout the MTB genome (Sun et al. 2004; Allix-Béguec et al. 2008). The ease with which this PCR-based technique can be performed, its adaptability to high-throughput automation, and the digital format of MIRU-VNTR patterns make this method suitable for the global study of the molecular epidemiology of MTB. In addition, MIRU-VNTR typing has a high discriminatory power for MTB strains with a low copy number of IS*6110* or that are devoid of IS*6110* (Y. Sun et al. 2004).

The evaluation of MIRU-VNTR in several genotypic studies has shown that the discriminatory power is equivalent to that of IS*6110* RFLP and is considered to be an alternative to RFLP for epidemiology purposes. However, other authors have found limitations in its discriminatory power and incomplete correlation with the IS*6110* RFLP analysis, indicating that MIRU analysis should be combined with an additional genotyping method (Alonso-Rodríguez et al. 2008).

In order to improve the standard of the most recent MIRU-VNTR technique, a standardized MIRU-VNTR format composed of 24 loci with a high discriminatory power has been proposed. This is on the basis of analyzing the clonal stability and evolutionary rates of MIRU-VNTR marker in primary genetic lineages of tubercle bacilli from around the world. Of the 24 loci, 15 are defined as composing a discriminatory subset based on a higher variability within the different clonal complex as studied (Allix-Béguec et al. 2008).

1.8.3 Molecular Epidemiology of TB in South Africa

The management of TB, especially MDR-TB and XDR-TB depends on an understanding of the population structure, as well as the prevalence and spread of TB strains driving the epidemic (Said et al. 2012a). This is mainly achieved through molecular epidemiological studies, which generate data regarding the strains that are circulating in a particular area. Unfortunately, despite South Africa ranking third on the world's 22 high burden TB countries (Said et al. 2012b), there is only limited data regarding the locally circulating strains. Based on the literature search, the majority of the molecular epidemiological studies that have been done in the country are on strains from the Western Cape Province (WCP), done by the Centre for Molecular and Cellular Biology at the University of Stellenbosch. As a result, the available data does not represent all the regions of South Africa. Of the few studies done in WCP and in some other parts of South Africa, the findings demonstrate the usefulness of molecular epidemiology.

To date, there is only one national molecular epidemiological study, which has been conducted. A national molecular epidemiological study (from eight provinces; Eastern Cape, Free State, Gauteng, KwaZulu – Natal, Northern Province, Mpumalanga, North West and Western Cape) on MTB isolates which were obtained during a national survey in 2001 – 2002, demonstrated a high diversity in the MTB genotypes in the country as 10 different lineages were found (Stavrum et al. 2009a). The most predominant lineages were found to be the T family ST53 (25.8%), followed by Beijing (10.3%). All the lineages were observed in MDR-TB except MANU2 and H37Rv. Despite the survey suggesting that there is no specific lineage that contributes to the spread of TB disease in the country, the sample size was too small (n=252) to give a true

indication on the TB dynamics in the different provinces. For example, Gauteng province with a population of more than 11 million was only represented with 35 isolates making 13% of the study (Stavrum et al. 2009a). Therefore, more information is needed in order to better understand the distribution of strains circulating within specific regions in the country.

Of the few molecular studies done in the country, some have demonstrated that TB may be due to specific lineages. The MTB genotype LAM3 (F11), characterized by a lack of spoligotype spacers 9 to 11, 21 to 24, and 33 to 36, and appear to be a unique marker of this strain family, represented the largest proportion of all isolates from TB patients in one of the studies from WCP (Streicher et al 2004). The authors suggested it may be at least as successful as the Beijing genotype in contributing to the TB problem in WCP of South Africa (Victor et al. 2004). The predominance of one family was also shown in a different study in the same province where the sub-group of Beijing family, termed R220 based on IS*6110* RFLP banding pattern, was overrepresented among drug-resistant isolates, giving an indication that the TB drug-resistance in the area during that time was largely caused by one lineage of MTB (Johnson et al. 2010).

Furthermore, studies done in KZN have also demonstrated that the incidences of drug-resistant TB are mostly due to clonality. It has been shown that LAM4 (SIT 60) is the most predominant genotype circulating in the area. In an XDR-TB study, of the 86 XDR-TB isolates, 92% (79) belonged to the genotype LAM 4 SIT 60 and this was found to be due to clonality after DNA sequencing of the 10 resistance determining regions of these isolates demonstrated identical mutations across all genes tested (Gandhi et al. 2013). In another study, analysis of 102 MDR-

TB isolates in 2005 from one district in KZN showed that 60 (59%) of these isolates belonged to the F15/LAM4/KZN (Pillay & Sturm, 2007).

Incidences of TB are very high in South African mines where close monitoring of spread of the disease is required (Hanifa et al. 2009). A molecular study conducted from a certain mine in South Africa, observed clustering of isolates to be more frequent among case-patients with MDR-TB than mono-resistant strains. Again, comparison of clustering rate among pre-XDR-TB and XDR-TB isolates showed that highly resistant strains were more likely to belong to a cluster than those with less resistance. It was also noted that 59% of clustered case-patients had a documented previous episode of TB, which suggested re-infection with a circulating strain. This suggested that the current TB control program was largely able to prevent the acquisition of drug-resistance to at least the first-line drugs but could not prevent the transmission of pre-existing MDR-TB in this highly vulnerable population (Victor et al. 2004).

Demonstration of mode of transmission through molecular techniques has been achieved in some studies. A recent study showed that Beijing strains among XDR-TB were evolving and were spreading adaptively rather than by clonal expansion while MDR-TB was being transmitted clonally. This clonal transmission of MDR was demonstrated by the observation that isolates in the Beijing R220 cluster shared the same *c-15t inhA* promoter mutation, and isolates in the Beijing R86 cluster shared the same *katG S315T* mutation. Unlike in MDR-TB, in XDR-TB the mutations were not clonally related (Ioerger et al. 2010).

There have been limited molecular studies done in the Gauteng province of South Africa. An XDR-TB study done in Gauteng Province but on isolates from four different provinces (Limpopo, Gauteng, North West, and Northern Cape) to determine whether XDR-TB was primarily transmitted or acquired, demonstrated high diversity of XDR-TB strains with Beijing genotype family being the largest in the study contributing 34% of all the isolates. The analysis of the data further revealed that majority of XDR-TB cases was due to drug acquisition rather than transmission. This was supported by the high default rate (30%) from TB treatment for the MDR-TB patients (Mlambo et al. 2008). The Beijing family, also dominated (16%) in an epidemiological study of spread of multidrug-resistant tuberculosis in Johannesburg (Marais et al. 2013). In a similar study conducted in the same province of Gauteng in Pretoria, the Beijing family was the most predominant seconded by the T1 family (Hove et al. 2012).

A community based study done in the Western Cape Province of South Africa identified an outbreak of drug-resistant TB involving strains that belong to a newly describing genotype, the DRF150. This clone represented almost half of the cases of drug-resistant TB identified in the George sub-district during the study period. This shows the risk of widespread transmission of drug-resistant strains that are not of the Beijing/W lineage, and suggests that circulation of further currently unrecognized drug-resistant strains may contribute to the South African drug-resistant TB epidemic (Victor et al. 2007).

Studies have shown that the global TB epidemiology is caused by different genotypes, and these genotypes occur at frequencies that differ between district, cities, countries, and continents. In South Africa, the population structure of MTB strains differs among the provinces (Said et al. 2012b). However, little information is available from some parts of South Africa. Johannesburg is the most densely inhabited industrialized city in South Africa as well as the financial hub of Africa yet very little is known about the circulating strains types, compared to the vast amount of data forthcoming on the genotypic diversity of strains from the WCP. It is possible that the types of MTB strains/families in this area are not the same as those identified in the WCP. Though patient management may not vary according to strain type, the ability to differentiate genotype families between geographical locations may have an impact on clinical and epidemiological future plans for a particular location (Schürch & van Soolingen 2012).

1.9. Aim and objectives of the study

1.9.1 Aim

This study **aimed** firstly to determine the genetic diversity of drug susceptible and drug resistant MTB strains isolated from patients with TB residing in the Johannesburg area; secondly to compare the genetic diversity of these strains to national as well as international databanks and finally to determine whether there is a predominant clone that is circulating in the area.

1.9.2 Study Objectives

The study objectives are summarized as follow:

1. To collect and categorize the isolates according to the drug susceptibility pattern.
2. To detect and type MTB and determine groups of related strains through spoligotyping.
3. To characterize groups of related strains by IS*6110* RFLP.
4. To compare the genotypic data to national and international databanks.

Chapter two: Spoligotyping of *Mycobacterium tuberculosis* strains

2.1 Introduction

TB has gained critical attention (Guernier et al. 2008) being one of the deadly disease for decades and remains a problem in developing countries particularly in Sub-Saharan Africa which is fuelled by HIV epidemic (Pang et al. 2012). The TB problem in South Africa has further been exacerbated by the emergence of MDR-TB and XDR-TB which is believed to be due to either non-adherence to drug regimens or the use of inappropriate drug regimens (Stavrum et al. 2009a).

A clear understanding of the transmission of the disease and the establishment of infection contributes to the development of effective prevention and control measures for TB. The use of molecular techniques to characterize Mycobacterium strains, particularly the MTB strains and the concomitant accumulation of epidemiological data has increased the possibility to trace transmission routes of MTB outbreaks (Guernier et al. 2008)**.** By providing insight into the transmission dynamics, source and spread of MTB, molecular studies have enhanced our knowledge of TB epidemiology (Wang et al. 2012; Pang et al. 2012). Through a genotypic study, patients with identical MTB genotypes can be identified and are designated as cluster cases which may be indicative of recent transmission (Guernier et al. 2008). This estimation of recent transmission through molecular epidemiologic methods is regarded as an important indicator for surveillance and TB control programs (Wang et al. 2012). Through molecular studies, it is possible to establish the existence and spread of particular strain families in defined geographic

regions (Wang et al. 2012), which may significantly increase our capacity to monitor the transmission and to prevent secondary cases (Blanca et al. 2010).

Spoligotyping is a reliable and informative technology for characterizing the genetic structure of MTB populations (Pang et al. 2012). The method is suitable for large studies on phylogeny and identification of genotype families (Abadía et al. 2009), and has gained widespread acceptance because it is a simple, rapid, and robust method. Results can be expressed in a simple digital format that is easier to compare and store, in comparison with those of other available techniques. Thus it has been extensively applied, alone or in conjunction with other techniques (Bezos et al. 2004).

2.1.1 The General overview of Spoligotyping

Spoligotyping is a PCR – based technique first described in the mid 1990s and is based on DNA polymorphisms at a single chromosomal locus characterized by the presence of a high number of conserved direct repeats. This so-called Direct Repeat (DR) region consists of directly repeated sequences that are interspersed by DNA spacers (Figure 2.1). When the direct repeat regions of several isolates were compared it was noted that the order of spacers was nearly the same in all isolates, but that many deletions and/or insertions occurred in different strains. Because spoligotyping is a PCR driven technique, only small amounts of DNA are required for analysis, so the method is particularly suitable for the analysis of slowly growing Mycobacteria. It also permits the comparison of isolates which are not suited for re-culture after prolonged storage (Boer et al. 2004).

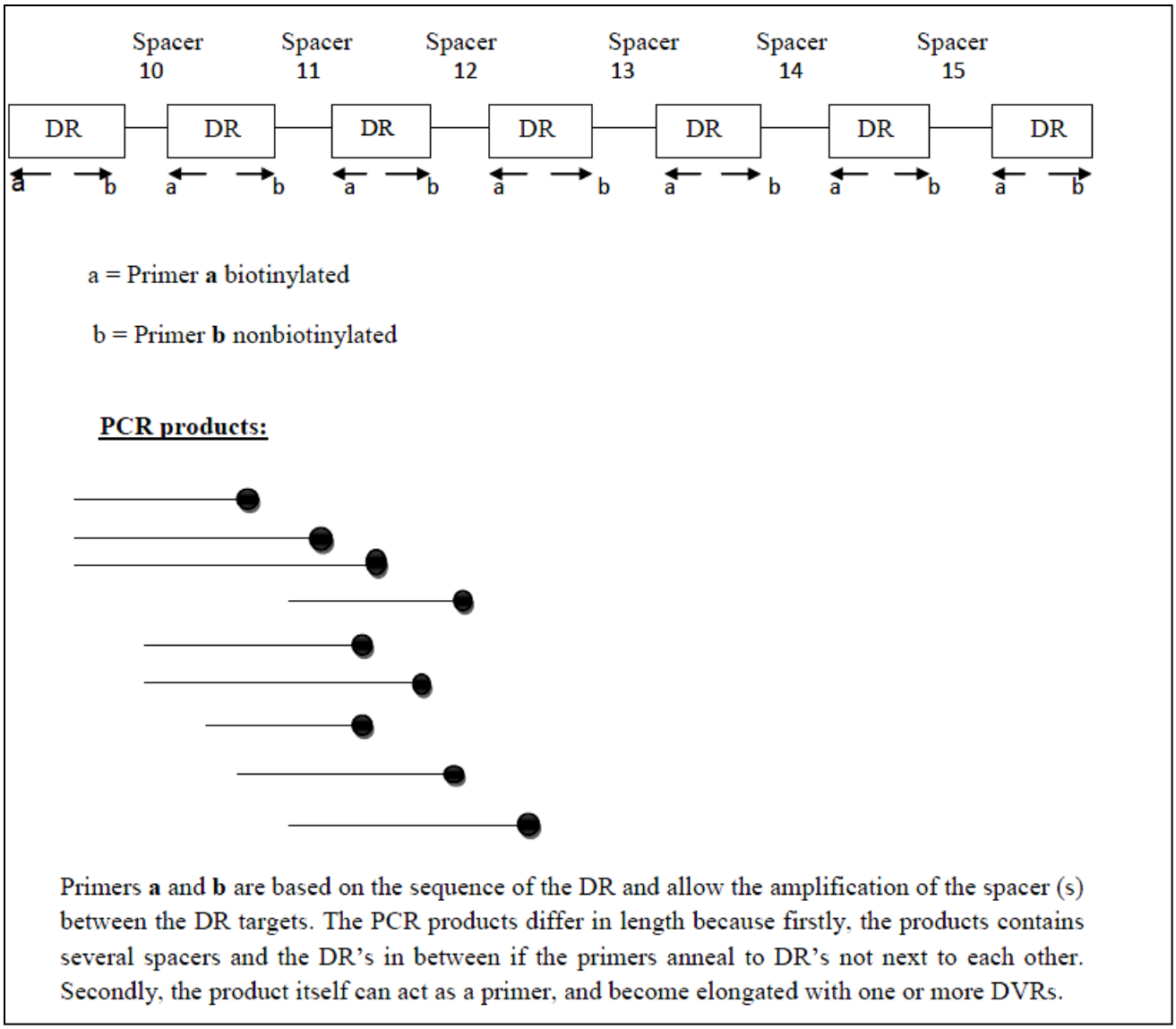


Figure 2.1: Annealing of the primers to the DRs and PCR products. Adapted from Honisch et al. 2010

The spacer nucleotides between the direct repeats can be amplified simultaneously using a single set of primers that target the DR sequences. The presence or absence of spacers in a given strain is determined by hybridization with a set of 43 oligonucleotides derived from spacer sequences of MTB H37Rv. These (oligonucleotide) spacers derived from the known spacers in the DR cluster are covalently linked to an activated membrane in parallel lines. PCR products are hybridized perpendicular to the oligo lines. After hybridization the membrane is incubated in streptavidin peroxidase, which binds to the biotin label on the PCR products. Detection of the hybridization signals are optimized by the enhanced chemiluminescence (ECL). The peroxidase present on the streptavidin catalyzes a reaction resulting in the emission of light, which can be detected by autoradiography of the membrane. The presence of the spacers is visualized on film as black squares after incubation with streptavidin-peroxidase and ECL-detection (Narayanan, 2004; Bezos et al. 2004).

Although the overall discriminatory power of spoligotyping is lower than that of IS*6110* RFLP typing that is still considered as the gold standard for typing, it has the specific advantage of higher discrimination of strains with low copy numbers of IS*6110* (Figure 2.2) (Narayanan, 2004). Other advantages of spoligotyping over IS*6110* RFLP method is that spoligotyping only requires a small amount of sample DNA and this can be obtained from liquid culture or direct form the specimen i.e. sputum. Furthermore spoligotyping data, which is represented in absolute terms, can be readily shared among laboratories thereby enabling the creation of a large international database (SpolDB) (Warren et al. 2002; Millet et al. 2009; Kehinde, 2012). This

development allows investigators to survey strain diversity and uncover global strain families such as the Beijing and Latin American Mediterranean (LAM) families. The method has been very successful in providing a tool for the rapid acquisition of MTB genotyping information and for the establishment of a global picture of MTB diversity. It is highly reproducible and has been developed into a high-throughput assay for large molecular epidemiology surveys (Kehinde, 2012; Millet et al. 2009). The strengths of this method include its low cost, digital data results and good correlation of results with other genetic markers. It further offers a fair level of overall differentiation of MTB strains, has high-throughput capacity, and is able to provide species information within the MTBC (Kehinde, 2012; Warren et al. 2002).

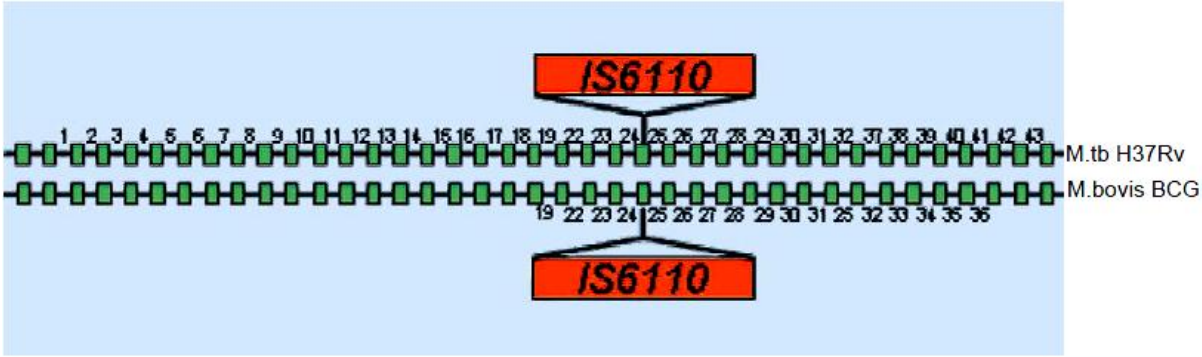


Figure 2.2: structure of the Direct Repeat locus in the genome of MTB H37Rv and *M.bovis* BCG P3. The green rectangles depict the 36 bp Direct Repeat region while the strait black lines in between Direct Repeats, represents the unique spacers. Adapted from Boer et al. 2004

2.2 The aim of the study

The purpose of this study was to genotype the MTB isolates through spoligotyping and to determine the predominant strain types circulating in the Johannesburg area. Furthermore the aim was to identify whether the TB incidences are due to clonality or due to different strain types. In addition we aimed to determine if there are certain strain types were more associated with resistance than others. Lastly, we aimed to characterize a subset of isolates through IS*6110* RFLP in order to setup the RFLP procedure in the department as well as to correlate the results for the two methods mainly for the clustered MTB isolates.

