Association of *Mycobacterium tuberculosis* genotypes and treatment outcome in pulmonary tuberculosis patients in Tshwane Metropolitan area

SIPHIWE RUTHY MATUKANE

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

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by

SIPHIWE RUTHY MATUKANE

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DECLARATION

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

Signed:……..……..this……day of……2015

I can do all things through Christ,

He strengthens me . . .

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ABBREVIATIONS

AIDS	Acquired Immune Deficiency
Anti-TB	Antituberculous
ART	Antiretroviral therapy
BCG	Bacille Calmette-Guerin
CAS	Central Asian
CDC	Centers for Disease Prevention and Control
DNA	Deoxyribonucleic acid
DOTS	Directly Observed Treatment Short-course
DR	Direct Repeat
DST	Direct Susceptibility Testing
DVR	Direct Variable Repeat
EAI	East-African-Indian
H	Haarlem
HGDI	Hunter Gaston Discriminatory Index
HIV	Human immunodeficiency virus
hr	Hour
IS	Insertion Sequence
IUATLD	International Union Against Tuberculosis and Lung Disease
KZN	KwaZulu-Natal
LAM	Latin-American-Mediterranean
M. tuberculosis	*Mycobacterium tuberculosis*
MAC	*Mycobacterium avium complex*
MDR-TB	Multidrug-resistant tuberculosis
MGIT	Mycobacteria Growth Indicator Tube
MHC II	Major Histocompatibility Complex class II
Min	Minute
MIRU-VNTR	Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats
MIT	MIRU international type

Ml	millilitre
MTBC	*Mycobacterium tuberculosis* complex
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NHLS	National Health Laboratory services
NTM	Non-tuberculous mycobacteria
NTP	National TB programme
PCR	Polymerase Chain Reaction
RFLP	Restriction fragment length polymorphism
Sec	second
SIT	shared International Type
Spoligotyping	Spacer oligonucleotide typing
TB	Tuberculosis
T-cells	Thymus cells
TDH	Tshwane District Hospital
UP	University of Pretoria
VNTR	Variable number tandem repeat
WHO	World Health Organisation
XDR-TB	Extensively drug-resistant tuberculosis

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SIPHIWE RUTHY MATUKANE

Supervisor: **Dr MR Lekalakala**

Co-supervisor: **Dr HM Said**

Department: **Medical Microbiology, Faculty of Health Sciences**

University of Pretoria

Degree: **MSc (Medical Microbiology)**

SUMMARY

Summary

Tuberculosis (TB) is a major cause of death worldwide, especially in Asia and Africa. Genotyping methods such as insertion sequence 6110-restriction fragment length polymorphism (IS6110-RFLP), spoligotyping and mycobacterial interspersed repetitive units variable number of tandem repeats (MIRU-VNTR) have expanded the ability to investigate and understand TB. Genotyping of *M. tuberculosis* has shown that certain *M. tuberculosis* strains are more prevalent in certain geographic regions, thus raising questions about bacterial factors in pathogenesis, presentation of disease and treatment response. Given the diversity in insertions, deletions and single nucleotide polymorphisms seen in *M. tuberculosis,* it is plausible that the genetics of the pathogen play a role in presentation of disease and response to treatment. However, there is very limited evidence on the influence of *M. tuberculosis* genotypes on treatment outcome of TB around the world. Whether response to TB treatment differs among the different genotypes is unknown. It is important to investigate the influence *M. tuberculosis* genotypes may have on treatment response; to develop effective control strategies for tuberculosis. This study aimed to determine the genetic diversity of *M. tuberculosis* strains in Tshwane metropolitan area and to

determine the association of those genotypes with the laboratory based-treatment response using the eight weeks culture result as a marker for treatment response. In addition the study explored the association of genotypes with drug resistance and patient demographics (age and gender).

In this study a total of 108 consecutive *M. tuberculosis* isolates resistant to either rifampicin and/or isoniazid were collected between September 2011 and December 2011from the National Health Laboratory Services (NHLS), Tshwane academic division laboratory . Two isolates were excluded from the study due to contamination. The study population included 50 females (47.17 %) and 47 males (44.34 %) with 9 patients (8.49 %) that did not have gender available. The age range for the study population was from 2 days to 79 years. Genotyping was performed using two different PCR-based methods; spoligotyping, and 24 loci MIRU-VNTR typing.

Comparison of Spoligotyping results of the 106 *M. tuberculosis* isolates with the SpolDB4 database showed that 73% (83/106) isolates belonged to 21 previously described shared types (ST); while 27% (23/106) were not found in the SpolDB4 database and were considered as orphans. Spoligotyping identified seven major distinct families of TB including Beijing, East African Indian (EAI), Latin American and Mediterranean (LAM), T family, X family, CAS as well as Manu 2 families. Spoligotyping identified 14 clusters, giving a clustering rate of 50%. However no cluster was identified with 24-loci MIRU-VNTR typing. The study did not find any as association of. In this study only 46 patients out of the 106 of the study population had the 8 weeks follow up result and no genotypes were associated with treatment outcome in this population. The study also did not find any association of specific genotype with gender, age or drug resistance to INH or Rif. Resistance to RIF was associated with treatment failure (patients failed to convert after 8 weeks), with P value of 0.006. However, no significant association was found between INH resistance and treatment outcome. In conclusion the study showed high genetic diversity of strains in Tshwane Metropolitan area. No association was found between genotypes and treatment response. However more than 50% did not have eight weeks follow up result. The study did however find Rifampicin (Rif) mono resistance to be associated with treatment failure. Further studies are needed to confirm the present study finding.

CHAPTER 1

1.1 INTRODUCTION

Tuberculosis (TB) is a disease of major public health concern worldwide. The World Health Organisation (WHO) reported that in 2012 the five countries with the largest number of incidences were India with a reported 2 million to 2.4 million people infected, China (0.9 million -1.1 million), South Africa (0.4 million to 0.6 million), Indonesia (0.4 million to 0.5 million) and Pakistan (0.3 million to 0.5 million). The TB problem is increasing due to multidrug-resistant (MDR) TB, i.e. tuberculosis resistant to at least isoniazid and rifampicin (RIF) and extensively drug resistant (XDR) TB, i.e. MDR-TB with additional resistance to any fluoroquinolone (FLQ) and to at least one of the three injectable second-line drugs: kanamycin (KAN), amikacin (AMK) and/or capreomycin (CAP) (WHO, 2003).

The burden of TB remains high in Africa with South Africa as one of the countries with the highest TB incidence, with a rate of 993/100 000 people in 2011 and a prevalence of 768/100 000 people (WHO, 2012). South Africa was one of the countries to first report XDR-TB after an outbreak of XDR-TB among HIV-infected patients in 2011 in KwaZulu-Natal, where 52 of the 53 patients died (WHO, 2012).

Conventional diagnostic methods for TB are solid-based techniques such as Lowenstein Jensen (LJ) agar or Middlebrook agar for the identification, culture and drug susceptibility testing (DST) of *M. tuberculosis.* Faster techniques are needed for early detection of TB in order for the patient to start treatment. The introduction of liquid culture-based methods for diagnosis of TB has decreased *M. tuberculosis* detection time as these methods are faster than solid culture-based methods. These methods include the automated BACTEC MGIT 960 which is used for culture and DST of *M. tuberculosis*. Molecular techniques for diagnosis and DST are also available. These methods can identify *M. tuberculosis* rapidly with identification of drug resistant genes which include the Genotype® MTBDRplus assay (Raveendran *et al*., 2012) and the GeneXpert® system (Pai and Ling, 2008).

Mycobacterium tuberculosis treatment is taken over a period of six months for susceptible TB, but can take up to 24 months or more with MDR-TB and XDR-TB (Caminero *et al*., 2010). The standard regimen for susceptible TB include RIF, INH, pyrazinamide (PZN), ethambutol (E) and streptomycin (S) all administered for the first two months in the intensive phase, followed by the continuation phase during which the patient receives INH and RIF for the next four months if the TB isolate is known to be fully susceptible (Caminero *et al*., 2010).

In addition to the management and treatment of TB, understanding the population structure, diversity and spread of *M. tuberculosis* strains is crucial (Nicol and Wilkinson, 2008). Many molecular methods have been applied in efforts to study and understand the disease and have improved epidemiological studies. These techniques have shed light on the most common strains that are circulating in certain parts of the world. Information from strain typing has been used to supplement traditional epidemiological methods and provided insight into the transmission dynamics of TB and major circulating strains around the world (Affolabi *et al*., 2009). A number of genotyping methods are available that are currently in use in molecular typing of *M. tuberculosis*: these include insertion sequence 6110-restriction fragment length polymorphism (IS6110-RFLP) (Thierry *et al*., 1990; van Soolingen, 2001 and Affolabi *et al.*, 2009) and polymerase chain reaction (PCR)-based techniques, including spoligotyping (Kamerbeek *et al*., 1997) and mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) (Supply *et al*., 2000 and Rohani *et al*., 2009).

The IS6110-RFLP is based on analysis of the distribution of the IS elements in different *M. tuberculosis* strains. Due to its high discriminatory power, the IS6110-RFLP is the current gold standard method. Spoligotyping is a PCR-based method that interrogates a direct repeat sequence comprising a repetitive 36-basepair element separated by short, unique, non-repetitive sequences (Daley, 2005; Warren *et al*., 2002). The direct repeat has been determined to be an important chromosomal domain for studying the evolution of *M. tuberculosis* (Small *et al*., 1994). Different strains differ in terms of the presence or absence of a specific spacer in different regions; (Barnes and Cave, 2003; Filliol *et al.*, 2000; Kamerbeek *et al*., 1997; Groenen *et al*., 1993 and Hermans *et al*., 1990).

Mycobacterial interspersed repetitive units-variable number of tandem repeats is the other PCR-based method commonly used with spoligotyping (Roetzer *et al*., 2011). It is based on PCR amplification of DNA segments containing tandem repeated sequences (Barnes and Cave, 2003) and identification and sizes the different alleles on *M. tuberculosis* genome, determining the number and the sizes of the variable number of tandem repeats (VNTRs) on the different loci of the *M. tuberculosis* genome (Supply *et al*., 2000).

Environmental and host factors that contribute to the outcome of treatment in *M. tuberculosis* infection are well recognised (Nahid *et al*., 2010). Important factors such as age, chest radiographic features, and higher sputum smear and culture grading at diagnosis have been associated with treatment failure in pulmonary tuberculosis patients (Veen *et al*., 1998). However, the impact of the *M. tuberculosis* genotypes on treatment outcome has not been well investigated. Data regarding the impact of those genotypes on treatment response is lacking. A small number of studies have examined whether the mycobacterial genotype influences treatment outcome, often with conflicting results. Therefore, whether response to TB treatment differs among the different *M. tuberculosis* genotypes is unknown.

This study aimed to determine the genetic diversity of *M. tuberculosis* in the Tshwane metropolitan area and the impact of those genotypes on treatment outcome using the eight weeks culture result as a marker for treatment response. In addition the study will explore the association of genotypes with drug resistance and patient demographics (age and gender). Such information could have the potential to influence control of and prevention strategies against TB. Monitoring the clinical outcome in patients is important in order to evaluate the effectiveness of the intervention (Veen *et al*., 1998) and to provide information on the association of specific genotypes with treatment failure. Treatment outcome analysis and interpretation can allow a more appropriate intervention and improved quality of care.

1.2 Aim

To determine the genetic diversity of *M. tuberculosis* strains in the Tshwane metropolitan area and to determine the association of those genotypes with the laboratory-based treatment response of tuberculosis, drug resistance and patient demographics (age and gender).

1.3 Objectives

The objectives of this study were to determine:

- the genetic diversity of *M. tuberculosis* isolates in the Tshwane metropolitan area;
- the impact of *M. tuberculosis* genotypes on treatment response (using sputum culture conversion within the first two months as a marker for treatment response); and
- If any of the genotypes are associated with drug resistance and patient demographics (age and gender).

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Tuberculosis (TB) was first described by Robert Koch in 1882 (Barnes, 2000) as a chronic infectious disease caused by the obligate human pathogen, *M. tuberculosis* mainly affecting the lungs (pulmonary tuberculosis) but which can also affect other parts of the body (extra-pulmonary tuberculosis) (WHO, 2013). Tuberculosis is transmitted via the air when infected persons sneeze, talk or cough, propelling infective bacilli into the air (WHO, 2012). Immune response after exposure to *M. tuberculosis* remains incompletely understood (Sharma *et al*., 2012).

Mycobacterium tuberculosis has a very complex, lipid-rich cell wall containing a peptidoglycan layer with many branched-chain polysaccharides, proteins and lipids attached (Ryan *et al*., 2010). The mycobacterial cell wall is responsible for *M. tuberculosis* pathogenicity, for its intracellular survival ability and virulence (Armstrong and Hart, 1971). In addition to its complicated cell wall, this bacterial pathogen has evolved into strains resistant to certain essential antibiotics (first line and second line drugs) important for the treatment of TB. Many studies have been done in different parts of the world which have shed more light on the burden of TB and drug resistant TB and the molecular genotype diversity of *M. tuberculosis* around the world.

There are factors leading to drug resistance which need to be understood to develop appropriate control strategies for national programs. The global picture of TB shows continued progress but not fast enough: in 2012, around 8.6 million people had developed TB (WHO, 2013), but was declared a global emergency in 1993 by World Health Organisation (WHO) (McConnell and Hargreaves, 2013). Appropriate and early diagnosis of TB relies on the available methods of diagnosis like microscopy, bacteriological cultures, radiological, serological and molecular tests, but nonetheless TB remains a widespread disease.

