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SUSCEPTIBILITY TESTING AND RESISTANCE MECHANISMS TO SECOND-LINE AGENTS AGAINST *MYCOBACTERIUM TUBERCULOSIS* IN PRETORIA, SOUTH AFRICA

by

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SUMMARY

Multidrug-resistant (MDR) and extensively drug-resistant tuberculosis (XDR-TB) is a serious problem in South Africa. Standardised and accurate drug susceptibility testing for first-line and second-line anti-TB drugs are critical elements in battling the MDR- and XDR-TB epidemic. Furthermore, understanding of the population structure and transmission patterns of circulating drug-resistant *Mycobacterium tuberculosis* (*M. tuberculosis*) strains is of utmost importance. Previous studies have shown that different genotypes of *M. tuberculosis* strains predominate in different geographical regions and strain to strain variations may have important consequences when it comes to transmissibility, diagnostics and management. Despite the high prevalence of MDR- and XDR-TB in South Africa, data with regard to the population structure of drug-resistant strains is lacking in many regions. Therefore, the aim of the study was to evaluate phenotypic and genotypic methods for susceptibility testing of first-line and second-line anti-TB drugs and molecular characterisation of drug-resistant strains from high-burden TB areas in South Africa.

Consecutive MDR-TB isolates from four provinces were included in this study. The results of routine drug susceptibility testing of ethambutol, streptomycin, kanamycin and ofloxacin using the BACTEC MGIT 960 system in MDR-TB isolates was compared to the agar proportion method. The GenoType® MTBDR*sl* assay was evaluated for the detection of resistance against ethambutol, capreomycin, kanamycin and ofloxacin. Finally, the study

investigated the population structure and transmission patterns of drug-resistant *M. tuberculosis* isolates using spoligotyping and MIRU-VNTR typing. Additionally, the possible association of genotypes with drug-resistance patterns and demographic information were explored.

The sensitivity and specificity of the BACTEC MGIT 960 system using the agar proportion method as a gold standard was 18.8% and 96.5% for ethambutol, 95.2% and 37.4% for streptomycin, 54.6% and 91.8% for kanamycin and 100% and 89% for ofloxacin, respectively.

The GenoType® MTBDR*sl* assay gave comparable results for ofloxacin; however, a low performance was found for the detection of resistance to capreomycin, kanamycin and ethambutol. In order to resolve the discrepancies between the two methods, DNA sequencing was done for the target genes (*gyr*A, *rrs*, *emb*B) from the discrepant results as well as two additional genes (*gyr*B, *tly*A) that were not included in the assay. The DNA sequencing identified mutations in the *gyr*A, *gyr*B, *rrs* and *tlyA* genes that were not detected by the GenoType® MTBDR*sl* assay.

The prevalence of XDR-TB and pre-XDR TB was 7.1% and 9.5%, respectively. A high-diversity of *M. tuberculosis* strains were found in this study, with the Beijing and EAI1_SOM families being predominant. No association was found between genotypes and specific drug-resistance or demographic information.

The sensitivity of the BACTEC MGIT 960 system for ofloxacin and streptomycin was excellent; however, the sensitivity was low for ethambutol and kanamycin. The GenoType® MTBDR*sl* assay was promising for the detection of ofloxacin; however, the sensitivity of the assay needs to be improved for capreomycin, kanamycin and ethambutol. The high-level of diversity and the geographical distribution of the drug-resistant *M. tuberculosis* isolates in this study suggested that the transmission of drug-resistant TB in these study settings is not caused by clonal spread of a specific *M. tuberculosis* strain.

LIST OF ABBREVIATIONS

AMK	Amikacin
AP	Alkaline phosphotase
BCG	Bacillus Calmette-Guérin
CAP	Capreomycin
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
DOTS	Directly Observed Treatment Short course
DR	Direct Repeats
DST	Drug Susceptibility Testing
EAI	East African Indian
EMB	Ethambutol
ERDR	*Emb*B Resistance Determining Region
ETH	Ethionamide
FIND	Foundation for Innovative New Diagnostics
FLQ	Fluoroquinolone
FMO	Flavin Monooxygenase
FRET	Fluorescent Resonant Energy Transfer
GI	Growth Index
GLI	Global Laboratory Initiative
H	Haarlem
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
INH	Isoniazid
IS	Insertion Sequence
IUATLD	International Union Against Tuberculosis and Lung Disease
KAN	Kanamycin
LAM	Latin American Mediterranean
LJ	Löwenstein-Jensen
LSP	Large Sequence Polymorphisms
M. africanum	*Mycobacterium africanum*
M. bovis	*Mycobacterium bovis*
M. tuberculoisis	*Mycobacterium tuberculosis*
MDR-TB	Multi Drug-Resistant Tuberculosis
MGIT	Mycobacteria Growth Indicator Tube
MIRU-VNTR	Mycobacterial Interspersed Repetitive Unit-Variable Number of Tandem Repeats
MODS	Microscopic Observation Drug Susceptibility

MOX	Moxifloxacin
NHLS	National Health Laboratory Service
OFX	Ofloxacin
PAS	Para-aminosalicylic Acid
PCR	Polymerase Chain Reaction
POA	Pyrazinoic Acid
PPD	Purified Protein Derivative
PRO	Prothionamid
PZA	Pyrazinamide
PZase	Pyrazinamidase
QRDR	Quinolone Resistance Determining Regions
RFLP	Restriction Fragment Length Polymorphism
RIF	Rifampicin
RRDR	Rifampin Resistance Determining Region
SSCP	Single Strand Conformational Polymorphism
SIT	Shared International Types
ST	Shared Types
STR	Streptomycin
TB	Tuberculosis
TLA	Thin Layer Agar
VIO	Viomycin
WHO	World Health Organization
XDR-TB	Extensively Drug-Resistant Tuberculosis

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LIST OF PUBLICATION AND CONFERENCE CONTRIBUTION

PUBLICATIONS:

- Halima M Said, Marleen M Kock, Nazir A Ismail, Kamaldeen Baba, Shaheed V Omar, Ayman G Osman, Anwar A Hoosen, Marthie M Ehlers. Evaluation of the Genotype® MTBDR*sl* assay for susceptibility testing of second-line anti-tuberculosis drugs. *Journal of Tuberculosis and Lung disease*, 2012. 16:104-110.

- Halima M Said, Marleen M Kock, Nazir A Ismail, Kamaldeen Baba, Shaheed V Omar, Ayman G Osman, Anwar A Hoosen, Marthie M Ehlers. Molecular characterization and second-line anti-TB drug-resistance patterns of MDR-TB isolates from the northern region of South Africa. Accepted for publication in the *Journal of Clinical Microbiology*, 2012

- Halima M Said, Marleen M Kock, Nazir A Ismail, Kamaldeen Baba, Shaheed V Omar, Anwar A Hoosen, Marthie M Ehlers. Comparison between the BACTEC MGIT 960 system and the agar proportion method for susceptibility testing of multidrug-resistant tuberculosis strains in a high burden setting of South Africa Manuscript submitted for publication to *BMC Infectious Diseases*.

CONFERENCE CONTRIBUTIONS:

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- Halima M Said, Marleen M Kock, Nazir A Ismail, Anwar A Hoosen, Marthie M Ehlers. Determining the performance of BACTEC MIGIT 960 for susceptibility testing of ethambutol and streptomycin. **Poster presentation** at TB colloquium, Cape Town, South Africa, 3-4 June, 2009.

- Halima M Said, Marleen M Kock, Nazir A Ismail, Anwar A Hoosen, Marthie M Ehlers. Evaluation of the Genotype® MTBDR*sl* assay for susceptibility testing of second-line anti-tuberculosis drugs. **Oral presentation** at the South African National TB Conference, ICC, Durban, South Africa, 1-4 June 2010.

CHAPTER 1

INTRODUCTION

Tuberculosis (TB) is a serious public health problem in South Africa. The incidence of TB in South Africa is high with an estimated TB incidence rate of 998 cases per 100 000 of the population (WHO, 2010a). The TB problem in South Africa is worsened by the high prevalence of Human Immunodeficiency Virus (HIV) and the emergence and spread of drug-resistant *Mycobacterium tuberculosis* strains (*M. tuberculosis*) especially, multidrug-resistant (MDR) [ie, resistant to at least isoniazid (INH) and rifampicin (RIF)] and extensively drug-resistant (XDR) TB strains [ie 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)] (CDC, 2006a; WHO, 2010b). It is estimated that 9.6% of all TB cases are MDR-TB, thus ranking South Africa as one of the high-burden MDR-TB countries in the world (WHO, 2010b). This represents a dramatic increase (three-fold) since 2002, when it was shown that 3.1% of all TB cases (new and re-treatment) had MDR-TB (WHO, 2008). Data from the National Health Laboratory Service (NHLS) showed that 6.3% of the diagnosed MDR-TB cases over the period January 2004 to October 2010 in South Africa were XDR-TB, while the World Health Organization (WHO) estimated that 10.5% of the MDR-TB cases in South Africa were XDR-TB (NHLS, 2010; WHO, 2010a).

Rapid and reliable drug susceptibility testing (DST) is needed for the design and success of treatment regimens. Using conventional methodologies; culture, identification of *M. tuberculosis* and DST may take weeks or even months (Palomino, 2009; WHO, 2009). New rapid phenotypic DST methods (eg liquid culture-based methods, colorimetric methods, phage-based methods) and genotypic DST techniques (eg nucleic acid amplification assays, resistance mutation detection and sequence-based assays) have been developed for first-line anti-TB drugs to shorten the time to both detection and DST (Palomino *et al*., 2008; Palomino, 2009; Richter *et al*., 2009; Parsons *et al*., 2011). However, DST methods for second-line anti-TB drugs are less standardised than tests for first-line anti-TB drugs (Shah *et al*., 2007; Richter *et al*., 2009). Most of these methods are either still in developmental phases, or in early validation stages (Richter *et al*., 2009; Parsons *et al*., 2011). Even supranational reference laboratories, organised in the WHO/IUATLD SRL network, reported a variety of methods used and drug concentrations tested (CDC, 2006b; Shah *et al*., 2007).

There is, therefore, an urgent need for rapid, standardised and accurate DST methods for second-line anti-TB drugs.

In addition to the rapid detection of drug-resistance, the control of TB requires methods for tracing sources of infection, so that further transmission can be prevented. Developments in molecular biology have resulted in techniques that allow prompt identification and tracking of specific strains of *M. tuberculosis* as these strains spread through the population. Molecular genotyping tools for *M. tuberculosis*, such as IS*6110* based restriction fragment length polymorphism (RFLP), spoligotyping and mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing have become invaluable in the investigation of disease transmission dynamics, outbreaks and phylogenetics (Mathema *et al*., 2006). The IS*6110*-RFLP typing is considered the gold standard, although it is a laborious method and requires large amounts (2 µg) of DNA (Van Embden *et al*., 1993). Spoligotyping is a PCR-based method for simultaneous detection and typing of the *M. tuberculosis* complex using one particular chromosomal locus with high polymorphism, named the direct repeat (DR) region (Kamerbeek *et al*., 1997). Spoligotyping is simple, rapid and highly reproducibile and can be performed directly on clinical samples, without the need for prior culture (Kamerbeek *et al*., 1997; Mathema *et al*., 2006). The MIRU-VNTR typing based on VNTR of genetic elements named MIRU that are located mainly in intergenic regions dispersed throughout the *M. tuberculosis* genome (Supply *et al*., 1997, Supply *et al*., 2000; Supply *et al*., 2001). Each MIRU includes strings of short repetitive sequences and the number of repeats varies among strains (Mathema *et al*., 2006). The PCR amplification of each MIRU generates fragments of different sizes for different strains and the number of repeats at each locus can be determined (Supply *et al*., 1997, Supply *et al*., 2000; Supply *et al*., 2001). The MIRU-VNTR typing is a faster genotyping method and it can be performed by amplifying a panel of 12, 15 or 24 loci (Alonso-Rodríguez *et al*., 2008).

Despite the advances in the development of rapid diagnostics for drug-resistance and molecular epidemiological analysis; significant challenges persist. Although DST for RIF and INH is fairly standardised and accurate, DST for STR, EMB and PZA is less reliable and reproducible (Laszlo *et al*., 1997; Parsons *et al*., 2004). The determination of DST patterns to second-line drugs remains a substantial challenge (Johnson *et al*., 2006; Palomino *et al*., 2008; Palomino, 2009; Parsons *et al*., 2011). Moreover, use of molecular methods for DST requires a more detailed understanding of the mutations that lead to first-line and second-line

anti-TB drugs (Johnson *et al*., 2006; Palomino, 2009, Richter *et al*., 2009; Parsons *et al*., 2011). Currently, not all the genetic mutations associatedwith resistance is for anti-TB drugs are known, especially second-line for anti-TB drugs (Johnson *et al*., 2006; Palomino, 2009; Parsons *et al*., 2011). Even though, South Africa is one of the high-burden MDR-TB countries, relatively little is known with regard to the population structure, diversity and predominant circulating drug-resistant strains, especially in the northern region of South Africa. Previous studies in South Africa were mainly conducted in the Western Cape province, thus are not representative. Studies have shown that *M. tuberculosis* strains differ in different geographical regions (Filliol *et al*., 2003; Brudey *et al*., 2006). Therefore, studies on the characterisation of drug-resistant strains are needed in order to develop effective control strategies for MDR- and XDR-TB.

The aim of this study was as follows: (i) to compare the performance of the BACTEC MGIT 960 system for the routine drug susceptibility testing of MDR-TB isolates to two first-line drugs (EMB and STR) and two second-line drugs (OFX and KAN) against the standard agar proportion method (ii) to evaluate the GenoType® MTBDR*sl* assay (Hain Lifescience, Germany) for drug susceptibility testing of MDR-TB isolates to second-line drugs against the standard agar proportion method (iii) to genotype all MDR-TB isolates using two PCR-based techniques, spoligotyping and MIRU-VNTR typing in order to understand the population structure of drug-resistant *M. tuberculosis* from four provinces (Gauteng, Limpopo, Mpumalanga and North-West) in the northern region of South Africa (iv) to determine the transmission patterns of drug-resistant *M. tuberculosis* strains in this region. Furthermore, to explore the possible association of genotypes (clusters) with specific drug-resistance patterns and demographic characteristics (age and gender).

The objectives of the study were:

1. To collect consecutive MDR-TB isolates submitted to the National Health Laboratory Service (NHLS), tertiary laboratory, Dr George Mukhari hospital
2. To confirm all the MDR-TB isolates against first-line ant-TB drugs using the standard agar proportion method
3. To compare the performance of the BACTEC MGIT 960 system for susceptibility testing of two first-line anti-TB drugs (EMB and STR) and two second-line drugs (OFX and KAN) against the standard agar proportion method

4. To evaluate the GenoType® MTBDR*sl* assays against the standard agar proportion method for detection of resistance to three second-line anti-TB drugs
5. To amplify and sequence selected genes (*gyr*A, *gyr*B, *rrs* and *tlyA)* associated with second-line anti-TB drug-resistance from MDR-TB isolates
6. To perform spoligotyping for all MDR-TB isolates
7. To perform MIRU-VNTR typing for all MDR-TB isolates
8. To determine possible association of genotypes (clusters) with demographic information (age and gender) and drug-resistance
9. To determine the transmission patterns of drug-resistant *M. tuberculosis* isolates by comparing genotyping data with the geographical origin of each isolate.