2.3 Materials and Methods

2.3.1 Study design

This was a retrospective and descriptive study of MTB isolates that were one year or two years (2009 and 2010) old. These isolates were stored in the routine culture laboratory after routine testing. Thus drug susceptibility results were already available from routine culture and drug susceptibility testing of TB suspects within the National Department of Health TB Control programme, in Gauteng.

2.3.2 Ethics approval

The study received ethical approval from the University of Witwatersrand Human Research Ethics Committee. The study clearance certificate number is M111113.

2.3.3 Sample collection procedure and demographics

The *Mycobacterium tuberculosis* complex (MTBC) isolates were collected from the National Health Laboratory Services (NHLS) Mycobacteriology Referral Laboratory in Braamfontein, Johannesburg. This laboratory serves as the routine diagnostic mycobacterial culture and drug susceptibility testing laboratory for Gauteng, with the majority (about 60%) of specimens primarily submitted from the Chris Hani Baragwanath, Charlotte Maxeke Johannesburg Academic, Helen Joseph hospital (all tertiary academic hospital linked to the University of the Witwatersrand) and Sizwe drug resistant TB hospital (where all newly diagnosed MDR-TB patients are admitted in the Gauteng province. It also provides culture services to about 100 TB clinics in Gauteng province, as well as to prisons. The laboratory also receives specimens from TB culture laboratories in other provinces of South Africa such as Limpopo, Mpumalanga, and from outside the country or second-line drug susceptibility testing. It performs drug susceptibility testing for first line (Rifampicin, Isoniazid, Streptomycin, Ethambutol, and Pyrazinamide) as well as second line (Ethionamide, Kanamycin and Ofloxacin). The laboratory is accredited with SANAS (South African National Accreditation System) and subscribes to External Quality Assurance (EQA) programmes (e.g. College of American Pathologist CAP) thrice a year.

2.3.3.1 Specimen origin

The study tested MTB isolates from specimens that originated from TB suspects from within the seven regions of Johannesburg (Figure 2.3). This city is the most densely populated city in South Africa, and with a population of 3,888,180 is the provincial capital of Gauteng province and the financial hub of South Africa (Community Survey report; 2007).

2.3.4.2 Specimen identification and selection

The MTB isolates collected from the NHLS Mycobacteriology referral laboratory were identified through searching the laboratory information system (LIS). The isolates were consecutively selected so that no bias in selection process occurred. Based on the demographic data, each specimen obtained was categorized according to its geographical region of origin (patient residential address). Only specimens originating from the seven regions of Johannesburg (Figure 2.3) were included in this study. These included specimens from males and females, all age groups and all specimen types. The ethnicity or nationality was not considered, as we were blind to the information. Isolates were excluded if neither the physical address nor the clinic name was indicated as it could then not be ascertained if the specimen originated from one of the seven mentioned regions. All patient duplicate samples were removed. The collection of isolates from patients from all the seven regions of Johannesburg was to ensure that the study sample was well represented, even though analysis of results will not focus on the individual regions. The number of isolates tested from each region of Johannesburg is indicated in Figure 2.3.

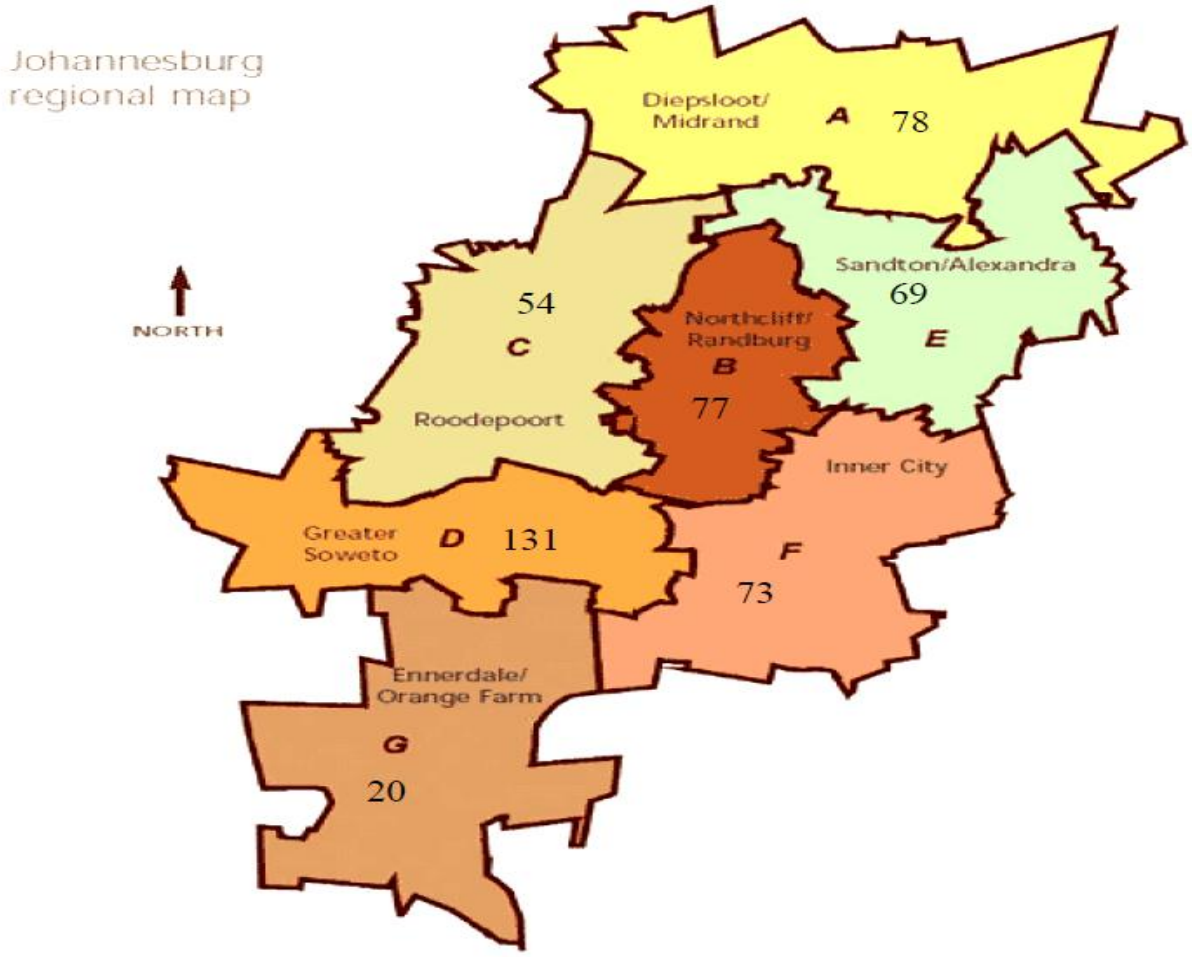


Figure 2.3: Map illustrating the seven administrative regions of Johannesburg (A-G) where of MTB isolates originated from. Total numbers of isolates are shown as per region. Map taken from www.joburg.org

Isolates were tested from tubes of positive Mycobacterial Growth Indicator Tube (MGIT) culture (BD BioSciences, Sparks, MD, USA). Each MGIT tube had a unique laboratory identification number that is linked to demographic data such as date of birth, gender and physical address. Each specimen was re-assigned a unique study number (SN) and hence delinked from patient identifiers, once regions were documented. No patient information was collected. By the time of selection, the drug susceptibility testing (DST) information of each isolate was available from the LIS. Based on this information, isolates were grouped into 5 drug susceptibility result categories as shown in Table 2.1.

Table 2.1: Description of drug susceptibility categories

Drug susceptibility category	**Definition**
Susceptible MTBC	MTBC with no resistance to RIF and INH
Mono – resistant MTBC	MTBC resistant to RIF or INH but not to both
Multidrug – resistant MTBC (MDR-TB)	MTBC resistant to at least both of RIF and INH (but not ofloxacin or to one of the three injectable second line drugs [AMK, KM or CPM]).
Pre – XDR MTBC	MDR-MTBC that is also resistant to one of the three second line injectable drugs or to ofloxacin.
XDR MTBC	MDR-MTBC that is also resistant to one of the three second line injectable as well as resistant to ofloxacin.

The total number of isolates collected per drug susceptibility category is shown in **figure 2.4**.

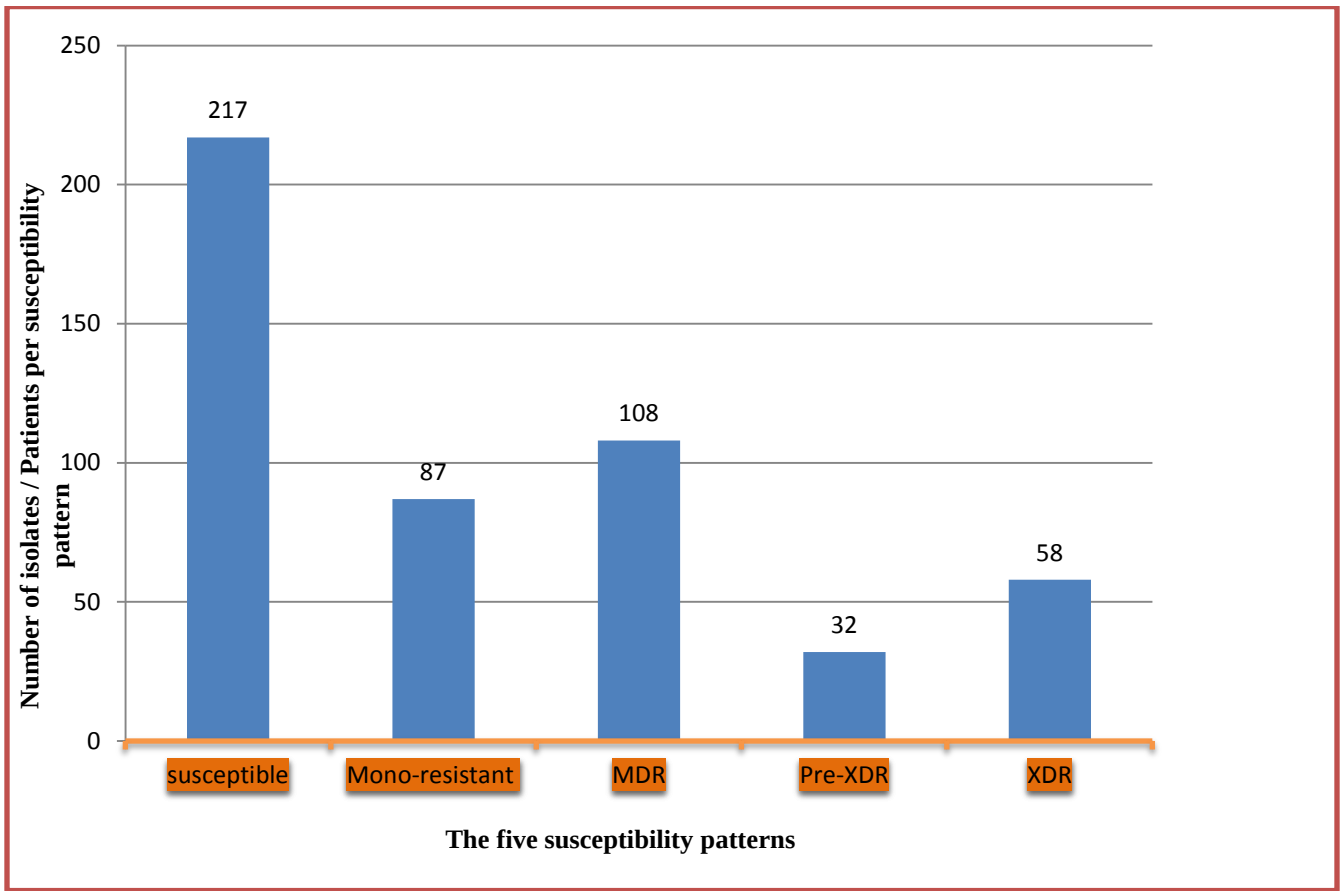


Figure 2.4: Number of samples collected per drug susceptibility category.

Resistance of MDR isolates in other MTB drugs such as STR, EMB, ETHIO and PZA, differed considerably as shown in figure 2.5.

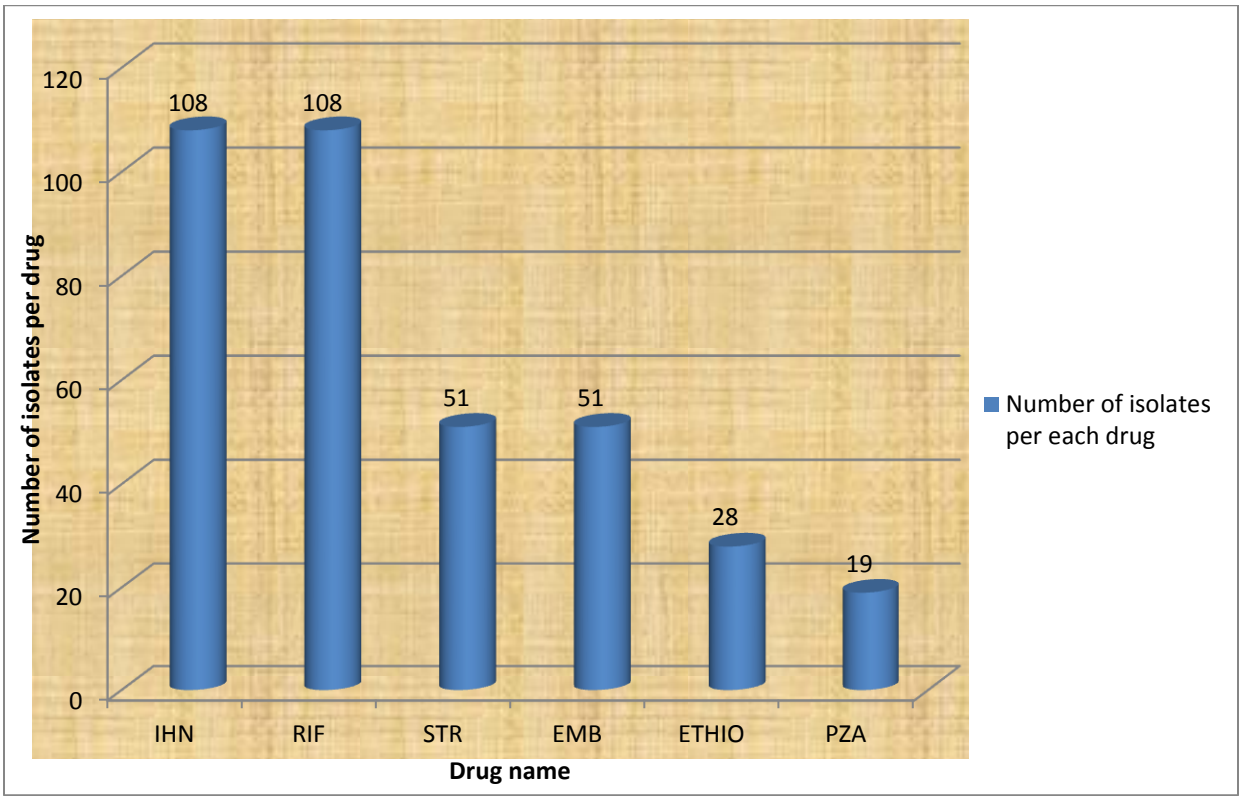


Figure 2.5: Presents 108 isolates and six TB drugs showing the extent of drug resistant for MDR-TB isolates included in this study.

2.4 Spoligotyping

Spoligotyping was performed out using a commercially available kit (Isogen Bioscience BV, Maarssen, Netherlands) according to the manufacturer's instructions. As mentioned, spoligotyping targets the direct repeats (DRs) present in multiple copies in the genomic DR locus of MTB complex bacteria. The well-conserved 36 bp DRs are interspersed with nonrepetitive spacer sequences of 34 to 41 bp in length. The variation of these spacer sequences among strains of the MT complex allows for the genotyping of the *Mycobacterium tuberculosis* complex. Spoligotyping was performed using the primers DRa (5'GGTTTTGGGTCTGACGAC3') and DRb (5'CCGAGAGGGGACGGAAAC3') as originally described by Kamerbeek *et al* 1997. The method involves three main steps; (A) *in vitro* amplification of spacer DNA by PCR, (B) Hybridization of the PCR product and detection, and (C) Regeneration of the membrane for next Hybridization.

2.4.1 Specimen processing for spoligotyping

Using aerosol resistant tips to avoid contamination, 400µl of culture from each MGIT was transferred to a screw-cap tube and incubated at 80˚C for 30 minutes using the Accu Block^{tm} Digital Dry Bath (Lab-Net) in order to lyse the bacterial cells. The screw-cap tubes were used to prevent aerosol formation during the heat killing process. The process of heat killing the mycobacterial cells was done in a Biosafety level III laboratory. The heat killed isolates were stored frozen in microcentrifuge tubes at -20˚C until the time of use.

2.4.2 Preparation of the PCR Mixture

DNA amplification was performed as previously described (Kamerbeek et al. 1997) using DRb (5'-CCGAGAGGGGACGGAAAC-3') and primer DRa (5'-GGTTTTGGGTCTGACGAC-3'). The PCR reaction mixture used in all spoligotyping applications contained 1X Master Mix: 0.05U/µl Taq DNA polymerase, 2mM $MgCl_2$, 0.2mM each of the deoxynucleotide triphosphates (dNTPs) (Fermentas Life Science, Glen Burnie, MD, USA), 2µM of the primer pair (DRa, DRb) and, 1µg DNA made up to the final volume of 25µl with sterile water. Amplification was performed using an iCycler Thermocycler (Bio-rad Hercules, CA, USA) starting with a denaturing step for 3mins at 96˚C, followed by 30 cycles of 1min at 96˚C, 1min at 55˚C and 30sec at 72˚C. This was followed by a final extension step of 72˚C for 10mins. DNA from MTB strain H37Rv and *M.bovis* BCG were used as positive controls and sterile water was used as a negative control in each run.