Molecular techniques have made it possible to look at the clinical outcome associated with different *M. tuberculosis* genotypes, as studies have shown that mycobacterial virulence depends on the identification of genes and gene products that contribute to the pathogenesis of TB (Nicol and Wilkinson, 2008).

2.2 Historical background of tuberculosis

Written descriptions of TB can be found in Greek literature, and the earliest evidence of TB in Egypt was documented around 5000 years ago (Daniel, 2006). Robert Koch's ground-breaking work on TB was done around the early 1880s onwards (Gradmann, 2006). Tuberculosis has probably killed over 100 million people over the past 100 years (Frieden *et al*., 2003). It is the second worst killer in the world after the human immuno-deficiency syndrome (WHO, 2013). During the 19thcentury a sanatorium was opened to host people infected with TB in order to remove them from the community (*Figure 2.1*). During that time there was intense research which finally lead to the discovery of para-aminosalicyclic acid (PAS) and streptomycin (Brennan, 1999) for treatment of TB.

Figure 2.1 Sanatorium for tuberculosis patients (Brennan, 1999).

More than three million years ago the early progenitor of TB is thought to have been present and probably co-evolved with early homids (Gutierrez *et al*., 2005). The genus *Mycobacterium* originated more than 150 years ago (Daniel, 2006) but to date, this *Mycobacterium* still continues to cause disease and death amongst humans.

2.3 Classification and characteristics of mycobacteria

Mycobacterium tuberculosis belongs to the mycobacteria family, which are distinctive rod-shaped bacteria, non-motile, do not form spores and do not produce toxins (Ryan *et al*., 2010). They have a lipid-rich cell wall which retains carbol fuchsin dye even in the presence of acid alcohol (acid fast-staining) (Glickman and Jacobs, 2001). The cell wall is covered with a thick, waxy mixture of lipids, polysaccharides and mycolic acids (Ahmad, 2010). The cell wall contains a peptidoglycan layer similar to other Gram-positive bacteria with many branched chain polysaccharides, proteins, and lipids attached (Ryan *et al*., 2010). The cell wall also has mycolic acids which are high-molecular weight X-alkyl, B-hydroxy fatty acids covalently attached to arabinogalactan (Kolyva and Karakousis, 2012). *Mycobacterium tuberculosis* is a slow growing, acid fast staining, intracellular organism, which takes up to eight weeks to grow on solid media.

Mycobacterium, *Corynebacterium*, *Actinomyces* and Nocardia are genetically related and all appear as gram-positive irregular non sporing rods (Van Soolingen, 2001). *Mycobacterium avium*, *M. intracellulare* and *M. kansasii* are examples of non-tuberculous mycobacteria (NTM) found in the environment and may cause disease in the immuno-compromised (Ryan *et al*., 2010). *Mycobacterium tuberculosis* complex (MTBC) refers to a genetically closely related group of Mycobacterium species that can cause tuberculosis, which includes *M. tuberculosis*, *M. africanum*, *M. bovis*, *Mycobacterium bovis BCG*, *M. microti*, *M. canettii*, *M. pinnipedii* and *M. mungi* (Brosch R *et al*.,2001).

2.4 Pathogenesis of *M. tuberculosis*

Tuberculosis is transmitted by aerosolised droplets from infected persons which, when inhaled by a new susceptible host, will lodge in the terminal air spaces of the lung (Frieden, 2003) where they will enter and replicate within the alveolar macrophages (Glickman and Jacobs, 2001; Frieden, 2003). Once inside the host macrophages *Mycobacterium* resides within a membrane-bound vacuole and it is capable of modifying the maturation of the phagosomal compartment in order to enhance its own intracellular survival (Armstrong and Hart, 1971).

Mycobacterium tuberculosis has distinct phases of replication that include dissemination, establishment and maintenance of latency and reactivation, which involve the replication of the organism at the initial pulmonary site of infection, from where it will spread to local lymph nodes within the lung and will eventually disseminate to remote sites of the body (Glickman and Jacobs, 2001).

2.5 Immunity to tuberculosis

The immune response to *M. tuberculosis* is complex and still not yet fully understood (Sharma *et al*., 2012). Strain variation of *M. tuberculosis* has shown to be a probable cause factor to elicit variable degrees of severity of the disease in the human host (Sharma *et al*., 2012). The impact of this variation on the clinical manifestation of the disease remains largely unknown; there also exists significant variability in the immune status of the host governing susceptibility to tuberculosis (Sharma *et al*., 2012). What is currently known is that the cell-mediated immune (CMI) response plays the most important part in the control of *M. tuberculosis* infection (Sharma *et al*., 2012). Antibody-mediated immunity (AMI) is not as effective in the control of *M. tuberculosis* because it is an intracellular pathogen (Todar, 2005). Inherited and acquired host factors can predispose one to mycobacterial infection; this knowledge has enhanced our understanding of the mechanism of protective immunity (Nicol and Wilkinson, 2008). An overview of Immune response to *M. tuberculosis* is shown in *Figure 2.2.*

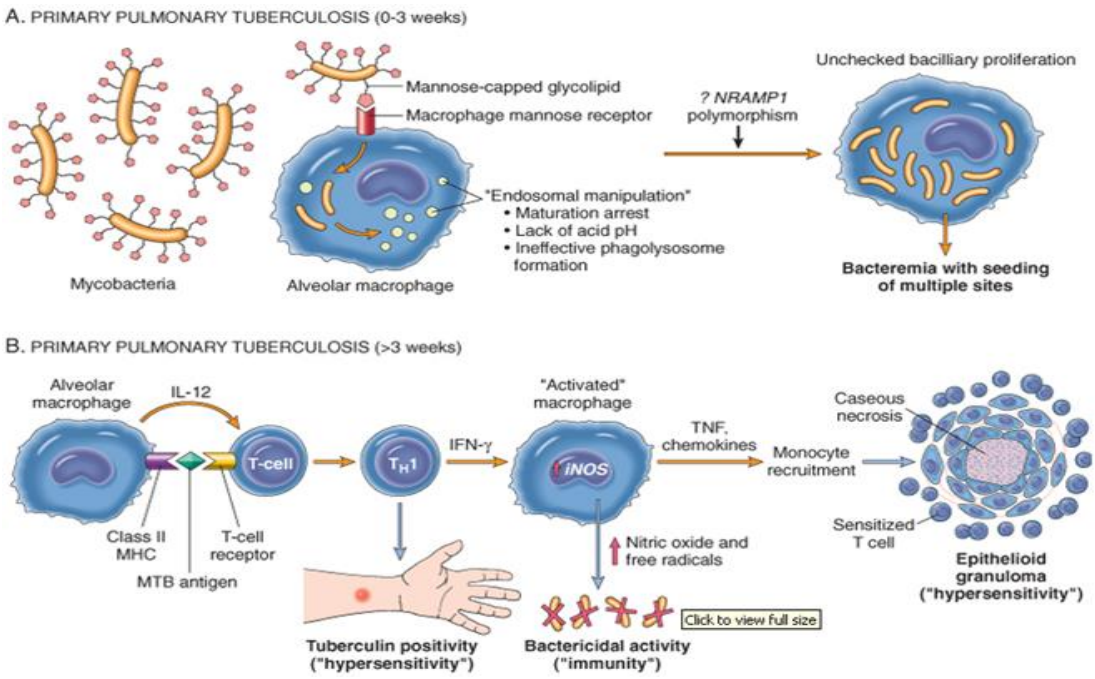

Figure 2.2 Immune response to *Mycobacterium tuberculosis* infection (Todar, 2005)

2.6 Virulence factors of *M. tuberculosis*

Mycobacterium tuberculosis resides in the alveoli after being inhaled; alveolar macrophages then produce inflammatory cytokines and chemokines that serve as a signal for infection (Ahmad, 2010). *Mycobacterium tuberculosis* can resist the bactericidal mechanism of the macrophages by preventing phagosome-lysome fusion, then continue to multiply sustaining long-term survival within the host macrophages (Glickman and Jacobs, 2001) and finally escape from the phagosome/phagolysome and cause macrophage necrosis (Ahmad, 2010). This occurs the same way in each macrophage that is newly infected by the pathogen. In addition, the cell wall of mycobacteria is a very complex one, rendering it resistant to chemical injury, dehydration and certain antibiotics (Clifton, 2001). The cell wall has low or limited permeability, due to the presence of mycolic acids which makes it retain dyes following acid treatment during staining; its ability to form cords is also due to the presence of a coating of mycolic acid on the cell wall (Clifton, 2001).

2.7 Epidemiology of *M. tuberculosis*

The majority of TB cases worldwide in 2012 were in the South-East Asian (29%), African (27%) and Western Pacific (19%) regions; India and China alone accounted for 26% and 12% of total cases respectively (WHO, 2013). According to the 2013 report of the World Health Organisation, the current global picture of TB shows continued progress, but TB still remains one of the world's deadliest communicable diseases. In 2013 an estimated 9.0 million people developed TB and 1.5 million died from TB. Globally in 2012 450 000 people developed MDR-TB with an estimated 170 000 deaths from MDR-TB (WHO, 2013).

Africa has the highest incidence rates, for example in Lesotho, South Africa and Swaziland one in every 100 people develops active TB each year (WHO, 2013). The African region also accounts for the highest HIV co-infection cases in the world with about four in every five individuals with TB in Africa being HIV positive (WHO, 2013). An estimated 1.1 million (13%) of the 8.6 million people who had developed TB in 2012 were HIV-positive (WHO, 2013).

About 60% of TB cases and deaths occur among men but women are also affected as reported in 2013 with an estimated 510 00 women who died as a result of TB and in 2012 530 000 TB cases were diagnosed among children under 15 years of age (WHO, 2013).

2.7.1 Tuberculosis in South Africa

South Africa makes up 0.7% of the world's population but contributes 20% of the number of HIV-positive TB cases worldwide and 33% of HIV-positive TB cases in Africa (Barnard *et al*., 2012). In most parts of Southern Africa more than 50% of tuberculosis cases are co-infected with HIV (WHO, 2014). South Africa and other countries (India, China, and the Russian Federation) comprise almost 60% of MDR-TB cases in the world. According to the WHO 2012 report in 2011 there were 10 085 cases of MDR-TB reported in South Africa (WHO, 2012).

According to the TB treatment outcome in South Africa reported in 2011, in 15% of the cases treatment was successful, 40% of the patients died and 36% of the patients were lost to follow up or were not evaluated (WHO, 2014). The high mortality of MDR-TB and XDR-TB patients in South Africa is most likely to be associated with a high level of HIV co-infection in TB patients (WHO, 2014). South Africa has a high TB rate, and more than 500/100 000 of the population is estimated to contract TB annually (*Figure 2.3*).

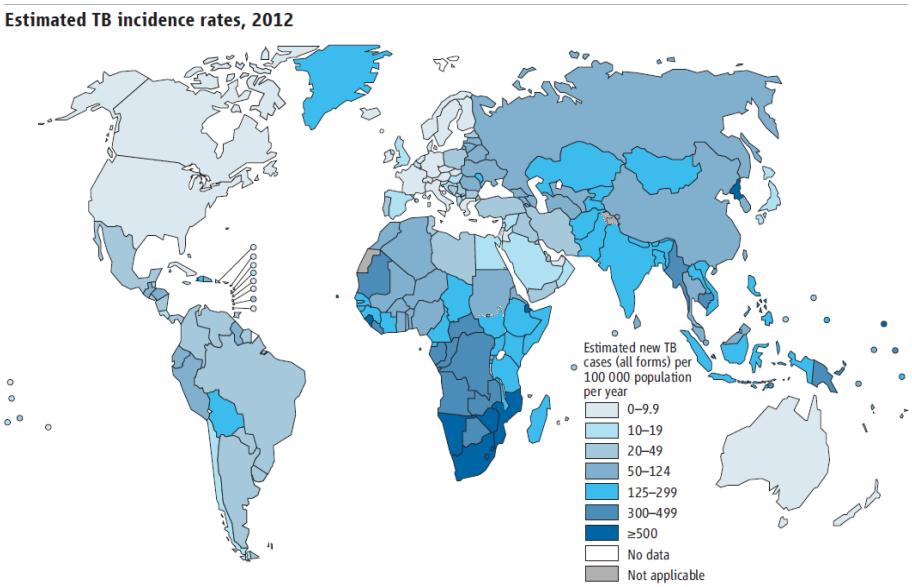

Figure 2.3 Estimated new TB cases (WHO, 2013).

2.8 Development of drug-resistant tuberculosis

Most *M. tuberculosis* strains were initially susceptible to first-line anti-TB drugs, and global control of the disease seemed a realistic goal then (McConnell and Hargreaves, 2013). With the emergence of drug resistance this reality is now hard to achieve. Around 630 000 cases of MDR-TB occur worldwide, and XDR-TB is now reported in 84 countries (WHO, 2012). Laboratories in highly burdened countries are ill-equipped to detect and diagnose the extent of drug resistance (Zumla *et al*., 2013). Although a cure for TB has been available for many decades (Zumla *et al*., 2013), drug resistance and several human and structural failures cause the disease to remain widespread and not properly manageable.