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CHAPTER 2

LITRATURE REVIEW

2.1 Introduction

Tuberculosis (TB) is a disease of major public health concern worldwide. The World Health Organization (WHO) estimated that 9.4 million new cases of TB occurred in 2009 (WHO, 2010a). The high incidence of TB is further compounded by the increasing problem of multidrug-resistant (MDR) *Mycobacterium tuberculosis* (*M. tuberculosis*) strains that are resistant to isoniazid (INH) and rifampicin (RIF) (WHO, 2010a). In 2008, an estimated 440 000 cases of MDR-TB were diagnosed, of which approximately 40 000 were extensively drug-resistant (XDR)-TB [ie MDR-TB with additional resistance to any fluoroquinolon (FLQ) and to at least one of the three injectable second-line drugs, kanamycin (KAN), amikacin (AMK) and/or capreomycin (CAP)] (CDC, 2006; WHO-IUATLD, 2008; WHO, 2010b). In 2005, outbreaks of XDR-TB have been reported in Russia and South Africa (WHO, 2008a). By January 2010, XDR-TB cases had been reported in 58 countries around the world (WHO, 2010b).

Treatment of MDR-TB is complex and uses second-line anti-TB drugs that are less effective, and toxic that must be administered for a longer duration than for drug-susceptible TB patients (Orenstein *et al*., 2009). Patients with MDR-TB have lower cure rates and higher mortality than patients with drug-susceptible TB (Orenstein *et al*., 2009). However, successful outcomes for MDR-TB are achievable in about two-thirds of patients (Orenstein *et al*., 2009). Outcomes of treatment for XDR-TB vary in different countries (Gandhi *et al*., 2006; Mitnick *et al*., 2008; Orenstein *et al*., 2009; Dheda *et al*., 2010).

With the global rise in MDR-TB strains, there is an increasing need to determine susceptibility to first and second-line anti-TB drugs accurately. The available conventional methods for mycobacterial drug susceptibility testing (DST) are based on solid media (Heifets, 1991; Heifets and Desmond, 2005; Parsons *et al*., 2011). The proportion method using either Lowenstein-Jensen (LJ) or agar medium is universally accepted as the ‘‘gold standard’’ although it has longer turnaround times before final results are available (Heifets, 1991; Heifets and Desmond, 2005; Parsons *et al*., 2011). Rapid DST methods including phenotypic and genotypic assays have been developed for first-line anti-TB drugs to shorten

the time of susceptibility testing of *M. tuberculosis*. However, DST methods for second-line anti-TB drugs are not yet standardised and are less reproducible than methods for first-line anti-TB drugs (Shah *et al*., 2007). Critical drug concentrations for second-line anti-TB drugs have not been completely established for all the drugs (Shah *et al*., 2007). As a result, the methods and drug concentrations used for second-line anti-TB drugs vary between different laboratories (Shah *et al*., 2007). This highlights the urgent need for studies on accurate susceptibility testing in order to standardise methods and drug concentrations for second-line drugs.

In addition to the management of drug-resistant strains, understanding the population structure, diversity and spread of resistant *M. tuberculosis* strains is crucial. Molecular characterisation of drug-resistant strains is helpful to gain insight in the major circulating strains of *M. tuberculosis* (Mathema *et al*., 2006). A number of genotyping methods based on various genetic markers have been developed (Moström *et al*., 2002). These methods include IS*6110*-based restriction fragment length polymorphism (RFLP), spoligotyping and mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing. The IS*6110*-RFLP typing has been the most widely used and is internationally accepted as the gold standard and has become invaluable in investigations of disease transmission dynamics and outbreaks (Mathema *et al*., 2006). However, IS*6110*-RFLP typing is a laborious method that requires large amounts of DNA per isolate and has poor discriminatory power for *M. tuberculosis* isolates with a low copy number of IS*6110* (Van Embden *et al*., 1993). It is important to combine various typing methods to increase the power of strain differentiation as a single genotyping method cannot define all unique isolates (Barlow *et al*., 2001; Cowan *et al*., 2005).

2.2 Classification of mycobacteria

The genus *Mycobacterium* is the only genus in the family *Mycobacteriaceae* (Barrera, 2007; Pfyffer, 2007). The genus *Mycobacterium* is classified into several species (Pfyffer, 2007; Euzéby, 2011). However, for the purpose of the diagnosis and treatment, *Mycobacterium* species can be grouped into *M. tuberculosis* complex and nontuberculous mycobacteria (Barrera, 2007; Pfyffer, 2007). The *M. tuberculosis* complex members are causative agents of human and animal TB (Pfyffer, 2007). Species in this complex include: *M. tuberculosis*, (the major cause of human TB), *M. bovis* (cattle strain), *M. bovis* BCG (vaccine strain), *M. africanum*, *M. canetti*, *M. caprae*, *M. microti* and *M. pinnipeddii* (Pfyffer, 2007; Euzéby,

2011). Nontuberculous mycobacteria include *M. avium, M. kansasii, M. intracellulare* and *M. fortuitum* (Pfyffer, 2007). Nontuberculous mycobacteria can cause pulmonary disease resembling tuberculosis, lymphadenitis and skin disease or disseminated disease (Pfyffer, 2007; Euzéby, 2011).

2.3 General characteristics of mycobacteria

Mycobacteria are aerobic, non-motile, non-sporulated rods and do not contain a capsule nor produce any toxins (Barrera, 2007; Pfyffer, 2007). All *Mycobacterium* species share a characteristic cell wall, thicker than in many other bacteria, which is hydrophobic, waxy and rich in mycolic acids/mycolates (Draper and Daffé, 2005; Barrera, 2007; Bhamidi, 2009). The high content of complex lipids of the cell wall prevents access of common dyes (Pfyffer, 2007; Bhamidi, 2009; Todar, 2011). Therefore, the *Mycobacterium* species, along with members of a related genus *Nocardia*, are classified as acid-fast bacteria (Barrera, 2007; Todar, 2011). Despite this, once stained, acid-fast bacteria will retain dyes when heated and treated with acidified organic compounds (Barrera, 2007; Pfyffer, 2007; Todar, 2011).

Mycobacteria are widespread bacteria, typically living in water (including tap water treated with chlorine) and food sources (Barrera, 2007). However, mycobacteria such as *M. tuberculosis* and *M. leprae* are obligate pathogens; *M. avium* is opportunistic pathogens while other species are saprophytes (Pfyffer, 2007). *Mycobacterium tuberculosis* can infect several animal species, although humans are the principal hosts (Grange, 2009). *Mycobacterium tuberculosis* grows most successfully in tissues with high oxygen content, such as the lungs (Grange, 2009; Lawn and Zumla, 2011).

Colony morphology of mycobacteria varies among species, ranging from smooth to rough and from nonpigmented to pigmented (Pfyffer, 2007). Two types of media are used to grow *M. tuberculosis* namely Middlebrook medium, which is an agar-based medium and Löwenstein-Jensen (LJ) medium which is an egg-based medium (Barrera, 2007; Pfyffer, 2007). Both types of media contain inhibitors to prevent contaminants from out-growing *M. tuberculosis* (Barrera, 2007; Pfyffer, 2007). *Mycobacterium tuberculosis* colonies are small and buff coloured when grown on either LJ or Middlebrook media (Barrera, 2007; Pfyffer, 2007). The generation time of *M. tuberculosis* is 15 to 20 hours, which is slow compared with other bacteria [*Escherichia coli* (E. coli) divides every 20 minutes] (Pfyffer, 2007; Lawn and Zumla, 2011). It takes 4 to 6 weeks to get visual colonies on either type of

media and *M. tuberculosis* tends to grow in parallel groups, producing serpentine cording (Barrera, 2007; Pfyffer, 2007).

2.4 Virulence factors of *M. tuberculosis*

Unlike other bacteria, *M. tuberculosis* does not possess the classic bacterial virulence factors, such as toxins, capsules and fimbriae (Todar, 2011). However, a number of structural and physiological properties of the *M. tuberculosis* have been described that contributes to mycobacterial virulence and the pathology of TB, even though the exact role in *M. tuberculosis* virulence is unclear (Todar, 2011). These include: (i) *M. tuberculosis* have special mechanisms for cell entry by binding directly to mannose receptors on macrophages (ii) *M. tuberculosis* can grow intracellularly in macrophages. Resistance to killing by macrophages is critical to the virulence of *M. tuberculosis*. Macrophages produce reactive oxygen species and reactive nitrogen species that have potent antimicrobial activity. *Mycobacterium tuberculosis* has two genes encoding superoxide dismutase proteins, sodA and sodC. SodC is a Cu, Zn superoxide dismutase responsible for only a minor portion of the superoxide dismutase activity of *M. tuberculosis*. However, SodC has a lipoprotein binding motif, which suggests that it may be anchored in the membrane to protect *M. tuberculosis* from reactive oxygen intermediates at the bacterial surface. (iii) *M. tuberculosis* interferes with the toxic effects of reactive oxygen intermediates produced in the process of phagocytosis (iv) antigen 85 complex: this complex is composed of a group of proteins secreted by *M. tuberculosis* and these proteins help in walling off the bacilli from the immune system and may facilitate tubercle formation (v) slow generation time, the immune system may not readily recognize the bacilli or may not be triggered sufficiently to eliminate the *M. tuberculosis* (vi) high lipid concentration in cell wall which accounts for impermeability and resistance to antimicrobial agents (vii) cord factor: the cord factor is a surface glycolipid which blocks macrophage activation by IFN-γ, induces secretion of TNFα and causes *M. tuberculosis* to form cords *in-vitro*. This is the main virulence factor which helps *M. tuberculosis* to become resistant to anti-TB drugs (Todar, 2011).

2.5 Pathogenesis of *M. tuberculosis*

Mycobacterium tuberculosis is the causative agent of most cases of TB (Barrera, 2007; Todar, 2011). Tuberculosis is spread by inhaling droplet nuclei measuring 1 µm to 5 µm containing bacilli from infectious patients (Ducati *et al*., 2006; Meya and McAdam, 2007; Todar, 2011). Infection occurs when a susceptible person inhales the droplet nuclei (Pfyffer,

2007). The infectious droplets settle throughout the airways (Frieden *et al*., 2003; Knechel, 2009). The majority of the *M. tuberculosis* are trapped in the upper parts of the airways where the mucus-secreting goblet cells exist (Frieden *et al*., 2003; Knechel, 2009). Droplets that bypass the mucociliary system and reach the alveoli penetrate into the terminal alveoli of the lungs, where the tubercle bacilli multiply, spread to the local lymph nodes and to the rest of the body (Ducati *et al*., 2006; Meya and McAdam, 2007; Knechel, 2009).

In the majority (90%) of immunocompetent individuals the initial host immune response usually arrests and limits infection such that the patient does not develop TB (Russell, 2007; Eley and Beatty, 2009; Paige and Bishai, 2010). Alternatively, the bacteria can multiply within alveolar macrophages after infection, causing primary TB (Dheda *et al*., 2005; Pfyffer, 2007; Paige and Bishai, 2010). Some *M. tuberculosis* bacilli can remain viable but dormant within well-organized granulomata for many years after initial infection (Pfyffer, 2007; CDC, 2009). Stable granulomata may never cause disease at all, so-called latent infection, manifesting only by a positive tuberculin skin test (TST) (Pfyffer, 2007; CDC, 2009). However, the latent bacteria can eventually grow, with resultant clinical disease, known as reactivation TB (Pfyffer, 2007). Although co-infection with HIV is the most notable cause for progression to active disease (Lawn and Bekker, 2009; Knechel, 2009), other factors, such as uncontrolled diabetes mellitus, sepsis, renal failure, malnutrition, smoking, chemotherapy, organ transplantation and long-term corticosteroid usage, that can trigger reactivation of a remote infection are more common in the critical care setting (CDC, 2009).

2.6 Treatment of TB

Chemotherapy for TB began in the late 1940s with streptomycin (STR), closely followed by *p*-aminosalicylic acid (PAS) and the key treatment drug INH (Saltini, 2006). Rifampicin, the other key treatment drug, was introduced in the late 1960s (Saltini, 2006). Pyrazinamide (PZA), which was already known in the 1950s but caused side effects in the dosages used, was introduced as short course chemotherapy given in lower doses, in the 1970s (Petrini and Hoffner, 1999; Saltini, 2006). Ethambutol (EMB) is mainly an adjunct drug, preventing development of drug-resistance (Saltini, 2006). In the 1980s, the International Union Against Tuberculosis and Lung Disease (IUATLD) recommended the modern short course chemotherapy consisting of INH, RIF and PZA for the two month induction phase followed by INH and RIF for the four month continuation phase of the six month daily regimen (Stratton and Reed, 1986; IUATLD, 1988).

The slow replication rate and ability to persist in a latent state of *M. tuberculosis* results in the need for long durations of both drug therapy of TB and for preventive therapy in people with *M. tuberculosis* infection (Lawn and Zumla, 2011). The use of multiple drugs helps in preventing the appearance of drug-resistant TB (Lawn and Zumla, 2011).

In 1993, the WHO announced a global strategy for TB control known as the Directly Observed Therapy Short course (DOTS) (WHO, 1997; Saltini, 2006). The treatment regimen for all adults with previously untreated TB should consist of a two month initial phase of INH, RIF, PZA and EMB (WHO, 1997; Saltini, 2006; WHO, 2010c). If drug susceptibility test results are known and the bacteria are fully susceptible, EMB need not be included (Saltini, 2006; WHO, 2010c). The DOTS programme focuses on assessing progress in the achievement of 70% case detection and 85% treatment success rates as defined by the World Health Assembly (WHO, 1997; WHO, 2010c).

Table 2.1: Recommended doses for first-line drugs for treatment of adults with *M. tuberculosis* (WHO, 2010c)

	Recommended doses			
	Daily		Three times per week	
Drugs	Dose and range	Maximum (mg)	Dose and range	Daily maximum (mg)
INH	5 (4-6)	300	10 (8-12)	900
RIF	10 (8-12)	600	10 (8-12)	600
PZA	25 (20-30)		35 (30-40)	
EMB	15 (15-20)		30 (25-35)	
	15 (15-20)		15 (12-18)	1 000

INH=Isoniazid, RIF= Rifampicin, PZA= Pyrazinamide, EMB= Ethambutol, STR= Streptomycin

Although DOTS is highly effective in the management of drug-susceptible TB, it is insufficient for controlling MDR-TB (Di Perri and Bonora, 2004; Saltini, 2006). In 1999, the WHO recommended the DOTS Plus programme (Farmer and Kim, 1998; WHO, 2003; WHO, 2010c). The DOTS-Plus refers to the DOTS programmes that add components for MDR-TB diagnosis, management and treatment (WHO, 2003). The first WHO endorsed DOTS-Plus programmes began in 2000 (WHO, 2010c). A typical MDR-TB regimen should contain at least four, preferably five or even six drugs, with either certain or almost certain efficacy based on drug susceptibility testing results and/or treatment history (Farmer and Kim, 1998; WHO, 2003; WHO, 2010c). The commonly used drugs in the treatment regimen for MDR-TB include, aminoglycosides (KAN and AMK), polypeptides (CAP), viomycin (VIO) and enviomycin (E), FLQ (OFX, CIP and gatifloxacin), D-cycloserine and thionamides

[ethionamide (ETH) and prothionamide (PTH)] (WHO, 2003; WHO, 2010c). Many of these drugs are less effective, more expensive and toxic than first-line anti-TB drugs (White and Moore-Gillon, 2000; Rajbhandary *et al*., 2004; WHO, 2006). The treatment of MDR-TB requires prolonged periods, usually 24 months, compared with the six to eight months required for drug-susceptible TB (Pérez-Guzmán *et al*., 2002; WHO, 2006).