2.4.3 Hybridization

After the PCR was completed, 20µl of the PCR product was added to 150µl 2 X SSPE/0.1%SDS in a microcentrifuge tube. The mixture was placed at 99˚C (Dry Block Heater HB_2 hägar designs) for 10 mins in order to denature the diluted PCR product. Immediately after denaturing, the tube was placed on ice until the process of washing the membrane and assembling of the miniblotter was complete. The spoligotyping membrane (Isogen, Bioscience BV, Utrecht, The Netherlands) was washed in 2 X SSPE/0.1% SDS for 5 minutes in a hybridization oven (MWG-Biotech AG, Ebersberg, Germany) at 57˚C. The membrane was then placed on a support cushion in a miniblotter (Isogenic, Bioscience BV, Utrecht, Netherlands).

After preparation of the PCR product and assembly of the miniblotter/membrane, any excess buffer remaining after pre-treating the membrane was aspirated. The slots were then slowly filled with the diluted denatured PCR product, with careful attention to avoid forming air bubbles in the slots. The first and last slots on the miniblotter were loaded with 2 X SSPE/0.1% SDS buffer, leaving the remaining lanes for samples and controls. After loading, the miniblotter/membrane was placed in a hybridization oven (MWG-Biotech AG, Ebersberg, Germany) for 60 minutes at 57˚C without shaking to avoid cross flow as this could lead to contamination.

2.4.4 Detection

PCR products were removed by aspiration from the miniblotter slots after hybridization was complete. The membrane was washed twice in 2 X SSPE/0.5 SDS for 7 minutes at 57˚C after which it was placed in a rolling bottle (GATC TUBE detection system-Germany). Thirty millilitres of 2 X SSPE/0.5 SDS (42˚C) was added with 7.5µl streptavidin peroxidase conjugate (500U/ml) in a 50 ml tube and gently mixed. The mixture was poured onto the membrane in a rolling bottle and incubated for 60 minutes at 42˚C. After incubation, the membrane was washed twice with 2 X SSPE/0.5% SDS for 10mins at 42˚C, followed by rinsing with 2 X SSPE for 5mins at room temperature. The detection step was achieved with the use of ECL detection liquid (Amersham Biosciences, Buckinghamshire, UK). The membrane was incubated in 10ml detection mixture (5ml of ECL detection solution 1 and 5ml ECL detection solution 2) for 90 seconds then transferred to a clear plastic bag. After heat-sealing, the bag was transferred into a 25 x 30 cm film cassette (Amersham Biosciences, Buckinghamshire, UK). The film was exposed

to the membrane for periods ranging 20 seconds to 1 minute in a dark room at the Radiology Department at the Charlotte Maxeke Johannesburg Academic Hospital, and developed using a Kodak RPX-OXMAT Processor (Eastman Kodak Company, New York, USA).

2.4.5 Stripping the Membrane

Removal of the PCR products from the membrane (stripping) was done by washing the membrane twice in 1% SDS at 80°C for 40 minutes. The membrane was further washed in 20mM EDTA, pH 8, for 15 minutes at room temperature, and then sealed in a plastic bag containing a small amount of 20mM EDTA, pH 8, to keep the membrane moistened and stored at 4°C until further use.

2.4.6 Interpretation of the results

Results from the spoligotype membrane autoradiographs were analyzed by recording the presence or absence of signals at the sites of DNA/DNA hybridizations. The presence of spacers was represented on film as black squares after incubation with streptavidine-peroxidase and ECL detection. Results were entered in a binary format into excel spreadsheets and compared with the published spoligotyping database SpolDB4 of the Pasteur Institute, Guadeloupe (Brudey *et al*. 2006) as well as with the updated version SITVIT 2 from the Pasteur Institute in Guadeloupe.

2.4.6.1 Estimation of recent transmission

The estimation of transmission was done according to the n-1 method: $RTI_{n-1} = (n_c\text{-}c)/n$, where **n** is the total number of cases in the sample, $\mathbf{n_c}$ is the total number of cases in cluster and **c** is the number of genotypes represented by at least two cases. The concept is that a more genetically homogenous data set would represent a more severe extent of disease transmission i.e. an outbreak (Guernier et al. 2008).

2.5 Statistical analysis

Pearson's Chi-square test was used to determine statistical association between predominant spoligotyping families and drug-resistance. Statistical Package for Social Science Software (SPSS, USA) was used for analysis. P values <0.05 was considered significant.

2.6 Spoligotyping results

The study included a total of 500 MTC isolates as shown in Figure 2.6.

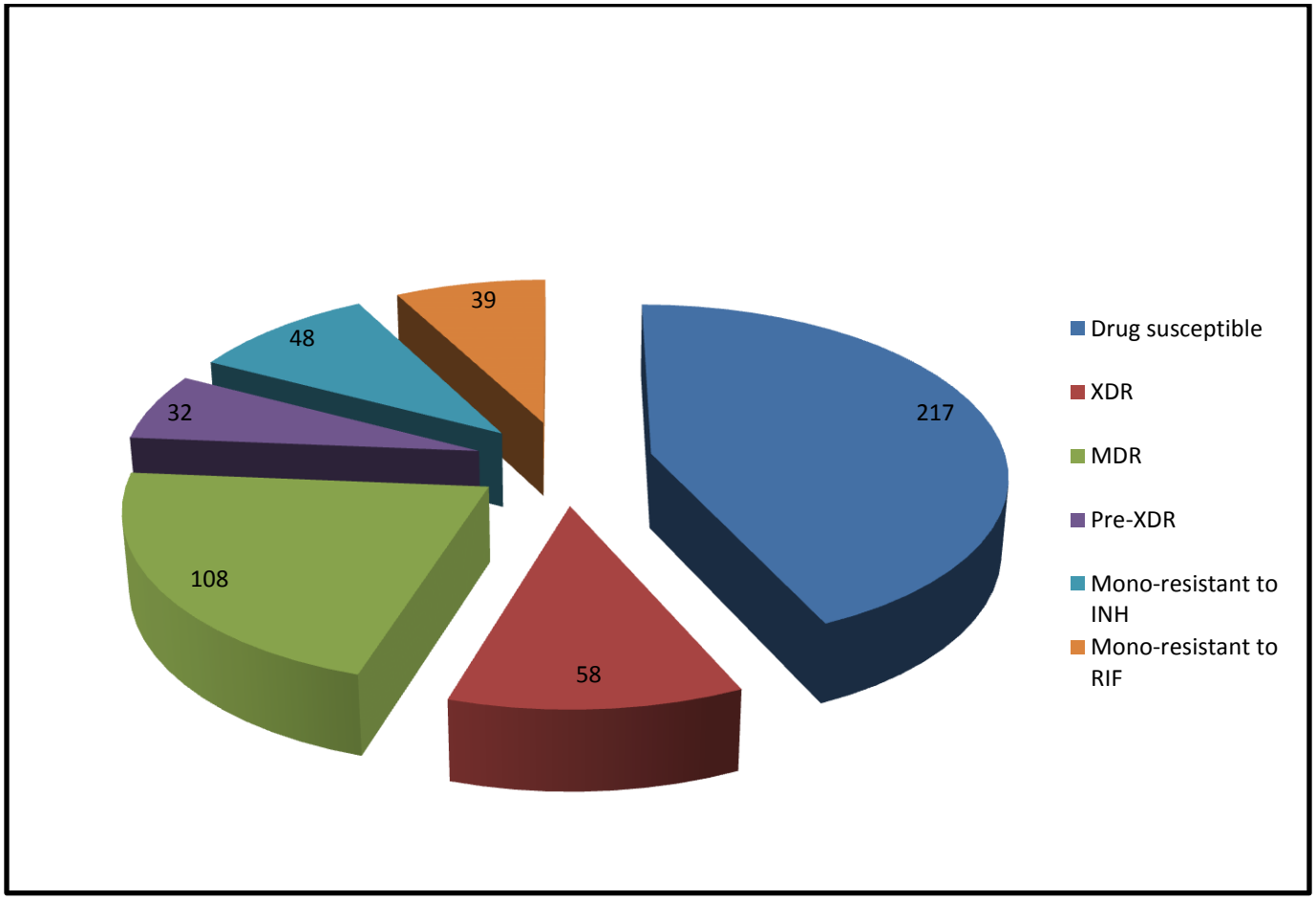


Figure 2.6: Total number of MTC isolates analyzed from each sensitivity category

Spoligotyping of the 500 isolates generated 62 shared types i.e. an identical pattern shared by two or more patients worldwide (within this study, or matching another strain in the SITVIT2 database). Ninety two percent of our sample matched the pre-existing shared types. Thirty one [467/500 (93%)] shared types were clustered (2 to 135 per cluster). Detailed spoligotyping results are summarized in Table 2.2. Of the thirty one clustered shared types, eight were predominant (clustering from 16 to 135 per cluster) constituting 68% (340/500) of all (500) typed isolates and 73% (340/467) of all shared types found.

The other 20 shared types clustered 2 to 8 representing 17% (87/500) while 31 (6.2%) shared types (non clusters) represented the single isolate (Table not shown). Forty two (8.4%) isolates were found to be undefined (absent in the SpolDB4.0 data base) and were termed orphans (Table 2.5).

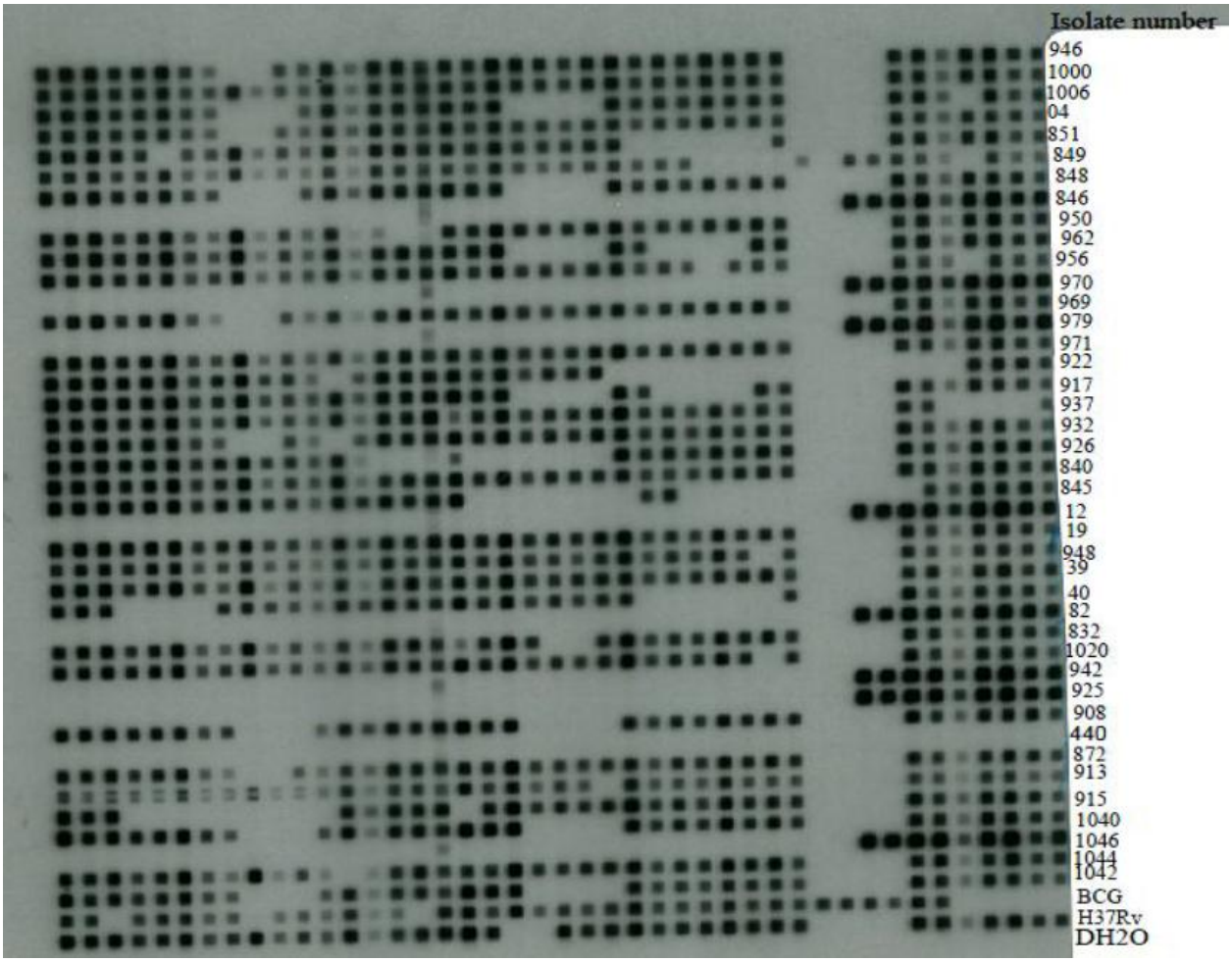


Figure 2.7: Presence (black blocks) and absence (empty spaces) of spacers in each isolate displayed on the X – ray film.

Table 2.2: Description of 59 (SIT) shared types

SITn	Clade	Binary (Spoligotype) Description	**Total strains**	% in this study
1	Beijing	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	135	27 %
190	Beijing	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■□■■■	1	0.2
255	Beijing	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■□■■■■	1	0.2
53	T1	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	38	8.2
766	T1	■■■■■■■■■■■■■□□□■□□□□□□■■■■■■■□□□□■■■■■■■	1	0.2
771	T1	■■■■■■■■■■■■■■■■■■■■■□□■■■■■■■■■□□□□■■■■■■■	3	0.6
136	T1	■■■■■■■■■■□□□□□■■■□□□□□■□■■■■■■□□□□■□□■■■■	1	0.2
1597	T1	■■■□□□■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	3	0.6
719	T1	■■■■■■■□□□■■■■■■■□□□□□■■■■■■■■□□□□■■■■■■■	6	1.2
358	T1	■■■□□■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	1	0.2
244	T1	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■	6	1.2
154	T1	■■■■□■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	1	0.2
1210	T1	□□□□□■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■	1	0.2
373	T1	■■■■■■■■■■■■■■■■■■■■■□■■■■■■■□□□□■■■■■■■	1	0.2
191	T1	□□■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	1	0.2
649	T1	□■■■■■■■■■■■■■■■■■□□□□■□□■■■□■■■□□□□■■■■■■■	4	0.8
613	T1	■■■■■■■■□□■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	1	0.2
52	T2	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	5	1
37	T3	■■■■■■■■■■■□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	3	0.6
73	T2-T3	■■■■■■■■■■■□■■■■■■■■■■■■■■■■■□□□□■■■□■■■	3	0.6

Table 2.2: continues.

SITn	Clade	Binary (Spoligotype) Description	**Total strains**	% in this study
33	LAM 3	■■■■■■■□□□■■■■■■■■□□□□■■■■■■■□□□□■■■■■■	30	6
1295	LAM 3	■■■■■■■□□□■■■□□■■■■□□□□■■■■■■■□□□□■■■■■■	1	0.2
60	LAM4	■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■□■■■	41	8.2
811	LAM4	■■■■■■■■■■■■■■■■■■■□□□□■□□□□□■■□□□□■■■□■■■	6	1.2
1710	LAM4	■■■■■■■■■■■□■■■■■■■□□□□■■■■■■■□□□□■■■□■■■	1	0.2
59	LAM11_ZWE	■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■■□□□□■■■■■■	8	1.6
815	LAM11_ZWE	■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■■□□□□■■■□■■■	3	0.6
813	LAM11_ZWE	■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■■□□□□■■□□■■■	1	0.2
1607	LAM11_ZWE	■■□□□□□□■■■■■■■■■■■□□□□■■□□□□■■□□□□■■■■■■	1	0.2
42	LAM9	■■□□□□□□■■■■■■■■■■■□□□□■■□□□□■■□□□□■■■■■■	7	1.4
207	LAM9	■■■■■□■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■	1	0.2
95	LAM6	■■■■■□■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■	1	0.2
20	LAM1	■■□■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■	4	0.8
1092	CAS1_DELHI	■■■□□□□■□■■■■■■■■■■■■■□□□□□□□□□□□□□■■■■■■	1	0.2
26	CAS1_DELHI	■■■□□□□■■■■■■■■■■■■■■□□□□□□□□□□□□□■■■■■■	2	0.4
21	CAS1_KILLI	■■■□□□□■■□■■■■■■■■■□□□□□□□□□□□□□□□■■■■■■	6	1.2
334	CAS1_KILLI	■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■	1	0.2
776	CAS	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□□□■■■■■■	2	0.4
34	S	■■■■■■■□□■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■	42	8.4
1296	S	■■■■■■■□□■■■■■□□■■■■■■■■■■■■■■■□□□□■■■■■■	1	0.2
71	S	■■■■■■■□□■■□■■■■■■■■■■■■■■■■■■□□□□■■■■■■	6	1.2

Table 2.2: continues.

SITn	Clade	Binary (Spoligotype) Description	**Total strains**	% in this study
88	S	■■■■■■■■□□■■□■■■■■■■■■■■■■■■■■■■■□□□□□■■■□■■■	1	0.2
48	EAI1_SOM	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□■□■■■■■□■■■	17	3
806	EAI1_SOM	■■■■□■■■■■■■■■■■■■■■■■■■■■■■■□□□□□■□■■■■■□■■■	1	0.2
625	EAI5	■□□■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□■□■□□□□□■■■■	2	0.4
129	EAI5	■■■□□□□□□■■■■■■■■■■■■■□□■■■■□□□□□■□■■■■■■■■■	2	0.4
4	LAM3 & S/convergent	□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■□□□□■■■■■■■	3	0.6
92	X3	■■■□□□□□□□□□■■■■■□■■■■■■■■■■■■■■□□□□■■■■■■■	16	3
62	H1	■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■□■■■	1	0.2
49	H3	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■□■■■	1	0.2
50	H3	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	21	4.2
1533	H3	■■■■■■■■■■■■■□□■■■■■■■■■■■■■■■□■□□□□■■■■■■■	1	0.2
36	H3_T3	■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	1	0.2
54	MANU2	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□■■■■■■■■■	1	0.2
451	H37Rv	■■■■■■■■■■■■■■■■■■□□■■■■■■■■■■■□□□□■■■■■■■	1	0.2
784	T2-S	■■■■■■■■□□■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	1	0.2
29	Unknown	■■■■■□□□□□□□□□□□■■□□□□□□□□□□□□□□□□□□□■■■■■	1	0.2
32	Unknown	■■■■■■■■□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■	1	0.2
563	Unknown	■■■■■■■■■■■■□■■■■■■■■■■■□□□□□□□□□□□□□□□■■■■	1	0.2

Eight spoligotyping families formed the predominant group of the study. Of these eight families, Beijing SIT1 was the most predominant at 27% seconded by S SIT 34 at 8.4%. The LAM4 SIT 60 and T1 SIT 53 both represented 8.2%, LAM3 SIT 33 represented 6%, H3 SIT 50 represented 4.2% while EAI1- SOM SIT 48 and X3 SIT 92 both represented 3%. The Beijing family dominated in all susceptibility patterns while LAM 4 SIT 60 was well represented in XDR – TB (Figure 2.9). The H3 and EAI1-SOM were almost equally distributed in all sensitivity patterns while LAM 3 and T1 families formed good representation in susceptible isolates. The S family had over 40% of the MDR –TB isolates, zero XDR and drug-susceptible and mono-resistant patterns were equally distributed. The results of these eight predominant families are summarized in Table 2.3 and figure 2.7. There were no major differences in distribution of the 8 predominant families within the seven regions of Johannesburg (Table 2.4).