The increase in drug-resistant TB strains poses a great challenge to global TB control efforts (Kolyva and Karakousis, 2012). Multidrug resistant TB is defined as resistance to at least INH and RIF, while XDR-TB is defined as MDR-TB with additional resistance to fluoroquinolones and at least one of the injectable second line drugs (capreomycin, kanamycin and amikacin) (Streicher *et al*., 2012). Drug resistance can develop in a host due to improper use of the anti-TB drugs either by the patient or the health care provider (Streicher *et al*., 2012). Drug resistance can also be acquired by susceptible individuals when they get infected by an already resistant *M. tuberculosis* strain (Streicher *et al*., 2012). Drug resistance can be intrinsic or acquired: some bacilli have the innate ability to resist the activity of a particular antimicrobial agent through their inherent structural or functional characteristics (Streicher *et al*., 2012). This intrinsic drug resistance ability of *M. tuberculosis* is due to its cell wall properties, including the presence of mycolic acids. Acquired drug resistance occurs when a bacillus previously susceptible to a particular anti-microbial becomes resistant mainly due to selective growth of the drug-resistant mutants (Kolyva and Karakousis, 2012).

2.9 Diagnosis and detection of drug resistance of *M. tuberculosis*

A number of diagnostic techniques are available for the detection of *M. tuberculosis* including microscopy, bacteriological culture, radiological, tuberculin skin tests, serological diagnosis and molecular assays (Wanchu, 2005). In recent years there has also been rapid progression in the development of new diagnostic assays for TB, e.g. Xpert MTB/RIF which was endorsed by the World Health Organisation (WHO) in December 2010 (Lawn *et al*., 2013). Molecular methods have enhanced the detection and identification of *M. tuberculosis* but smaller laboratories do not have the necessary resources to accurately identify these bacteria (Parsons *et al*., 2011). The most common method for diagnosis worldwide still remains sputum smear microscopy (WHO, 2014).

2.9.1 Clinical diagnosis

To get the full picture during TB diagnosis a complete medical history of the patient is needed, including a chest X-ray and a physical examination (Wanchu, 2005). A chest X-ray can help determine if the patient has active TB; the X-ray will show changes and can help confirm the diagnosis), but cannot be used as the only diagnostic tool (Wanchu, 2005). Chest X-rays of HIV-positive people may appear normal even if infected with *M. tuberculosis* (Wanchu, 2005).

2.9.2 Microscopy detection of *M. tuberculosis*

Microscopy has been used for diagnosis of TB for more than 100 years ago (WHO, 2012). Currently there are two staining methods used for detection of *M. tuberculosis* including fluorochrome acid-fast stain (auramine staining) and Ziehl-Neelsen staining. The Ziehl-Neelsen staining uses a light microscope while the fluorescence microscope and the light emitting diode (LED) are used for auramine stain. The Ziehl-Neelsen stain covers only a small area of the specimen (100X magnification) while the auramine staining technique gives a 45X or 50X magnification which covers a larger surface area of the specimen (Dezemon *et al*., 2014). Fluorescent microscopy has a higher sensitivity and specificity (Steingart *et al*., 2006); its use in clinical settings can improve TB case-findings.

Microscopy is a rapid presumptive diagnosis of TB because *M. tuberculosis* is a slow grower (Narayan, 2012). Microscopy is cheaper and faster than culture and other techniques; the disadvantage is the low sensitivity and specificity of the method (50-80%) (Narayan, 2012). Ziehl-Neelsen staining is done on heat-fixed smears on microscope slides, with the use of carbol fuchsin and read under a microscope using the immersion lens (100X) (Dezemon *et al*., 2014).

2.9.3 Solid culture based methods

Various solid media have been developed for the isolation and DST of *M. tuberculosis* complex such as the Lowenstein Jensen (LJ) medium (Cummings MM, 1949 and Joloba *et al*., 2014) and

the Middlebrook culture media, with a detection period of four to six weeks (Parsons *et al*., 2011).

2.9.3.1 Lowenstein Jensen culture media

The LJ medium is used for both identification (Anargyros *et al*., 1990) and DST of *M. tuberculosis* (Rastogi *et al*., 1989). It is a solid egg-based medium which can help to differentiate between different colonies of different *Mycobacterium* species, but has a 2 to 6 weeks detection time period. The Lowenstein Jensen medium contains homogenised whole egg, organic salts, asparagine and glycerol with malachite green dye used to inhibit contaminating microorganisms.

Figure 2.4 *Mycobacterium tuberculosis* growing on Lowenstein Jensen agar

The LJ medium is relatively cheap to prepare in local laboratories and fairly resistant to contamination and does not depend on incubation with supplemental carbon dioxide for mycobacterial recovery (Joloba *et al*., 2014). This medium is also one of the conventional methods for DST; it gives DST results in four to five weeks after initial inoculation (Rastogi et *al*., 1989). This bacterium grows on LJ media producing buff to yellow, rough wrinkled colonies (*Figure 2.4*)

2.9.3.2 Middlebrook culture medium

This agar is used for isolation, cultivation and sensitivity testing of *M. tuberculosis*. It contains oleic acid, catalase and albumin, which protects *Mycobacterium* from toxic agents (Joloba *et al*., 2014). The Middlebrook 7H10 and 7H11 are mostly used and are different in that the latter has casein hydrolysate which improves recovery of some of the fastidious strains of *M. tuberculosis* but both are supplemented with polymyxin B, amphotericin B, carbenicillin and trimethoprim (Joloba *et al*., 2014).

2.9.4 Liquid culture based methods

Liquid media is preferred for isolation of *M. tuberculosis*; the most commonly used is the modified Middlebrook 7H9 broth (Joloba *et al*., 2014). There are a number of liquid culture based methods including BACTEC 960 MGIT and versa TRECK systems. Liquid based systems such as MGIT are more rapid and detect more mycobacterial isolates than solid culture methods; however, morphological examination of colonies alone can no longer be used for species identification (Chihota *et al*., 2011).

2.9.4.1 The BACTEC 960 MGIT system

The BACTEC 960 MGIT system is a non-radiometric automated system used for the isolation and DST of *M. tuberculosis* (Tortoli *et al*., 2003). The system is based on the presence of an oxygen-quenched fluorochrome, tris-4, and 7-diphenyl-1, 10-phenothroline ruthenium chloride pentahydrate, embedded in silicone at the bottom of the tube. Bacteria grown uses oxygen displacing with carbon dioxide, when the oxygen level drops the fluorochrome is not inhibited and it starts to fluoresce. Drug sensitivity testing is done according to the same principle but in the presence of antibiotics (first and second line drugs) and the intensity is directly proportional to oxygen levels (http://www.laboratorystack.com/bactec-mgit-960-system-principle-manual-brochure/). *Mycobacterium tuberculosis* DST on BACTEC MGIT 960 (Tortoli *et al*., 2003) provides results for streptomycin (SM), INH, RIP, and EMB (SIRE kit) and PZA (PZA kit) in a

time frame close to the BACTEC 460TB system (Tortoli *et al*., 2003). The system performs a qualitative susceptibility test for the drugs within four to 19 days.

The BACTEC MGIT system is fast and was recommended by the centres for disease prevention and control (CDC) to use as one of the most rapid methods available for diagnosing mycobacteria. The only limitation would be the cost as compared to conventional methods and liquid-based methods are known to have a high percentage of contamination.

2.9.4.2 Versa TREK System

Versa TREK (formerly ESP Culture System II) is an automated method that was first developed for blood cultures and later adapted for the recovery and DST of mycobacteria (Yuksel *et al*., 2011). The Versa TREK system combines liquid culture medium (versa TREK *Myco* Media), a growth supplement (*Myco* GS) and for potentially contaminated species an antibiotic supplement (*Myco* AS or *Myco* PVNA) with a detection system that automatically incubates and continuously monitors culture bottles with specimens. It is based on the detection of headspace pressure changes within a sealed bottle, monitoring the changes in either gas production or gas consumption due to microbial growth (Yuksel *et al*., 2011). It has been validated for performing qualitative susceptibility testing with INH, RMP, and EMB with *M. tuberculosis* clinical isolates, while testing for SM and PZA has not been cleared by the FDA.

2.9.5 Molecular based assays for the detection and identification of *M. tuberculosis*

Polymerase chain reaction (PCR) can be used to directly detect *M. tuberculosis* on clinical specimens. It targets specific genes in the mycobacterial genome. Multi drug-resistant TB and XDR-TB diagnoses are based on the detection of the mutations in corresponding genes.

2.9.5.1 Nucleic acid amplification technique (NAAT)

Molecular probes target both DNA and ribosomal RNA sequences in clinical specimens. These methods include the use of either PCR, transcription-mediated amplification (TMA) or loop-mediated isothermal amplification (LAMP) (CDC, 2009). Nucleic acid amplification methods can also be used for identification of mycobacteria isolated in culture with rapid detection (can be performed within six to eight hours). These methods have a high specificity for *M. tuberculosis* (>98%) as it is extremely susceptible to contamination from amplicons derived from positive specimens (CDC, 2009).

2.9.5.2 Line probe assay

Reverse hybridisation-based assays, referred to as line probe assays (LPAs), are available commercially and recommended in endemic areas for rapid detection of mutations resulting in resistance to INH and RIF (Barnard *et al*., 2012). Line probe assays are based on the hybridisation of PCR products from patient specimens to specific probes for wild-type and mutant alleles of genes involved in drug resistance (Barnard *et al*., 2012). Line probe assays have shown high specificity and sensitivity in detecting *M. tuberculosis* and identifying the specific mutations. Examples of LPA include the INNO-LiPA® MYCOBACTERIA V2 (Fujirebio, Europe) and the Genotype® MTBDRplus (Hain Lifescience, Nehren, Germany) (Barnard *et al*., 2012).

2.9.5.3 INNO-LiPA® MYCOBACTERIA V2

INNO-LiPA® MYCOBACTERIA V2 is a line probe assay that simultaneously detects and identifies the genus *Mycobacterium* and 16 different mycobacterial species. The test is based on the nucleotide differences in the 16s-23-r rRNA spacer region, followed by hybridisation to DNA probes covering the core region of the *rpo*B gene of *M. tuberculosis* that are immobilised on a nitrocellulose strip. The nitrocellulose strip contains 10 oligonucleotide probes: one specific for *M. tuberculosis*, five wild-type probes (S1 to S5) and four probes (R) for the detection of the most frequent mutations that cause resistance to RIF (Neonakis *et al*., 2008).

The assay can be performed from a liquid or solid culture. The test has a sensitivity of 100% and a specificity of 94% (Tortoli *et al*., 2003).

2.9.5.4 The Genotype®MTBDR*plus* assay

The Genotype®MTBDR*plus* assay is a Hain Life Science assay based on PCR amplification and PCR reverse hybridisation, used for first line drug susceptibility testing (WHO, 2012). It is based on a DNA trip test that allows simultaneous molecular identification of TB and the most common genetic mutations causing resistance to RIF and INH (WHO, 2012). It can diagnose MDR-TB directly from smear-positive sputum sample and culture-based isolates (Hillemann *et al*., 2007) providing results in just five hours (WHO, 2012).

2.9.5.5 GeneXpert® system

The geneXpert system is a system that utilises real-time PCR to amplify and detect *M. tuberculosis* by targeting DNA (Pai and Ling, 2008). The geneXpert® system is a fully automated system that involves sample preparation, DNA amplification and detection at the same time (Pai and Ling, 2008). If there are TB bacteria in the sample, the machine will detect the DNA and mutations are detected by real-time PCR assay for rifampicin with results available within two hours (Pai and Ling, 2008). The limitation of using the GeneXpert® system is that it is more expensive than the standard sputum-smear tests like microscopy; and the machines require an uninterrupted supply of electricity and need to be connected to a computer. Diagnosis of TB by microscopy in many countries has been replaced by the use of geneXpert® system, which has made it easier and faster to diagnose TB and to get the appropriate TB treatment regimen early.

2.9.6 Serological assays for the detection of mycobacteria

Serological test assays rely on the detection of a humoral antibody immune response to *M. tuberculosis* (Steingart *et al*., 2007). Common antibody detection test designs include the enzyme-linked immunosorbet assay (ELISA) format and the immunochromatographic test

format (Steingart *et al*., 2007). Serological tests are fast, simples and can be a cheaper alternative for low income countries (Steingart *et al*., 2007, WHO, 2013). However, serological assays lack of sensitivity and specificity and they do not work well enough to replace sputum-smear microscopy as the presence of antibodies can sometimes only mean TB exposure and not necessarily active disease.

2.9.6.1 IGRAS- Interferon- Gamma release Assays

The assays are whole blood tests for TB infection and can aid in diagnosis of *M. tuberculosis* infection, but they do not differentiate latent TB from active TB disease (Rangaka *et al*, 2012), these tests include QuantiFERON®-TB Gold (QFT-GIT) and the T-spot®.TB test (T-spot). Interferon-Gamma release assays measure a person's immune reactivity to *M. tuberculosis* (Rangaka *et al*, 2012). White blood cells from most persons that have been infected with *M. tuberculosis* will release interferon-gamma (IFN-g) when mixed with antigens derived from *M. tuberculosis*. They have a few advantages and disadvantages, like prior BCG vaccination will not cause a false positive; false positives may occur with children less than 5 years, in persons recently exposed to *M. tuberculosis* and in immunocompromised persons. Neither IGRAS nor the Tuberculin skin test has a high accuracy for the prediction of active disease (Rangaka *et al*, 2012).