The WHO revised international guidelines for the treatment of TB in 2010 (WHO, 2010c), The recommendations included: (i) rapid molecular testing for INH and RIF is advisable even in previously untreated patients if resources make it possible (ii) monthly culture for the monitoring of treatment response is preferred (iii) an intensive phase of eight months' duration is conditionally recommended instead of the previous minimum of six months (iv) the addition of PZA to a minimum of four second-line anti-TB drugs that are likely to be effective is recommended (v) the use of FLQ and ETH is strongly recommended and (vi) all patients with drug-resistant TB and HIV who are on second-line medications should be placed on antiretroviral therapy (ART) as soon as they can tolerate it (WHO, 2010c).

2.7 Drug-resistance in *M. tuberculosis*

Drug-resistance to TB occur as a result of treatment non-compliance including lack of adherence to therapy, poor medical or nursing practices in which incorrect treatment regimens were prescribed or less commonly, because of physiological problems, such as drug interaction or malabsorption (Mahmoudi and Iseman, 1993; Bastian *et al*., 2003; Lienhardt and Ogden, 2004). Drug-resistance in TB may be primary or acquired (secondary) (WHO, 2008b; WHO, 2010c). Drug-resistance in a patient who has never received anti-TB treatment previously is termed primary drug-resistance (WHO, 2008b; WHO, 2010c). Acquired drug-resistance occurs when patients who are infected with drug-sensitive bacilli develop resistance to drugs (WHO, 2008b; WHO, 2010c). A new TB case is defined as a patient who has never taken TB treatment or who has taken anti-TB drugs for less than one month (WHO, 2003; WHO, 2008b; WHO, 2010c). In contrast, previously treated cases are defined as patients that have been treated for TB with combination chemotherapy for four weeks or more (WHO, 2003; WHO, 2010c). Acquired drug-resistance is more common than primary resistance (WHO, 2003; WHO, 2010c).

2.8 Epidemiology of drug-resistant TB

According to the global project on anti-TB drug-resistance surveillance conducted by the WHO and the IUATLD (1999 to 2002), drug-resistant TB, including MDR-TB, was found in all regions of the world (WHO, 2004). In 2004, the estimated number of MDR-TB cases occurring worldwide was about 424 000 (4.3%) of all new and previously treated TB cases (Zignol *et al.*, 2006). In the same year, 181 408 MDR-TB cases were estimated to have occurred among previously treated TB cases alone (Zignol *et al.*, 2006). Three countries, including China, India and Russia, accounted for 62% of the estimated global burden of MDR-TB cases (Zignol *et al.*, 2006). Other hot spots of MDR-TB were found in South America and Latin America (CDC, 1998; Espinal *et al.*, 1998; García-García *et al.*, 2000), South Africa (Karstaedt *et al.*, 1998; WHO, 2004), the Middle East (Surucuoglu *et al.*, 2005; Masjedi *et al.*, 2006) and many areas of Asia, including Vietnam (Quy *et al.*, 2006), Thailand, (Tansuphasiri *et al.*, 2003) Myanmar (Phyu *et al.*, 2005), Korea (Kim *et al.*, 1999) and the Philippines (Mendoza *et al.*, 1997). In 2007, approximately 511 000 new MDR-TB cases were reported worldwide (WHO, 2009).

Multidrug-resistance is a risk factor for the emergence of XDR-TB (Ahmad and Mokaddas, 2009). In 2006, nearly 10% of all MDR-TB cases in the former Soviet Union countries (ranging from 4% in Armenia to 24% in Estonia) were XDR-TB (WHO, 2008b). In South Africa, 996 of the 17 615 (5.6%) of MDR-TB cases were also XDR-TB (WHO, 2008b). In 2008, an estimated 440 000 cases of MDR-TB emerged worldwide (WHO, 2010b). The 27 countries (15 in the European Region) that account for 86% of all cases have been termed the 27 high-burden MDR-TB countries (WHO, 2010b). The four countries that had the largest number of estimated cases of MDR-TB in absolute terms in 2008 were China (100 000; range, 79 000 to 120 000), India (99 000; range, 79 000 to 120 000), the Russian Federation (38 000; range, 30 000 to 45 000) and South Africa (13 000; range 10 000 to 16 000) (WHO, 2010a; WHO, 2010b). The full extent of XDR-TB is unknown because many countries lack sufficient laboratory system capacity; however, the available data show that 58 countries had reported at least one case of XDR-TB in July 2010 (WHO, 2010a; WHO, 2010b).

2.9 Genetic basis of resistance against first and second line anti-TB drugs

Unlike other bacteria, resistance of *M. tuberculosis* is caused by random chromosomal mutations in different genes, such as nucleotide insertions, deletions or substitutions (Petrini

and Hoffner, 1999; Zhang and Yew, 2009). Individual nucleotide changes confer resistance to single drugs and the stepwise accumulation of these mutations leads to MDR-TB (Petrini and Hoffner, 1999; Richter *et al*., 2009). *Mycobacterium tuberculosis* strains show different mutation rates for the different anti-TB drugs (Schluger *et al*., 1996; Zhang and Yew, 2009). The genes associated with drug-resistance in *M. tuberculosis* for first-line and second-line anti-TB drugs are shown in Table 2.2.

Table 2.2: Mechanisms of drug-resistance in *M. tuberculosis* (Almeida Da Silva and Palomino, 2011)

Drug	MIC (mg/L)	Gene	Role of gene product
INH	0.02-02 (7H9/7H10)	*kat*G	catalase/peroxidase
		*inh*A	enoyl reductase
		*ahp*C	alkyl hydroperoxidase reductase
RIF	0.05-0.1 (7H9/7H10)	*rpo*B	β-subunit of RNA polymerase
PZA	16-50 (LJ)	*pnc*A	Pyrazinamidase
STR	2-8 (7H9/7H10)	*rps*L	S12 ribosomal protein
		rrs	16S rRNA
		*gid*B	7-methylguanosine methyltransferase
EMB	1-5 (7H9/7H10)	*emb*B	arabinosyl transferase
FLQ	0.5-2 (7H9/7H10)	*gyr*A/*gyr*B	DNA gyrase
KAN/AMK	2-4 (7H9/7H10)	*rrs*	16S rRNA
CAP/VIO	2-4 (7H9/7H10)	*tly*A	rRNA methyltransferase
ETH	10 (7H11)	*inh*A	enoyl reductase
PAS	0.5 (LJ)	*thy*A	thymidylate synthase A

INH=Isoniazid, RIF=Rifampicin, PZA=Pyrazinamide, EMB=Ethambutol, STR=Streptomycin, FLQ=Fluoroquinolones, KAN=Kanamycin, AMK=Amikacin, CAP=Capreomycin, VIO=Viomycin, ETH=Ethionamid, PAS=P-amino salicylic acid

It is essential to understand the mechanisms by which *M. tuberculosis* strains acquire multidrug-resistance, since it can help in the development of improved diagnostic techniques and control of drug-resistant TB (Zhang and Yew, 2009). Considerable progress has been made in understanding the molecular mechanisms for resistance of *M. tuberculosis* to first-line anti-TB drugs (Johnson *et al*., 2006a; Zhang and Yew, 2009; Almeida Da Silva and Palomino, 2011). At least one gene for each of the first-line anti-TB drugs has been identified in which specific mutations lead to a resistant phenotype (Johnson *et al*., 2006a; Zhang and Yew, 2009; Almeida Da Silva and Palomino, 2011). However, knowledge regarding the molecular genetic basis of resistance to second-line anti-TB drugs is limited (Zhang and Yew, 2009; Almeida Da Silva and Palomino, 2011).

2.9.1 Isoniazid

Isoniazid is an important component of the current TB treatment regimen (Stratton and Reed, 1986; Zhang and Yew, 2009). Isoniazid is a bactericidal agent, only active against growing *M. tuberculosis* and is not active against non-replicating bacilli or under anaerobic conditions (Zhang and Yew, 2009). It inhibits the synthesis of mycolic acid, required for the mycobacterial cell wall (Zhang and Yew, 2009) (Fig 1). It is a pro-drug requiring activation by the catalase/peroxidase enzyme encoded by the *kat*G gene (Zhang *et al*., 1992; Zhang and Yew 2009; Almeida Da Silva and Palomino, 2011). The molecular basis of resistance to INH is more complex and is caused by a variety of mutations associated with four different genes of *M. tuberculosis*: (i) *kat*G encoding catalase peroxidase, (ii) *inh*A encoding the enoyl acyl carrier protein (ACP) reductase, an enzyme that synthesizes mycolic acid (iii) *kas*A encoding b-ketoacyl ACP synthase and (iv) *ahp*C encoding alkylhydroperoxide reductase (Telenti *et al*., 1997a; Ramaswamy and Musser, 1998; Zhang and Yew, 2009). Mutations associated with INH resistance occur at an estimated frequency of 1 in 10^5 to 10^6 (Zhang and Yew, 2009).

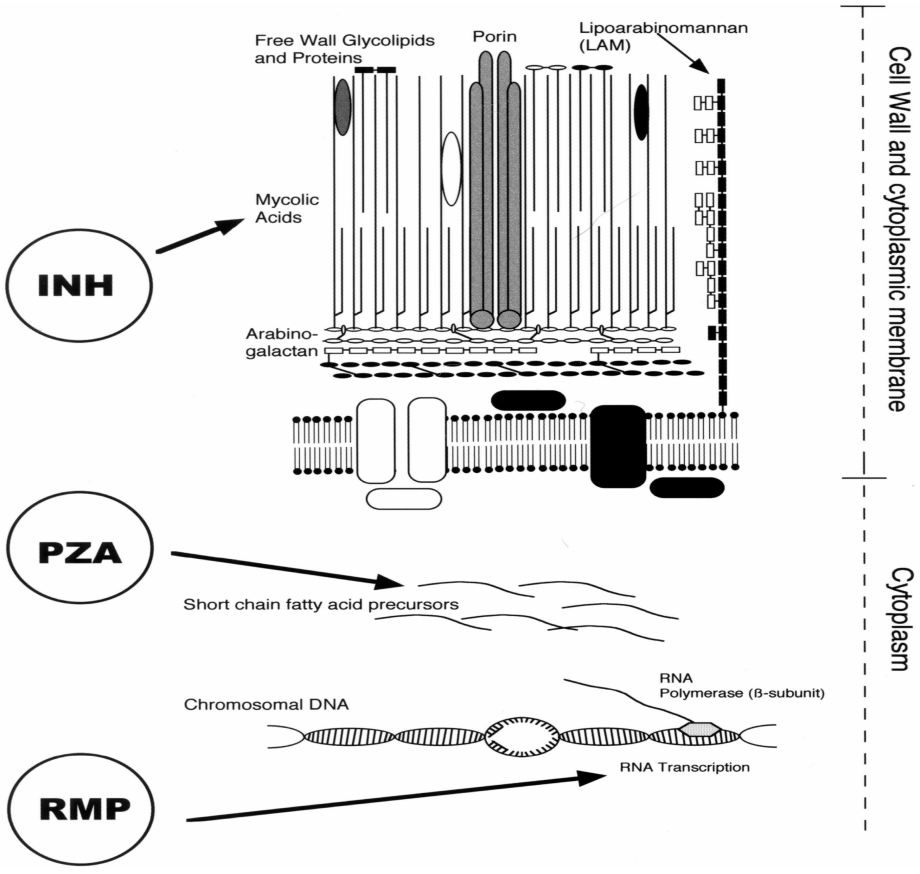

Figure 2.1: Sites of action for INH, PZA and RIF (RMP) in the *M. tuberculosis* cell (Parsons *et al.*, 1997)

Mutations of the *kat*G gene are a major mechanism of INH-resistance in *M. tuberculosis* (Heym *et al*., 1995; Rouse *et al*., 1995; Musser *et al*., 1996; Zhang and Yew, 2009). Mutations in the *kat*G gene cause an altered enzyme structure, which results in a decreased conversion of INH to its biologically active form; leading to high level (MIC > 5 µg/ml) INH-resistance (Heym *et al*., 1995; Rouse *et al*., 1995; Musser *et al*., 1996; Zhang and Yew, 2009). Most mutations of the *kat*G were found between codons 138 and 328, with 50% to 95% of the INH-resistant strains being found to contain mutations in codon Ser315Thr of the *kat*G gene (Slayden and Barry, 2000; Mokrousov *et al*., 2002a; Zhang and Yew, 2009).

Alterations or an overexpression of the *inh*A gene can also cause INH-resistance (Banerjee *et al*., 1994; Slayden and Barry, 2000; Johnson *et al*., 2006a). Approximately 20% to 35% of INH-resistant *M. tuberculosis* strains contain mutations in the *inh*A structural gene or promoter region (Slayden and Barry, 2000; Johnson *et al*., 2006a). Mutations in the *inh*A promoter are more frequent (8% to 20%) than mutations in the structural gene (0% to 5%) (Musser *et al*., 1996; Zhang, 2005; Almeida Da Silva and Palomino, 2011). Mutations in the *inh*A region are usually associated with low-level resistance (MICs = 0.2 to 1 µg/ml) and are less frequent than k*at*G mutations (Zhang and Yew, 2009). Simultaneous mutations in the two genes, k*at*G and *inh*A, are associated with resistance in 75% to 85% of TB cases (Guo *et al*., 2006; Jiao *et al*., 2007).

Mutations in the *kas*A gene can cause INH-resistance (Mdluli *et al*., 1998); however, studies found that the *kas*A gene mutations were also detected in INH-susceptible strains (Lee *et al*., 1999; Ramaswamy *et al*., 2003). In 10% to 15% of INH-resistant *M. tuberculosis* strains, mutations in the the *oxy*R gene and the neighbouring *aphC* gene, as well as in the intergenic region has been reported (Telenti *et al*., 1997a; Johnson *et al*., 2006a). However, it has been reported that an increase in the expression of the *ahp*C gene seems to be more a compensatory mutation for the loss of catalase/peroxidase activity than the basis for INH-resistance (Sherman *et al*., 1996; Almeida Da Silva and Palomino, 2011).

It has been described that mutations in the *ndh* gene reduce the activity of NADH dehydrogenase and produce resistance to INH and ETH (Miesel *et al*., 1998; Almeida Da Silva and Palomino, 2011). Mutations in the *ndh* gene have been associated with INH resistance alone or in combination with other gene mutations, such as the *ndh* and *kat*G genes (Almeida Da Silva and Palomino, 2011). Although A13C and V18A mutations in the *ndh* gene were reported in INH-resistant *M. tuberculosis* strains, the V18A mutation was

previously described in an INH-susceptible strain (Ramaswamy *et al*., 2003; Cardoso *et al*., 2007).