Table 2.3: Description of 8 predominant families (Clustered from 16 to 135 per Clade) from a total of 340 isolates.

SITn	Clade	Binary (Spoligotype) Description	Total strains
1	Beijing	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	135
34	S	■■■■■■■■□□■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	42
60	LAM4	■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■□■■■	41
53	T1	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	38
33	LAM3	■■■■■■■■□□□■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	30
50	H3	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	21
48	EAI1_SOM	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■□■■■	17
92	X3	■■■□□□□□□□□□■■■■■□■■■■■■■■■■■■■■□□□□■■■■■■■	16

The eight predominant families represented (340/500) 68% of the total 500 isolates. The percentage for each of the eight predominant families is presented in figure 2.7

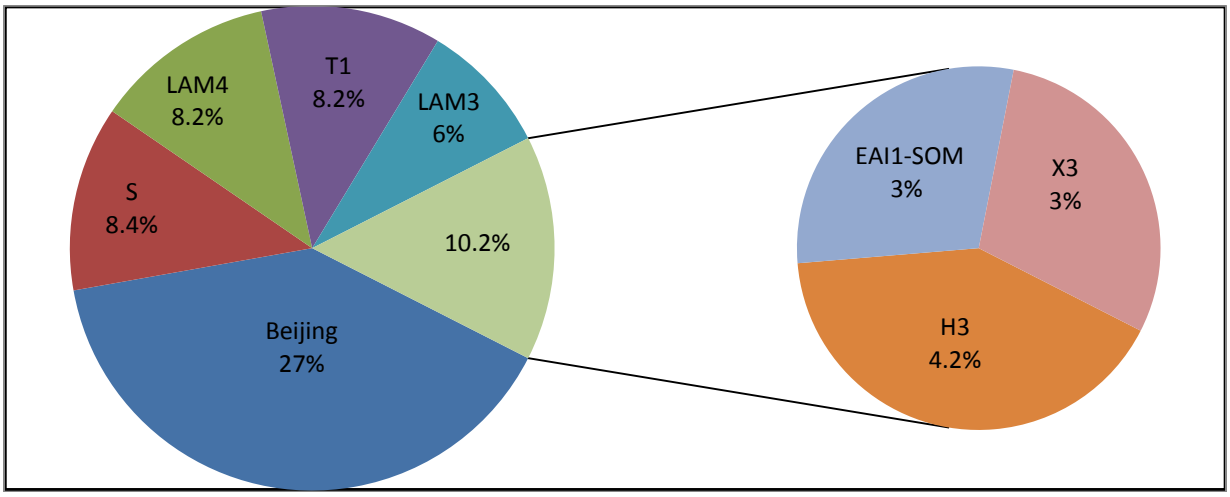


Figure 2.8: Percentage representation for the eight predominant families.

Table 2.4: Distribution of the 8 predominant families in seven regions (A-G) of Johannesburg

SIT	Clade	Region A	Region B	Region C	Region D	Region E	Region F	Region G
1	Beijing	21	20	14	37	19	22	2
34	S	7	10	6	11	3	4	1
60	LAM4	2	4	4	13	8	8	2
53	T1	8	4	5	11	3	7	0
33	LAM3	5	5	3	10	3	2	2
50	H3	0	4	3	5	5	4	0
48	EAI1_SOM	1	3	3	7	3	2	0
92	X3	2	2	2	5	0	5	0

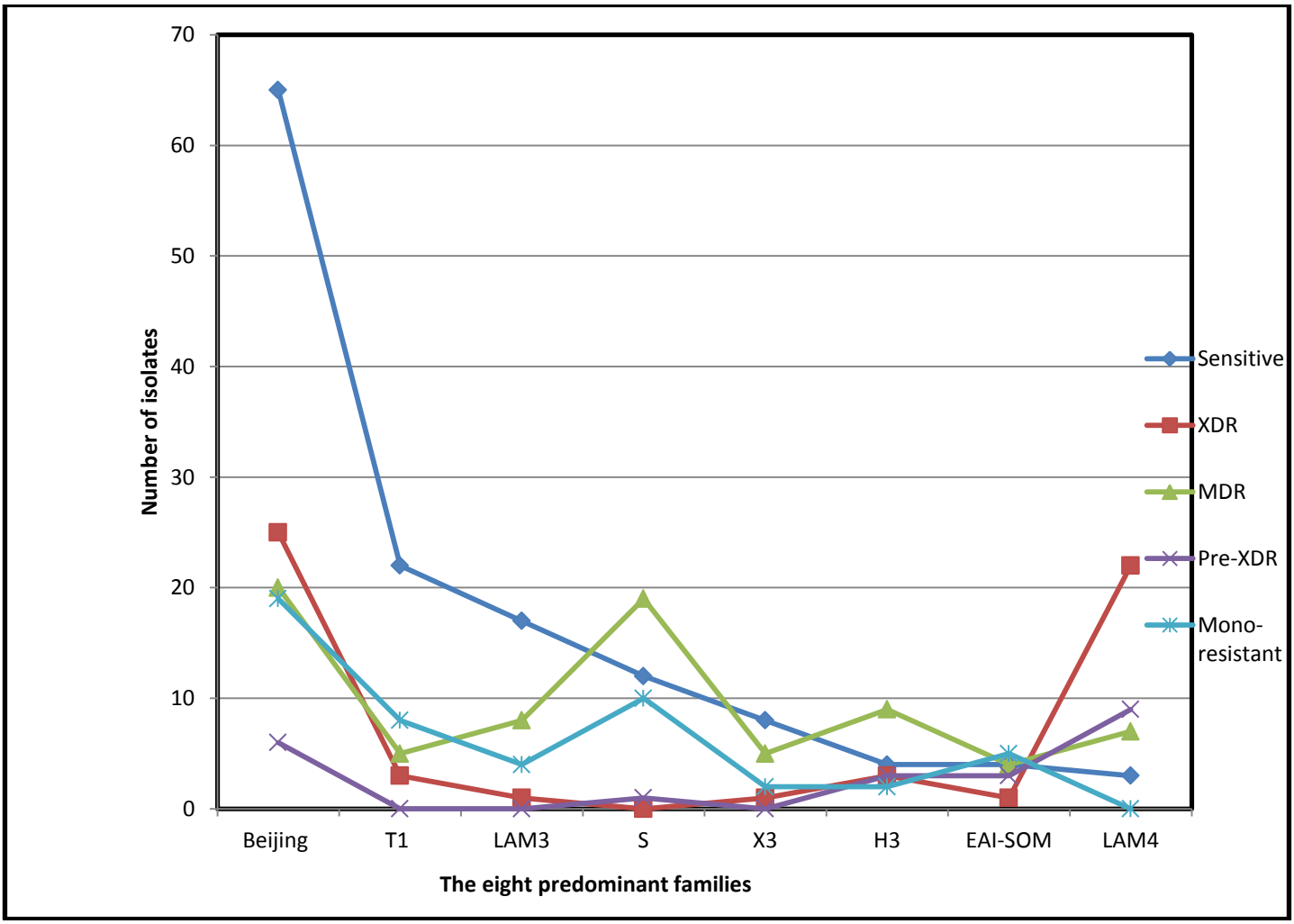


Figure 2.9: Distribution of the eight predominant families within the five susceptibility patterns

These eight predominant families were found to be greatly associated with drug-resistance with a p value of <0.001 when was statistically tested by chi-square test. Despite the fact that there were only eight predominant families, a number of small clustering (2 to 8) families were identified and the number of isolates for each family found in the study is presented in figure 2.9.

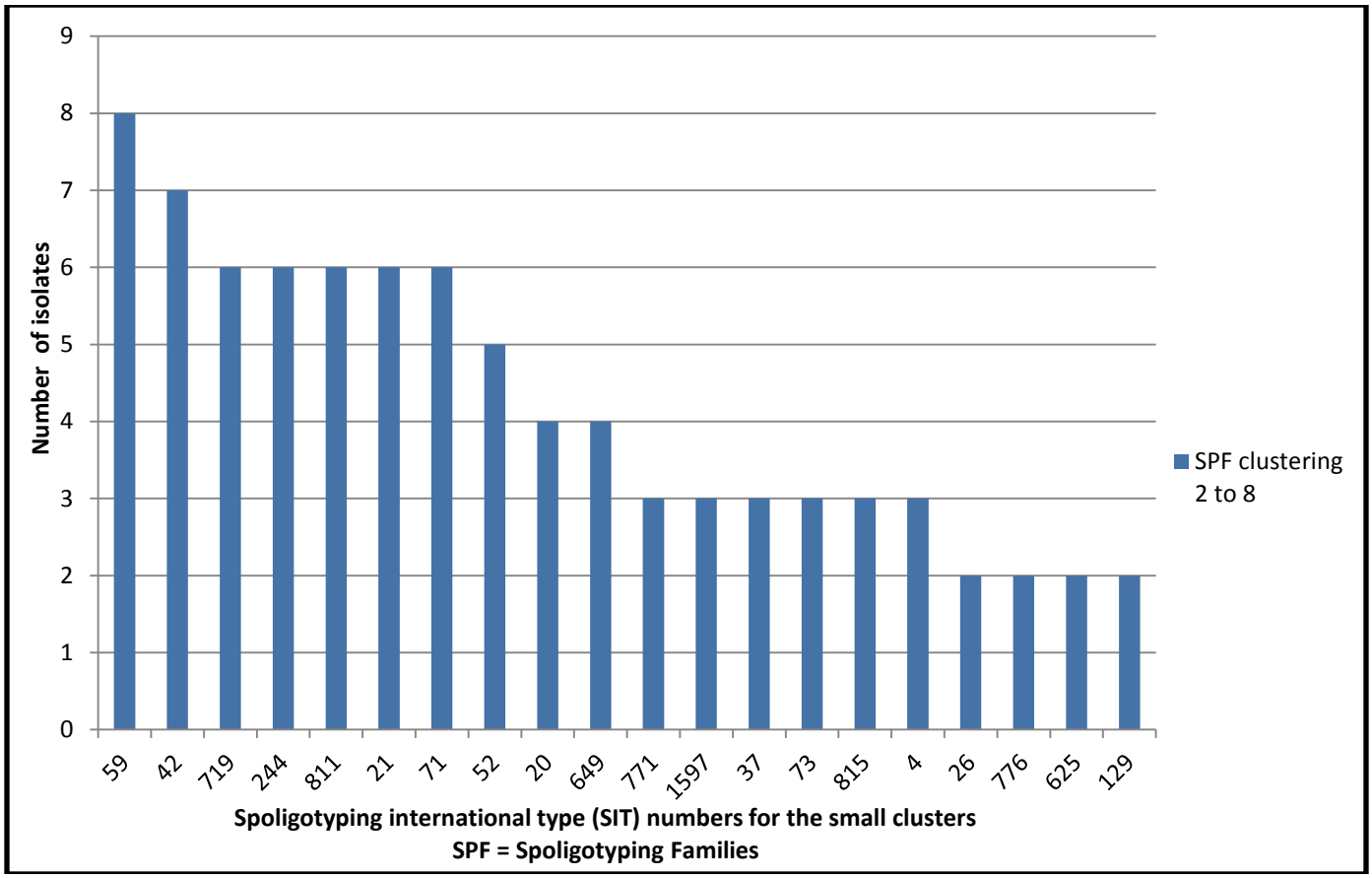


Figure 2.10: Distribution of the small clustering isolates

Table 2.5: Description of orphan isolates (**n = 42**).

Binary (Spoligotype) description	No. of isolates
■■■■■ □■■■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■■■■■	5
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■■■■■ ■■■■■■■■■■■■■□■■■□□□□■■□□□□■■□□□□■■■□■■■	2
■■□■■ ■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■	1
■■■■■ ■■■■■■■■■□□□□■□□□□□□■■■■■■■■■□□□□■■■■■■■	1
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■■■■■ ■■■■■■■■■■■■■■■■■□□□□■■□□□□□■□□□□■■■□■■■	1
■■■■□ □□□□□□□□□□□■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	1

Table 2.5: continues.

Binary (Spoligotype) Description	No. of Isolates
■□□□□ □□□□□□□□■■■■■□■■■■■■■■■■■■■■□□□□■■■■■■■	1
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2.6.1 Recent transmission index results

RTI_{n-1} = (n_c-c)/n (where n is the total number of cases in the sample, n_c is the total number of cases in cluster while c is the number of (cluster) genotypes represented by at least two cases) (Guernier et al. 2008). n = 500, n_c = 436, c = 31. Therefore: ([436 – 31]/500) X 100 = 81%. Based on the n-1 method as shown above and as explained in section **2.5.6.1**, the recent transmission index showed that 81% of the isolates were recently transmitted. Out of 81%, 62% ([340 - 31/500] x 100) represented the eight predominant families.

2.7 Discussion

This study presents the largest amount of molecular epidemiological data on MTB isolates from seven regions of Johannesburg and is the first study to have typed the isolates of MTB that have varying patterns of susceptibility, from fully-susceptible to INH/RIF-mono-resistant, MDR, pre-XDR and XDR. People from other regions of South Africa and other countries in Africa migrate to Johannesburg looking for work, and this migration may have an impact on the TB strains that are circulating in the area as some of the immigrants come from areas of high TB and drug-resistant TB prevalence such as KwaZulu-Natal (KZN) in South Africa, (Houlihan et al. 2010) and Nigeria (Lawson et al. 2012a).

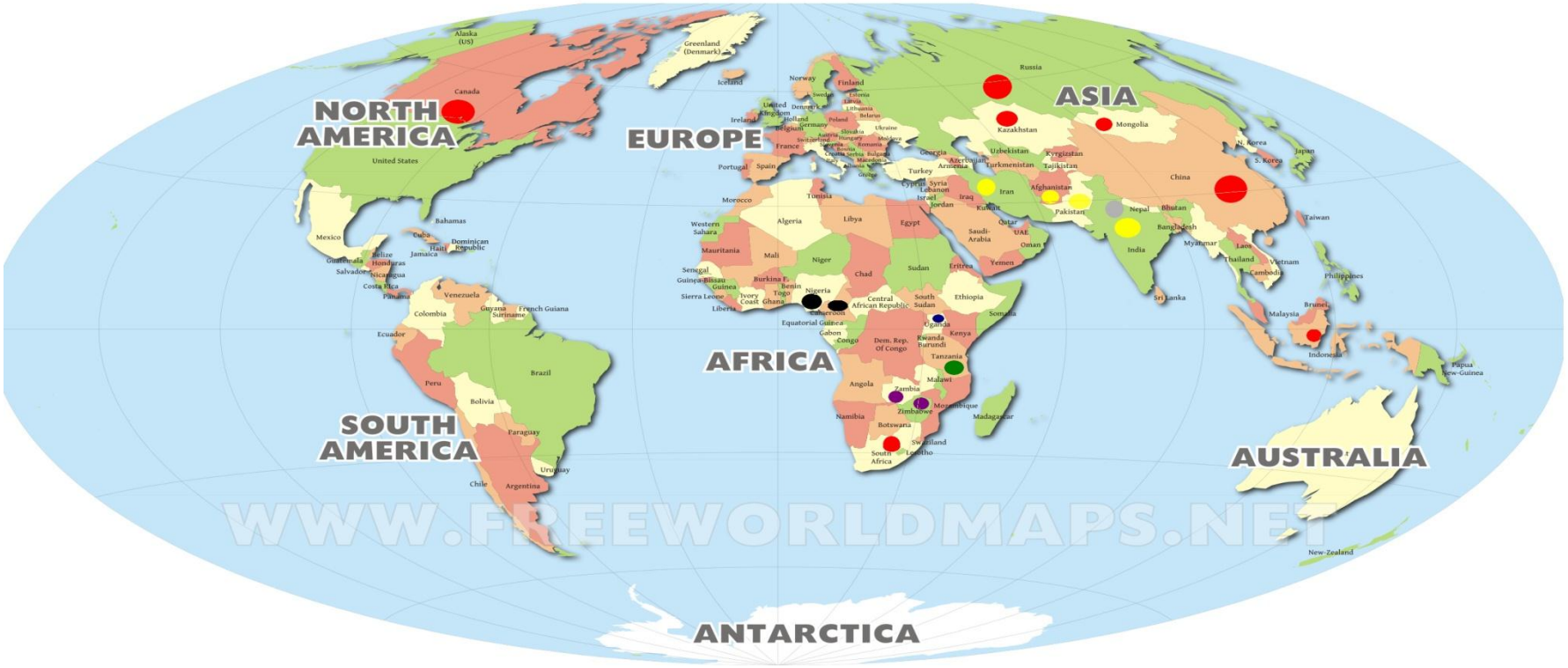
Previous studies based in Johannesburg, have documented a high prevalence of MDR-TB in children (Fairlie et al. 2011) as well as a high prevalence of TB/HIV co-infection in adults (John et al. 2007). There is a need for information on the molecular epidemiology of MTB strains that are circulating in Johannesburg to determine which of them are associated with the high prevalence of drug resistant MTB and HIV. The findings would assist in tracing the source of infection and allow appropriate preventative measures that could potentially minimize or control the spread of the disease. We thus ought to describe the genetic diversity of 500 MTB isolates by spoligotyping.

Genotyping of the 500 isolates by spoligotyping revealed a wide genetic diversity. Comparison of the spoligotyping results with the SPolDB4 showed that the majority 92% (n=458) of the isolates belonged to 59 previously described shared types while 8% (n=42) were not found in the SPolDB4 database and were termed orphans. Of the 42 orphan isolates, 10 isolates were clustered into 3 groups (ranging 2 to 5) while 32 isolates were unique orphans. The 59 previously described shared types and the 3 shared types from orphan isolates formed 62 shared types.

Of these 62 shared types, 31 shared types were clustered representing a cluster rate of 93%. Eight of the 62 clustered shared types (clustered from 16 to 135) constituted 68% (n=340/500) of the total number of isolates spoligotyped; these, were by far the most prevalent and they were comprised of Beijing (SIT 1), S (SIT 34), LAM4 (SIT 60), T1 (SIT 53), LAM3 (SIT 33), H3 (SIT 50), EAI1_SOM (SIT 48) and X3 (SIT 92). The same predominant families were also identified in a genotypic study conducted in eight of the nine provinces from South Africa in 2009 (Stavrum et al. 2009b).