2.9.7 Differentiation of mycobacteria

When tuberculosis is suspected, either pulmonary or extra pulmonary TB, a specimen is taken (tissue, fluid or sputum) and sent to the laboratory for diagnosis with the conventional use of acid fast staining methods, culture and phenotypic characterisation (Machado *et al*,2014). A number of diagnostic tests are available for diagnosis of TB directly from the specimen, which include the use of the GeneXpert® system (Cepheid, USA), an automated system that detects *Mycobacterium tuberculosis* complex (*M. tuberculosis* complex) and Rif resistance within two hours (Pai and Ling, 2008). The *M. tuberculosis* complex includes *M. bovis*, *M. africanum*, *M. pinnipedii*, *M. microti*, *M. caprae*, *M. canettii* and *M. tuberculosis* (Brosch *et al*., 2001). Fast staining methods have available options that include conventional light microscopy which uses

carbolfuchsin Ziehl-Neelsen or Kinyoun acid fast stain and fluorescence microscopy which uses acid-fast fluorochrome dye (e.g. auramine O or auramine-rhodamine) (Steingart *et al*, 2006). Microscopy will only show the presence of any acid fast bacilli which could be any of the *M. tuberculosis* complex species or any of the non-tuberculous mycobacteria species. Ziehl-Neelsen can at least show the cord characteristic of *M. tuberculosis* complex, *M. tuberculosis* complex species tend to aggregate forming structures known as cords that appear as rope-like structures, an intrinsic property of the human tubercle bacilli (Gao *et al*, 2004) and the loose and clustered characteristic of non-tuberculous mycobacteria under the microscope. The accuracy in detection and characterisation of these organisms' feature under the microscope will depend on the experience of the person performing the test.

Further differentiation of mycobacteria may be achieved by serological tests like the BD MGIT™ TBc identification test which can detect *M. tuberculosis* complex: *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*, as it is very important to distinguish non-tuberculous mycobacteria from *M. tuberculosis* complex (BD MGIT™ TBc identification test pamphlet, 2015). This test identifies *M. tuberculosis* complex from culture grown in liquid media within 15 minutes (Yu *et al*, 2009). The test is a chromatographic immunoassay for the qualitative detection of *M. tuberculosis* complex from AFB-positive MGIT tube, it detects the MPT64 a mycobacterial protein fraction that is secreted from MTBC cells during culture (Yu *et al*, 2009 and Machado *et al*, 2014). This test does not rule out the presence of other mycobacterial or mixed bacterial infections. A negative test result by the test does not always rule out the possibility of infection with *M. tuberculosis* complex, the test is unable to detect *M. tuberculosis* complex when a mutation arises in the MPT64 gene and some sub-strains of *M. bovis* BCG among *M. tuberculosis* complex produce no MPT64 antigen and will therefore result in a negative test result (BD MGIT™ TBc identification test pamphlet, 2015).

Molecular methods are available which enable the detection of *M. tuberculosis* complex and non-tuberculous mycobacteria like the GenoType Mycobacterium CM/AS (HAIN Lifesciences, Germany) that further differentiate mycobacterial species. The test is based on a PCR technique targeting a 23S rRNA gene region, the GenoType CM is capable of identifying 23 species from

the *M. tuberculosis* complex and the Genotype AS can identify a further 14 non-tuberculous mycobacteria (Richter *et al*, 2006).

2.10 Treatment of tuberculosis

Treatment of *M. tuberculosis* requires the use of multiple drugs for several months (six months) and this is only with susceptible TB which does not include any resistance to any of the anti-TB drugs. The treatment for drug sensitive TB includes two phases; the intense phase that requires RIF, pyrazinamide (PZN), INH, ethambutol (E) or streptomycin (S) for two months and the continuation phase with RIF and INH for four months.

Drug resistant *M. tuberculosis* strains complicate treatment especially MDR-TB which now needs to be treated with a variety of drugs including some first-line and second-line drugs (kanamycin, prothionamide, para-aminosalicylic acid, thiacetazone, capreomycin, ofloxacin, cycloserine and amikacin) (Leimane *et al*., 2005). Treatment of MDR-TB and XD-TB strains requires at least three effective drugs in combination to successfully treat and prevent development of further resistance (Zhang *et al*., 2009).

The WHO recommendations for treatment of MDR-TB is composed of at least pyrazinamide and four second-line drugs considered to be effective (based on DST) and /or previous use and/ or drug resistance surveillance data, with a fluoroquinolone (preferably later-generation), a second-line injectable agent and two bacteriostatic drugs, preferably prothionamide or ethionamide and cycloserine or p-aminosalicyclic acid. Treatment of XDR-TB is with more expensive, less effective second-line antibiotics resulting in a longer treatment course with a more aggressive treatment approach with daily supervised therapy of at least five antibiotics including cycloserine, an injectable drug and fluoroquinolones administered at the highest tolerated doses (Haydel, 2010).

Drug-resistant TB has low cure rates, MDR-TB cure rates range from 50% to 70% (Wright *et al*., 2009) and XDR-TB has cure rates between 30%-60% (Dye *et al*.,2000; WHO, 2008; Kim *et al*., 2008). The susceptibility of the bacterial strain to anti-TB drugs, the regimen employed and

its duration, the availability of drugs and the adherence of both treatment provider and patient to recommended standards of care will determine the treatment outcome in patients (Veen *et al*., 1998).

2.11 Control and eradication of tuberculosis

The WHO implemented the *Stop TB Strategy* from 1995 to 2008 (WHO, 2013) in an effort to control TB globally. This was estimated to have cured 36 million people with TB and averted six million deaths worldwide (WHO, 2013). Despite all the effort, TB is still a burden and still continues to kill and cause morbidity in millions of people. Current control efforts need to be intensified if eradication is to be achieved. The increase in drug-resistant TB is making it difficult to eradicate the disease. For better control of the disease and possible eradication, new highly effective and widely accessible diagnostics, new drugs and vaccines are needed to support TB control globally, and technical and structural challenges which include logistics, that delay detection, treatment and prevention of all forms of TB must be overcome (WHO, 2013).

2.12 Vaccines

Tuberculosis has been around for millions of years. At first it was thought that the disease could be conquered by vaccination with *M. bovis* Bacille Calmette-Guerin (BCG) vaccine (Gheorghiu M, 1990). The BCG vaccine is an attenuated strain of *M*. bovis that was derived from a virulent strain (Anderson and Doherty, 2005). The vaccine protects children efficiently against early manifestation of TB but protection in adults is different in different populations, ranging from 0 to 80% (Tom and Kaufmann, 2012). This difference in adult protection is thought to be due to the interaction between the vaccine and mycobacteria in the environment (Anderson and Doherty, 2005). Vaccines that prevent establishment of *M. tuberculosis* infection or those that prevent progression of established infection towards active TB disease are needed. The BCG vaccine is the only available vaccine against TB (Tom and Kaufmann, 2012). This vaccine is not safe in HIV-positive infants, causing disseminated disease (Talbot *et al*., 1997).

There is a lot of research into vaccine development against TB; attempts are being made to design a new vaccine or to enhance the efficacy of the BCG vaccine. So far none of the vaccine being development has successfully completed the clinical trial pipeline; many are still under advanced clinical assessment (Kaufmann, 2013 and MacShane, 2011). These vaccines aim at the prevention of active TB over a long period of time ideally lifelong (Kaufmann, 2013).

2.13 Molecular epidemiology of M. *tuberculosis*

Molecular characterisation of *M. tuberculosis* strains is helpful to gain insight in the major circulating strains of *M. tuberculosis* around the world (Affolabi *et al*., 2009). Information from strain typing has been used to supplement traditional epidemiological methods and has provided insight into the transmission dynamics of TB (Affolabi *et al*., 2009).

A number of genotyping methods including insertion sequence 6110-restriction fragment length polymorphism (IS6110-RFLP) (Thierry *et al*., 1990), spoligotyping (Kamerbeek *et al*., 1997) and mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) (Supply *et al*., 2000) have expanded the ability to investigate and understand TB. These techniques can give insight of *M. tuberculosis* strain distribution and possible associations between patient demographics, clustering of strains, clinical manifestations and drug resistance.

2.14 Restriction fragment length polymorphism (RLFP)

The standard approach to genotyping *M. tuberculosis* isolates is RFLP analysis of the distribution of the insertion sequence IS6110 (*Figure 2.15*) in different strains (Stavrum *et al*., 2009). The IS6110 is a transposable element and was first described by Thierry *et al* (1990). The IS6110 element is able to make copies of itself and can insert the copy anywhere in the *M. tuberculosis* genome (transposition) (Barnes and Caves, 2003). The differences in the IS6110 copy numbers per strain range from 0 to about 25 and RFLP can identify variability in the chromosomal positions of these IS6110 insertion sequences (Van Soolingen, 2001). Isolates from patients with epidemiologically unrelated strains of *M. tuberculosis* have different RFLP patterns (Barnes and Cave, 2003).

The IS6110 banding pattern may change over time (Niemann *et al*., 2000; de Boer *et al*., 1999; Warren *et al*., 2002). The stability of the transposable element IS6110 on *M. tuberculosis* can last for 3.2 years (de Boer *et al*., 1999) or around 8.7 years (Rhee *et al*., 2000), the difference in IS6110 half-life is due to the change that may occur during active growth prior to treatment with the low rate reflecting a change occurring during or after treatment (Mohammad and Hossein, 2007).

The IS6110-RFLP provides excellent discrimination, highly reproducible and is internationally accepted as the gold standard. However, IS6110-based RFLP genotyping requires a high concentration (i.e. 2 µg) of pure DNA from each *M. tuberculosis* culture; it is also a slow, labour-intensive, and technically demanding. In addition, the RFLP method has a low discriminatory power for isolates that have six or fewer copies of IS6110 and should be supplemented with other methods (see *Table 2.1*).

2.15 Mycobacterial interspersed repetitive units-variable number of tandem repeats

Variable Number of Tandem Repeats was first found in Eukaryotic cells and now also in prokaryotic cells, in Mycobacteria which are 40-100bp. The genome of *M. tuberculosis* contains many mycobacterial interspersed repetitive units (MIRUs), some containing identical repeat units and others containing repeats that vary slightly in sequence and length (Supply *et al*., 1997). The MIRU's are repetitive sequences found in non-coding regions of the *M. tuberculosis* genome (Jagielski *et al*., 2014). These MIRU's are called minisatellites mainly because of their size of 40-100 bp and sequence repeats. These minisatellite are homologous DNA sequences found as tandem repeats and are dispersed in intergenic regions on the *M. tuberculosis* genome (Supply *et al*., 2002). Supply *et al.* (2000) identified three major types I-II of MIRUs dispersed within 41 variable number tandem repeat (VNTR) located on the chromosome of the H37Rv, CDC1551 and AF2122/97 *M. tuberculosis* strains. Out of the 41 MIRU loci, 12 to 24 MIRU loci are commonly used for the genotyping of *M. tuberculosis* clinical isolates (Mathema *et al*., 2006; Supply *et al*., 2006). Most MIRU-VNTRs are relatively stable and evolve slowly in mycobacterial populations.

This technique is based on mini-satellite loci detection of the MIRU-VNTR allele, numbering and sizing of the repetitive units in each locus calculated by the size of the fragment and PCR amplification of these repetitive units (Jagielski *et al*., 2014). An allele is one of a number of alternative forms of the same gene. Different *M. tuberculosis* strains have different distinguishable VNTR'S at different loci on the *M. tuberculosis* genome (Jagielski *et al*., 2014). The MIRU-VNTR determines the number of mycobacterial interspersed repetitive units on each of the different loci with the number of repetitions determined by PCR (Supply *et al.*, 2000). Primers will bind to the non-repetitive sequences flanking the known repetitive sequences, amplified and quantified with the automated system or by electrophoretic migration technique (Jagielski *et al*., 2014). Tandem repeat polymorphism can occur due to nucleotide changes between the individual repeat units or due to variation in the number of repeats. The MIRU-VNTR is based on the number of variation of these repetitive units occurring at different loci on the *M. tuberculosis* genome.

The MIRU-VNTR typing can be done using the 12-loci, 15-loci and 24-loci formats and MIRU-VNTR typing scheme has been proposed for international standardization. The method has high discriminatory power close/equal to IS6110 RFLP and the results are expressed as digital codes, easily exchangeable between different centres. Unlike IS*6110*-RFLP, which is labour-intensive and requires the preparation of high quality DNA from cultured specimens, PCR-based MIRU-VNTR can be performed on clinical samples and can be done rapidly at high-throughput. Some studies, however, have reported a lower discriminatory power in settings where Beijing strains are dominant. The detailed advantages and disadvantages of MIRU-VNTR typing are shown in T*able 2.1*.