In 5% to 10% of INH-resistant *M. tuberculosis* isolates no mutation has been identified (Zhang and Yew, 2009). Therefore, other molecular mechanisms mediating INH-resistance remain to be discovered (Johnson *et al*., 2006a; Zhang and Yew, 2009). Reliable clinical application of genotypic testing for all mutations associated with INH-resistance is not currently available due to the diversity of these mutations (Johnson *et al*., 2006a; Zhang and Yew, 2009).

2.9.2 Rifampicin

Rifampicin has early bactericidal activity on metabolically active *M. tuberculosis* and has late sterilizing action against semi-dormant bacteria (Johnson *et al*., 2006a; Zhang and Yew, 2009). Rifampicin inhibits bacterial DNA-dependent RNA synthesis by inhibiting bacterial DNA-dependent RNA polymerase (Almeida Da Silva and Palomino, 2011) (Fig 1). The highly effective bactericidal action of this drug against *M. tuberculosis* has made it a key component of therapy and along with INH it forms the backbone of the short-course *M. tuberculosis* chemotherapy worldwide (Stratton and Reed, 1986; Almeida Da Silva and Palomino, 2011). Rifampicin is bactericidal for *M. tuberculosis* with MICs ranging from 0.05 to 1 µg/ml on solid or liquid media, but the MIC is higher in egg media (MIC = 2.5 to 10 µg/ml) (Zhang and Yew, 2009). *Mycobacterium tuberculosis* strains with MICs < 1 µg/ml in liquid or agar medium or MICs < 40 µg/ml in LJ medium are considered RIF-susceptible (Zhang and Yew, 2009).

Almost 95% to 98% of RIF-resistance is associated with point mutations in the 'hot-spot' region of the 81 bp core known as the RIF-resistance determining region (RRDR) of the *rpo*B gene, corresponding to codons 507 to 533 (Telenti *et al*., 1993; Telenti *et al*., 1997a; Zhang and Yew, 2009; Almeida Da Silva and Palomino, 2011). Mutations in codons 526 and 531 are the most frequently reported mutations and usually lead to a high-level of phenotypic resistance (MIC > 32 µg/ml) as well as cross-resistance to other rifampicins (Riska *et al*., 2000; Zhang, 2005; Zhang and Yew, 2009; Almeida Da Silva and Palomino, 2011). Mutations at codons 511, 516, 518 and 522 result in low-level resistance (MIC < 2 µg/ml) to RIF and rifapentine but some susceptibility to rifabutin and rifalazil (Zhang, 2005; Zhang and

Yew, 2009; Almeida Da Silva and Palomino, 2011). Mutations for RIF occur at an estimated frequency of 1 out of 10^7 to 10^8 (Johnson *et al*., 2006a; Zhang and Yew, 2009).

Mono-resistance to RIF is rare, resistance most commonly develops in conjunction with INH resistance, thus determination of RIF-resistance is a useful surrogate marker for MDR-TB (Vareldzis *et al*., 1994; Ramaswamy and Musser, 1998; Johnson *et al*., 2006a; Almeida Da Silva and Palomino, 2011). Rifampicin-dependent strains of *M. tuberculosis* have been reported in clinical settings (Zhang and Yew, 2009). Unlike the strictly STR-dependent *M. tuberculosis* strains that only grow in the presence of STR, RIF-dependent *M. tuberculosis* strains could still show very poor growth in the absence of RIF (Zhang and Yew, 2009). These RIF-dependent *M. tuberculosis* strains have not been widely reported, probably because current diagnostic practices use only drug-free media (Zhang and Yew, 2009). The RIF-dependent *M. tuberculosis* strains occur as MDR-TB and seem to develop upon repeated treatment with RIF in re-treatment patients but the circumstances under which the RIF-dependent strains arise remain unclear (Zhang and Yew, 2009). Further studies are needed to determine the mechanisms of RIF-dependence and to assess the role of such strains in treatment failure (Zhang and Yew, 2009).

2.9.3 Pyrazinamide

Pyrazinamide is a nicotinamide analog and is highly specific for *M. tuberculosis*, as it has no effect on other mycobacteria (Johnson *et al*., 2006a; Zhang and Yew, 2009). The mechanism of action of PZA is not fully understood (Johnson *et al*., 2006a; Zhang and Yew, 2009; Almeida Da Silva and Palomino, 2011). Pyrazinamide targets an enzyme involved in fatty-acid synthesis and has no bactericidal activity against rapidly growing mycobacteria (Heifets and Lindholm-Levy, 1992; Zhang and Yew, 2009) (Fig 1). However, the drug has optimum activity against dormant or semi-dormant mycobacteria in an acidic environment contained inside phagolysosomes (Somoskovi *et al*., 2001; Johnson *et al*., 2006a; Zhang and Yew, 2009). The use of PZA along with RIF shortened the chemotherapeutic regimen from 12 months to 6 months (Zhang and Yew, 2009; Almeida Da Silva and Palomino, 2011). Pyrazinamide, like INH, is a pro-drug requiring activation to pyrazinoic acid (POA) by the enzyme pyrazinamidase (PZase) encoded by the *pnc*A gene (Zhang and Yew, 2009; Almeida Da Silva and Palomino, 2011). Studies have shown that mutations in *pnc*A confer PZA-resistance in more than 70% PZA-resistant *M. tuberculosis* (Hirano *et al*., 1997; Scorpio *et al*., 1997; Sreevatsan *et al*., 1997; Zhang and Yew, 2009). Most of the mutations occur in a

561 bp *pnc*A region of the open reading frame or in an 82 bp region of its putative promoter (Scorpio *et al*., 1997; Sreevatsan *et al*., 1997; Riska *et al*., 2000; Zhang, 2005; Juréen *et al*., 2008). However, some PZA-resistant *M. tuberculosis* strains do not show mutations in the *pnc*A gene or in its promoter region (Zhang and Yew, 2009; Almeida Da Silva and Palomino, 2011). It has been postulated that resistance to PZA in these strains could be due to mutations occurring in an unknown *pnc*A regulatory gene (Cheng *et al*., 2000; Almeida Da Silva and Palomino, 2011).

2.9.4 Streptomycin

Streptomycin is an aminoglycoside used as an alternative first-line drug to PZA in TB therapy (Moazed and Noller, 1987; Zhang and Yew, 2009). Streptomycin inhibits protein synthesis by interacting with the small 30S ribosome subunit (Musser, 1995; Johnson *et al*., 2006a; Zhang and Yew, 2009). Streptomycin-resistance is associated with mutations in the *rrs* or *rps*L genes (Finken *et al*., 1993; Meier *et al*., 1996; Johnson *et al*., 2006a; Zhang and Yew, 2009). Point mutations in the *rrs* gene occur in the loops of the 16S rRNA around nucleotides 530 and 912 (Meier *et al*., 1996; Zhang and Yew, 2009). Both 530 and 912 are situated close to the STR-binding site and confer moderate levels of STR resistance (Meier *et al*., 1996; Zhang and Yew, 2009). A mutation at lysine residue 42 or lysine residue 87 of the S12 ribosomal protein, which interacts with the 16S rRNA near the STR-binding site, confers high-level resistance to the drug (Meier *et al*., 1996; Zhang and Yew, 2009). The majority of mutations in the *rps*L gene occurs at positions 43 and 88 and are usually associated with high-level resistance (MIC > 1000 µg/ml) (Meier *et al*., 1996; Johnson *et al*., 2006a). Thirty percent of STR-resistant strains lack mutations in the *rps*L or *rrs* genes which suggest that other molecular mechanisms of STR-resistance in *M. tuberculosis* might exist (Johnson *et al*., 2006a; Zhang and Yew 2009; Almeida Da Silva and Palomino, 2011). It has been suggested that mutations in the *gid*B gene, encoding a conserved 16S rRNA specific 7-methylguanosine methyltransferase, or the presence of efflux pump inhibitors, appear to be involved in low-level STR-resistance (Okamoto *et al*., 2007; Spies *et al*., 2008).

2.9.5 Ethambutol

Ethambutol is a bacteriostatic agent that is active against growing mycobacteria and has no effect on non-replicating bacilli (Zhang and Yew, 2009; Almeida Da Silva and Palomino, 2011). Ethambutol inhibits arabinosyl transferase involved in the incorporation of essential

mycolic acids into the mycobacterial cell wall (Takayama and Kilburn, 1989; Johnson *et al*., 2006a). Ethambutol resistance is associated with a gene cluster, *emb*CAB, which encodes homologous arabinosyl transferase enzymes (Johnson *et al*., 2006a). Resistance to EMB arises either through increased expression of these genes, thus overcoming low-levels of EMB or by mutations at codon 306 of the *emb*B gene (Telenti *et al*., 1997b; Johnson *et al*., 2006a). Almost 50% to 70% of EMB-resistant *M. tuberculosis* strains contain mutations in the *emb*B resistance determining region (ERDR) of the *emb*B gene with the majority (47% to 60%) of the *M. tuberculosis* strains carrying mutations at codon 306 (Sreevatsan *et al*., 1997; Telenti *et al*., 1997b; Johnson *et al*., 2006a; Zhang and Yew, 2009). Five mutations have been identified in codon 306, which result in three different amino acid substitutions (Val, Leu and Ile) in EMB-resistant *M. tuberculosis* strains (Johnson *et al*., 2006a). Mutations that confer EMB-resistance occur at a frequency of 1 x 10^{-5} (Johnson *et al*., 2006a). About 35% of EMB-resistant *M. tuberculosis* strains (MIC < 10 µg/ml) do not have *emb*B gene mutations, suggesting that there may be other mechanisms of EMB-resistance (Zhang and Yew, 2009). Further studies are needed to identify other mechanisms of EMB-resistance (Zhang and Yew, 2009; Almeida Da Silva and Palomino, 2011).

Some reports have suggested that the *emb*B codon 306 mutation is not involved in EMB-resistance but is instead associated with the development of other drug-resistance, including MDR-TB (Mokrousov *et al*., 2002b; Hazbón *et al*., 2005). A Russian study found mutations of *emb*B306 in 48.3% of *M. tuberculosis* resistant strains and in 31.2% of susceptible *M. tuberculosis* strains (Mokrousov *et al*., 2002b). Other studies (Hazbón *et al*., 2005; Plinke *et al*., 2009) found mutations in *emb*B codon 306 in up to 20% of EMB-susceptible *M. tuberculosis* isolates. It has been postulated that mutations in *emb*B306 may be related to variable degrees of EMB-resistance and that this molecular alteration could be necessary, but not sufficient, for high-level EMB resistance (Almeida Da Silva and Palomino, 2011).

An interesting observation was reported that resistance to EMB is frequent among MDR-TB strains (Pablos-Méndez *et al*. 1998; Johnson *et al*., 2006b). Johnson *et al*. (2006b) reported a strong association of EMB-resistance and MDR-TB, with 87% of the MDR-TB isolates being EMB-resistant. More studies are needed to determine the association between EMB-resistance and MDR-TB.

2.9.6 Kanamycin, amikacin, capreomycin and viomycin

Kanamycin and AMK are two closely related injectable aminoglycosides while CAP and VIO are cyclic peptides (Perri and Bonora, 2004; Johnson *et al*., 2006a). All four drugs are used for the treatment of patients with MDR-TB (WHO, 2010c). Although aminoglycosides and peptides belong to two different antibiotic families, all these drugs inhibit protein synthesis (Johnson *et al*., 2006a; Almeida Da Silva and Palomino, 2011). Little is known regarding the molecular basis of resistance of *M. tuberculosis* to these drugs (Johnson *et al*., 2006a). Mutations in the *rrs* gene particularly at positions 1401, 1402, and 1484, have been associated with resistance to each of the drugs (Alangaden *et al*., 1998; Suzuki *et al*., 1998; Maus *et al*., 2005; Johnson *et al*., 2006a). In addition, mutations of the *tly*A gene, which encodes a putative rRNA methyltransferase, have been reported to cause resistance to CAP and VIO in *M. tuberculosis* (Zhang and Yew, 2009).

Kanamycin and AMK are deoxystreptamine aminoglycosides and cross-resistance does not occur with STR, a streptidine drug (Zhang and Yew, 2009). In contrast, cross-resistance occurs between AMK, KAN, CAP and VIO (Almeida Da Silva and Palomino, 2011). Variable degrees of cross-resistance have been reported between KAN, AMK, CAP or VIO (Maus *et al*., 2005; Zhang and Yew, 2009). Mutants resistant to CAP and VIO could have *tly*A, C1402T, or G1484T *rrs* mutations, while mutants resistant to CAP but not VIO could have an A1401G *rrs* mutation (Maus *et al*., 2005; Zhang and Yew, 2009). Mutants with an A1401G mutation could cause resistance to KAN and CAP but not VIO (Maus *et al*., 2005; Zhang and Yew, 2009). Mutants resistant to CAP, KAN and VIO could have either a C1402T or a G1484T mutation in the *rrs* gene (Maus *et al*., 2005; Zhang and Yew, 2009).

Mutations in the promoter region of the *eis* gene in *M. tuberculosis*, which encodes an aminoglycoside acetyltransferase, was reported to cause an overexpression of the protein and conferred low-level resistance to KAN but not AMK (Almeida Da Silva and Palomino, 2011). Similar results have been obtained in two recent studies that assessed low-level KAN-resistant clinical isolates (Engström *et al*., 2011; Campbell *et al*., 2011).

2.9.7 Fluoroquinolones

Ciprofloxacin (CIP) and ofloxacin (OFX) are the two FLQ used as second-line anti-TB drugs in MDR-TB treatment (Berning, 2001; WHO, 2001; Perri and Bonora, 2004).

Fluoroquinolones act by introducing negative supercoiling, thus preventing replication and cell division (Johnson *et al*., 2006a; Almeida Da Silva and Palomino, 2011). Fluoroquinolones block topoisomerase II (called DNA gyrase) of *M. tuberculosis*, a heterotetramer with two A and B subunits coded by the *gyr*A and *gyr*B genes (Johnson *et al*., 2006a; Zhang and Yew; 2009). Fluoroquinolone resistance is associated with mutations in the conserved region known as the quinolone resistance determining region (QRDR) of *gyr*A (320 bp) and *gyr*B (375 bp) genes (Berning, 2001; Johnson *et al*., 2006a; Almeida Da Silva and Palomino, 2011). Mutations in *gyr*B occur less frequent than *gyr*A mutations (Aubry *et al*., 2006; Mdluli and Ma, 2007; Wang *et al*., 2007). Approximately 70% to 90% of FLQ-resistant strains show mutations in the *gyr*A gene (Takiff *et al*., 1994; Musser, 1995; Xu *et al*., 1996; Ramaswamy and Musser, 1998; Ginsburg *et al*., 2003). Most of the *M. tuberculosis* strains [with high-levels of resistance (MIC > 2 µg/ml to ofloxacin)], showed mutations at codons 88, 89, 90, 91, 94 and 95 of the *gyr*A gene (Zhang and Yew, 2009). The most frequent mutations occur in codon 94 (D94G or D94A) and codon 90 (A90V) of the *gyr*A gene (Siddiqi *et al*., 2002; Cheng *et al*., 2004; Bozeman *et al*., 2005; Aubry *et al*., 2006; Shi *et al*., 2006). Codon 95 has been reported to occur in both FLQ-susceptible and FLQ-resistant strains (Musser, 1995; Almeida Da Silva and Palomino, 2011).