The origin of some of the eight predominant shared types is known while others are uncertain (figure 2.9). The Beijing family is considered to have originated from China where they were first identified in 1995 and were termed Beijing because the vast majority of these Chinese strains originated from TB patients residing in Beijing (van Soolingen et al. 1995; Wan et al. 2011). Other studies have indicated that the Beijing genotype was already highly prevalent in areas surrounding Beijing from 1956 to 1960 and that these strains disseminated to the neighbouring Asian countries such as Mongolia, Thailand, South Korea and Vietnam, and later

to other countries (Toungoussova et al. 2002). The Haarlem family (SIT 50) is thought to have originated from Central America and the Caribbean and provides a possible link to the post-Columbus European colonization. The T family remains an ill-defined family of MTB and although found worldwide was thought, to be more specific to Russia. The X type is thought to have originated from the UK and the USA (Brudey et al. 2006). The LAM 3 (SIT 33) and EAI1-SOM (SIT 48) were first isolated in South Africa and Somalia respectively (Abadía et al. 2009; Said et al. 2012). The S (SIT 34) lack specificity, but LAM 4 (SIT 60) is frequently regarded as a South African strain since it was responsible for the TB outbreak in Tugela Ferry from KZN (Pillay & Sturm, 2007). This strain is a member of Euro-American family and was first described in this region in 1994 (Gandhi et al. 2013)



Beijing family
Showing areas where it is more prevalent

Isolates which were not identified in this study but have caused major outbreaks in other African countries

LAM10 – Cameroon

T2 – Uganda

SIT families which did not dominate in this study but have caused major outbreaks in other African countries and outside Africa

LAM11 – ZWE

CAS1 – DELHI

CAS1 – KILI

CAS

Figure 2.11 Demonstrates differences in geographical distribution of spoligotyping families. Sources; (Demay et al. 2012; Easterbrook et al. 2004; Mulenga et al. 2010; Ani et al. 2010; Godreuil et al. 2007; Bazira et al. 2010)

This study reveals that Beijing was the most predominant family in Johannesburg representing 27% of all the isolates, and 40% of the eight predominant shared types. The Beijing family was further well represented in all seven regions of Johannesburg with minor variations in numbers. This family has also been identified in other parts of South Africa, Africa, Asia, Europe and South America (Said et al. 2012; Viegas et al. 2010; Abadía et al. 2009; Pang et al. 2012; Liu et al. 2011). In a study conducted in Cape Town, the Beijing family was well represented. Of the 291 isolates obtained from children, 23% were of Beijing (SIT 1) genotype making it the most predominant family and its prevalence was noted to have increased from 13% in 2002 to 32.8% in 2003 (Cowley et al. 2008; Mlambo et al. 2008) indicating that this family is fast emerging. In another study conducted in the northern region of South Africa, the Beijing family was found to dominate in both North-West and Limpopo provinces (Said et al. 2012a). Similarly, in a study conducted in Pretoria, 28% of the study isolates belonged to the Beijing family, and formed the largest group (Hove et al. 2012). This indicates that the Beijing family is well represented in South Africa although it may not necessarily form a major group in all of the provinces (Stavrum et al. 2009b).

The high proportion of Beijing strains in Johannesburg is a worrisome situation since there are numerous studies that have linked this family to rapid transmission of MTB (Cowley et al. 2008) and to drug-resistant TB (Judith et al. 2006; Johnson et al. 2010; Ghebremichael et al. 2010; Kubica et al. 2005; Toungoussova et al. 2002; Kisa et al. 2012). In this study, 48% of all Beijing isolates were drug susceptible, 14% were mono-resistant to either RIF or INH, 15% were MDR, 4% were pre-XDR and 19% were XDR. This shows that approximately 55% of the Beijing

isolates were associated with drug-resistance and we may be tempted to link this family to drug-resistance. In a study conducted in Cape Town a similar association was found, with 18% of the Beijing isolates being drug-resistant (Marais et al. 2006). In South Africa in 2008, 34% of XDR-TB isolates were found to be the Beijing family and formed the largest group within the XDR-TB isolates described (Mlambo et al. 2008). Despite the majority of studies showing an association of the Beijing family to drug-resistance, there are some studies which fail to confirm this (Alonso et al. 2010; Toungoussova et al. 2003) and in a recent South African study not even a single Beijing isolate was found to be drug-resistant (Pillay & Sturm, 2010).

Despite the Beijing family being well represented in all susceptibility patterns, the proportion of LAM (LAM 4) in pre-XDR and XDR-TB isolates showed an interesting trend. Ninety three percent of LAM 4 isolates were associated with drug-resistance (17% were MDR, 22% were pre-XDR and 54% were XDR isolates), while only 7% were fully susceptible and no mono-resistant isolates were found. The spoligotype pattern of the LAM 4 isolates in this study is identical to that of the pattern described in the F15/LAM4/KZN strain (Pillay & Sturm 2007; E M Streicher et al. 2007) and shows a lack of spacers 21 – 24, 33 – 36, and 40.

The LAM 4 (SIT 60) (F15/LAM4/KZN) family, has been associated with drug-resistance in the KZN province of South Africa where it is believed to have been in circulation since 1994 (Pillay & Sturm 2007). In a recent study from KZN, 83% of TB reinfection was due to LAM4 (Pillay & Sturm, 2007). Furthermore, this family was found to significantly contribute to the nosocomial infection in both HIV-positive and negative patients (Pillay & Sturm, 2010) and was responsible for the outbreak of XDR-TB in KZN where 98% of the patients, died. (Gandhi et al. 2006).

The circulation of S, T1 and LAM3 family strains in this study was not surprising since some of these families have been found dominating in other studies done in South Africa. Their presence in Johannesburg is likely as a result of movement of people from different regions. The S family was responsible for the exogenous reinfection for 63% of the patients with MDR-TB from a South African rural community (Andrews et al. 2008). The S family formed the second most predominant family in this study (8%), after the Beijing family. The association of this family with drug-resistance as suggested by Stavrum et al. 2009 was supported by the findings of our study where 71% of the isolates belonging to this family were of drug-resistant. Of these, 63% were MDR. It was found that no isolate belonging to the S family was XDR indicating that the S family is less associated with XDR. Our findings are in line with the study done on XDR-TB patients from four provinces (Limpopo, North West, Northern Cape and Gauteng) of South Africa where none of the S family was found to be XDR (Mlambo et al. 2008). In contrast only one isolate of S family was found to be XDR in a recent study done in the in the Eastern Cape (Klopper et al. 2013). There is a high possibility that 63% of the S family which were MDR might become XDR. Therefore, we cannot rule out this family from becoming one of the families contributing to the transmission of XDR-TB in Johannesburg in the near future.

It seems likely that the S family is widely spread throughout South Africa as it was also found to be one of the predominant families in a study from the Northern Province of South Africa (Said et al. 2012a). Similarly, the T1 family seems to be prevalent throughout South Africa especially within the Gauteng province (Stavrum et al. 2009; Hove et al. 2012). Hence circulation of T family in Johannesburg is not surprising.

We detected the presence of LAM 3 (SIT 33) which is likely to be the same as F11 which was first described in Cape Town. Both, LAM3 (SIT 33) and F11 are characterized by lack of spacers 9 to 11, 21 to 24 and 33 to 36 which appear to be a unique marker for this strain (Streicher et al. 2007; Victor et al. 2004). The LAM3 (SIT 33) family was first described in South Africa in the Western Cape where it has shown to be a successful strain contributing to the TB epidemic (Victor et al. 2004). Currently, the LAM 3 family seems to be widely spread in South Africa as evidenced through several studies (Hove et al. 2012; Klopper et al. 2013; Said et al. 2012), and it has also been largely associated with drug-resistance (Streicher et al. 2004; Victor et al. 2004). Our findings could not support the association of LAM 3 (SIT 33) with drug-resistance as 13%, 27% and 3% were mono-resistant, MDR and XDR respectively, while 57% were drug-susceptible.

Despite 23 families (designated as small clusters ranging from 2 to 8 isolates) being not well represented (they formed 17% of the total isolates in this study), they were families which have been described in TB epidemics in other African countries. Examples include the LAM11-ZWE (SIT 59) which is interchangeably called the Southern Africa 1 (SAF1) strain (Eldholm et al. 2006) and the CASI-Kili (SIT 21) strain. The LAM11-ZWE is known to contribute to the TB epidemic mainly in Zimbabwe where it was first described and in Zambia. From a study done in Zambia, a total of 22% of isolates belonged to the LAM11-ZWE SIT 59 family (Mulenga et al. 2010). The same isolate was found circulating in a study performed in Zimbabwe (Easterbrook et al. 2004). The circulation of this isolate in both Zimbabwe and Zambia can be due to easy migration of people between these countries. South Africa is a popular destination for migrant Zimbabweans. However, the scarcity of the LAM11-ZWE family amongst isolates evaluated here could be due to the time needed before transmission can become well established. The CASI-Kili family which was not well represented in this study, is more prevalent in Tanzania as demonstrated in the two studies (Kibiki et al. 2007; Eldholm et al. 2006).

The orphan strains were compared with other orphans found in different studies. Four isolates in our study matched orphan isolates found in Zambia (Mulenga et al. 2010) and three of these clustered together. The remaining thirty eight orphan isolates did not match any isolate from other studies. Further comparison of these orphan strains with other studies is necessary in order to establish whether these isolates are specific to Johannesburg or South Africa.

Studies of the same nature have been conducted in different African countries including South Africa's neibouring countries. These studies have demonstrated that different geographical locations are dominated by different strains which are responsible for the TB epidemic in that particular area. From a study done in Mozambique, the LAM9 and Beijing families were the most predominant genotype (Viegas et al. 2010). Similarly, in Zimbabwe, the LAM11-ZWE family was the most predominant (Easterbrook et al. 2004); this was also found in a study in Zambia (Mulenga et al. 2010). However, CASI-Kili and LAM11-ZWE families dominated in a study conducted in Tanzania (Kibiki et al. 2007). The geographical specificity of MTB strains (Figure 2.10) has also been demonstrated from other studies done from Nigeria, Cameroon, Chad and Burkina Faso where LAM10-CAM is the most predominant family (Ani et al. 2010; Kamgue et al. 2013; Diguimbaye et al. 2006; Lawson et al. 2012b; Godreuil et al. 2007).

Some of the predominant genotypes which we found in our study, such as the Beijing and LAM 4 (SIT 60) families were not predominant in other African countries. It was noted that in all of the above mentioned studies done in Zimbabwe, Zambia, Tanzania, Uganda, Nigeria, Cameroon and Chad, the LAM4 family was not identified. It was detected in a study performed in Mozambique (Viegas et al. 2010). We may be tempted to say LAM4 (SIT 60) is not present in these countries but rather that this family is more prevalent in South Africa and particularly KZN and Johannesburg. Despite the Beijing family's wide spread, it does not seem to be predominant in many other African countries (Ghebremichael et al. 2010).

Our study did not identify some of the families which are dominating in other African countries such as LAM 10 (The Cameroon type) that dominates in Cameroon and Nigeria (Ani et al. 2010; Kamgue et al. 2013) and in other West African countries including Benin, Senegal and Ivory Coast (Godreuil et al. 2007). Our study also did not detect the most predominant family in Uganda, the T2-Uganda family (Bazira et al. 2010). The geographical specificity of these strains probably indicate that the difference in geographical location have an impact on the epidemiology of TB. It may be suggested that adaptation of particular lineages of MTB to human populations or geographical areas are different. It is thus suggested that local strains are more likely to transmit in a given local setting than compared to others (Bazira et al. 2010).

It is a surprising that the Cameroon strain (LAM 10) which dominates in Nigeria was not found in this study since Johannesburg accommodates a large number of Cameroonians and Nigerians. The explanation could be that, these individuals, as foreigners, might prefer to seek health services in private settings which do not necessarily refer specimens to the NHLS TB referral Laboratory and as a result, they did not form part of this study.

A high cluster rate of 93% was found in this area indicating that these spoligotype families greatly contribute to the transmission of TB in Johannesburg and that there is an ongoing transmission of TB taking place in this area. However, this has to be interpreted with caution since spoligotyping used alone is less discriminatory than when used in combination with other genotyping methods such as RFLP IS*6110* and Mycobacterial Interspaced Repetitive Unit

(MIRU) (Kisa et al. 2012) even though some studies have demonstrated a strong correlation between results of Spoligotyping, MIRU and RFLP IS*6110* genotyping results (Dou et al. 2008; Toungoussova et al. 2002).

The study has shown a wide genetic diversity of strains circulating in the Johannesburg area. This could indicate that the incidence of TB in Johannesburg is not due to clonal transmission but rather that there is a wide range of strains contributing to TB transmission in the area. All strains were well represented in all seven regions, with minor variation in numbers and as a result there was no strain found to be specific to a particular area.

Despite that the effort was made to have good sample selection criteria and a well represented study sample number, we still had some limitations. Firstly, we couldn't be able to select equal number of isolates from each region. This was due to the fact that other regions had few isolates per sensitivity pattern which qualified for the study. Never the rest, results were well represented. Secondly, we could not be able to justify if all patients entered in this study indeed got infected during their stay in Johannesburg to know if the transmission index we are having is a true representation of what is happen in the Great Johannesburg Metropolitan Area. Thirdly, we could not be able to link patients who had identical genotypes despite having demographic data. There was no actual visit of the patients to gain more information i.e. where he/she has lived before, history of hospitalization, etc in order to establish transmission routes. This was a big challenge as it could have formed another big study requiring additional financial support and extending study duration. We propose that in future, a similar kind of study should be conducted.

Chapter three: DNA Fingerprinting of *Mycobacterium tuberculosis* through IS*6110* RFLP

3.1 Introduction

Mycobacterium tuberculosis has challenged medical researchers for decades by their failing to completely eradicate this disease as it continues to kill millions of people each year. The disease has been more extensively studied than any other infection caused by a bacterium. Despite all the effort, there is little sign and hope that the disease will soon end. The situation is more complicated with the emergence of Multidrug-resistant tuberculosis (MDR-TB) and the extensively drug-resistant tuberculosis (XDR-TB) (Brudey et al. 2006). In depth understanding of TB transmission dynamics, source and spread is need and this can be essential to control or stop the spread of the disease. Molecular epidemiological studies for TB are giving more hope than traditional studies to shade light on disease dynamics (Wang et al. 2012).

The IS*6110* RFLP fingerprinting was included in the study to confirm the relatedness of a subset of clustered isolates by spoligotyping. We also wanted to see the correlation of results between the two methods; IS*6110* RFLP and spoligotyping. Only a subset of isolates were fingerprinted because this was the first study to setup RFLP procedure in the department as a result a lot of challenges were met. This made it impossible to include a large sample size.

Various DNA fingerprinting methods differentiating strains belonging to the *Mycobacterium tuberculosis* (MTBC) have been developed after the discovery of the transposable elements and other repetitive DNA elements in MTBC in the early 1990s. Some of these are PCR based which is achieved by the amplification of repetitive DNA sequences, while other methods are based on the visualization of the restriction fragments containing particular repetitive DNA elements such as insertion sequence (IS) elements (Friedman et al. 1995; Plikaytis et al. 1993; van Soolingen et al. 1994; Wiid et al. 1994). A comparison of these two methods, the spoligotyping and Restriction Fragment Length Polymorphism (RFLP) typing methods, showed that IS*6110* RFLP has a high discriminatory power and is reproducible (Wiid et al. 1994; Narayanan 2004).

The international standardization of the RFLP method with regard to the choice of restriction enzyme, probe, and size markers has enabled interlaboratory comparison through computerization of DNA fingerprints (van Embden, 1993). The presence of the IS*6110* element in different copy numbers, integrated at various chromosomal sites and the molecular weights of the DNA fragments, provides enough information regarding transmission dynamics of MTB (Kim et al. 2010). It is because of this high discriminatory power that the IS*6110* RFLP has been applied in different geographical locations for the management of MDR-TB and in population based molecular epidemiological studies (Edlin et al. 1992) . With several studies done worldwide, the molecular epidemiology of MTBC through IS*6110* RFLP provided insight into risk factors for the transmission of TB and the spread of particular MTB genotypes families in different regions (Small et al. 1994; Alland et al. 1994; Yang et al. 1995; Pfyffer et al. 1998).

3.1.1 General overview of the IS*6110*

The IS*6110* is a genomic insertion element containing 1,361 bp that is found only in species within the organisms *Mycobacterium tuberculosis* complex (MTBC) (Kim et al. 2010). The IS*6110*, IS986 and IS987 limited to mycobacterial species belonging to the MTBC, have been characterized and sequence analysis has revealed that they differ in only a few base pair. However, they are considered to be virtually identical (van Embden et al. 1993; Hermans et al. 1991; Thierry et al. 1990). The copy number of the IS*6110* element varies between the members of the MTBC. While the *M. bovis* BCG genome contains one or two copies of IS*6110* (van Soolingen et al. 1991; Hermans et al. 1991), the genome of *Mycobacterium tuberculosis* has between 0 and 25 copies of this element (Kim et al. 2010). The sequence of the element designated IS*6110*, was first reported by Thierry et al. in 1990 and was shown to be related to the IS3 family of insertion sequences which were discovered in members of the family Enterobacteriaceae.

The structural organization of the 1,361 bp long has 28 bp of imperfect inverted repeats (IRs) and duplications that are 3 or 4 bp in length next to the insertion site. It has two partially overlapping reading frames (ORFs), *orfA* and *orfB*, that encode a transposase allowing the insertion of IS*6110* at multiple sites (figure 3.1) (Kim et al. 2010). The ORFs (orfA and orfB) of IS*6110* are similar to the ORFs (*orfA* and *orfB*) of IS3 found in prokaryotes (Mendiola et al. 1992). The physical map of the IS*6110* sequence (figure 3.1) indicates that various restriction enzymes; (*BamHI*, *SstII*, *PstI*, *BstEII*, *BssHII*, and *PvuII*) cleave within the 1,361-bp element.

The *PvuII* enzyme has successfully been used in RFLP and is considered to be the standard enzyme used in the majority of laboratories performing RFLP as it cleaves the IS*6110* sequence only once. The *PvuII* digestion of IS*6110*-containing genomic DNA leads to IS*6110*-hybridization fragments of at least 0.90 or 0.46 kb, depending on the IS*6110* probe that is used (van Embden et al. 1993).