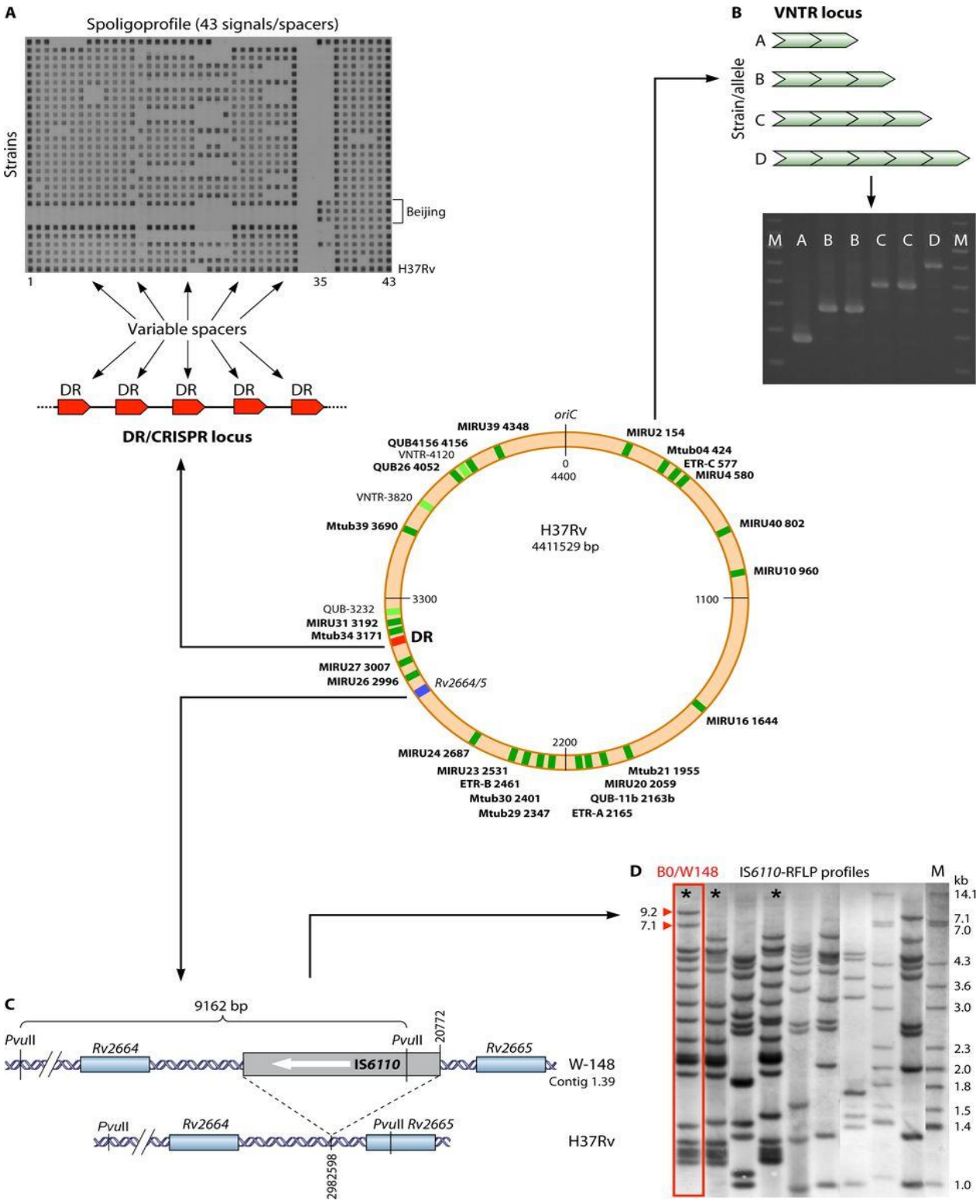

Figure 2.15 Molecular genotyping target sites on *M. tuberculosis* H37Rv, Structure of the DR locus in the mycobacterial genome of *M. tuberculosis* H37Rv and *M. bovis* BCG contain 48 and 41 DRs, respectively (depicted as red), which are interspersed with unique spacers varying in length from 35 to 41 bp The site of integration of insertion element IS*6110* is depicted in blue and the VNTR's are depicted in green.

2.16 Spacer Oligonucleotide Typing

Spoligotyping is a method based on DNA polymorphism present at one chromosomal locus, the direct Repeat (DR) region which is uniquely present in *M. tuberculosis* complex. It is based on PCR, which analyses the DR sequence comprising 10 to 50 copies of 36-basepair element separated by short, unique, non-repetitive sequences (Daley, 2005). The DR region in *M. bovis* BCG consists of DR sequences of each 35- 41 base pairs in length. The DR region occurs in different *M. tuberculosis* strains in the same location with varying locations of the spacers separating the direct repeat sequences (Kamerbeek *et al*., 1997). The deletions of the spacers on the DR region alter the spacer composition in each strain (Jagielski *et al*., 2014).

The DR region undergoes genetic rearrangements in different *M. tuberculosis* complex strains (Groenen *et al*., 1993). Strains differ in terms of the presence or absence of specific spacer (Barnes and Cave, 20003; Filliol *et al*., 2000; Kamerbeek *et al*., 1997; Groenen *et al*., 1993 and Hermans *et al*., 1990). The number of DR's in other *M. tuberculosis* complex strains vary significantly and the order of spaces in different strains is similar but the deletions and/or insertions of these spacers and DR's vary significantly making it possible to genotype *M. tuberculosis* based on this difference. The amplified regions/fragments are hybridised to a set of immobilised, complementary oligonucleotides and the presence of each spacer is detected by chemiluminescence. Spoligotyping assay produces a series of bands much like a bar code, a 43-digit binary code that is converted to the 15-digit octal code. For example, the Beijing family lacks spacers (1-34), and most *M. tuberculosis* TB strains lack spacers 33-36 and *M. bovis* (BCG) stains have 33-36 but lack spacers 39-43.

Spoligotyping is technically simple, reproducible, and easily coded, but its discriminatory power is lower than IS*6110*-RFLP typing and MIRU-VNTR. This is because spoligotyping targets a single locus that accounts for less than 0.1% of the *M. tuberculosis* genome, unlike IS*6110*-RFLP typing, which examines the distribution of IS*6110* throughout the entire genome. However, spoligotyping is better than IS6110 for distinguishing isolates of *M. tuberculosis* with few copy numbers of IS6110 (Goyal *et al*., 1997 and Goyal *et al*., 1999) (see *Table 2.1*). The advantages and disadvantages of spoligotyping are summarized in *table 2.1*. Major changes in

spoligotype due to single deletion events are not always useful for defining the relationship between strains, nor are they sufficiently discriminatory to differentiate closely related strains (Gori *et al.*, 2005).

Table 2.1 Comparison of techniques for molecular typing or evaluating genetic diversity within *Mycobacterium tuberculosis* (Nicol and Wilkinson, 2008).

Comparison of techniques for molecular typing or evaluating genetic diversity within *Mycobacterium tuberculosis*					
	IS6110	Spoligotyping	MIRU-VNTR	LSP analysis	SNP analysis
Discriminatory Power	Excellent	Fair	Good to excellent	Poor	Poor, likely to improve with increased SNP identification
Ease of use	Time consuming and technically demanding; requires extracted chromosomal DNA	Rapid and simple; can be performed directly on specimens or heat-killed cultures	Rapid and fairly simple automation; requires access to sophisticated equipment; can be performed on heat-killed cultures.	Simple and robust	Rapid and fairly simple; high-throughput analysis requires access to sophisticated equipment
Interpretation	Simple, but not easily standardised	Simple visual interpretation	Simple visual interpretation	Straightforward	Straightforward
Data sharing	Complex lack of standardised nomenclature	Straightforward, standardised binary or octal coding	Straightforward, standardised numerical coding	Straightforward, standardised nomenclature	Straightforward, standardised nomenclature
Utility for epidemiological investigations	Excellent due to high discriminatory power	Useful for rapid cluster identification but requires secondary confirmation	Excellent and rapid with newer protocols	Poor due to low discriminatory power	Poor at present due to low discriminatory power
Utility for phylogenetic -’4]analysis	May be limited by irregular rates transposition and favoured sites	Fairly good correlation with SNP-based phylogeny; large deletions may bias analysis	Relatively poor correlation with SNP-based phylogeny	Useful for evolution history (sequential deletions) and for identifying major lineages	Gold-standard; low rate of SNPs in *M. tuberculosis* necessitates large-scale sequencing to identify informative SNPs
MIRU-VNTR: Mycobacterial repetitive unit-variable number of tandem repeats; LPS: Large sequence polymorphism. SNP: single nucleotide polymorphism					

2.17 Treatment outcome associated with different *M. tuberculosis* genotypes

Mycobacterium tuberculosis has the ability to survive in diverse environments; it can survive for years in the stationary phase in cultures in vitro and can go into dormancy (latent infection). Mycobacterial virulence depends on the identification of genes and gene products that contribute to the pathogenesis of TB (Nicol and Wilkinson, 2008). Evidence that proves that genetic variation may influence the outcome and infection with *M. tuberculosis* is inconclusive (Nicol and Wilkinson, 2008). Phylogenic relationship studies between strain lineages help in determining the association between outcome and strain lineage (Nicol and Wilkinson, 2008).

There are many risk factors that may affect treatment outcome in TB patients, which may include *M. tuberculosis* genotype (Marais *et al*., 2013), drug resistance (Holtz *et al*., 2006), patient demographics (Orenstein *et al*., 2009), clinical presentation (Ahuja *et al*., 2012), host factors, HIV, previous TB treatment (Ahuja *et al*., 2012). Multidrug-resistant TB treatment is frequently associated with high rates of treatment failure (Marais *et al*., 2013). The success of anti-TB drugs in pulmonary TB is directly linked to the microbiological status of a patient's sputum specimen during treatment (Holtz *et al*., 2006). Studies have suggested that the resistance to and use of certain drugs can prolong sputum culture conversion (Holtz *et al*., 2006).

Certain *M. tuberculosis* genotypes, such as the Beijing genotype, have been associated with poor treatment outcome (Caws *et al*., 2006), while other studies found the Haarlem strain type to be associated with poor treatment outcome (Marais *et al*., 2013). There are a few studies that have evaluated factors that affect treatment outcome. More studies in this area are needed to find more risk factors associated with the variety of strains circulating in the world.

CHAPTER 3

ASSOCIATION OF *MYCOBACTERIUM TUBERCULOSIS* GENOTYPES AND TREATMENT OUTCOME IN PULMONARY TUBERCULOSIS PATIENTS IN TSHWANE METROPOLITAN AREA

3.1 ABSTRACT

BACKGROUND: Tuberculosis remains the major health problem worldwide. Understanding the population structure, diversity and impact of *M. tuberculosis* strains on treatment is crucial in the management of tuberculosis. Little data is available regarding the association of genotypes to treatment outcome and often with conflicting results.

OBJECTIVES: This study aimed to determine the genetic diversity of *M. tuberculosis* in the Tshwane metropolitan area and the impact of those genotypes on treatment outcome using the eight weeks culture result as a marker for treatment response. In addition the study explored the association of genotypes with drug resistance and patient demographics (age and gender).

METHODS: A total of 108 consecutive *M. tuberculosis* isolates resistant to either rifampicin and/or isoniazid were collected between September and December 2011 from Tshwane Academic Division. The treatment outcome in this study was assessed using the 8 weeks culture conversion results after the patient started TB treatment, as a marker for treatment outcome. Two isolates were contaminated and were excluded from the study. All isolates were genotyped using spoligotyping and 24-loci MIRU-VNTR typing.

RESULTS: Comparison of spoligotyping results of the 106 *M. tuberculosis* isolates with the SpolDB4 database showed that 73% (83/106) isolates belonged to 21 previously described shared types (ST), while 27% (23/106) were not found in the SpolDB4 database and were regarded as orphans. Spoligotyping identified 14 clusters, giving a clustering rate of 50%. The Beijing family was the predominant family in the study. No cluster was identified with 24-loci MIRU-VNTR typing, and no association of *M. tuberculosis* genotypes with poor treatment outcome.

CONCLUSION: The study did not find any association of genotypes with treatment outcome; however only 46 patients out of the 106 of the study population had the eight weeks follow-up results. The study also did not find an association of any specific genotype with gender, age or drug resistance to INH or RIF. Resistance to RIF was associated with treatment failure (patients

failed to convert after eight weeks), with P value of 0.006. However, no significant association was found between INH resistance and treatment outcome. More extensive studies are needed to verify these study findings.

3.2 Background

Tuberculosis (TB) is a disease of major public health concern worldwide. The World Health Organisation (WHO) estimates that in 2011 there were 8.7 million new cases of TB and 1.4 million people died from TB, including almost 1 million deaths among people who were HIV positive (WHO, 2012). The TB problem is increasing due to multidrug-resistant (MDR) TB and extensive drug-resistant tuberculosis (XDR) TB (Wright *et al*., 2009; WHO, 2006). South Africa is one of the high TB-burdened countries and the TB burden in South Africa is worsened by the concurrent epidemic of HIV and the emergence of MDR-TB and XDR-TB (Chihota *et al*., 2011).

The introduction of DNA genotyping techniques has increased the understanding of pathogenesis, the underlying resistance mechanisms, and global distribution of *M. tuberculosis* (Nicol and Wilkinson, 2008). Tuberculosis strain typing methods when combined with epidemiological data can help to identify TB patients who may be involved in the same chain of recent TB transmission (Parra *et al*., 2011). Molecular epidemiology results assist in the initiation of timely and appropriate control measures following a cluster or an outbreak (Parra *et al*., 2011).

The current gold standard genotyping method is restriction fragment length polymorphism (RFLP) which relies on the detection of the transposable element IS6110 that is able to insert itself in different sites on the *M. tuberculosis* genome over time (Groenen *et al*., 1993). The IS*6110*-RFLP has high discriminatory power; however, this method is labour intensive, has low discriminatory power for strains with low copy numbers of IS*6110* and requires large quantity of DNA (Warren *et al*., 2002).

To overcome the limitations of IS*6110*-RFLP, faster PCR-based techniques have been developed including spoligotyping (Kamerbeek *et al*., 1997) and mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) (Supply *et al*., 2006). Spoligotyping is based on DNA polymorphism present at one particular chromosomal locus (direct repeat region) detecting the presence or absence of spacers on *M. tuberculosis* chromosomal DNA. Mycobacterial interspersed repetitive units-variable number of tandem repeats is commonly used

in combinations with spoligotyping (De Beer *et al.*, 2012; Kamerbeek *et al*., 1997). This method is used to type microbial isolates upon the variable copy numbers of tandem repeats which typically exhibit a large range of copy numbers, even among highly-related bacterial strains (supply *et al*., 2006).