Some strains with low-level resistance have no mutation in the *gyr*A or *gyr*B gene, which suggests mutations in another target gene or the use of other mechanisms, such as decreased cell-wall permeability to drugs, drug efflux pumps, drug sequestration, or perhaps even drug inactivation (Riska *et al*., 2000). A new mechanism of FLQ resistance mediated by the *Mfp*A gene was identified (Zhang and Yew, 2009; Almeida Da Silva and Palomino, 2011). The *Mfp*A is a member of the penta-peptide repeat family of proteins from *M. tuberculosis* and its expression causes resistance to FLQ drugs (Zhang and Yew, 2009; Almeida Da Silva and Palomino, 2011). The *Mfp*A binds to DNA gyrase and inhibits its activity in the form of a DNA mimicry, which explains its inhibitory effect on DNA gyrase and FLQ-resistance (Zhang and Yew, 2009). Currently, the new generation of FLQs, such as moxifloxacin and gatifloxacin, are under clinical evaluation and are being proposed as first-line anti-TB drugs with the goal of shortening the duration of TB treatment (Nuermberger *et al*., 2004; Rustomjee *et al*., 2008; Almeida Da Silva and Palomino, 2011).

2.9.8 Ethionamide

Ethionamide (ETH) is a thioamide and is among one of the most effective and frequently used second-line drugs (Johnson *et al*., 2006a). It is bactericidal only against *M. tuberculosis, M. avium-intracellulare* and *M. leprae* (Zhang and Yew, 2009). The MICs of ETH for *M. tuberculosis* are 0.5 to 2 µg/ml in liquid medium, 2.5 to 10 µg/ml in 7H11 agar and 5 to 20 µg/ml in LJ medium (Zhang and Yew, 2009). Ethionamide is mechanistically and structurally analoguous to INH, requiring activation by the mycobacterial enzyme, flavin monooxygenase (FMO) to exert its antimicrobial activity (Ramaswamy and Musser, 1998; Johnson *et al*., 2006a). It disrupts cell wall biosynthesis by inhibiting mycolic acid synthesis (Ramaswamy and Musser, 1998; Johnson *et al*., 2006a). The exact mechanism of ETH-resistance is unknown but some isolates resistant to ETH harbour mutations in a putative regulatory region located upstream of the *orf1* gene in the *inh*A gene (Ramaswamy and Musser, 1998; Morlock *et al*., 2003; Johnson *et al*., 2006a). In addition, mutations in the drug-activating enzyme EtaA/EthA confer resistance to ETH and other thioamides (Zhang and Yew, 2009). Mutations of the *eth*A gene are spread out along the entire gene (Almeida Da Silva and Palomino, 2011). In the study by Morlock *et al*. (2003), approximately 76% of high-level ETH-resistant strains showed such mutations. Recently, *msh*A, a gene encoding a glycosyltransferase involved in mycothiol biosynthesis, has been suggested as a possible target for ETH (Vilchèze *et al*., 2008; Almeida Da Silva and Palomino, 2011).

2.9.9 Serine analogues

D-cycloserine (DCS) is a second line drug used for the treatment of patients with MDR-TB (Perri and Bonora, 2004; Johnson *et al*., 2006a). It is a cyclic analog of D-alanine, which is one of the central molecules of the cross linking step of peptidoglycan assembly (Perri and Bonora, 2004; Johnson *et al*., 2006a). D-cycloserine inhibits cell wall synthesis by competing with D-alanine for the enzymes D-alanyl-D-alanine synthetase (Ddl) and D-alanine racemase (Alr) and inhibits the synthesis of these proteins (Johnson *et al*., 2006a). Transversion of G to T in the alanine racemase (Alr) promoter may lead to the overexpression of Alr resulting in DCS resistance (Johnson *et al*., 2006a).

2.10 Diagnosis of drug-resistant TB

Multi-drug-resistant and XDR-TB is a major concern worldwide (WHO, 2010b). The rapid determination of drug susceptibility patterns is needed as a guide for the treatment of patients and to reduce morbidity, mortality and the duration of patient infectiousness and thereby decreasing the public health threat (Ahmad and Mokaddas, 2009). In general, there are two different strategies for determining drug-resistance: phenotypic and genotypic techniques (Palomino *et al*., 2008; Ahmad and Mokaddas, 2009).

2.10.1 Phenotypic susceptibility testing techniques for *M. tuberculosis*

Phenotypic techniques are based on the determination of growth or the inhibition of growth in the presence of anti-TB drugs (Palomino *et al*., 2008; Parsons *et al*., 2011). These techniques are either solid culture-based or liquid culture-based methods (Palomino *et al*., 2008; Richter *et al*., 2009; Parsons *et al*., 2011).

2.10.1.1 Conventional solid culture-based drug susceptibility testing methods for *M. tuberculosis*

The primary isolation of *M. tuberculosis* from clinical specimens is the first step before performing DST of *M. tuberculosis* (Palomino *et al*., 2008). The traditionally accepted solid culture-based methods include: the proportion method, the absolute concentration method and the resistance ratio method (McClatchy *et al*., 1978; WHO, 2001; Mitchison, 2005). These methods are based on the measurement of growth on solid culture media containing anti-TB drugs and can be performed on either egg-based (LJ medium) or agar-based (Middlebrook) media to sub-culture bacteria after the initial positive culture is obtained for diagnosis (WHO, 2003; Kim, 2005; Mitchison, 2005). The proportion method is the most commonly used method based on determination of the proportion of resistant isolates to a certain drug (Canetti *et al*., 1963; Heifets, 1991; Parsons *et al*., 2011). The absolute concentration method is based on determination of resistance in terms of the lowest concentration of a certain drug that inhibits all or almost all the growth of the strain (Canetti *et al*., 1963; Heifets, 1991; Parsons *et al*., 2011). In the resistance ratio method the unknown clinical isolate is compared with a susceptible standard laboratory strain (Canetti *et al*., 1963; Heifets, 1991; Parsons *et al*., 2011). These three methods are well standardised for clinical specimens (Heifets, 1991; WHO, 2003). However, the inoculum size must be carefully standardised to obtain reliable

results, since the number, dispersion and viability of the bacteria in the inoculum have a significant effect on the DST results (Canetti *et al*., 1963; WHO, 2003).

Growth of mycobacteria tends to be slightly better on egg-based medium but more rapid on the agar-based medium (WHO, 2003; Mitchison, 2005). The Clinical and Laboratory Standards Institute (CLSI) considers LJ medium to be unsuitable for susceptibility testing due to uncertainty about the potency of drugs following inspissations and also because components present in the eggs or the medium may negatively affect some drugs (CLSI, 2003; Parsons *et al*., 2011). Both the CDC and CLSI recommend that Middlebrook agar supplemented with oleic acid albumin dextrose and catalase (OADC) should be used as the standard medium for the agar proportion method (Woods *et al*., 2007, Parsons *et al*., 2011).

Solid culture-based methods are relatively inexpensive and do not require sophisticated equipment (Palomino *et al*., 2008). It also allows examination of colony morphology and quantitative measurement (Canetti *et al*., 1963; Palomino *et al*., 2008). However, solid culture-based methods are slow; usually taking three to six weeks to obtain the initial positive culture with an additional three weeks for susceptibility testing (Drobniewski *et al*., 2007; Palomino *et al*., 2008).

2.10.1.2 Rapid solid culture-based susceptibility testing of *M. tuberculosis*

Several new rapid approaches have been developed to shorten the long turnaround time of conventional solid-based DST methods (Palomino *et al*., 2008). The majority of these methods have been developed as in-house assays (Palomino *et al*., 2008). These methods include:

***(i) Phage-based assay for susceptibility testing of M. tuberculosis*:** The phage-based assay utilizes bacteriophages to infect and detect the presence of *M. tuberculosis* in clinical specimens and culture isolates (Mole and Maskell, 2001; Trollip *et al*., 2001; Palomino *et al*., 2008). Propagation and replication of these mycobacteriophages in rapidly-growing mycobacteria will be directly proportional to the number of *M. tuberculosis* bacilli (Mole and Maskell, 2001; Trollip *et al*., 2001; Palomino *et al*., 2008). Two formats of phage-based methods have been proposed for drug-resistance detection of *M. tuberculosis*, namely the phage amplification method and the luciferase reporter method (Pai *et al*., 2005; Palomino *et*

al., 2008). Amplification based assays are commercially available whilst light based assays are still in-house assay (Kim, 2005; Palomino, 2005; Palomino *et al*., 2008).

***(ii) The E-test for susceptibility testing of M. tuberculosis*:** The E-test is a commercial test, which is based on strips with impregnated gradients of drugs for the determination of DST (Yeung *et al*., 1993; Wanger and Mills, 1994; Palomino *et al*., 2008). The E-test has been evaluated against the agar proportion method and the BACTEC system for DST of first-line drugs and OFX (Wanger and Mills, 1994; Hausdorfer *et al*., 1998; Joloba *et al*., 2000; Freixo *et al*., 2004), showing sensitivity and specificity ranging between 90% to 100% (Wanger and Mills, 1994, Joloba *et al*., 2000).

***(iii) The nitrate reductase assay for susceptibility testing of M. tuberculosis*:** The nitrate reductase assay (NRA) is based on the capacity of *M. tuberculosis* to reduce nitrate to nitrite, which is detected by adding the Griess reagent to the medium (Angeby *et al*., 2002; Drobniewski *et al*., 2007; Palomino *et al*., 2008). The assay is simple and rapid and has demonstrated a sensitivity of 95% or greater for detection of resistance to RIF and INH (Angeby *et al*., 2002; Montoro *et al*., 2005; Lemus *et al*., 2006; Martin *et al*., 2008a). However, the sensitivity and specificity of the NRA assay were reported to be lower for STR and EMB (Affolabi *et al*., 2008; Shikama *et al*., 2009).

***(iv) The TK medium for susceptibility testing of M. tuberculosis*:** The TK medium is a rapid, solid-culture medium used as an alternative to LJ medium (Palomino *et al*., 2008; Parsons *et al*., 2011). The TK medium contains a magnesium salt, an iron salt, an amino acid, a carbohydrate, an indicator, a dye and a gelling substance (Baylan *et al*., 2004). The TK medium detects the metabolic activity of mycobacteria, which changes colour from red to yellow for a positive isolate, while any contaminating bacteria will change from red to green (Baylan *et al*., 2004; Palomino *et al*., 2008). The colour change occurs long before the colonies become visible (Baylan *et al*., 2004; Palomino *et al*., 2008). There are several types of TK medium available including: identification (TK SLC), speciation (TK PNB) and drug susceptibility testing (TK Anti TB and PNB) (Baylan *et al*., 2004; Palomino *et al*., 2008).

***(v) Thin-layer agar for susceptibility testing of M. tuberculosis*:** Thin layer agar (TLA) also known as the microcolony method is one of the low cost methods used for culture and DST of *M. tuberculosis* (Palomino *et al*., 2008; Robledo *et al*., 2008; Richter *et al*., 2009). This method uses Middlebrook 7H10 or 7H11 and the identification of *M. tuberculosis* isolates is

based on the characteristic cording morphology that is observed under the microscope (10 x objective) (Robledo *et al*., 2006; Robledo *et al*., 2008). The TLA method was evaluated for the detection of resistance to RIF, INH, KAN and OFX, showing senstivity and specificity greater than 95% (Robledo *et al*., 2006; Robledo *et al*., 2008; Martin *et al*., 2009).

2.10.1.3 Liquid culture-based susceptibility testing for *M. tuberculosis*

In order to shorten the turnaround time of DST, several new liquid-based techniques have been introduced (Palomino *et al*., 2008; Richter *et al*., 2009; Parsons *et al*., 2011). These methods reduced the prolonged incubation time required for solid culture media from three to eight weeks to one to three weeks (Palomino *et al*., 2008; Richter *et al*., 2009; Parsons *et al*., 2011). Currently, there are a number of commercially available liquid culture systems of which some are simple manual methods while others rely on more sophisticated automated instruments (Palomino *et al*., 2008; Richter *et al*., 2009; Parsons *et al*., 2011).

***(i) BACTEC radiometric system for susceptibility testing of M. tuberculosis*:** The radiometric BACTEC 460 TB culture system (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD, USA) is an automated system, which uses Middlebrook 7H12 broth medium containing ^{14}C-labeled palmitic acid for the radiometric detection of mycobacterial growth (Morgan *et al*., 1983; Sewell *et al*., 1993; Palomino *et al*., 2008; Parsons *et al*., 2011). It is rapid and sensitive compared to conventional solid-culture based methods (Morgan *et al*., 1983; Sewell *et al*., 1993; Palomino *et al*., 2008). Several studies have evaluated the BACTEC radiometric system for DST of first-line and second-line anti-TB drugs and the method has shown a good correlation with the proportion method using LJ medium with the sensitivity and specificity ranging between 97% to 98% and 90% and 99% for first-line and second-line drugs, respectively (Pfyffer *et al*., 1999; Palomino, 2005; Guillerm *et al*., 2006; Rodrigues *et al*., 2007). However, the BACTEC radiometric system relies on radioactive materials, which limits its use on a routine basis especially in resource poor countries (Palomino, 2005; Guillerm *et al*., 2006; Rodrigues *et al*., 2007; Parsons *et al*., 2011).

***(ii) The mycobacteria growth indicator tube system for susceptibility testing of M. tuberculosis*:** The mycobacteria growth indicator tube (MGIT) system is develped for the rapid culture and DST of *M. tuberculosis* (Palomino *et al*., 2008; Richter *et al*., 2009). The MGIT system uses a modified Middlebrook 7H9 broth combined with fluorescence quenching-based oxygen sensor to detect growth. The indicator fluoresces under UV light if

there is viable growth (Walters and Hanna, 1996; Hanna *et al*., 1999; Palomino, 2005; Palomino *et al*., 2008). The method can be used as a manual or automated technique. Drug susceptibility testing can be performed using prepared kits, which is available for susceptibility testing of INH, RIF, EMB, STR and PZA (Palomino *et al*., 2008).

Several studies (Reisner *et al*., 1995; Palaci *et al*., 1996; Palomino *et al*., 1999) have evaluated the MGIT manual system for DST of *M. tuberculosis* against the first-line drugs: INH, RIF, EMB and STR showing a sensitivity and specificity above 90%. The MGIT manual system was also evaluated for DST of second-line drugs (OFX, CAP, KAN and ETH) (Martin *et al.,* 2008b). In this study, the sensitivity and specificity of the MGIT manual system was 100% for OFX and CAP, 98.6% and 100% for KAN and 96.2% and 77.6% for ETH, respectively (Bastian *et al*., 2001; Palomino *et al*., 2008).