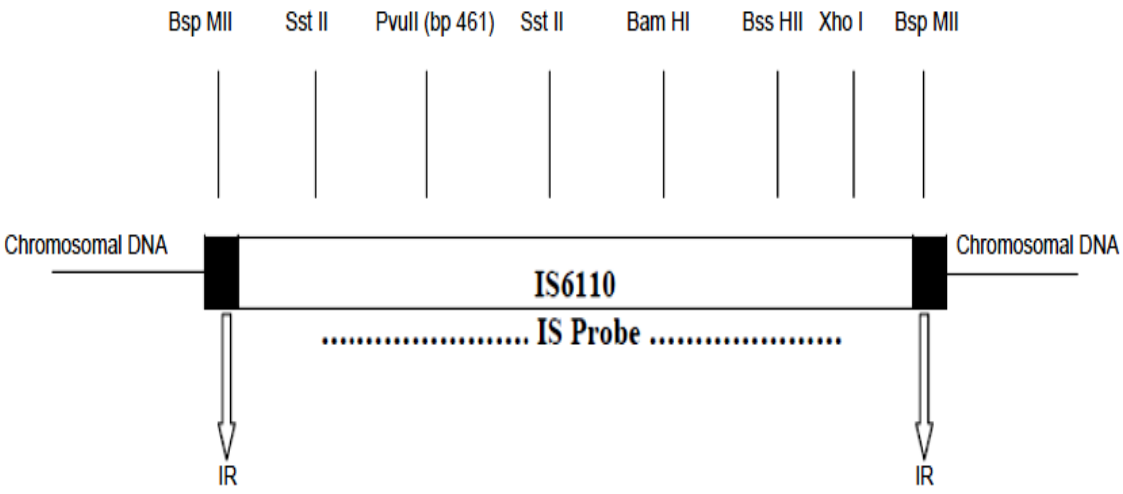


Figure 3.1: Physical map of the 1.35-kb M. tuberculosis insertion element IS6110. The cleavage sites of several restriction enzymes are depicted. PvuII cleaves the element at base pair 461. Therefore, any chromosomal mycobacterial DNA fragment obtained by the recommended standard typing method is larger than 0.9 kb. The closed bars represent the 28-bp inverted repeats (IR) bordering IS6110 DNA. Chromosomal DNA is represented by the lines to the left and right

Adapted from van Embden et al. 1993.

Although the IS*6110* is a transposable element, with the potential to translocate within the chromosome, it is stable enough to provide identical restriction fragments for mycobacterial strains from within small clusters of infection (Yuen et al. 1993) and several studies have demonstrated the stability of this element (Hermans et al. 1991; Mathema et al. 2006b). Apart from being used as a molecular epidemiological marker, the IS*6110* is considered to have different functions, including the activation of genes during infection (Safi et al. 2004; van Embden et al. 1993; Soto et al. 2004). Finally, it has been suggested that the presence of IS*6110* in *M.bovis* could participate in the adaptation of this bacteria to a particular host, either animal or human (Otal et al. 2008).

Despite IS*6110* RFLP typing being considered the gold standard for characterizing the molecular epidemiology of MTBC, the method is technically demanding and requires 4-8 weeks to culture isolates in order to obtain pure and sufficient DNA. In addition, it provides insufficient discriminatory power particularly for isolates with less than 6 copy numbers of IS*6110*. Although this method provides excellent differentiation, it requires specialized software for analysis of the data and results in a relatively long turnaround times for reporting of the results. (Kehinde, 2012; Narayanan, 2004).

The epidemiological analysis of MTB by IS*6110* DNA fingerprinting is based on the observation that the polymorphism of the IS*6110* RFLP patterns among unrelated clinical isolates is very high, whereas epidemiologically related MTB strains show identical or similar (one band variation) fingerprint patterns. Hence, MTB isolates with identical fingerprint patterns represent strains that have possibly been recently transmitted, and these strains are likely to involved in a chain of transmission (Niemann et al. 1999)

The purpose of this RFLP portion of this study was to fingerprint a subset of the related MTB isolates previously found through spoligotyping to confirm the isolate relatedness and categorize them according to their respective families. Furthermore to correlate the IS*6110* RFLP fingerprinting results with the spoligotyping results and to establish transmission routes through clustered predominant clones circulating in the great Johannesburg metropolitan area.

3.2 Materials and Methods

3.2.1 Sample identification and selection

Sample identification and selection was done as previously described. Please refer to chapter 2, sections 2.2.3.1 and 2.2.4.2

3.2.2 Sub-culturing of Mycobacterium tuberculosis on Löwenstain Jensen medium

Seven drops (50µl) of each isolate from a MGIT vial was inoculated onto Löwenstain Jensen slants using a sterile 1 ml Pasteur pipettes. The slants were left facing upwards and the lids slightly loose and incubated (Labcon incubator, Laboratory marketing services, RSA) at 37°C for 24 hours, after which the lids were kept tightly closed. The LJ slants were aerated after every 2 days for 2 weeks, two times a week during the third and fourth week, and once a week from the fifth week to the eighth week depending on the growth observed. Growth was indicated by formation of granular white/cream colonies on the slants.

3.2.3 Isolation of genomic DNA

3.2.3.1 Extraction

The LJ slant cultures were heat-inactivated at 80˚C for 1 hour in a Biological safety class II cabinet before DNA extraction was performed. The heat-inactivated growth from a slant culture was scraped off and suspended in 1.5 ml of distilled water thereafter it was transferred into a sterile 2ml conical screw cap microtube which contained three 5 mm glass beads. The bacterial clumps were broken up by vigorous vortexing of the tube for approximately 2 minutes. The tube was then centrifuged (Eppendorf, Hamburg, Germany) at a speed of 7000 xg for 5 minutes and the supernatant removed. Cell lysis was achieved by resuspending the cell pellet in 400 ul TE buffer (5ml 1M Tris-Cl, pH 8.0 and 1ml 0.5M EDTA) into which 50 ul of Lysozyme [10mg/ml (0.5g Lysozyme plus 50ml 10mM Tris-Cl, pH, 8.0)] was added and gently mixed by inverting the tube several times before overnight incubation (MWG-Biotech Mini Oven MKII) at 37˚C.

3.2.3.2 Purification of DNA

After overnight incubation, purification and precipitation of the DNA was performed. Seventy five microliters of SDS/Proteinase K (10%SDS plus 10mg/ml of proteinase K. 14:1 ratio respectively) was added into each tube containing the cells. The contents were mixed by gentle inversion and then incubated at 65˚C for 1 hour. After incubation, 100 µl 5M NaCl and 100 µl CTAB/NaCL (pre-warmed to 70˚C) was added to each tube. The tubes were vortexed for 10 seconds until the solution became milky, and then incubation for 20 minutes at 65˚C. It was then allowed to cool at room temperature for 5 minutes before the addition of 750 µl Chloroform-

Isoamyl alcohol (24:1 ratio respectively to remove CTAB-protein/polysaccharide complexes). The tubes were thoroughly mixed by inversion followed by centrifugation at 7000 xg for 15 minutes. The upper/aqueous phase containing the DNA was transferred into a new sterile conical screw cap microtube.

Precipitation of the DNA was achieved by adding 500 µl of ice-cold isopropanol into each tube. This was carefully mixed by inverting and DNA precipitation could be seen at this stage before freezing at - 80˚C for 30 minutes.

After the freezing process, the precipitated DNA was left at room temperature for 30 minutes before being centrifuged for 15 minutes at 7000 xg (Eppendorf, Hamburg, Germany). The supernatant was gently removed by pipetting. A volume of 1ml of cold 70% ethanol was added and centrifuged at 7000 xg for 15 minutes. The supernatant was again carefully removed with a sterile Pasteur pipette. The pellet was left to dry at room temperature for 4 hours. Finally, the DNA was dissolved in 50 µl 1 X TE buffer at 37˚C for 2 to 3 three hours. The quality and quantity of DNA was determined spectrophotometrically (Nanodrop 2000 Spectrophotometer) and by running the DNA on 1% (TBE buffer) agarose gel.

3.2.4 Digestion of genomic DNA

RFLP was performed using the standardized IS*6110* technique (van Embden et al. 1993). The extracted genomic DNA was digested with *PvuII* in a reaction mixture consisting of 6 µg DNA, 10 µl 10X buffer (Fermentas, Life Sciences), 3 µl (30units) *PvuII* enzyme (Fermentas, Life Sciences) and made up to 100µl volume with distilled water. The restriction mixture was

incubated overnight (16 hrs) at 37˚C. The *PvuII* enzyme activity was inactivated by incubating the digested sample at 65˚C for 15 minutes.

An aliquot of 10 ụl of *PvuII* digest products mixed with 4 ụl 6 X loading buffer, was run on 1% (w/v) Seakem® ME agarose gel (BioWhittaker molecular applications, USA) at 30 Volts in 1 X TBE (g/l 21.6g Tris, 11 g Boric acid, 1.5 g EDTA, pH 8.3) buffer for (16hrs) overnight to test for the completion of digestion. The gel was stained with ethidium bromide solution (50 ụl of 10 mg/ml of ethidium bromide, 500 ml of 1 X TBE buffer) and DNA pictures were visualised using the BIO-RAD Gel documentation system (BIO-RAD, USA).

3.2.5 Sample preparation for the final gel and electrophoresis

The *PvuII* digested DNA was neutralized and precipitated by adding 10ụl 3M Sodium acetate and 330ụl ice cold 100% ethanol to the remaining 90 ụl of the *PvuII* digested product and incubated for overnight at -20˚C. Samples were centrifuged at 4˚C using refrigerated centrifuge (Hettich Zentrifugen, Universal 16R, 31603-M13-01, Germany) at a speed of 10 000 xg for 30 minutes. The supernatant was aspirated 500 ụl of 70% ice cold ethanol was added to the DNA pellet. This followed another centrifugation and aspiration of the supernatant step. The DNA pellet was air dried overnight. Re-dissolving of DNA was achieved by adding loading buffer containing Internal Molecular Weight Marker (IMWM). The amount of buffer to add into each sample was calculated based on the intensity of DNA seen in the test gel. A 0.8% Seakem® ME agarose gel (20 cm^2) was prepared and loaded with 15ul of the re-dissolved DNA. The reference strain (Mt 14323) was used as external marker which was loaded in the first well and middle well of the gel. Fragments of DNA were separated by gel electrophoresis in 1 X TBE buffer at

30 V for 18 hrs. Denaturing of DNA in the gel was achieved by agitating the gel in 500 mL depurination solution (0.25N HCL) for 10 minutes, then in 500 mL of denaturing solution (0.4N NaOH / 0.6M NaCL) for 40 minutes and finally in 500 mL of neutralization buffer (1.5M NaCL / 0.5M Tris pH 7.5) for 30 minutes. The gel was rinsed with distilled water between each step. The gel was stained with ethidium bromide and visualized as mentioned in section 3.2.3.

3.2.6 Southern transfer of the DNA

Transfer of the DNA fragments from the gel onto a Hybond-N^+ membrane (Amersham Life Science) was achieved through the Southern transfer technique. Whatman 3MM paper pre-soaked in 20X SSPE was set in a clean plastic tray where the inverted agarose gel was placed on with edges as well as the wells removed with a sterile surgical blade. Air bubbles were removed by rolling a 10 mL serological pipette over the gel. Orientation of the membrane was achieved by spotting it with an orientation marker (3 µl of the Mt 14323 strain, 3 µl of IMWM, 45 µl of 0.8% of NaOH) on the top and bottom of the membrane. The membrane was hydrated in distilled water before being placed on top of the gel. Two layers of Whatman 3MM blotting paper, pre-wetted in 20X SSPE (3M NaCL, 0.2 M NaH_2PO_4, 20mM EDTA) pH 7.4 were placed onto the Hybond-N^+ membrane and again air bubbles were removed. To ensure fluid flow through the agarose gel, folded paper towels were stacked onto the Whatman 3MM papers. The contact was enhanced by the addition of a glass plate and a 1 kg weight. The blotting tray was filled with 20X SSPE (pH 7.4) with the first layer of blotting papers three-quarter immersed in the transferring solution. The Southern transfer was run for at least 16 hours. After transfer, the Hybond-N^+

membrane was washed in 2X SSPE for 10 minutes thereafter it was baked at 80˚C (between 2 sheets of Whatman 3MM blotting paper) for 2 hours to fixed the DNA to the membrane.

3.2.7 Preparation of the IS*6110* Probe

Two microliters of a (Mt 14323) DNA probe was used as template for amplification of 245-bp fragment. The reaction was carried out in 50 ul volume consisting of 25 ul PCR master mix (Fermentas-Life Sciences), 5 ul of forward primer (INS 1, 5´ CGTGAGGGCATCGAGGTGGC), 5ul of reverse primer (INS 2, 5´GCGTAGGCGTCGGTGACAAA), and 13 ul of distilled water. Thermal cycling (Bio-rad, iCycler Thermal Cycler, USA) were as follows: 94˚C for 5 minutes (denaturing) followed by 30 cycles of 94˚C for 30 seconds, and 65˚C for 30 seconds. A final extension step at 72˚C for 10 minutes followed. Amplification was confirmed by running a small volume of the product on a 0.8% agarose (Seakem, Lonza, Rock land, ME USA) gel. After confirmation of amplification, the amplified probe was purified using the GeneJET^{TM} Gel Extraction Kit (Fermentas, Vilnius, Lithuania). The purified DNA probe was diluted to 10ng/mL with 0.1X TE buffer.

3.2.8 Hybridization with ECL Kit (Amersham Biosciences)

3.2.8.1 Labelling of the probe

Twenty microliters of the diluted DNA probe was placed in a microfuge tube and denatured by boiling for 10 minutes in a beaker containing water. It was immediately cooled on ice for 10 minutes and briefly spun to collect the contents at the bottom of the tube. Twenty microliter of the labelling reagent was added to the cooled DNA probe followed by 20 ul of glutaraldehyde solution. The mixture was incubated in a heating block (Labnet, Edson, NJ) for 20 minutes at 37˚C.

3.2.8.2 Hybridization

Hybridization was performed in a rotating hybridization oven (MWG-Biotech Mini Oven MKII). The membrane was briefly rehydrated in 500mL of distilled H_2O before it was placed in a hybridization tube (MWG – Biotech AG, Ebersberg, Germany). The membrane was then covered with 50mL of pre-warmed (42˚C) hybridization buffer (Amersham Biosciences, Buckinghamshire, UK) and prehybridized for one hour at 42˚C. The labelled IS*6110* probe was then added and hybridization continued overnight at 42˚C. Hybridization of the membrane with the IMWM was done in a sealed plastic bag containing 50mL of hybridization buffer. A 500mL of water in a sealed plastic back was placed on top of the bag containing the membrane. Pre-hybridization was done at 42 ˚C for one hour in an incubator (Incubator Shaker, 25 series, Edison, New Jersey, USA). The labelled IMWM probe was added to the buffer and hybridized for overnight.

3.2.8.3 Post-hybridization

Stringency wash of the membrane was done immediately after hybridization. The hybridization buffer was removed and replaced with 100mL pre-warmed(42˚C) primary buffer (360g Urea, 25mL 20X SSC, 20mL 20% SDS, 1000mL distilled H_2O) and washed twice for 30 minutes each in the hybridization oven at 42˚C. Finally, it was washed twice with 500mL secondary washing buffer (100ml 20X SSC, 800mL DH_2O) at room temperature for five minutes each on a shaking platform (Labcon shaker, Laboratory marketing services, RSA).

3.2.8.4 Signal generation and detection

Signal generation and detection was done according to the ECL kit (Amersham Biosciences, Buckinghamshire, UK) following manufacturer's instructions. After washing the membrane with the secondary buffer, the membrane was put in a clean plastic bag with the DNA facing upwards. Excess secondary wash buffer was removed by rolling a 10mL serological pipette over the membrane. Equal volumes of ECL detection reagents one (5mL) and two (5mL) were mixed and poured directly on the membrane on the DNA side. It was evenly spread by rolling with a 10 mL serological pipette for 90 sec. Excess detection fluid was removed as explained above. The sealed membrane was placed in an X-ray cassette. Exposure to the film (Amersham Biosciences, Buckinghamshire, UK) was done in a darkroom at the Radiology Department at the Charlotte Maxeke Johannesburg Hospital, and developed using a Kodak RPX – OXMAT processor

(Eastman Kodak Company, New York, USA). The exposure time ranged from 1 hour to 16 hours.

3.2.8.5 Stripping the membrane

The probe was effectively removed by placing the membrane in a flat plastic container containing 500 mL of boiling 0.1% SDS. It was incubated with gentle shacking for 60 minutes or until the solution reached the room temperature.

3.2.9 Analysis of IS*6110* RFLP patterns

The analysis of RFLP profiles was achieved by using the computer software Gelcompar II, Version 2.5 (Applied Maths, Kortrijk, Belgium). The DNA fingerprints were analyzed using the Dice coefficient unweighted pair-group method with arithmetic averages (UPGMA). Analysis of the RFLP profiles was done at University of Stellenbosch, Division of Molecular Biology and Human Genetics, Cape Town.

3.3 Results for the IS6110 RFLP

A subset of 74 MTB isolates from 500 isolates were randomly selected from each sensitivity category as presented in chapter two section 2.6, figure 2.5. These isolates were previously genotyped through spoligotyping. RFLP analysis of 74 MTB isolates was performed using IS*6110* as a probe. The copy number of IS*6110* for each isolate was determined from the number of bands hybridizing with the probe. The fingerprint patterns on the resulting autoradiographs were scanned and analyzed using Gelcompar II, Version 2.5 (Applied Maths, Kortrijk, Belgium).

The study showed that eighty percent (59/74) of the isolates had their fingerprint patterns available in the database and the predominant families identified through IS*6110* RFLP corresponds to the families identified through spoligotyping.

Thirty distinct RFLP patterns were revealed from 74 MTB isolates and results showed highly variable IS*6110* fingerprinting patterns (figure 3.2 and figure 3.3) with copy number ranging from 0 to 22. Fifteen isolates could not be identified and were assigned a study identification family name (E-T) (figure 3.2) Six of these isolates designated FOE, FOF, FOG, FOH, FOI and FOJ, were previously classified as orphans through spoligotyping.

A figure below presents isolates which did not exist in the database. Three isolates had zero copy number of IS*6110* of which one was an orphan.