Given the diversity in insertions, deletions and single nucleotide polymorphisms seen in *M. tuberculosis*, it is plausible that the genetics of the pathogen play a role in presentation of disease and response to treatment (Nahid et *al*., 2010). However, there is very limited evidence of the influence of *M. tuberculosis* genotypes on treatment outcome of TB worldwide. Therefore, studies investigating the influence of *M. tuberculosis* genotypes on treatment response are needed in order to develop effective control strategies for TB.

The present study used spoligotyping and MIRU-VNTR typing to characterise *M. tuberculosis* isolates, in order to assess the impact of *M. tuberculosis* genotypes on treatment response by using bacteriologic culture conversion at eight weeks as a marker. In addition the study determined the genetic diversity of *M. tuberculosis* isolates in the Tshwane Metropolitan area and the association with drug resistance and patient demographics (age and gender).

3.3 MATERIALS AND METHODS

3.3.1 Clinical isolates and setting

A total of 108 consecutive *M. tuberculosis* isolates resistant to either rifampicin (RIF) and/or isoniazid (INH) by BACTEC MGIT 960 system were collected between September 2011 and December 2011 from the National Health Laboratory Service (NHLS), Tshwane Academic Division. The laboratory is located in central Pretoria, next to Steve Biko Academic Hospital, which offers services to patients from surrounding suburbs and referrals from clinics and schools as well as in-patient referrals.

The experimental procedures were all carried out at the Centre for TB at the National Institute of Communicable Diseases (NICD) in Johannesburg. This laboratory functions as a national reference laboratory for South Africa and neighbouring countries.

The sample size was determined with the help of the statistician to a number appropriate for statistical analysis.

3.3.2 Data collection

Data including demographics (age and gender), drug susceptibility and eight weeks culture results were collected from the NHLS information system data base.

3.3.3 DNA extraction

The GenoLyse® method was used for DNA extraction in this study, a method that allows for the manual extraction of genomic bacterial DNA from direct patient material and both solid and liquid mycobacterial culture media. In Brief, a thousand microliters of liquid culture was centrifuged for 15 min at 10, 000 x g in an aerosol-tight rotor. The supernatant was discarded and the pellet was resuspended into 100ul of the yellow lysis buffer (A-LYS) by physically dislodging the pellet by means of consecutive up and down pipetting with the lysis buffer. Once resuspended, the tube was incubated for 5 min at 95°C in a dry-block. After 5 minutes the lysate was spun for 5 minutes in a centrifuge at 13, 000 x g after which an equal amount (100ul) of the neutralization buffer (A-NB) was added. After vortexing, the neutralized lysate, 100ul of the supernatant was aliquoted into a clean tube and stored until further use at a -/+2 ^{o}c fridge.

3.3.4 Spoligotyping

All the isolates were typed using spoligotyping, a commercially available kit (Ocimum BioSolution, India) according to manufacturer's instructions (see detailed methodology in appendix A). The method is based on PCR amplification of the direct repeats sequences of *M. tuberculosis* isolates, followed by hybridisation to a membrane containing covalently linked

oligonucleotides that correspond to the various spacer sequences. Individual strains are distinguished by the number of spacers missing from the complete spacer set (see detailed methodology in appendix A).

The resulting spoligotypes were entered in an Excel sheet/ as a binary code representing either a positive or negative hybridisation result (n and o, respectively). Spoligotypes in binary format were entered in the previously released SpolDB4 database. In this database, Spoligotype International Type (SIT) designates spoligotyping shared by two or more patient isolates, as opposed to "orphan" which designates patterns reported for a single isolate.

3.3.5 MIRU-VNTR typing

The MIRU-VNTR typing was performed using the 24 MIRU-VNTR typing kit quadruplex versions (GenoScreen, France) according to the manufacturer's instruction (see detailed methodology in appendix A). The results were entered into the database as numerical codes corresponding to the number of alleles at each locus. A dendrogram was constructed for the combination of both methodologies (spoligotyping and MIRU-VNTR). The genetic distance was built using the unweight pair group method with arithmetic averages (UPGMA) algorithm. The MIRU-VNTR*plus* database was used for construction of the dendrogram (www.miru-vntrplus.org/).

3.3.6 Quality Control

Mycobacterium bovis (BCG, H37Rv) was used as a positive control and sterile water was used as a negative control. These were used as quality control for all the methods and were used for both spoligotyping and MIRU-VNTR. The BCG and H37Rv control used in this study never failed to yield expected results for both methods when used.

3.3.7 Statistical analysis

Two month treatment outcome that is culture conversion was assessed using random effects logistic regression with covariates spoligotypes. The chi-square test or Fisher's exact test was performed to determine statistical association between genotypes and age, gender and drug resistance. A cluster was defined as two or more genotypes sharing the exact pattern for spoligotyping and MIRU-VNTR typing.

3.4 Ethical Approval

Permission was received from the business manager of the National Health Laboratory Services, Tshwane Academic Division, Medical Microbiology to collect the isolates and to collect patient's culture conversion results from their data base. Ethical approval was obtained from the Student Ethics Committee of the Faculty of Health Sciences, University of Pretoria and preceded the experimental work.

CHAPTER 4

Results and Discussion

4.1 RESULTS

In this study a total of 108 confirmed *M. tuberculosis* isolates were collected. Two of the isolates were contaminated and were excluded from the study. The treatment outcome in this study was assessed using the 8 weeks culture conversion results after the patient started TB treatment, as a marker for treatment outcome. The study population included 50 females (47.17 %) and 47 males (44.34 %) with 9 patients (8.49 %) whose gender was not known. The age range for the study population was from 2 days to 79 years. The isolates were from 39 hospitals and clinics around Pretoria.

Of the 106 isolates, the DST result was available for 97 isolates (91.5%). A total of 42 isolates were mono-resistant (26 RIF-mono and 16 INH-mono), 31 MDR-TB (31.96 %) and 4 isolates had inconclusive result for INH and 26 inconclusive for RIF.

4.1.1 Spoligotyping and 24-loci MIRU-VNTR typing results

Comparison of spoligotyping results of the 106 *M. tuberculosis* isolates with the SpolDB4 database showed that 73% (83/106) isolates belonged to 21 previously described shared types (ST); while 27% (23/106) were not found in the SpolDB4 database and were regarded as orphans. Spoligotyping identified seven major distinct families of TB including Beijing, East African Indian (EAI), Latin American and Mediterranean (LAM), T family, X family, CAS as well as Manu 2 families. The Beijing family (17%) were the most frequent followed by T1, LAM4 (10%) each and LAM 3 (6%) (*Figure3.1*). Spoligotyping identified 53 unique spoligotype patterns and 14 clusters, giving a clustering rate of 50%.

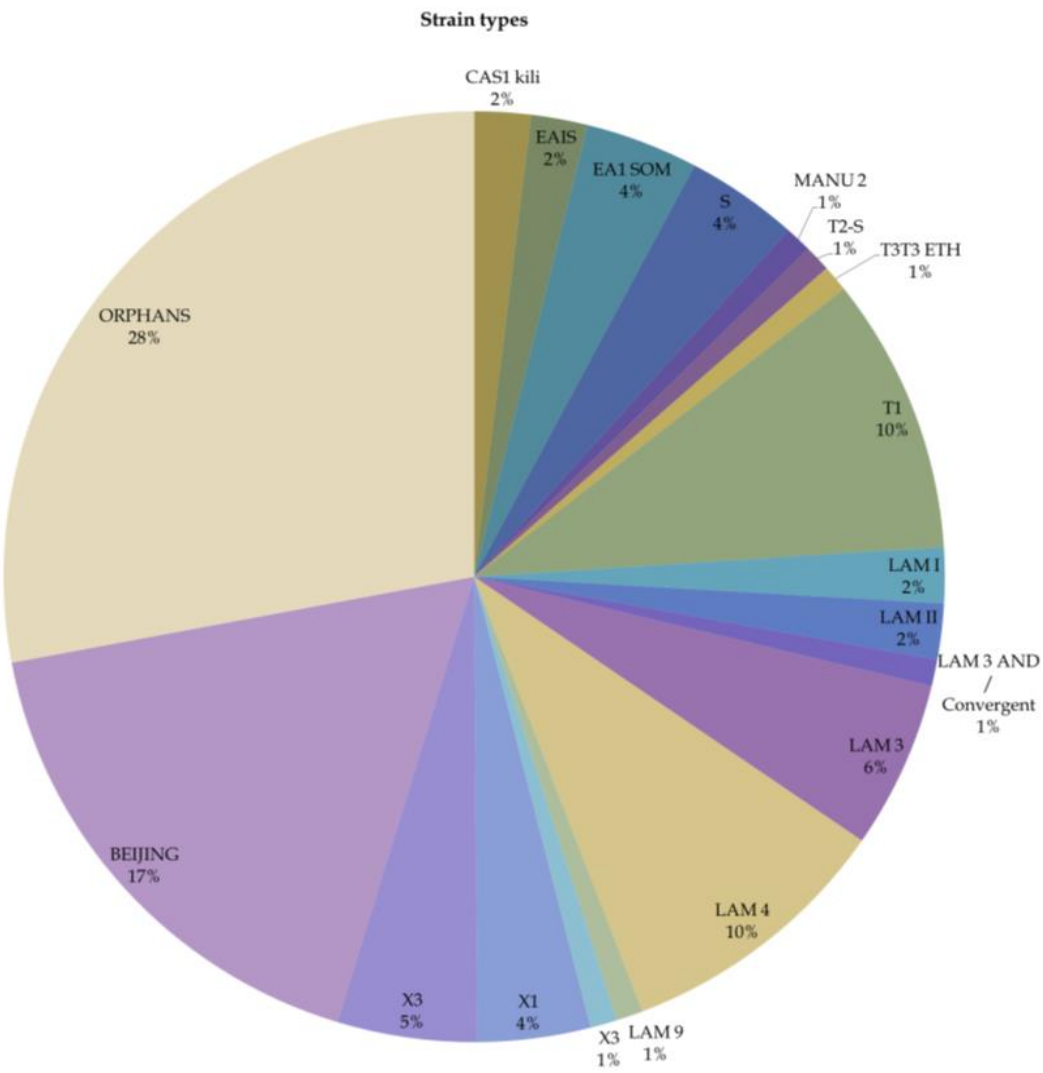

Figure 4.1 The different strains in Tshwane district area according to spoligotyping.

In this study no cluster was identified with 24-loci MIRU-VNTR typing; all the isolates had MIRU-unique patterns. Two isolates did not work with the MIRU-VNTR typing even after repeat testing.

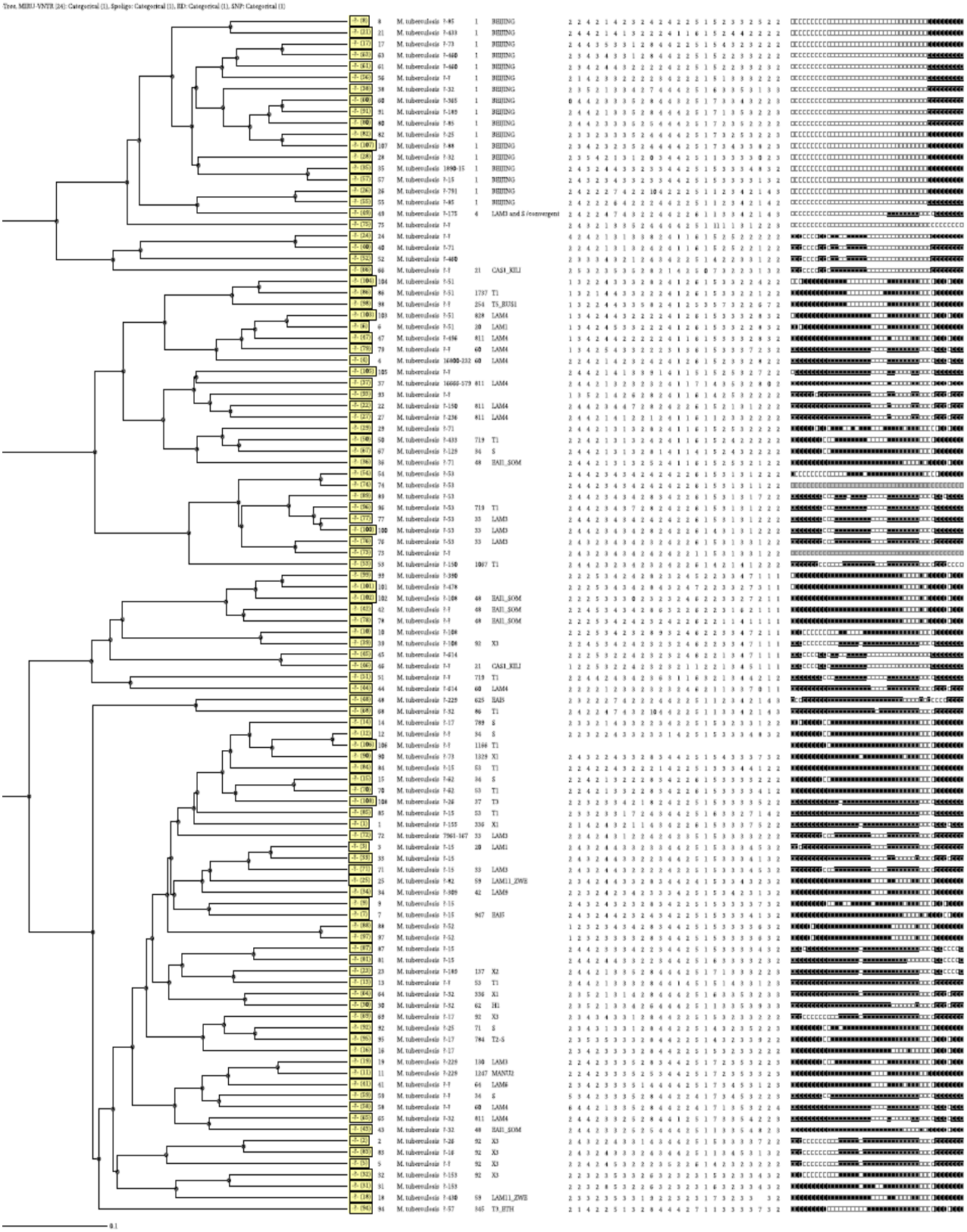

Figure 4.2 A dendrogram for the combination for spoligotyping and MIRU-VNTR

Table 4.3 The discriminatory power for *M. tuberculosis* of spoligotyping and MIRU-VNTR typing.