The automated MGIT system has been evaluated for first-line anti-TB drugs against the standard proportion method on LJ and the BACTEC 460 TB system and has shown a sensitivity of 100% for RIF and INH and a specificity ranging from 89% to 100% (Rüsch-Gerdes *et al*., 1999; Tortoli *et al*., 1999; Bergmann *et al*., 2000; Heifets *et al*., 2000; Ardito *et al*., 2001; Tortoli *et al*., 2002). However a slightly lower sensitivity and specificity was reported for the DST of EMB and STR (Richter *et al*., 2009). The automated method was also evaluated for second-line anti-TB drugs in order to establish breakpoint concentrations for testing (Kruuner *et al*., 2006; Rüsch-Gerdes *et al*., 2006). Critical concentrations were formulated for AMK (1.0 µg/ml), CAP (2.5 µg/ml), ETH (5.0 µg/ml), PRO (2.5 µg/ml), OFX (2.0 µg/ml) rifabutin (0.5 µg/ml) and linezolid (1.0 µg/ml) (Kruuner *et al*., 2006; Rüsch-Gerdes *et al*., 2006). The MGIT system is rapid, reliable and yield reproducible results within eight to twelve days (Palomino, 2005; Guillerm *et al*., 2006; Palomino *et al*., 2008). The disadvantage of the MGIT system is that it is relatively expensive compared to conventional methods and liquid-based methods are known to have a high percentage of contamination leading to unreliable results (Palomino *et al*., 2008; Richter *et al*., 2009).

***(iii) The BacT/Alert 3D system for susceptibility testing of M. tuberculosis*:** The BacT/Alert 3D system (bioMérieux, Durham, NC, USA) is a non-radiometric system based on the measurement of CO_2 as an indicator of bacterial growth in cultures in a closed and a fully automated system (Rohner *et al*., 1997; Angeby *et al*., 2003; Palomino *et al*., 2008). A multicentre evaluation study of the MB/BactT® system against the BACTEC 460 TB system showed an overall level of agreement of 96% for first-line drugs (Garrigó *et al*., 2007). The

BacT/Alert 3D system has also been evaluated against the BACTEC 460 TB for AMK and OFX and showed good concordance with 100% sensitivity for AMK (80.8% to 100%), OFX (78.1% to 100%) and 100% specificity for AMK and OFX (86.7% to 100%) compared to the BACTEC system (Barreto *et al*., 2003).

***(iv) VersaTREK® system for susceptibility testing of M. tuberculosis*:** The VersaTREK® system is a fully automated, non-radioactive system providing continuous monitoring of growth of mycobacteria (Richter *et al*., 2009). The VersaTREK® system uses enriched 7H9 Middlebrook broth for the detection of growth of *M. tuberculosis* by measuring pressure changes inside the culture vial due to mycobacterial metabolism (Guillerm *et al*., 2006; Richter *et al*., 2009). The VersaTREK® system has been evaluated for DST of first-line anti-TB drugs against the proportion method (LJ) and BACTEC radiometric system (Bergmann *et al*., 1998; Ruiz *et al*., 2000; LaBombardi *et al*., 2002). The assay showed a sensitivity and specificity ranging between 95% and 100% (Bergmann *et al*., 1998; Ruiz *et al*., 2000; LaBombardi *et al*., 2002).

***(v) Microscopic observation drug susceptibility testing for M. tuberculosis*:** The microscopic observation drug susceptibility (MODS) assay is based on the observation of the characteristic cord-formation of *M. tuberculosis* in liquid culture (Middlebrook 7H9) medium using an inverted light microscope (Caviedes *et al*., 2000; Moore *et al*., 2004; Moore *et al*., 2006). The MODS assay can be performed from culture or directly from decontaminated sputum specimens (Palomino *et al*., 2008; Richter *et al*., 2009). The MODS assay has been evaluated for RIF and INH, showing excellent sensitivity (> 95%) when compared to the proportion method using LJ medium and the BacT/Alert 3D system (Moor *et al*., 2006; Shiferaw *et al*., 2007). The MODS assay is cheap and can be used as an alternative method for routine TB testing in high-burden, low-resource settings (Palomino *et al*., 2008; Richter *et al*., 2009). However, it is laborious and time-consuming as reading of the plates has to be performed daily (Palomino *et al*., 2008; Richter *et al*., 2009). Furthermore, cord-formation can sometimes be seen in nontuberculous mycobacteria species (eg *M. kansasii*), making definitive identification of *M. tuberculosis* difficult (Richter *et al*., 2009).

***(vi) Colorimetric methods for susceptibility testing of M. tuberculosis*:** Colorimetric methods are based on the colour change by an indicator dye, induced of mycobacterial growth (Martin *et al*., 2007; Palomino *et al*., 2007). Colorimetric methods have been evaluated for the detection of RIF and INH using different indicators, such as tetrazolium salts: XTT [2, 3 b

is (2 methoxy 4 nitro 5 sulfophenyl) 2H tetrazolium 5 carboxanilide] and MTT [3 (4, 5 dimet hylthiazol 2 yl) 2,5 diphenyltetrazolium bromide] (Mshana *et al*., 1998; Foongladda *et al*., 2002) and the redox indicators, Alamar blue (Yajko *et al*., 1995; Franzblau *et al*., 1998) and resazurin (Palomino *et al*., 2002; Banfi *et al*., 2003). These colorimetric methods gave comparable results with the agar proportion method (LJ) (Yajko *et al*., 1995; Franzblau *et al*., 1998; Mshana *et al*., 1998; Foongladda *et al*., 2002; Palomino *et al*., 2002; Banfi *et al*., 2003). The redox indicator resazurin has been used for DST of PZA and second-line anti-TB drugs (Martin *et al*., 2003; Martin *et al*., 2006).

2.10.2 Molecular detection of drug-resistance in *M. tuberculosis*

Phenotypic methods for the detection of resistance in *M. tuberculosis* based on conventional solid media take several weeks to give results (Palomino *et al*., 2008). The introduction of liquid culture-based techniques was an improvement for diagnosis, shortening the time of detection (Palomino *et al*., 2008; Richter *et al*., 2009; Parsons *et al*., 2011). However, the time to obtain susceptibility testing results is still seven to ten days, beginning from the time that a positive culture is obtained (Palomino, 2005; Guillerm *et al*., 2006; Richter *et al*., 2009). Molecular methods can be used as faster alternatives for rapid DST of *M. tuberculosis* (Richter *et al*., 2009; Parsons *et al*., 2011). These methods are based on the identification of specific mutations in mycobacterial genes that confer resistance to anti-TB drugs (Richter *et al*., 2009). However, molecular methods will not replace phenotypic susceptibility testing, but it can provide preliminary results faster in positive cases thus improving the turnaround time (Guillerm *et al*., 2006; Richter *et al*., 2009). Conventional testing will still be required in cases that are negative by this approach (Guillerm *et al*., 2006; Richter *et al*., 2009). A number of molecular methods have been developed for DST especially for first-line drugs, including:

(i) Sequencing: Sequencing of DNA is a widely used method and is considered as the gold standard for defining genetic resistance for DST (Garcıa de Viedma, 2003). Sequencing can detect mutations and can be predictive of drug-resistance (Garcıa de Viedma, 2003; Johnson *et al*., 2006a). Sequencing has been widely used to characterise mutations in the *rpo*B gene in RIF-resistant *M. tuberculosis* isolates and to detect mutations responsible for resistance to other first-line drugs (Palomino, 2005; Johnson *et al*., 2006a). However, DNA sequencing is expensive and challenging if multiple genes are involved in resistance or if resistance mutations are not clustered in the target gene (Palomino, 2005; Johnson *et al*., 2006). In addition it requires sequencing capacity and a sophisticated laboratory, which makes it impractical for use

in routine diagnostic laboratories, especially in developing countries (Palomino, 2005; Johnson *et al*., 2006a).

***(ii) Inno-LiPA RifTB for susceptibility testing of M. tuberculosis*:** The Inno-LiPA RifTB is a line-probe assay based on PCR amplification of the mycobacterial 16S-23S rRNA spacer region of *M. tuberculosis* isolates, 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 (De Beenhouwer *et al*., 1995; Neonakis *et al*., 2008). 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). In a meta-analysis study, the Inno-LiPA RifTB assay has shown sensitivity greater than 95% and 100% specificity for the majority of studies using culture isolates (Morgan *et al*., 2005). The Inno-LiPA RifTB assay was also evaluated for sputum specimens showing sensitivity between 80% and 100% and it specificity of 98% and 100% (Morgan *et al*., 2005; Traore *et al*., 2006; Tortoli and Marcelli, 2007).

(iii) GenoType MTBDR plus for susceptibility testing of M. tuberculosis: The GenoType MTBDR *plus* assay (Hain Lifescience, Germany) uses a conventional multiplex PCR and reverse-hybridisation to probes immobilised on a plastic strip (Hillemann *et al*., 2007; Neonakis *et al*., 2008; Richter *et al*., 2009). The GenoType MTBDR *plus* assay offers the simultaneous identification of the *M. tuberculosis* complex and detection of the most common resistance mutations in the *rpo*B gene (RIF resistance), *kat*G and *inh*A gene (INH resistance) (Hillemann *et al*., 2007; Neonakis *et al*., 2008). The assay detects mutations in the 81-bp hotspot region of *rpo*B, at codon 315 of the *kat*G gene and in the *inh*A promoter region (Hillemann *et al*., 2007; Neonakis *et al*., 2008; Richter *et al*., 2009). The old version of the GenoType MTBDR *plus* assay referred to as GenoType MTBDR assay detects mutations only in the *kat*G and *rpo*B genes (Mäkinen *et al*., 2006). The GenoType MTBDR *plus* assay has been evaluated in different settings on both clinical isolates and sputum specimens demonstrating excellent specificity and good concordance with phenotypic DST results (Hillemann *et al*., 2007; Barnard *et al*., 2008; Miotto *et al*., 2008). Based on these findings, the WHO released a policy in 2008, recommending the use of the GenoType MTBDR *plus* assay for the rapid screening of MDR-TB in low and middle income settings (WHO, 2008c).

***(iv) GenoType MTBDRsl assay for susceptibility testing of M. tuberculosis*:** The GenoType MTBDR*sl* assay (Hain Lifescience, Germany) was developed for the detection of

FLQ, CAP, AMK, KAN, VIO and EMB resistance by targeting the *gyr*A, *rrs* and *emb*B genes for the rapid identification of patients with XDR-TB (Hillemann *et al*., 2009). The assay is based on a conventional multiplex PCR, followed by reverse hybridisation and line probe technology (Hillemann *et al*., 2009; Richter *et al*., 2009; Parsons *et al*., 2011). The assay contains 22 probes, including 16 probes for gene mutation detection and six probes for the control of the test procedure. Three *gyr*A wild-type probes (WT1, WT2 and WT3), which encompass the region of the gene encoding amino acids 85 to 97 and six probes, *gyr*A MUT1 (A90V), *gyr*A MUT2 (S91P), *gyr*A MUT3A (D94A), *gyr*A MUT3B (D94N/Y), *gyr*A MUT3C (D94G) and *gyr*A MUT3D (D94H) specifically targeting the most common mutations associated with resistance are included for the detection of FLQ-resistance. To detect CAP/AMK/KAN and VIO resistance, two wild-type probes covering the region of the *rrs* gene and two mutation probes *rrs* MUT1 (A1401G) and MUT2 (G1484T) are included. The *emb*B wild-type probe WT1, which targets the *emb*B 306 codon was included for the detection of EMB resistance, while the *emb*B MUT1A and *emb*B MUT1B probes were designed to bind to the following mutations, ATG/ATA (M306I) and ATG/GTG (M306V) (Hillemann *et al*., 2009; Richter *et al*., 2009).

The GenoType MTBDR*sl* assay has been evaluated in Germany against the proportion method using LJ in Germany, analysing 106 culture isolates and 64 sputum specimens (Hillemann *et al*., 2009; Richter *et al*., 2009). High specificity values were obtained; 100% for OFX, AMK and EMB and 99% for CAP (Hillemann *et al*., 2009). The sensitivity; however, varied between 90.2% (OFX), 86% (CAP) and 83.3% (AMK) to 59% (EMB) (Hillemann *et al*., 2009; Richter *et al*., 2009). A similar study was done in Vietnam using 64 culture isolates (Kiet *et al*., 2010). The sensitivity was 75% for FLQ, 100% for KAN and 64% for EMB. The specificity was 100% for all the three drugs (Kiet *et al*., 2010). The GenoType MTBDR*sl* assay was also evaluated against DNA sequencing using 53 isolates (Brossier *et al*., 2010). The sensitivity and specificity of the MTBDR*sl* assay were as follows: 87% and 96%, respectively, for FLQ; 100% for both for AMK; 77% and 100%, respectively, for KAN, 80% and 98%, respectively, for CAP and 57% and 92%, respectively, for EMB (Brossier *et al*., 2010).

(v) Real-time PCR techniques for susceptibility testing of M. tuberculosis: Real-time PCR technology is based on the amplification of target genes and hybridisation with fluorescent-labeled probes to specific regions, while the process is monitored as it takes place using

various real-time PCR platforms (Shamputa *et al*., 2004). Real-time PCR assays have been evaluated for the detection of drug-resistance especially for RIF and INH using different probe chemistries, such as TaqMan probes (Espasa *et al*., 2005), fluorescence resonance energy transfer (FRET) probes (Saribas *et al*., 2005), molecular beacons (Varma-Basil *et al*., 2004) and biprobes (Edwards *et al*., 2001). Cepheid Inc (Sunnyvale, CA) in collaboration with FIND developed the single-tube, molecular beacon-based, real-time PCR Xpert MTB assay for the detection of RIF-resistance in *M. tuberculosis* isolates. The assay does not require sputum processing and can be used on chemically inactivated specimens directly (Bonnet *et al*., 2007; Helb *et al*., 2010; Parsons *et al*., 2011). The Xpert MTB assay is simple, rapid (can be performed within 2 h) and does not require special expertise and biosafety requirements (Bonnet *et al*., 2007; Helb *et al*., 2010; Parsons *et al*., 2011). The Xpert MTB assay has been evaluated in several studies (Bonnet *et al*., 2007; Helb *et al*., 2010) and showed a sensitivity ranging between 92% to 100% for smear positive specimens and 71% to 85% for smear negative specimens. The overall specificity of the Xpert MTB assay ranged between 99% and 100% (Bonnet *et al*., 2007; Helb *et al*., 2010). The main advantage of real-time PCR based assays are that results are available between one and a half to two hours as well as the lower risk of contamination, since the reaction and detection both occur in a single tube (Palomino, 2009).

(vi) Polymerase chain reaction-single strand conformation **polymorphism:** Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) has been used for the rapid detection of mutations in genes associated with resistance to first-line anti-TB drugs (Johnson *et al*., 2006a; Neonakis *et al*., 2008). The method is based on the detection of the altered mobility of a single strand of DNA in non-denaturing polyacrylamide gels (Johnson *et al*., 2006a; Neonakis *et al*., 2008). Changes in DNA mobility is caused by conformational changes that result from mutations in nucleotide sequences (Johnson *et al*., 2006a; Neonakis *et al*., 2008). The PCR-SSCP assay has frequently been used for the detection of mutations in the *rpo*B gene but rarely for genes associated with resistance to INH (Johnson *et al*., 2006). The PCR-SSCP is expensive, technically demanding and not sufficiently sensitive compared to DNA sequencing (Johnson *et al*., 2006a; Neonakis *et al*., 2008).