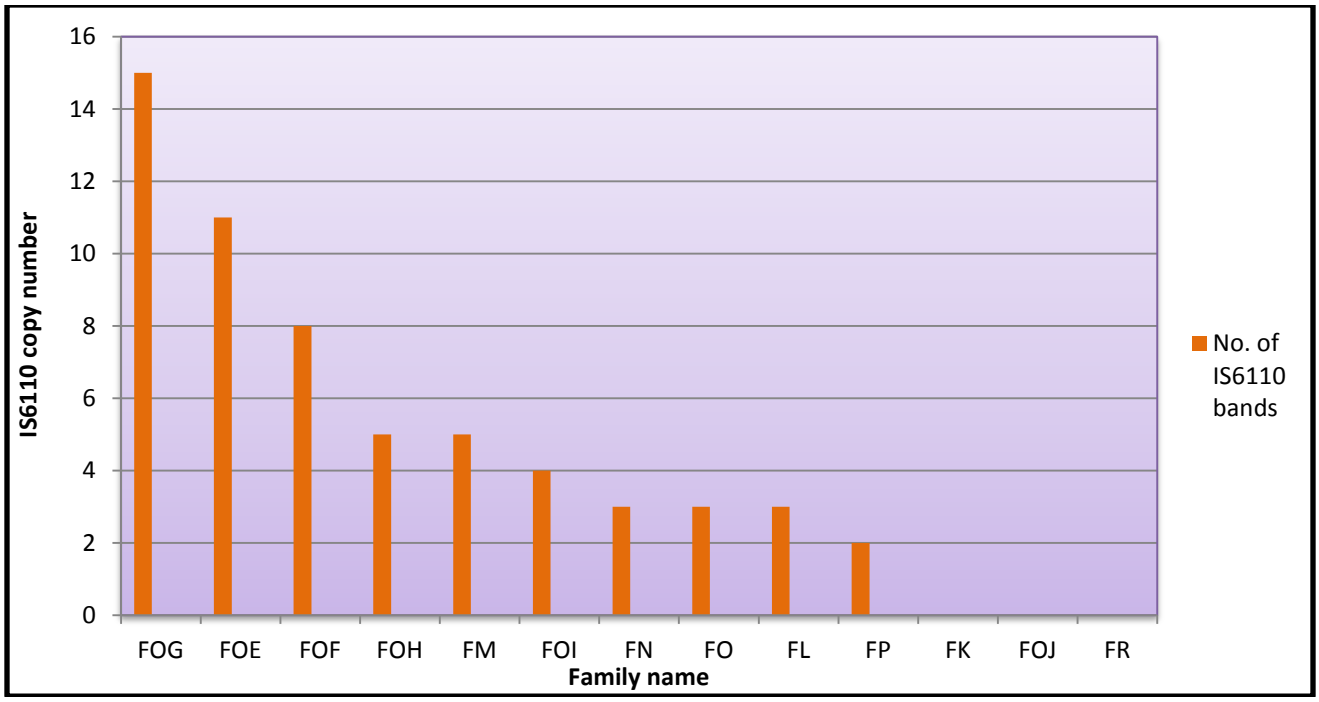


Figure 3.2: number of isolates which did not exist in the database.

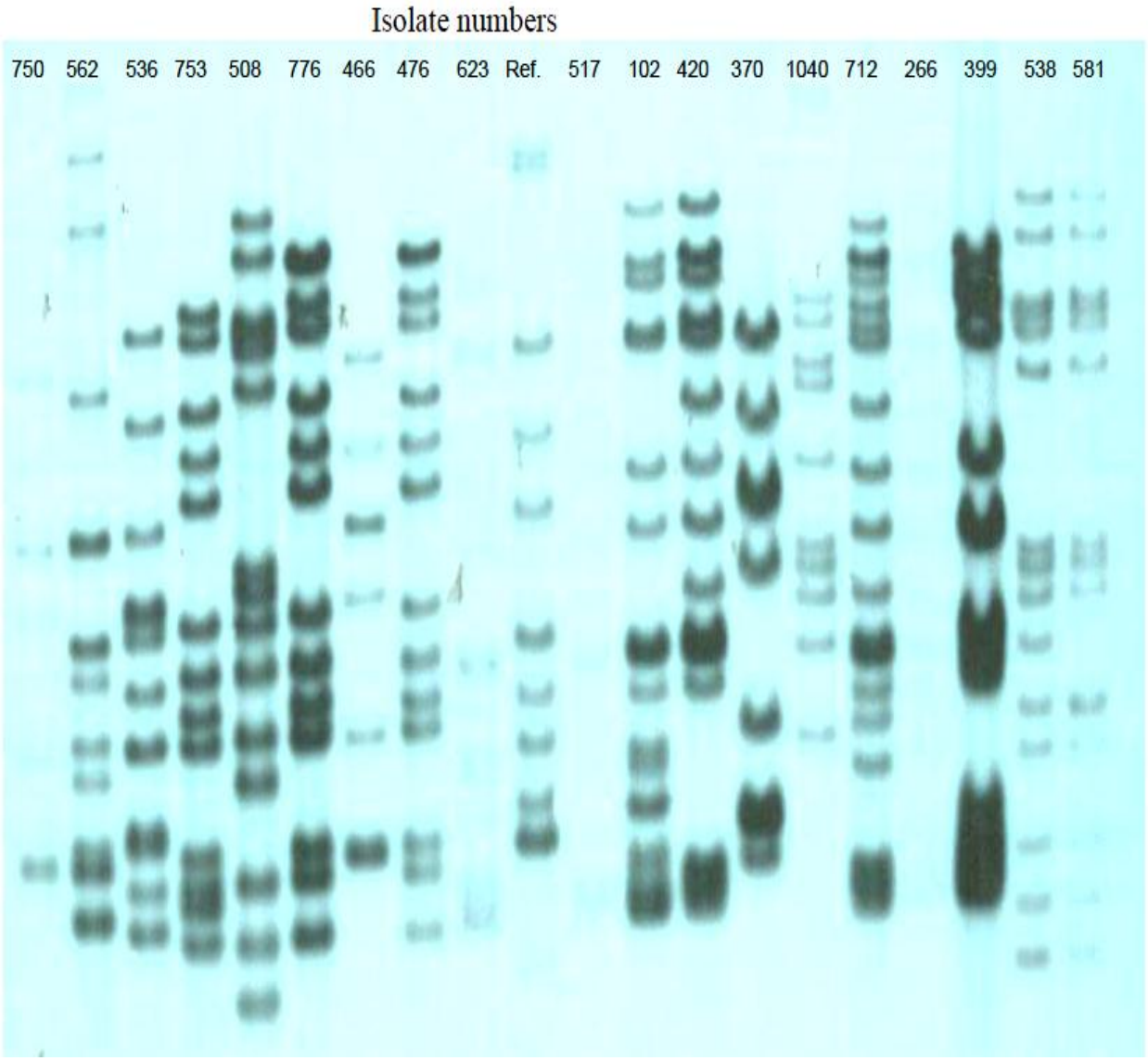


Figure 3.3: representation of IS*6110* fingerprints on the X-ray film.

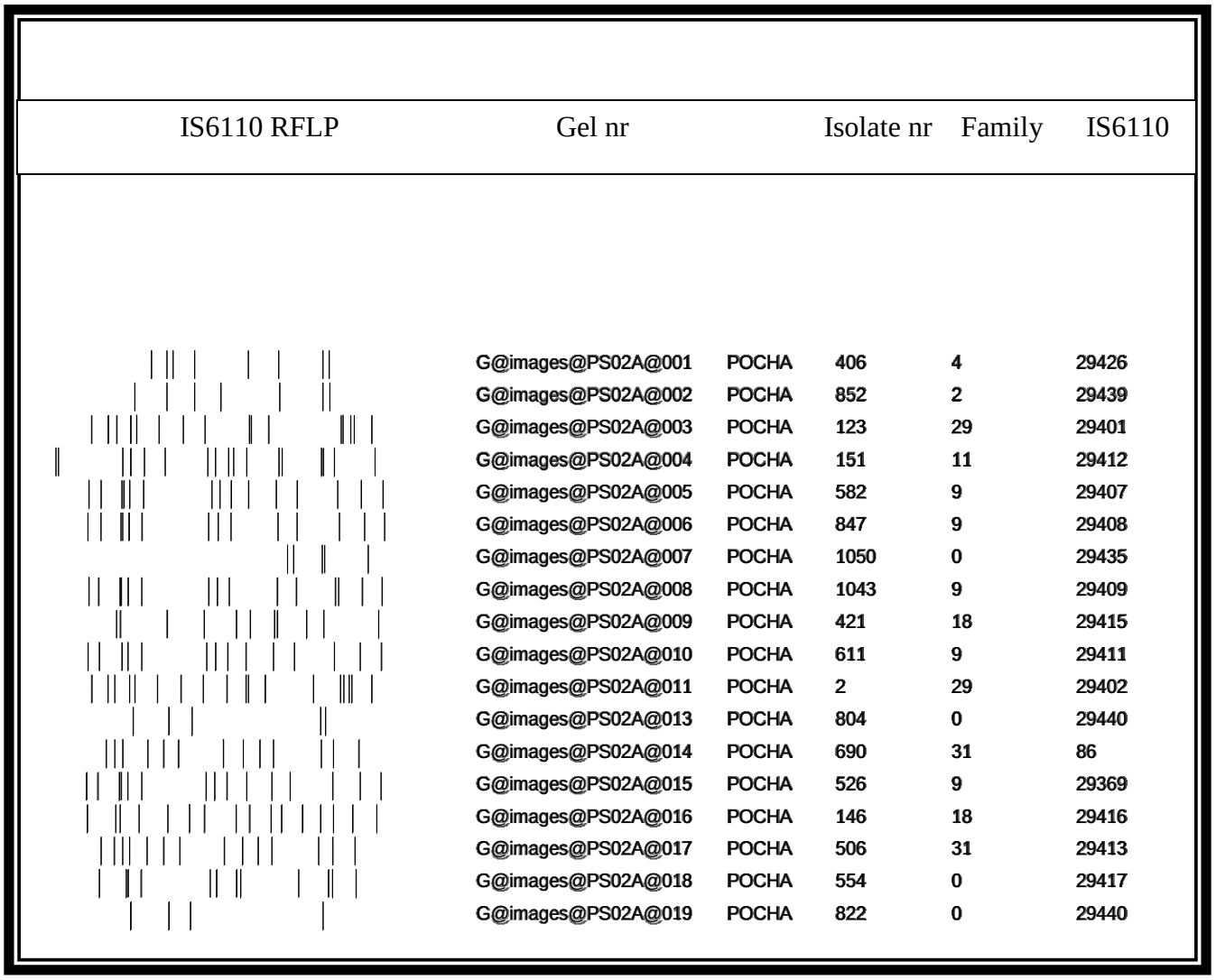


Figure 3.4: description of IS*6110* RFLP patterns and the corresponding families by Gelcompar II for one membrane.

Of the thirty obtained RFLP fingerprint patterns, 15 (20%) were represented by a single isolate from which 3 (4%) had zero copy number. The cluster range for the 12 RFLP patterns, was from 2 to 10 (figure 3.3). The largest RFLP cluster pattern, which comprised of 12 isolates, where designated F29, also known as Beijing family. This was closely followed by F15, commonly known as F15/LAM4/KZN and F9 which is also a LAM4 family. The fingerprints for all isolates which were not classified by Gelcompar II were visually compared.

The clustering percentage of the isolates was considerably high at 82% (61/74), which may suggest cases representative of recent transmission, while the 13 unique isolates may indicate epidemiological independence or recurrent infection. The proportion of isolates with low copy number of six or less was determined to be 9%.

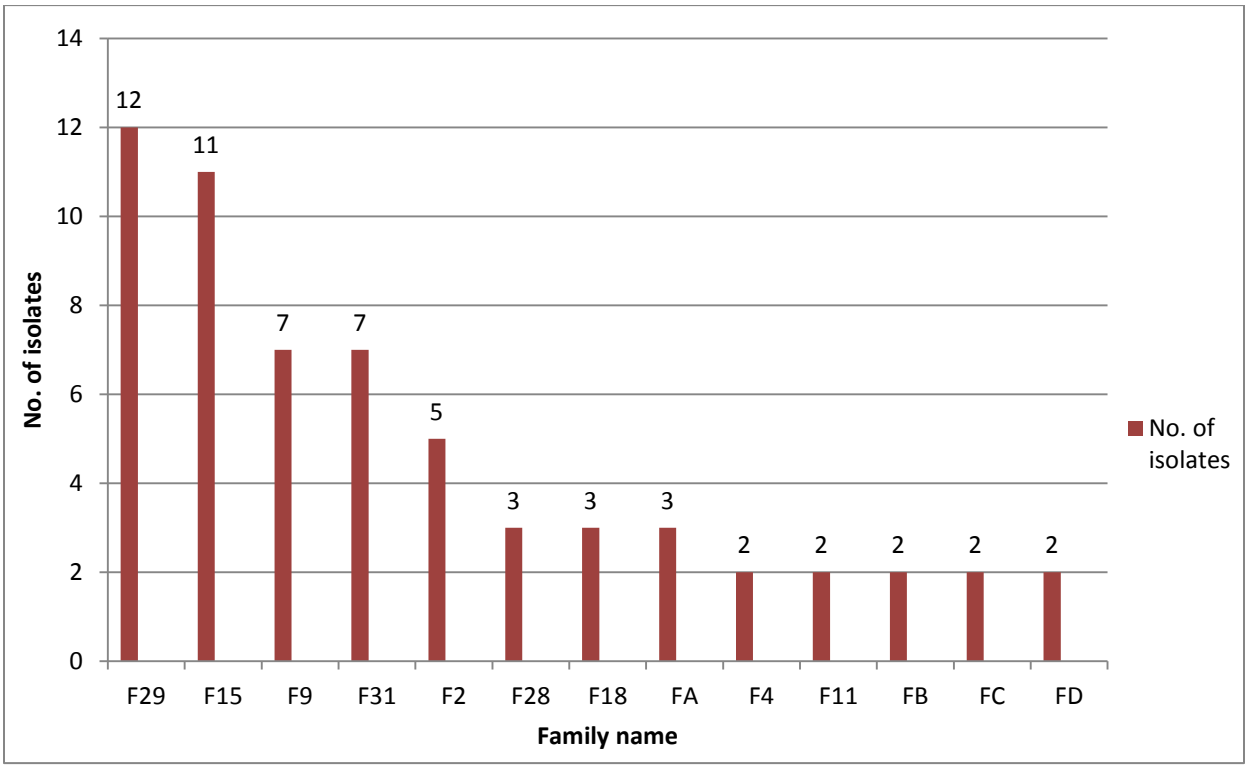


Figure 3.5: IS*6110* RFLP patterns clustered from 2 to 10.

Comparison of the results for the two methods showed that 56 isolates had identical IS*6110* RFLP patterns as well as spoligotypes. Nine isolates which were clustered by spoligotyping were later splinted by IS*6110* RFLP fingerprinting.

3.4 Discussion

Molecular epidemiology of MTB has gained worldwide acceptance over traditional techniques. DNA finger printing of *Mycobacterium tuberculosis* through the study of the IS*6110* element is still considered the gold standard despite the cumbersome and costly nature of the procedure. In the present study, the IS*6110* RFLP was used to fingerprint 74 MTB isolates. To the best of our knowledge, at the time of writing this report, this study is the first to have typed MTB isolates from the great Johannesburg metropolitan area through IS*6110* RFLP.

RFLP was performed on a subset of isolates previously classified by spoligotyping. The isolates were randomly selected from each spoligotype cluster and included a subset of isolates classified as orphans and a few isolates which did not cluster. Inclusion of the orphan and non-clustering isolates were to establish whether they could be distinguished through IS*6110* RFLP.

Results for spoligotyping of the same 74 MTB isolates which were again analysed through IS*6110* RFLP, revealed a total of 25 distinct patterns. Of these, 14 were singly represented with the remaining 60 isolates being grouped into 8 clusters. Six of the unique isolates were absent in the spoligotyping database and were described as orphans while 8 isolates belonged to different spoligotyping groups. The largest spoligotyping cluster contained 22 isolates belonging to Beijing family seconded by LAM4 SIT 60 with 20 isolates.

Analysis of these 74 MTB isolates by IS*6110* RFLP fingerprinting technique generated 30 RFLP patterns and showed that 80% (59/74) of the isolates from this study matched the fingerprints found in the IS*6110* RFLP database. The copy number for the IS*6110* element varied from 0 to 22 in each of the isolates. Thirteen isolates, however, did not exist in the database and of these; six isolates had a low copy number of IS*6110* element of six or less. Furthermore, five were isolates that were previously described as orphans by spoligotyping, of which one had zero copy number and two isolates belonging to Beijing family, also had zero copy number of the IS*6110* element. All the orphan isolates included in this study did not match any fingerprint in the IS*6110* database hence were not classified to a family. Only two isolates containing low copy number were able to be classified as F120 and F130 while the other 4 isolates were absent in the database.

The RFLP analysis grouped 59 isolates into 12 clusters, 8 of which are presented in figure 3.6. The largest cluster contained 12 isolates belonging to F29/Beijing family and was followed by 9 isolates within F15/LAM4 SIT 60 family. The correlation of the results between the two typing methods (figure. 3.6) was found to be 76% (56/74). It was further observed that the IS*6110* RFLP fingerprinting subclustered the spoligotyping clusters Beijing and LAM4 SIT 60 into two subclusteres each. The Beijing family was categorized into F29 and F31 while LAM4 SIT 60 was categorized into F15 and F9 even though the F29 and F15 were still the predominant families. It was also noted that spoligotyping had classified 5 isolates as H3 SIT 50 but these were subclustered by RFLP fingerprinting into three subclusters. Two isolates were classified as F2, the other two as F4 and one isolate was absent from the database. In another note, one isolate

classified by spoligotyping as an unknown isolate (SIT 34 U likely S) was identified as F28 together which paired with the S type families. The tendency of IS*6110* RFLP fingerprinting to split spoligotype clusters, has also been observed in other studies (Goyal & Saunders 1997) while it has also been demonstrated to have a good correlation between the two molecular typing techniques (Toungoussova et al. 2003; Gandhi et al. 2006).

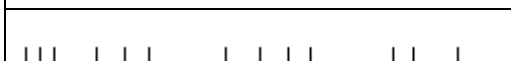
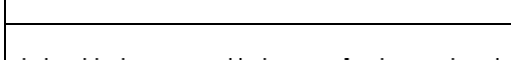
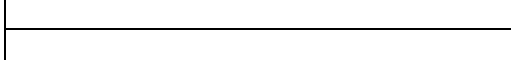
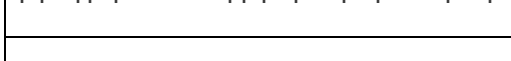
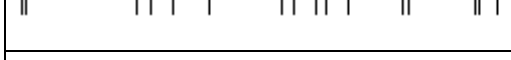
IS6110 RFLP description (Gelcompar II)	Binary description of spoligotyping (SpolDB4.0)	RFLP Family	Spoligo. family	# of Isolates in agreement
		F29	Beijing	12
		F31	Beijing	7
		F15	LAM4 SIT 60	11
		F9	LAM4 SIT 60	7
		F11	LAM3 SIT 33	2
		F2	H3 SIT 50	5
		F28	S SIT 88	3
		F18	T1 SIT 53	3
Total number of isolates for the eight clusters				50

Figure 3.6: Correlation of IS*6110* and Spoligotyping results. The relatedness of strains is shown by forming a cluster in both methods.