	Number of				
Methodology	**Distinct patterns**	**Clusters**	**Clustered isolates**	**Unique isolates**	**Clustering rate (%)**
Spoligotyping	53	15	68	38	50
MIRU-VNTR	106	0	0	106	0

4.1.2 Association of genotypes and drug resistance with treatment outcome

In this study only 46/106 (43.4%) patients provided the eight weeks follow-up result. Comparison of the available eight weeks result with the different genotypes found no significant association of the genotypes with the treatment outcome (*Table 4.4*).

Resistance to RIF was associated with treatment failure (patients failed to convert after eight weeks), with P value of 0.006. However, no significant association was found between INH resistance and treatment outcome (*Table 4.4)*.

Table 4.4 Association of genotypes, drug resistance (RIF and INH) with the treatment outcome

ASSOCIATION	Fisher's exact(P values)
All study genotypes with poor treatment outcome	0.488
Resistance to INH with poor treatment outcome	0.226
Resistance to RIF with poor treatment outcome	0.006

4.1.3 Association of genotypes with drug resistance and patient demographics

The study did not find an association of any specific genotype with gender (*Table 4.5*), age, (*Table 4.6)* or drug resistance to INH or RIF (*Table 4.4*).

Table 4.5 Association between genotype and gender

Genotype	P Value
Beijing	1.76
CAS 1 Kili	3.64
EAI 1 SOM	2.57
LAM 1	0.37
LAM II ZWE	3.64
LAM 3	0.81
LAM 4 (ST 60)	0.27
LAM 4 (ST 811)	5.12
S	0.37
T1 (ST 53)	0.27
T1 (ST 719)	0.002
X1	3.23
X3	1.03

Table 4.6 Association between genotype and age

Genotype	Age range	P value
Beijing	19- 35	0.22
	36-52	0.22
EAI 1 SOM	36-52	0.44
	53-68	0.63
CAS 1 Kili	19-35	0.63
	36-52	0.031
LAM 3	19-35	1.98
	36-52	1.90
LAM 4	19-35	1.05
	36-52	1.90
S	19-35	0.09
	Over 69	1.83
T1	Under 18	0.05
	19-35	0.88
	36-52	0.84
	53-68	3.18
X3	19-35	0.63
	19-35	0.031

Table 4.7 Association between genotype and INH/RIF drug resistance

Genotype	P value
Beijing	0.18
CAS 1 Kili	3.24
EAI 1 SOM	1.28
LAM II ZWE	3.02
LAM 3	0.63
LAM and S Convergent	3.02
LAM 4	0.074
S	0.78
T1	1.67
T3-ETH	0.21
X1	0.71
X3	3.76

4.2 DISCUSSION

Drug resistance, particularly MDR-TB, has a profound effect on patient treatment outcomes (Chihota *et al*., 2011). The success of treatment for TB depends on the susceptibility of the bacterial strain to anti-TB drugs, the regimen employed and its duration (Veen *et al*., 1998). Monitoring the clinical outcome in patients is important in order to evaluate the effectiveness of the intervention (Veen *et al*., 1998). Treatment outcome analysis and interpretation allows a more focused intervention and may contribute to improved quality of care (Veen *et al*., 1998).

Genotyping of *M. tuberculosis* strains has led to the identification of strain families associated with drug resistant TB (Chihota *et al*., 2011). However, there is no enough literature regarding *M tuberculosis* genotypes on treatment outcome, especially in South Africa. Most studies show conflicting results on the impact of genotypes on treatment outcome. This study aimed to determine the genetic diversity in the Tshwane metropolitan area and the association of genotypes with treatment outcome, using the eight weeks culture result as a marker for treatment response, as well as the association of the genotypes with drug resistance and patients' demographics (age and gender).

The study found a high genetic diversity in the Tshwane metropolitan area with the Beijing family (17%) being predominant. The Beijing genotype is thought to be the most common strain family in Western Cape and the Eastern Cape, South Africa (Chihota *et al*., 2011). However, in Gauteng an evenly equal distribution of genotypes that included Beijing, Lam4, S and T1 has been reported (Chihota *et al*., 2011). Another study done in Gauteng found Beijing, LAM 4 and H3 making up the largest groups in the area (Marais *et al*., 2014).

The clustering rate of spoligotyping found in this study was lower compared to other studies (Easterbrook *et al*., 2004; Guernier *et al*., 2008; Roetzer *et al*., 2013; Oelemann *et al*., 2006 and Nava-Aguilera *et al*., 2011). Spoligotyping gives a larger number of clusters compared to MIRU-VNTR and IS6110-RFLP (Roetzer *et al*., 20011 and Oelemann *et al*., 2006); it has low discriminatory power, but helps in grouping the strains in to their subsequent lineages. Interestingly in this study no clusters were found by the 24-lociMIRU-VNTR method; this could

be due to the lack of any epidemiological link between the patients. The isolates were collected from 39 different hospitals/clinics located at different regions and districts of Tshwane Metropolitan area. Genotyping by MIRU-VNTR typing failed for two isolates even after repeating the procedure. This could be due degradation of the DNA for these isolates.

The eight weeks sputum examination result was chosen as a marker for treatment response in this study since sputum conversion after eight weeks is considered the most important interim indicator of the efficacy of anti-TB drugs (Holts *et al*., 2006). In this study, less than 50% (46/106) of the study population had the eight weeks follow up result. Such low numbers of follow up should be of concern. This could be due to patients not coming for their eight weeks follow-up sputum examination (Botha *et al*., 2008). According to a study done by Botha *et al.* in 2008 in the Western Cape (Cape Town) patients default due to the long distance of their homes to the health facility where they receive their treatment. In addition patients could die of TB before the eight week follow up examination (de Valliére and Barker, 2006).

The comparison of available eight weeks culture results with genotyping results showed no association of *M. tuberculosis* genotypes with treatment outcome. Few studies have evaluated the sputum culture conversion as interim indicator of final treatment outcome. A study by Munoz-Sellart *et al*., (2010) has shown that failure to convert after 2-3 months was associated with a poor treatment outcome which resulted in treatment failure. A study done in Gauteng, South Africa by Marais *et al*. (2014) found that the H3 (Haarlem strain) genotype was significantly associated with poor treatment outcome, while the other strain families including LAM, T, and Beijing seemed to have had similar rates of successful treatment outcome. In another study the East Asian and Beijing lineages were found to be associated with shorter duration of illness in meningeal tuberculosis patients (Thwartes *et al*., 2008). However a study done in Vietnam found the Beijing genotype to be a significant risk factor for the treatment failure and relapse in TB patients (Lan *et al*., 2003).

Previous studies have reported that drug resistance is a risk factor for poor treatment outcome (Buu *et al*., 2010). In this study resistance to RIF was significantly associated with treatment failure (P value of 0.006). Similarly to this study, Meyssonner *et al* (2014) found RIF resistance

as risk factor for poor outcome in TB. Rifampicin resistance has been used as a marker for MDR-TB (Martens and Wilkinson, 2007).

This study also did not find any significant association of *M. tuberculosis* genotypes with drug resistance to either RIF or INH. Many studies found the Beijing genotype to be associated with drug resistance (Bifani, 2002; Caws *et al*.,2006; Drobniewski *et al*.,2005; Glynn *et al*., 2002; Hasan, 2006; Kubica *et al*., 2005; Park *et al*., 2005; Toungoussova *et al*., 2002 and Tracevska *et al*., 2003). In another study done by Buu *et al* (2010), researchers found no association of Beijing with treatment failure but rather suggested Beijing is not independently responsible for treatment failure alone. The Haarlem genotype has also been associated with drug resistance (Hanekom *et al*., 2011) causing drug-resistant TB outbreaks.

Previous studies reported that certain genotypes have been found to be associated with age and gender (Anh *et al*., 2000). In a study done in Vietnam the Beijing genotype was found to be significantly more prevalent among younger patients than older patients (Anh *et al*., 2000). However, in this study no association was found between genotypes and patient demographics (age and gender). Similarly to this study, Stephen *et al.* (2002) found no association between age, gender, race/ethnicity, and pattern of antimicrobial susceptibility. However, young age was found to be independently associated with East Asian/Beijing lineage in a study done in Indonesia (Thwartes *et al*., 2008).

The study had limitations: firstly we were not able to get all the eight weeks sputum culture conversion results for all the patients, and this resulted in a small number of samples to analyse. Secondly the isolates were from one laboratory in Tshwane Metropolitan area. Even though most clinics and other hospitals send their samples to Tshwane district hospital laboratory, not all the TB cases might receive treatment there and this might not be enough to represent the TB patients in the city. Lastly the sample size (106) could have been bigger which could have led to finding more eight weeks culture results for better analysis of the treatment outcome.

Clustering rate or the proportion of clustered strains in a population is assumed to reflect the rate of recent transmission of TB (Affolabi *et al*., 2009). The low level of the clustering rate in this study could be the fact that the patients were from different clinics and Hospitals around Tshwane, although possible contact was not determined the possibility of these patients meeting is slim accept for those who might be receiving treatment at the same clinic or hospital. Low clustering rate has been associated with low rate of recent transmission in a community, reactivation of old TB infections and early detection of TB cases (Affolabi *et al*., 2009). Clustering has been associated with young age, male gender, HIV status and Drug resistance especially with W-Beijing family, however these factors were not picked up or well investigated.

4.3 CONCLUSION

The study showed high genetic diversity of tuberculosis (TB) strains in the Tshwane Metropolitan area. No association was found between genotypes and treatment response, but more than 50% did not produce eight weeks follow-up results. The study did however find Rifampicin (RIF) mono-resistance to be associated with treatment failure. The study also showed no association between *M. tuberculosis* genotypes and drug resistance or patient demographics. Although the study was not able to find any association of genotypes with drug resistance and treatment outcome, it was however successful in determining the genetic diversity of *M. tuberculosis* in Tshwane. The Beijing genotype was slightly higher than the rest of the defined genotypes. The prevalence of the Beijing strain in Tshwane may be over or under estimated. The high prevalence of this strain in this setting according to our findings should be of concern because of Beijing genotypes being associated with fast transmission, drug resistance and poor treatment outcome. This study has also introduced a new way of monitoring treatment outcome which might help in designing a better treatment regimen for individuals based on the infecting genotype and treatment response at 8 weeks; although in this study was not able to prove an association of any *M. tuberculosis* genotypes with poor treatment outcome, we strongly believe that genotyping can be a new way of monitoring the possibility of poor treatment outcome, by determining the type of strain infecting an individual, as many studies have proved before that certain genotypes; like the Beijing strain as being associated with drug resistance which leads to poor treatment outcome.

This may also help target public health interventions and new knowledge on strain specific clinical outcome. Seeing the patient receive the most effective treatment early just after diagnosis.

Furthermore spoligotyping and MIRU-VNTR are useful when used together as they provide better resolution of the *M. tuberculosis* strains. Spoligotyping can over-cluster when used alone as observed in this study; however MIRU-VNTR was able to distinguish further the clusters identified by Spoligotyping. The study found an association of RIF resistance with treatment failure an important finding that has already been observed in other studies, Rif resistance is also considered as a good marker for poor treatment response like the 8 weeks culture conversion results, as it is known that patients who fail to convert after 8 weeks have a higher chance of relapse in the months to follow with poor treatment outcome. More studies with larger sample sizes with well-structured/described epidemiological data is needed to determine true clusters and to provide more patient information to determine the association better.

CHAPTER 5

CONCLUDING REMARKS

5.1 CONCLUSIONS

Tuberculosis (TB) still remains a major problem in most developing countries, with access to fast and accurate diagnosis still being part of the problem of TB control programme (Hanrahan *et al*., 2013). Sub-Saharan Africa remains burdened with the disease often in association with underlying HIV infection (Friedland, 2011). The diagnosis of TB often presents many difficulties. Failure of early diagnose can result in unrecognised drug resistance which also leads to prolonged infectivity, spread of infection and adverse clinical outcomes including death (Friedland, 2011). In recent years the understanding of TB has improved with the increase in use of molecular techniques used for diagnosis, drug susceptibility testing, and epidemiological studies.

Understanding of diversity and prevalence of predominant circulating *M. tuberculosis* strains will help in successful control of the TB epidemic. Molecular methods such as the IS*6110*-based restriction fragment length polymorphism (IS*6110*-RFLP) (Van Soolingen *et al*., 1991), and the PCR-based spoligotyping and mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) (Supply *et al*., 2006) (Kamerbeek *et al*., 1997) have become the most important tools used in molecular epidemiological studies.