(vii) Microarray techniques for susceptibility testing of M. tuberculosis**:** Microarrays are based on the hybridisation of nucleic acids to miniature glass microchips containing specific oligonucleotide probes (Neonakis *et al*., 2008). Several microarray based assays for the

detection of resistance are commercially available, such as the (i) Combichip Mycobacteria chip (DNA Microarray) (Pusan, South Korea), which detects specific mutations in the *rpo*B, *kat*G and *inh*A genes (Kim *et al*., 2006; Park *et al*., 2006), (ii) the TB-Biochip oligonucleotide microarray system (Engelhardt Institute of Molecular Biology, Moscow, Russia) designed to identify 29 codon substitutions and one codon deletion in the *rpo*B gene (Caoili *et al*., 2006) and (iii) the high-density DNA probe array for the detection of eight mutations in the *gyr*A gene (Sougakoff *et al*., 2004). The disadvantage of microarrays include that it is expensive, requires skilled personnel and specialised equipment (Richter *et al*., 2009).

2.10.3 Molecular epidemiology of *M. tuberculosis*

Molecular epidemiology is a combination of molecular biology, clinical medicine, statistics and epidemiology (Narayanan, 2004; Mathema *et al*., 2006). Molecular epidemiology of *M. tuberculosis* uses DNA fingerprinting methods to provide novel information regarding the spread of families of *M. tuberculosis* in mini-epidemics and outbreaks, to analyse the transmission dynamics of TB, to distinguish exogenous re-infection from endogenous reactivation, to identify the source of laboratory contamination and to determine the risk factors for TB transmission in a community (Narayanan, 2004; Lilleboek, 2005; Mathema *et al*., 2006).

2.10.4 Genotyping methods used for *M. tuberculosis*

The *M. tuberculosis* complex genome is highly conserved in relation to other bacterial pathogens (Mathema *et al*., 2006). However, insertion sequences (IS), repetitive elements, genomic deletions and single nucleotide polymorphisms cause genetic polymorphisms (Van Soolingen, 2001; Mathema *et al*., 2006). These polymorphisms can be visualized by various typing techniques, often referred to as DNA fingerprinting (genotyping) methods (Van Soolingen, 2001; Narayanan, 2004; Lilleboek, 2005; Mathema *et al*., 2006). Some of the most common genotyping methods currently used for the *M. tuberculosis* complex include: IS*6110*-RFLP typing, spoligotyping and MIRU-VNTR typing (Van Soolingen, 2001; Narayanan, 2004; Lilleboek, 2005; Mathema *et al*., 2006).

2.10.4.1 IS*6110*-RFLP genotyping of *M. tuberculosis*

The IS*6110*-RFLP typing is based on analysis of the distribution of the IS in different *M. tuberculosis* strains (Van Soolingen, 2001). Insertion sequences are small mobile genetic

elements, usually less than 2.5 kb in size, carrying only the genetic information related to transposition and regulation (Chandler and Mahillon, 2002). Insertion sequences are repetitive elements present at various sites and variable copy numbers in the genomic DNA of *M. tuberculosis* (Van Soolingen, 2001; Mathema *et al*., 2006). The IS*6110* is a member of the IS*3* family of transposable elements, which is specific to the *M. tuberculosis* complex (Van Soolingen, 2001). The IS*6110* is the most widely used IS for RFLP typing (Van Soolingen *et al*., 1995; Van Soolingen, 2001). The number of copies of IS*6110* differs from zero to 25 and the insertion position in the *M. tuberculosis* genome is variable between different *M. tuberculosis* strains (Van Embden *et al*., 1993; Mathema *et al*., 2006). *Mycobacterium tuberculosis* isolates from patients infected with epidemiologically unrelated strains of tuberculosis have different RFLP patterns, whereas those from patients with epidemiologically linked strains generally have identical RFLP patterns (Van Embden *et al*., 1993; Mathema *et al*., 2006).

The IS*6110*-RFLP genotyping method is considered the gold standard to which other techniques are evaluated against and has been standardised and is widely used (Van Soolingen, 2001; Mathema *et al*., 2006). Population-based molecular epidemiological studies reported that most *M. tuberculosis* strains contain between eight to 18 copies of the IS*6110* insertion element, a number that is adequate to allow discrimination between the majority of *M. tuberculosis* strains (Van Soolingen, 2001; Mathema *et al*., 2006). Strains with fewer than six IS*6110* insertion sites have a limited degree of polymorphism and supplementary methods of genotyping are used in these cases (Van Soolingen, 2001; Mathema *et al*., 2006). The disadvantages of the IS*6110*-RFLP genotyping method is that it is slow, cumbersome, labour intensive, technically demanding and requires a high concentration (ie, 2 µg) of pure DNA from each *M. tuberculosis* strains (Narayanan, 2004; Mathema *et al*., 2006).

2.10.4.2 Spoligotyping of *M. tuberculosis*

Spoligotyping (Spacer oligonucleotide typing) is one of the most widely used genotyping methods for epidemiological investigations of TB. Spoligotyping is based on hybridisation of amplified DNA to nylon membrane-immobilized oligonucleotides where the sequences are representative of the 43 spacer regions (Goyal *et al*., 1997; Kamerbeek *et al*., 1997; Mathema *et al*., 2006). Variations in the number of spacers as a result of deletions of adjacent blocks of repetitive units allow the differentiation of *M. tuberculosis* isolates (Goyal *et al*., 1997; Kamerbeek *et al*., 1997; Mathema *et al*., 2006). Spoligotyping is simple, rapid and highly

reproducible, results are expressed in a digital pattern, which can easily be interpreted and computerized and which make it possible for intra-laboratory comparisons (Brudey *et al*., 2006; Mathema *et al*., 2006).

Spoligotyping, unlike IS*6110*-RFLP genotyping, which requires approximately 2 µg of bacterial DNA, can be performed with considerably less DNA template and in a shorter period of time (Narayanan, 2004; Mathema *et al*., 2006). Spoligotyping can be performed on bacterial DNA obtained by the boiling method or on impure extracted DNA from non-viable specimens, such as from paraffin-embedded material and material from Ziehl-Neelsen stained slides (Van der Zanden *et al*., 1998; Mathema *et al*., 2006). Spoligotyping has been shown to discriminate further between IS*6110* low copy number *M. tuberculosis* strains (Bauer *et al*., 1999; Soini *et al*., 2001; Mathema *et al*., 2006). In some strains, spoligotyping can distinguish among members of the *M. tuberculosis* complex based on the species-specific presence/absence of spacers (Mathema *et al*., 2006).

Although spoligotyping is simple and highly reproducible, its discriminatory power is lower than IS*6110*-RFLP typing, except for *M. tuberculosis* strains with low copy numbers of the IS*6110* (Van Soolingen, 2001). 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 (Mathema *et al*., 2002; Mathema *et al*., 2006). *Mycobacterium tuberculosis* strains with identical spoligotype patterns but distinctly different IS*6110* fingerprint profiles are often encountered (Van Embden *et al*., 2000; Mathema *et al*., 2002). The W-Beijing family of *M. tuberculosis* strains is a large phylogenetically related group of *M. tuberculosis* strains that comprise of hundreds of similar yet distinct IS*6110* variations, all displaying an almost identical spoligotyping pattern lacking spacers one to 34 (Bifani *et al*., 2002; Kremer *et al*., 2004).

2.10.4.3 Mycobacterial interspersed repetitive unit-variable number tandem repeat typing of *M. tuberculosis*

The genome of *M. tuberculosis* contains many MIRUs, some containing identical repeat units and others containing repeats that vary slightly in sequence and length (40 bp to 100 bp) (Supply *et al*., 1997; Frothingham and Meeker-O'Connell, 1998; Mazars *et al*., 2001; Mathema *et al*., 2006). These DNA elements are often found as tandem repeats and are dispersed in the intergenic regions on the genome of the *M. tuberculosis* complex (Supply *et*

al., 1997; Frothingham and Meeker-O'Connell, 1998; Mazars *et al*., 2001). These tandem repeats are situated in different locations on the *M. tuberculosis* genome (Frothingham and Meeker-O'Connell, 1998; Mazars *et al*., 2001). Supply *et al.* (2000) identified 41 variable number tandem repeat (VNTR) of MIRUs 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).

The MIRU-VNTR genotyping method relies on PCR amplification of multiple loci (either 12, 15 or 24) using primers specific for the flanking regions of each repeat locus and on the determination of the sizes of the amplicons, which reflects the number of the targeted MIRU-VNTR copies (Supply *et al*., 2001; Mathema *et al*., 2006). The sizing of amplicons can be done using capillary (Cowan *et al*., 2002; Mathema *et al*., 2006) or gel electrophoresis or non-denaturing high-performance liquid chromatography (Supply *et al*., 2001). The results of MIRU-VNTR genotyping are expressed as numerical codes and can be catalogued on a computer database (Supply *et al*., 2001). A website (www.miru-vntrplus.org) has been established to create a worldwide database of MIRU-VNTR patterns (Supply *et al*., 2001; Mathema *et al*., 2006).

The discriminatory power of the MIRU-VNTR assay is typically proportional to the number of loci evaluated (Mathema *et al*., 2006). The MIRU-VNTR genotyping based on a 12-loci set is adequate for large-scale prospective studies but have lower discriminatory power as compared to IS*6110*-RFLP (Cowan *et al*., 2005; Kam *et al*., 2005; Scott *et al*., 2005; Van Deutekom *et al*., 2005; García de Viedma *et al*., 2006). The expanded set of 24 MIRU loci, with a highly discriminatory set of 15 loci have a higher discriminatory power than the 12 loci method (Supply *et al*., 2006). These 15 loci include six of the previous 12 loci with nine additional loci (Supply *et al*., 2006). The 15-loci and 24-loci set (MIRU-15 and MIRU-24) MIRU-VNTR analyses have been considered an alternative to IS*6110*-RFLP typing (Oelemann *et al*., 2007; Alonso-Rodríguez *et al*., 2008; Allix-Béguec *et al*., 2008; Valcheva *et al*., 2008). Nevertheless, contradictory data showing poor results have emerged from analysis of homogeneous lineages, such as the Beijing family (Blackwood *et al*., 2004; Scott *et al*., 2005). Inclusion of other hypervariable loci in standardised panels has been recommended in order to increase the quality of genotyping data (Velji *et al*., 2009). If MIRU-VNTR typing is combined with spoligotyping, the discriminatory power approximates

that of IS*6110*-RFLP analysis (Frothingham and Meeker-O'Connell, 1998; Supply *et al*., 2001; Cowan *et al*., 2002). The combination of the three methods, MIRU-VNTR, IS*6110* RFLP and spoligotyping, has shown maximum specificity for genotyping of *M. tuberculosis* strains (Cowan *et al*., 2002).

2.10.5 Strain families of *M. tuberculosis*

Mycobacterium tuberculosis strains are genetically diverse, which results in significant phenotypic differences between clinical isolates. *Mycobacterium tuberculosis* exhibits a biogeographical population structure and different strain lineages are associated with different geographical regions (Gagneux, 2009). Strain variation could affect the relative fitness and transmission dynamics of drug-resistant *M. tuberculosis* strains and this variation has implications for the development of new diagnostics and vaccines (Gagneux, 2009).

Mycobacterium tuberculosis species have been subdivided into strain families, also called clades or lineages, corresponding to specific genotypes or cluster or groups of genotypes (Sylvain *et al*., 2007). In the past, it was an assumed that *M. tuberculosis* has a clonal population structure and that TB is caused by a single strain of *M. tuberculosis* (Sylvain *et al*., 2007). However, with the introduction of genotyping methods in the 1990s this view has changed. To date, it is well known that TB is caused by different *M. tuberculosis* strains with varying degrees of virulence (Van Crevel *et al*., 2001; Barnes and Cave, 2003; Malik and Godfrey-Faussett, 2005; Gagneux and Small, 2007). Generally, a strain family can be described as a group of isolates that share specific biomarkers or properties indicative of a common recent ancestor (Mathema *et al*., 2006). The *M. tuberculosis* genome changes over time and give rise to strain variants that can be detected on the basis of subtle genetic changes (Bifani *et al*., 2002).

The most commonly used classification of *M. tuberculosis* strains is based on spoligotyping (Kremer *et al*., 2005; Mathema *et al*., 2006). A number of strain families having specific spoligotype patterns have been described in the international spoligotyping databases (Kremer *et al*., 2005; Mathema *et al*., 2006). Groups of related spoligotypes were interchangeably called (sub) families, (sub) clades, lineages and classes (Filliol *et al*., 2002; Filliol *et al*., 2003). Some of the most common strain families described in SpolDB4 are shown in Table 2.3.

Table 2.3: Common strain families of *M. tuberculosis* from the SpolDB4 database (Brudey *et al*., 2006)

ST No	Lineage/Sublineage	ST No	Lineage/Sublineage
	LAM		**T**
20	LAM 01	53	T1
17	LAM 02	280	T1-Russia/2 (T1-RUS2)
33	LAM 03	52	T2
60	LAM 04	135	T2-Uganda
93	LAM 05	37	T3
64	LAM 06	149	T3-ETH
41	LAM 07 -Turkey (LAM07-TUR)	40	T4
290	LAM 08	39	T4-Central Europe/1
42	LAM 09	44	T5
61	LAM 10	58	T5-Madrid/2
59	LAM 11	254	T5-Russia/1
209	LAM 12	1737	Tuscany
		627	T3-Osaka
	EAI		**X**
236	EAI5	119	X1
48	EAI1-SOM	137	X2
19	EAI2-Manilia	38	X2-variant
89	EAI2-Nonthabun	92	X3
11	EAI3-IND	91	X2-variant 1
139	EAI3-VNM	197	X2-variant 2
591	EAI6-BGD1		
1898	EAI7-BGD2		
109	EAI8-MDG		
	H		**CAS**
47	H1	26	CAS1-Deli type (CAS1-Deli)
62	H1-variant	21	CAS1-Kilimanjaro (CAS1-Kili)
2	H2	25	CAS1-variant
50	H3	288	CAS 2
127	H4		
	Manu		
100	Manu1		
54	Manu2		
1378	Manu3		

ST=Shared-type, EAI=East African Indian, LAM=Latin American and Mediterranean, H=Haarlem

The major strain families within the *M. tuberculosis* strains adapted to humans include East African Indian (EAI), Beijing, Latin American and Mediterranean (LAM), Haarlem (H), CAS, X, T and several minor groupings (Bifani *et al*., 2002; Filliol *et al*., 2003; Filliol *et al*., 2006; Brudey *et al*., 2006). These families have been further divided into subfamilies depending on where these families were first reported or based on predominance of these families in that particular location (Table 2.3).

2.10.5.1 The East African Indian Family

The EAI is the most ancient family, mainly prevalent in South-East Asia, India and East Africa (Kremer *et al*., 1999). This family is known to have a spoligotyping pattern with the absence of spacers 29 to 32, presence of spacer 33 and absence of spacer 34 (Rastogi and Sola, 2007). The EAI is characterised by a low number of IS*6110* copies (Rastogi and Sola, 2007). East African Indian family strains with only a single copy of IS*6110* were reported

from Malaysia, Tanzania and Oman (Fomukong *et al*., 1994; Rastogi and Sola, 2007). It harbours a specific region of difference, RD239 and was renamed as Indo-Oceanic in the work of Gagneux *et al.* (2006). It is speculated that this family may have originated in Asia, where TB could have historically found favourable spreading conditions (Brudey *et al*., 2006). The ancestral EAI is made up of at least six main subfamilies, EAI 1 to EAI 6, based on the location these subfamilies were first reported (Brudey *et al*., 2006) (Table 2.3).