Combination of the two molecular techniques in the study of TB epidemiology in order to confirm the strain relationship, is imperative as suggested by Niemann et al. 1999 and Filliol et al. 2002. This notion is also supported by our study findings reported here. It was observed that the discriminatory power for the two molecular techniques differ as indicated by Filliol et al. 2002 and Goyal & Saunders 1997. Spoligotyping may be seen as the method of choice for isolates with low copy number (six or less), as discrimination among these isolates tend to be good. Out of six isolates with a low IS*6110* copy number, only two were described which might be due to poor discriminatory power of the IS*6110* fingerprinting technique in isolates with a low copy number. In a different note from this study, spoligotyping never subclustered the Beijing SIT 1, LAM4 SIT 60 and H3 SIT 50 family, but these clustering families were subclustered by IS*6110* RFLP fingerprinting as explained above. Although the IS*6110* RFLP fingerprinting might have shown a high discriminative power in Beijing family, other studies recommend the use of spoligotyping when distinguishing Beijing isolates from other isolates since the IS*6110* RFLP pattern of the Beijing strains is not unique (Burgos & Pym 2002). Much as we know that the two molecular techniques target different molecular markers, then these minor differences should be accepted.

The presence of these isolates in the database shows that apart from Johannesburg, they are also in circulation elsewhere within South Africa and abroad. In addition, the IS*6110* RFLP findings are confirming the spoligotyping results. The F29, F15, F28 and F11 have shown to be common in South Africa. The F29, F15 and F11 are interchangeably called Beijing, LAM 4 SIT 60 and LAM 3 respectively after confirming the genomic similarity (Richardson et al. 2002; Nicol et al. 2005; Naidoo & M Pillay 2013; Ferry et al. 2011). The F29, F28 and F11 shows to have been in circulation at least a decade ago (Richardson et al. 2002). These families were the most predominant families in a study conducted in Western Cape, South Africa in which F29 was confirmed to be a member of Beijing family (Richardson et al. 2002). It now seems apparent that they are still in circulation as demonstrated by results presented here as well as from the recent studies (Naidoo & Pillay 2013). The presence of these strain families in Johannesburg is not a surprise with migration of people to and from different provinces of South Africa including immigrants from cross borders. Our study has indicated that these isolates are one of the most successful strains causing TB epidemic in Johannesburg and in other provinces from South Africa.

The Beijing family has shown to be the most successful isolate in the world. This family through IS*6110* RFLP fingerprinting can be divided into seven lineages progressing from ancestral to modern. This family is well established in Johannesburg and in other provinces of South Africa as mentioned earlier on. The F29/Beijing was found to dominate in a study done in Cape Town in patients with mix strain cultures and it formed a large endemic cluster (Richardson et al.

2002). The F29 belong to lineage 6 and it is considered to be modern lineage. Though lineage 7 is considered as the most prevalent in Western Cape, the F29 through its sublineage R220 is overrepresented among drug-resistant isolates in the Western Cape of South Africa (Ioerger et al. 2010). This family (F29) is also considered as part of the globally relevant Beijing family (Richardson et al. 2002).

The description of the F15 found in this study has been confirmed to F15/LAM4/KZN also described by Pillay & Sturm 2007 and Ferry et al. 2011. Both molecular markers (IS*6110* and Spoligotyping) used in this study matched the description of F15/LAM4/KZN. The presence of this family in Johannesburg poses a major challenge to the National TB program (NTP). This family is largely associated with XDR-TB which is very difficult to treat (Banerjee et al 2008). It has shown to be common in South Africa as it has been described in many studies conducted around country. From a study done in KZN the F15 family formed the largest group among MDR-TB patients. Of the 109 patients suffering from TB infected with the F15/LAM4/KZN, 59% were suffering from MDR-TB. Again, in 2005 in one district of KZN, 59% of the 102 TB were suffering from MDR-TB infected with the F15/LAM4/KZN strain (Pillay & Sturm 2007b). From another study of XDR-TB done in Tugela ferry where 98% (52/53) of the infected people died, it was confirmed that 85% were infected with this (F15/LAM4/KZN) family. Our study is in support of the above mentioned data as all the isolates belonging to F15 in this study were XDR.

The presence of the F11 family in our study is not a surprise as the isolate is known to have first been described in South Africa (Abadía et al. 2009) as explained earlier on in chapter one. This gives an impression that the F11 is well established in the country and is one of the families responsible for TB epidemic. From a study done in Cape Town (Victor et al. 2004), this family was the most predominant and was associated with drug resistant TB. It is considered to be one of the successful strains causing TB epidemic in the Western Cape. The fingerprints of the F11 isolate also match the isolates identified in the database from Netherlands giving an indication that this family is also present in other countries and might have been imported from these countries.

We found it difficult to compare our IS*6110* RFLP results with some of the studies conducted within South African, the neibouring countries and the rest of African countries as it were the case with spoligotyping. In addition, through data search, it shows that TB molecular epidemiological studies targeting the IS*6110* element are not commonly done in African as only few countries have presented their data. In South Africa, the molecular fingerprinting of MTB isolates through IS*6110* RFLP is largely performed by at University of Stellenbosch Department of Molecular Biology and Human Genetics. This probably explains why most of the publications we currently have in South Africa are on the isolates from the Western Cape.

The lack of this procedure in many countries may be due to labour intensive nature of the procedure, its cost effectiveness and the lack of skills in the field. The other drawback was that some of the studies conducted so far did not use the Gelcompar software to analyze their results but rather used visual analysis (Green et al. 2013). In addition, classification of fingerprint patterns has been observed to be different from each study (Abbadi et al. 2001; Green et al. 2013 van Soolingen et al. 1993). This means that such isolates did not form part of the database to which the results presented here were analyzed. Some studies used a different probe and a different restriction enzyme (Thierry et al. 1990) which had different restriction sites leading to different restriction fragments. However, the data presented here has shown the presence of predominant MTB isolates which are also in circulation elsewhere and highlights the benefits of using IS*6110* for molecular typing of isolates.

It was observed that the high incidence of the absence of our isolates from the database was mainly in terms of isolates with low copy number; orphan isolates and isolates lacking the element IS*6110*. Only two of the six low copy number isolates, were identified in the database while the remaining 5 isolates were absent. Similarly, none the orphan isolates were observed in the database. Only a single isolate with more than six IS*6110* copy numbers was absent in the database but after counting the fingerprints, it was noticed to contain 7 fingerprints which is closer to six. The observation of poor discriminatory power by IS*6110* RFLP in isolates containing IS*6110* copy number of less than six, was also observed in a study done by Flores et

al. 2010 and recommended the use of secondary typing method to such MTB isolates. This shall mean that, from our study any isolate with higher copy number was able to or nearly match the fingerprints in the database.

Though other studies have doubted the discriminating power of the spoligotyping technique, this study has demonstrated a good correlation between spoligotyping and IS*6110* RFLP fingerprinting technique. We support the use of spoligotyping as an initial typing technique as recommended by Goyal & Saunders 1997 and Warren et al. 2002. Spoligotyping can serve as a filtering technique to identify isolates that would benefit from further IS*6110* RFLP typing and thereby minimizing both cost and turn-around-time

With the limited sample size, the results presents here have demonstrated that isolates from the Johannesburg region are rich in molecular fingerprinting data and future studies should include these typing methods. It has further been shown that these isolates are not specific to the region but are also present in other provinces of South Africa and outside the South Africa borders. Frequencies of other isolate families such as Beijing and F15/LAM/KZN seem to be higher in Johannesburg than in other South African provinces. This is a major concern since the two families are highly associated with drug-resistance. Johannesburg is a highly industrialized and populated city whereby these conditions favour the spread of these families.

For future studies, we recommend IS*6110* fingerprinting all the isolates enrolled in this study in order to have a true representation of the isolates circulating in the area as the sample size included was too small. We would also like to propose that further studies should be done on all the isolates which were classified as orphans by spoligotyping and failed to be identified by RFLP fingerprinting because the infectivity of these isolates in the community is not known.

Finally, the study achieved its objectives to establish the RFLP procedure in the department and to compare subsets of isolates through the IS*6110* RFLP database in order to determine the predominant families circulating in Johannesburg area.

Chapter four: Conclusion and recommendations

This is the first molecular study to have typed the isolates of different susceptibility patterns from the great Johannesburg metropolitan area, through both spoligotyping and IS*6110* RFLP.

Spoligotyping of 500 isolates yielded 98 different patterns, of which 59 patterns from 458 isolates were present in the Spoligotyping database 4 (SpoliDB4). This showed that 92% of our sample matched the pre-existing shared types in SpoliDB4. It was determined that eight families were predominant and these were; Beijing SIT 1, S SIT 34, LAM 4 SIT 60, T1 SIT 53, LAM3 SIT 33, H3 SIT 50, EAI1-SOM SIT 48 and X3 SIT 92. The Beijing family was the most predominant and was almost equally distributed in all the seven regions of Johannesburg.

The Beijing SIT 1 and LAM 4 SIT 60 families were both associated with drug-resistance. The LAM 4 isolates on the other note were highly associated with XDR-TB. There was no sensitive isolate observed in this family. Furthermore, the Beijing SIT 1 and LAM 4 SIT 60 seems to be more prevalent in South Africa than any other African country indicating geographical specificity of isolates.

The analysis of 74 MTB isolates through IS*6110* RFLP generated 30 distinct RFLP fingerprinting patterns. The IS*6110* copy number ranged from 0 – 22. Sixty one isolates had their fingerprints available in the database while 13 isolates did not exist in the database. After classification of the isolates, it was revealed that strains from 12 families showed clustering. The

F29 family was found to be the most predominant by clustered with 12 isolates. The other clustering families were F15, F28, F31, F4, F2, F18, F9, FA, FB, FC and FD. The F29 and F31 are interchangeably called Beijing family while F15 is interchangeably called LAM 4 SIT 60 family.

The combination of spoligotyping and IS*6110* RFLP in the molecular typing of *Mycobacterium tuberculosis* is recommended. Discriminating powers differed mainly in isolates containing low copy number of IS*6110* of six and less. Spoligotyping discriminated well all the isolates containing a low copy number of six and less while IS*6110* RFLP showed poor discriminatory power. Despite this, the two methods showed a good correlation of the results with fifty six isolates having identical IS*6110* RFLP patterns as well as spoligotypes.

Not surprisingly the results showed that TB in Johannesburg is caused by different types of strains as there was no clonal circulation of the strain. However, the Beijing family seems to be well established and well represented in Johannesburg as it was the most predominant family in this study. In addition, LAM4 SIT 60 which is known as the KZN family is also in high circulation in the Johannesburg metropolitan area. Its frequencies were very high in XDR-TB confirming its association with drug-resistance.

A high clustering rate of isolates was observed. This may possibly indicate a large scale transmission of TB taking place in Johannesburg and we therefore recommend that the National Tuberculosis Program evaluates its current control measures to minimize the spread of the disease.

Despite the limited sample size analyzed through IS*6110* RFLP fingerprinting, this study has shed light on the fingerprint patterns of MTB isolates circulating in Johannesburg area. It is therefore recommended that after the initial typing of isolates through spoligotyping, further analysis should be done using IS*6110* RFLP. Further analysis of the orphan isolates to determine strain properties such as infectivity, is also recommended.

Appendices

Appendix 1: Ethical approval from the University of the Witwatersrand

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Mr Samuel K Pocha

CLEARANCE CERTIFICATE	M111113
PROJECT	Molecular Typing of Drug-Susceptible, Mono-Resistant and Extensivelt Drug-Resistant Mycobacterium Tuberculosis Stains in Johannesburg
INVESTIGATORS	Mr Samuel K Pocha.
DEPARTMENT	Clinical Micobiology and Infectious Diseases
DATE CONSIDERED	25/11/2011
M11111130DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 25/11/2011 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Dr L Blann

DECLARATION OF INVESTIGATOR(S)
To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**
PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

Appendix 2: Spoligotyping Solutions

2A: Stock solutions

2A.1 20X SSPE

- 27.6g Sodium Hydrogen Phosphate (monohydrate)
- 175.3g Sodium Chloride
- 7.6g EDTA
- dH_2O 1000ml
- Adjust pH to 7.4
- Autoclave
- Stable at room temperature for 1 year

2A. 2 2X SSPE

- Dilute 20X SSPE ten times with dH_2O (1/10)

2A.3 10X SSPE

- 13.8g Sodium Hydrogen Phosphate (monohydrate)
- 87.65g Sodium Chloride
- 3.8g EDTA
- dH2O 1000nl
- Adjust Ph to 7.4
- Autoclave
- Stable at room temperature for 1 year

2A.4 10% SDS

- 50g SDS (Mix in fume Hood)
- dH_2O 500ml

2A.5 0.5M EDTA

- 93g EDTA
- dH_20 500ml

2A.6 Detection solution 1 & 2 (Amersham)

- Ready for use

Appendix 2 continues: Spoligotyping solutions

2B: Working solutions

2B.1 500ml 2X SSPE/0.1% SDS

- 100ml 10X SSPE
- 5ml 10% SDS
- dH_2O 500ml

2B.2 500ml 2X SSPE/0.5% SDS

- 100ml 10X SSPE
- 25ml 10% SDS
- dH20 500ml

2B.3 200ml 1% SDS

- 20ml 10% SDS
- dH_2O 200ml

2B.4 500ml 20 mM EDTA

- 20ml 0.5 EDTA
- dH_2O 500ml
- pH 8

2B.5 Detection solution

- Prepare fresh solution each time
- Mix equal volumes of detection solution 1 and 2

Appendix 3: Solutions for RFLP

3A. *Lysozyme*

- Working [lysozyme]: 50mg/ml
- Dissolve 500mg of lysozyme in 10ml of dH_2O
- Store at - 20˚C

3B. *RNAse A*

- working [RNaseA]: 10mg/ml
- Dissolve 50mg of RNaseA in 5ml of 10mM Tris-HCl, pH 7.4; 15mM NaCl.
- Heat at 100 ˚C for 15 minutes
- Allow to cool slowly at room temperature and store in aliquots.
- Store at -20 ˚C

3C. 10X *Proteinase K* buffer pH 7.8

- 2.5g 5% SDS
- 5ml 100mM Tris-HCl pH 7.8 (from 1M stock)
- 5ml 50mM EDTA (from 0.5M stock)
- Autoclave
- Store at room temperature

3D. Chloroform: Isoamyl alcohol

- 384ml chloroform
- 16ml Isoamyl alcohol to make a 24:1 ratio
- Invert to mix
- Store between 2 and 8 degrees Celsius

Appendix 3continues: Solutions for RFLP

3E. 3M Sodium Acetate pH 5.2

- 204.12 g Sodium acetate
- Fill up with water to 400ml mark
- Adjust pH to 5.2 with glacial acetic (approx 60ml)
- Autoclave
- Store at room temperature

3F. TE buffer pH 8.0

- 1.211g 10mM Tris
- 0.372g 1mM EDTA
- Fill up to 1L with dH_2O
- Adjust pH to 8
- Autoclaving.
- Store at room temperature

3G. 1X Restriction Buffer (NEBuffer 2) pH 7.9

- 50 mM NaCl
- 10 mM Tris-HCl
- 10 mM $MgCl_2$
- 1 mM DTT
- Store at 25 °C
- Supplied as a 10X concentrated stock.

3H. 6X Loading Buffer

- 60mg bromophenol blue
- 0.6g sodium dodecyl sulfate
- Fill to 70mL with ddH20 and swirl to mix
- Add 30mL of 100% gylcerol and swirl to mix
- Store at room temperature

Appendix 3continues: Solutions for RFLP

3I. 10X TBE buffer pH 8.3

- 108g Tris
- 55g Boric Acid
- 7.44g EDTA
- Fill to 1000mL with ddH2O
- Add stirrer bar and place on stirrer to dissolve solutes
- Autoclave
- Store at room temperature

3J. Internal Molecular Weight Marker

- 6 ml TE pH 8.0
- 2 ml loading buffer
- 6.6 µl Marker X
- Mix eppendorf tube by inversion
- Store at -20 degrees Celsius

3K. Denaturing Buffer

- 87,66g 1.5M NaCl
- 20g 0.5M NaOH
- Fill up to 1L with dH_2O
- Add stirrer bar and place on stirrer to dissolve solutes
- Autoclave
- Store at room temperature

3L. Neutralizing Buffer

- 87.66g 1.5M NaCl (use stirring bar to dissolve it)
- 60.5g 0.5M Tris-HCl pH 7.5
- Fill up to 1L with dH_20
- Add stirrer bar and place on stirrer to dissolve solutes
- Autoclave
- Store at room temperature

Appendix 3continues: Solutions for RFLP

3M. Orientation Marker

- 2µl Marker X (250 ng/µl)
- 20µl MTBC 14323 DNA (1.25µg/µl),
- 45µl 0.8 M NaOH,
- 23µl TE (pH 8.0)

3N. 20X SSPE Solution pH 7.4

- 175.3g of NaCl
- 27.6g of NaH_2PO_4-H_2O
- 7.6 g of EDTA
- Add 800ml dH_20
- Adjust the pH to 7.4
- Adjust the volume to 1l with additional distilled H_2O.
- Autoclave

3O. 20X SSC Solution pH 7.

- Dissolve the following in 800ml of distilled H_2O.
- 175.3g of NaCl
- 88.2g of sodium citrate
- Adjust the pH to 7.0 with a few drops of 1M HCl.
- Adjust the volume to 1l with additional distilled H_2O.
- Autoclave

3P. Primary Wash Buffer

- 720g urea
- 8g sodium dodecyl sulphate (SDS)
- Make up to 2l with distilled water.
- Autoclave

3Q. 1% SDS

- 5g SDS
- dH_2O 500ml

- Place on shaker with stirrer bar to dissolve

Appendix 3continues: Solutions for RFLP

3R. 5M Nacl

- 584g NaCl dH_2O to 2000ml
- Place on shaker with stirrer bar to dissolve
- Autoclave.

3S. 70% Ethanol

- 70ml of absolute Ethanol
- dH_2O 100ml

3T. CTAB/ NaCl

- 10g CTAB + 4.1g NaCl,
- dH_2O to 80ml,
- dissolve at 65˚C
- Adjust volume to 100ml with dH_2O.
- Store at room temperature.

3U. ECL Hybridization buffer

- 100ml ECL hybridization buffer,
- 5.844g NaCl final concentration 0.5M
- 5g blocking reagent: final concentration of 5% (w/v).
- Add the blocking reagent slowly to the buffer solution while stirring.
- Continue mixing at RT for 1h on a magnetic stirrer, then heat to 42˚C for 30 to 60 minutes with occasional mixing.
- Store at -20˚C for three months

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