This study has shown the importance of spoligotyping and MIRU-VNTR typing for studying strain diversity as well as the impact of strains possible treatment outcome of TB. These methods are rapid, require minimal bacterial DNA compared to IS6110-RFLP, and are non-laborious and easy to work with. With the use of both methods this study was able to determine the genetic diversity of the strains in the Tshwane Metropolitan area, and association of genotypes with drug resistance and patient demographics. The study also showed that use of spoligotyping alone is insufficient to differentiate between clustered *M. tuberculosis* isolates which is as expected. A secondary typing method is required to accurately define clustering strains. Spoligotyping overestimated the link between *M. tuberculosis* isolates in this study; grouping 50% of isolates

into 15 clusters. The MIRU-VNTR typing discriminated all the clusters indicating that transmission of the strains in this setting is not related to the clonal spread of a specific *M. tuberculosis* strain.

In this study, more than 50% of the study population did not have eight weeks follow up result, indicating the high rate of patient default. Greater vigilance is required to contain TB and especially the drug-resistant epidemic and the national TB control programs should aim at providing rapid diagnostic assays and proper management of anti-TB drugs and providing support to patients to maximize adherence to prescribed regimens.

The limitation of the present study sample size and the time frame which may have provided a better picture of the genetic diversity of strains in the study setting if it was larger over a long period; respectively; this would have allowed retrieval for more numbers of isolates with follow up result for determining the association of genotypes with treatment outcome. However more studies are needed in this area to substantiate these findings further. Further studies based on this study can help with health care guidelines in detecting clinical outcome early on during treatment, with the possibility of preventing poor treatment outcome or relapse due to infections with specific strains.

5.2 FUTURE RESEARCH

Future research in TB should be directed towards improvements of the monitoring programmes already available, and improving surveillance programs. Finding newer drugs for the treatment of TB is of paramount importance. The use of molecular epidemiology for surveillance is important in detecting outbreaks and risk factors for TB including the impact of *M. tuberculosis* genotypes on treatment outcome. Such information will help with proper and precise interventions and treatment. Therefore, these kinds of studies can have a great impact on tuberculosis control in South Africa.

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APPENDICES

Appendix A: Detailed Methodology

1. DNA extraction with GenoType MTBDR*plus* 2.0 for spoligotyping

Mycobacterial genomic DNA was extracted from cultured *M. tuberculosis* isolate using the GenoLyse Kit (Hains life sciences , Germany) as described by the manufacturer. Briefly, 500 µl of the 7H9 broth culture was transferred into a two ml Eppendorf tube (Lasec, South Africa) and centrifuged (Spectrafuge, Labnet International, Inc) at 10.000 *x g* for 15 min to pellet the cells. The supernatant was discarded and the pellet re-suspended in 100 µl lysis buffer and incubated at 95°C in a dry heating block (AccuBlock Digital Bath, Labnet International, Inc) for 5 minutes. Finally, 100 µl of neutralisation reagent was added to the suspension and the tubes were vortexed for 5 sec. The suspension was then centrifuged (Spectrafuge, Labnet International, Inc) at 12 500 *x g* for 5 min. Then the supernatant (DNA) was transferred into a new tube and stored at -20°C until further analysis.

2. PCR amplification and hybridization for spoligotyping

The direct-repeat region of the mycobacterial genome was amplified using primers DRa (5' biotinylated) and DRb. The PCR reaction was performed in a total volume of 25 µl reaction mix consisting of 12.5 µl of HSTaq Master Mix (Qiagen, Hilden, Germany) 2 µl of each primer, 5 µl of template DNA and 3.5 µl of de-ionized water. *Mycobacterium tuberculosis* H37Rv and BCG DNA were used as a positive control and de-ionized water was used as a negative control. The PCR was performed using the following cycling profile including 15 min denaturation step at 96°C, followed by 30 cycles of 1 min at 96°C, 1 min at 55°C and 30 sec at 72°C and a final extension step at 72°C for 10 min. The amplified biotinylated products were hybridised to a set of 43 oligonucleotides covalently bound to a membrane. The hybridised PCR products were incubated with streptavidin-peroxidase conjugate, and signal detection was obtained with an enhanced chemiluminescence detection system. Hybridisation of the biotin-labelled PCR products to the immobilised space-oligos that represent spacers of known sequence was done after incubation with streptavidin-peroxidase and ECL-detection. All buffers were prewarmed

before use. Buffers from concentrated stock were prepared using demineralised water for dilution using 250 ml 2XSSPE 0.1% SDS (60^{o}C) 250 ml 2XSSPE 0.5% SDS (60 ^{o}C), 250 ml 2XSSPE 0.5% SDS (42 ^{o}C) and 250ml 2XSSPE at room temperature. Twenty microliters of the PCR products were added to 150 µl 2X SSPE 0.1 % SDS. The membrane and support cushion were placed into a miniblotter, in such a way that the slots were perpendicular to the line pattern of the applied oligonucleotides. Residual fluid was removed from the slots of the miniblotter by aspiration. The slots were then filled with the diluted PCR products and were hybridised for 60 minutes at 60 ^{o}C on a horizontal surface without shaking.

Then the PCR products were removed by aspiration from the miniblotter slots after hybridisation was complete. The membrane was washed in 2X SSPE 0.5 SDS for 15 minutes at 57^{o}C. Thirty millilitres of 2X SSPE 0.5 SDS (42^{o}C) was added with 7.5uL streptavidin peroxidase conjugate (500uL) in a 50 ml tube and gently mixed. The mixture was poured onto the membrane and incubated for 60 minutes at 42^{o}C in a rolling bottle. After the incubation, the membrane was washed twice with 2X SSPE 0.5% SDS for 10 minutes at 42^{o}C, followed by rinsing with 2X SSPE for 5 minutes at room temperature. The detection step was achieved with the use of ECL detection liquid (Amersham Biosciences, Buckinghamshire, UK). The membrane was incubated in 10 ml (5 ml detection solution 1 and 5 ml detection solution 2) for 90 seconds then transferred to a clear plastic sheet. The membrane was then transferred into a 25X30 cm film cassette. The film was exposed to the membrane for about 5 minutes in a dark room and developed.

3. Preparation of the PCR for MIRU-VNTR

The PCR reaction was performed in a total volume of 12 µl consisting of 8 µl of MIRU-VNTR quadruplex mix and 2 µl of template DNA, using H37Rv and BCG as positive controls and sterile water as a negative control. The plate was covered with an adhesive plate and spun down to remove air bubbles then the plate containing the PCR mix and DNA was put into a thermal cycler using the following cycling profile including 15 min denaturation step at 95°C, followed by 40 cycles of 1 min at 94°C, 1 min at 59°C and 1 min and 30 sec at 72°C and a final extension step at 72°C for 10 min. The marker that was used was diluted by adding 50 µl of 1200 LIZ-marker to (%) µl HiDi formamide. The diluted LIZ-marker was added into each well into a 96-well microtiter plate. Then 2µl of the PCR products were added with the LIZ-marker. The 24 loci MIRU-VNTR typing method was subjected to electrophoresis using an ABI genetic analyser (Applied Biosynthesis, USA). Sizing of PCR fragments and assignments of the alleles of the 24 loci was done using the MIRU-VNTR calibration kit (GenoScreen, France), as well as the Gene Mapper software version 4.0 (Applied Biosystem, USA).

Table 3.1 The PCR master mix preparation for identification of the Direct Repeats of *M. tuberculosis*

Component	Volume per well
Primer 1 Direct Repeat a	2 µl
Primer 2 Direct Repeat b	2 µl
De-ionised water	3.5µl
HSTaq master mix	12.5 µl
Total	25 µl

Table 3.2 Locus designations and PCR primer sequence used in this study for the 24-locus set (Supply *et al*., 2006)

Locus	PCR primer pairs (5´ to 3´, with labelling indicated)	2531	CTGTCGATGGCCGCAACAAAACG (VIC) AGCTCAACGGGTTCGCCCTTTTGTC
580	GCGCGAGAGCCCGAACTGC (FAM) GCGCAGCAGAAACGCCAGC	4348	CGCATCGACAAACTGGAGCCAAAC CGGAAACGTCTACGCCCCACACAT(NED)
2996	TAGGTCTACCGTCGAAATCTGTGAC CATAGGCGACCAGGCGAATAG (VIC)	2059	TCGGAGAGATGCCCTTCGAGTTAG(FAM) GGAGACCGCGACCAGGTACTTGTA
802	GGGTTGCTGGATGACAACGTGT(NED) GGGTGATCTCGGCGAAATCAGATA	2687	CGACCAAGATGTGCAGGAATACAT GGGCGAGTTGAGCTCACAGAA (VIC)
960	GTTCTTGACCAACTGCAGTCGTCC GCCACCTTGGTGATCAGCTACCT(FAM)	3007	TCGAAAGCCTCTGCGTGCCAGTAA GCGATGTGAGCGTGCCACTCAA (NED)
1644	TCGGTGATCGGGTCCAGTCCAAGTA CCCGTCGTGCAGCCCTGGTAC (NAD)	2347	GCCAGCCGCCGTGCATAAACCT (FAM) AGCCACCCGGTGTGCCTTGTATGAC
3192	ACTGATTGGCTTCATACGGCTTTA GTGCCGACGTGGTCTTGAT (NED)	2461	ATGGCCACCCGATACCGCTTCAGT (VIC) CGACGGGCCATCTTGGATCAGCTAC
424	CTTGGCCGGCATCAAGCGCATTATT GGCAGCAGAGCCCGGGATTCTTC(FAM)	3171	GGTGCGCACCTGCTCCAGATAA (NED) GGCTCTCATTGCTGGAGGGTTGTAC
577	CGAGAGTGGCAGTGGCGGTTATCT(VIC) AATGACTTGAACGCGCAAATTGTGA	4052	AACGCTCAGCTGTCGGAT(NED) CGCCGTGCCGGCCAGGTCCTTCCCGA
2165	AAATCGGTCCCATCACCTTCTTAT(NED) CGAAGCCTGGGGTGCCCGCGATTT	154	TGGACTTGCAGCAATGGACCAACT TACTCGGACGCCGGCTCAAAAT (FAM)
2401	CTTGAAGCCCCGGTCTCATCTGT (FAM) ACTTGAACCCCCACGCCCATTAGTA		
3690	CGGTGGAGGCGATGAACGTCTTC (VIC) TAGAGCGGCACGGGGGAAAGCTTAG		
4156	TGACCACGGATTGCTCTAGT GCCGGCGTCCATGTT (NED)		
2163b	CGTAAGGGGGATGCGGGAAATAGG CGAAGTGAATGGTGGCAT (FAM)		
1955	AGATCCCAGTTGTCGTCGTC (VIC) CAACATCGCCTGGTTCTGTA		

Appendix B : detailed Results

Table 1. The different genotypic patterns according to spoligotyping

Family designation	ST no	No. of isolates	% of spoligotype isolates
BEIJING	1	17	16
CAS1_kili	21	2	1.9
EAI5	625	1	0.9
EAI5	947	1	0.9
EAII SOM	48	5	4.7
H1	62	1	0.9
LAM 3	33	5	4.7
LAM 3	130	1	0.9
LAM 3 and/Convergent	4	1	0.9
LAM 4	828	1	0.9
LAM 4	811	5	4.7
LAM 4	60	4	3.8
LAM 6	64	1	0.9
LAM 9	41	1	0.9
LAM I	20	2	1.9
LAM II_ZWE	59	2	1.9
MANU 2	1247	1	0.9
orphans	Not typeable	24	22.6
S	34	4	3.8
S	789	1	0.9
S	71	1	0.9
T1	173	1	0.9
T1	719	3	2.8
T1	1067	1	0.9
T1	86	1	0.9
T1	1166	1	0.9
T1	53	4	3.8
T2-s	784	1	0.9
T3	37	1	0.9
T3 ETH	345	1	0.9
T5_RUS1	254	1	0.9
X1	1329	1	0.9
X1	336	2	1.9
X2	137	1	0.9
X3	92	6	5.7

Appendix c: statistical analysis

Table 2. Association of genotypes with treatment outcome

Genotype	Outcome: Remain+	Outcome: convert-	Total
BEIJING	7	0	7
	100.00	0.00	100.00
EAI1 SOM	2	1	3
	66.67	33.33	100.00
H1	0	1	1
	0.00	100.00	100.00
LAM1	1	0	1
	100.00	0.00	100.00
LAM11 ZWE	1	0	1
	100.00	0.00	100.00
LAM3	1	0	1
	100.00	0.00	100.00
LAM4	3	1	4
	75.00	25.00	100.00
S	3	1	4
	75.00	25.00	100.00
T1	6	2	8
	75.00	25.00	100.00
T3	1	0	1
	100.00	0.00	100.00
X1	2	0	2
	100.00	0.00	100.00
X3	1	0	1
	100.00	0.00	100.00
Orphan	12	1	13
	92.31	7.69	100.00
Total	40	7	47
	85.11	14.89	100.00

Fisher's exact = 0.488

Table 3. Association of Isoniazid with the clinical outcome

	Outcome	
DST_INH	Remain+	convert-	Total
I	1	0	1
	100.00	0.00	100.00
R	25	1	26
	96.15	3.85	100.00
S	13	3	16
	81.25	18.75	100.00
Total	39	4	43
	90.70	9.30	100.00

Fisher's exact = 0.226

Table 4. Association of Rifampicin with clinical outcome

	Outcome	
DST_RIF	Remain+	convert-	Total
I	6	2	8
	75.00	25.00	100.00
R	30	0	30
	100.00	0.00	100.00
S	3	2	5
	60.00	40.00	100.00
Total	39	4	43
	90.70	9.30	100.00

Fisher's exact = 0.006