2.10.5.2 The Beijing family

The Beijing family was first described in 1995 by Van Soolingen *et al.* (1995) when 86% of isolates from Beijing, China, were identified as a single spoligotype. However, the W-strain, which represents the same family, was concurrently identified in New York in the 1990s (Kremer *et al*., 1999; Bifani *et al*., 2002). The Beijing family is identified by having a specific spoligotype signature, absence of spacers 1 to 33 and presence of spacer 34 to 43 (Rastogi and Sola, 2007). These strains are characterised by the presence of an inverted IS*6110* copy within the DR region, an IS*6110* element at a particular insertion site (within the origin of replication) and one or two IS*6110* copies in a DNA region called NTF (Plikaytis *et al*., 1994, Kurepina *et al*., 1998; Rastogi and Sola, 2007).

The Beijing genotype family can be divided into seven lineages, including "ancestral/atypical" (sublineage 1) to "modern/typical" (sublineage 7) (Hanekom *et al*., 2007). These classifications are based on the comparison of various markers including IS*6110* insertion sites in the NTF region, regions of deletion and other markers (Hanekom *et al*., 2007; Reed *et al*., 2007; Rindi *et al*., 2009). The term typical/modern refers to strains containing one or several IS*6110* insertions in the NTF region (Kremer *et al*., 2004; Mokrousov *et al*., 2006; Mokrousov, 2008), while atypical/ancestral refers to strains with absence of IS*6110* insertions in the same region (Kremer *et al*., 2004; Mokrousov *et al*., 2006; Mokrousov *et al*., 2008).

Typical and atypical strains of the Beijing strains have been described in the Netherlands, Vietnam and Hong Kong (Kremer *et al*., 2004). Some typical Beijing strains have been associated with large outbreaks in many countries, such as the 'W' strain that caused an outbreak of MDR-TB in New York in the early 1990s (Bifani *et al*., 1996; Bifani *et al*., 1999), large clusters of MDR-TB in the Baltic States and other former Soviet Union countries (Devaux *et al*., 2009). The typical Beijing strains are more prevalent in Asia and Eastern

Europe and are also found in parts of Western Europe, South Africa and South American countries (Anh *et al*., 2000; Bifani *et al*., 2002; Glynn, 2006; Hanekom *et al*., 2007; Buu *et al*., 2009). The typical Beijing strains have often been associated with resistance to anti-TB drugs (Glynn, 2006; Mokrousov *et al*., 2006, Sun *et al*., 2007; Victor *et al*., 2007).

The Beijing genotype is one of the most successful families, associated most often with major TB outbreaks globally (Bifani *et al*., 2002; Glynn *et al*., 2002; Brudey *et al*., 2006). It is reported that one-third of global TB is caused by the Beijing family strains (Bifani *et al*., 2002; Glynn *et al*., 2002; Brudey *et al*., 2006). The prevalence of the Beijing family is remarkably high in the Asian continent which constitutes about 50% of strains and 13% of isolates globally (Brudey *et al*., 2006). The Beijing genotype is less common in Africa with the exception of the Western Cape province in South Africa (Brudey *et al*., 2006). A high and increasing incidence of the Beijing lineage has been described in this region (Jonson *et al*., 2010). One particular sub-group of the Beijing family, cluster R220 (based on IS*6110*-RFLP banding patterns); have been reported to be highly prevalent among drug-resistant *M. tuberculosis* strains in the Western Cape region of South Africa (Johnson *et al*., 2010). Cluster R220 is a member of sublineage 6, representing a typical/modern Beijing genotype (Johnson *et al*., 2010). Cluster R220 constituted over 75% of isolates of the Beijing family in 2005 to 2006 in the Western Cape and accounted for 42% of the increase in drug-resistant cases since 2001 (Johnson *et al*., 2010). However, the atypical Beijing strain, known as cluster 86, is the predominant drug-resistant *M. tuberculosis* strain in the Eastern Cape province, accounting for most of the transmission of MDR-TB in this region (Bifani *et al*., 2002; Glynn *et al*., 2002; Strauss *et al*., 2008).

The Beijing genotype may have a selective advantage and the ability to spread more efficiently compared to other genotypes (Van Soolingen *et al*., 1995; Caminero *et al*., 2001; Van Soolingen, 2001). Although the reasons for selection and wide dissemination of Beijing family strains is unknown, it has been hypothesised that the Beijing genotype has evolved unique properties, including the ability to evade the protective effect of *Mycobacterium bovis* BCG vaccination (Van Soolingen *et al*., 1995; Caminero *et al*., 2001; Van Soolingen, 2001; Bifani *et al*., 2002). Beijing genotypes are more common in areas where BCG vaccination coverage is extensive (Behr, 2002). Most countries in Southeast Asia have used BCG vaccination for the past two to six decades (Behr, 2002). It has been suggested that BCG

vaccination may have favoured the selection of *M. tuberculosis* strains that resist BCG-induced immunity (Behr, 2002).

Many reports from Germany, Italy, Russia, Estonia, South Africa and Columbia documented the association of the Beijing genotype with MDR-TB (Van Soolingen *et al*., 1995; Caminero *et al*., 2001; Drobniewski *et al*., 2002; Filliol *et al*., 2002; Glynn *et al*., 2002; Toungoussova *et al*., 2002; Filliol *et al*., 2003; Lari *et al*., 2004). The association of the Beijing genotype with XDR-TB was also reported from different parts of the world like Vietnam, Taiwan, China and Russia (Glynn *et al*., 2002; Mardassi *et al*., 2005; Mokrousov, 2008; Baranov *et al*., 2009; Buu *et al*., 2009; Chang *et al*., 2011). However, the association between Beijing strains and drug-resistance varies worldwide (Van Soolingen *et al*., 1995; Anh *et al*., 2000; Tsolaki *et al*., 2005). In addition the association of the Beijing genotype with young patients was reported in two studies from Vietnam (Anh *et al*., 2000; Buu *et al*., 2009).

2.10.5.3 The CAS or Delhi family

The CAS family has a specific spoligotype signature, the absence of spacers four to 27 and 23 to 34 (Rastogi and Sola, 2007). This family shows a characteristic band pair in the high molecular weight region (12.1 and 10.1 kilo bp) (Rastogi and Sola, 2007). The CAS family family has been subdivided into four subfamilies (Table 2.3), which have been defined on the basis of spoligotype signatures (McHugh *et al*., 2005; Eldholm *et al*., 2006). Strains of the CAS family are mainly found in the Indian subcontinent as well as in other regions, such as the Middle-East and Central Asia (Sola *et al*., 2001; Singh *et al*., 2004; Gutierrez *et al*., 2006; Singh *et al*., 2007). The CAS family is more prevalent in North India as compared to South India (Suresh *et al*., 2006). This family was also shown to be endemic in Sudan, other sub-Saharan countries and Pakistan (Brudey *et al*., 2006). The CAS family could be the ancestor of the Beijing family, since it clusters close to the Beijing family when analysed by a combination of MIRU, spoligotyping and VNTR typing methods (Sola *et al*., 2003).

2.10.5.4 The Haarlem family

The H family was described in the Netherlands in 1999 (Kremer *et al*., 1999). The H family is characterised by the absence of spacer 31 in its spoligotyping pattern (Groenen *et al*., 1993). On IS*6110*-RFLP typing, these strains have a double band at 1.4 kb (Rastogi and Sola, 2007). Three main spoligotyping patterns are found within the H family (H1 to H3) (Kremer

et al., 1999) (Table 2.3). The H family is highly prevalent in Northern Europe and represents about 25% of the isolates (Brudey *et al*., 2006). Outside Europe, the H strains were mainly found in Central America and the Caribbean and to a lesser extent in Central Africa, where it is believed to have been introduced during the European colonisation (Filliol *et al*., 2003). Studies from Vietnam, Taiwan, China and Russia reported the association of the H family with first-line drug-resistance and XDR-TB (Glynn *et al*., 2002; Mardassi *et al*., 2005; Mokrousov, 2008; Baranov *et al*., 2009; Buu *et al*., 2009; Chang *et al*., 2011).

2.10.5.5 The LAM family

The LAM family was defined by the absence of spacers 21 to 24 in the spoligotyping pattern (Rastogi and Sola, 2007). Based on the SpolDB4 international spoligotype database the LAM family is subdivided into LAM1 to LAM12 (Table 2.3) (Brudey *et al*., 2006). The LAM family is more frequent in Mediterranean countries and in Latin America (Brudey *et al*., 2006). In Latin America, about 50% of the strains belong to the LAM family (Brudey *et al*., 2006). The LAM3/F11 family belonging to the LAM family is highly prevalent in the Western Cape province (Streicher *et al*., 2004; Johnson *et al*., 2010). In contrast, the F15/LAM4/KZN strain is the main MDR and XDR-TB strain in the KwaZulu-Natal province (Pillay and Sturm, 2007; Streicher *et al*., 2011).

2.10.5.6 The X family

The X family is among the well-defined genotypes (Brudey *et al*., 2006). It is characterised by the absence of spacer 18 in spoligotyping and a low number of IS*6110* copies (Sebban, 2002). Some X genotypes with no IS*6110* elements have been reported in Vietnam (Soini *et al*, 2001; Brudy *et al*., 2006). The distribution of the X family appears to be linked to the Anglo-Saxon countries (Dale *et al*., 2003). It is highly prevalent in North and Central America, where it represents 21.5% and 11.9%, respectively (Brudey *et al*., 2006). The X family is also prevalent in the United Kingdom, Australia, the Caribbean and South Africa (Sebban *et al*., 2002; Sola *et al*., 2003; Brudey *et al*., 2006). Specific epidemic variants of the X genotype family were described in South Africa (Streicher *et al*., 2004).

2.10.5.7 The T families

The 'ill-defined' T family is characterised "by default". It includes strains without spacers 33 to 36 and cannot be classified in any other family (Rastogi and Sola, 2007). The T genotype

includes high as well as low banding patterns of the IS*6110*-RFLP (Rastogi and Sola, 2007). The T family is subdivided into T1 to T4 (Brudey *et al*., 2006). The names of some of these genotypes reflect areas where these genotypes have been found (Table 2.3). The T family is a common genotype found in all the different regions of the world and account for about one third of strains in the SpolDB4 database (Brudey *et al*., 2006).

2.11 Summary

The increase in MDR-TB and the emergence of XDR-TB present a serious problem for the TB control programme, stressing the need for rapid DST to first- and second-line anti-TB drugs. The available conventional methods for detecting drug-resistance in *M. tuberculosis* are slow and cumbersome. A number of rapid liquid-based methods have been developed. These methods allow for faster detection of drug-resistance as compared to solid media, the time to obtain susceptibility testing results with these methods is still seven to ten days, beginning from the time that a positive culture is obtained (Palomino *et al*., 2008; Richter *et al*., 2009). Further development of rapid techniques is needed for timely diagnosis of MDR and XDR-TB. Several rapid molecular tests have been proposed for the rapid DST of *M. tuberculosis* (Richter *et al*., 2009; Parsons *et al*., 2011). However, use of genotypic methods requires a more detailed understanding of the mutations that lead to drug-resistance and their relative frequency (Johnson *et al*., 2006a; Palomino, 2009, Richter *et al*., 2009; Parsons *et al*., 2011). Second-line DST is complex, expensive and less standardised as compared to the DST methods for first-line anti-TB drugs (Shah *et al*., 2007; Richter *et al*., 2009).

In addition to the improvement of TB diagnostics and treatment, a better understanding of the molecular epidemiology of TB is a pre-requisite to control TB epidemics (Mathema *et al*., 2006; Van Soolingen *et al*., 2007). Developments in molecular biology have resulted in techniques that allow prompt identification and tracking specific strains of *M. tuberculosis* as they spread through the population. Some of the most common genotyping methods currently used for *M. tuberculosis* complex include: IS*6110*-RFLP typing, spoligotyping and MIRU-VNTR typing (Narayanan, 2004; Lilleboek, 2005; Mathema *et al*., 2006; Van Soolingen *et al*., 2007). The IS*6110*-RFLP typing is considered the gold standard for *M. tuberculosis* genotyping due to its high discriminatory power (Van Embden *et al*., 1993; Mathema *et al*., 2006).

Despite the substantial advances in the development of rapid liquid-culture and molecular methods for susceptibility testing; significant challenges persist (Palomino; 2009). The development of enhanced and cost-effective methods for rapid and reliable diagnosis of drug-resistance is critical determinants for establishing effective control of MDR and XDR-TB. It is known that the intrinsic accuracy of susceptibility testing results varies with the drug tested. Drug susceptibility testing for first-line anti-TB drugs is most accurate for RIF and INH and less reliable and reproducible for STR, EMB and PZA (Laszlo *et al*., 1997; Parsons *et al*., 2004). The determination of DST patterns to second-line anti-TB drugs remains a substantial challenge (Palomino *et al*., 2008; Palomino, 2009; Parsons *et al*., 2011). Currently the genetic basis of resistance is not known for many anti-TB drugs, especially second-line anti-TB drugs (Johnson *et al*., 2006a; Palomino, 2009; Parsons *et al*., 2011). Therefore, studies are needed to standardise methods and drug concentrations for STR, EMB, PZA as well as second-line drugs. Furthermore, the distribution and prevalence of different drug-resistant *M. tuberculosis* strains is important for the proper management of tuberculosis. The frequency and distribution of *M. tuberculosis* genotypes and specifically MDR and XDR-TB genotypes across the different provinces of South Africa are limited. It has been reported that the population structure of MDR-TB in South Africa differs between provinces with certain lineages being overrepresented in certain regions (Stretcher *et al*., 2011).

The present study was done to address some of the challenges mentioned above. In the first theme of the study, (i) a routine phenotypic susceptibility method (BACTEC MGIT 960 system) was evaluated for EMB, STR, OFX and CAP. In the second theme of the study, (ii) a molecular assay (GenoType® MTBDR*sl* assay) was evaluated for the detection of resistance against second-line anti-TB drugs. In the third theme of the study, drug-resistant *M. tuberculosis* isolates from the northern region of South Africa were characterised using spoligotyping and MIRU-VNTR typing, in order to determine (iii) the population structure of drug-resistant *M. tuberculosis* in this region and (iv) diversity and transmission patterns of drug-resistant *M. tuberculosis* in this region as well as to determine the possible association of genotypes (clusters), with specific drug-resistance patterns and demographic characteristics (age and gender).

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CHAPTER 3

COMPARISON BETWEEN THE BACTEC MGIT 960 SYSTEM AND THE AGAR PROPORTION METHOD FOR SUSCEPTIBILITY TESTING OF MULTIDRUG RESISTANT TUBERCULOSIS STRAINS IN A HIGH BURDEN SETTING OF SOUTH AFRICA

Editorial style of the Biomedical Journal of Infectious Diseases was followed in this chapter

Abstract

Background: The increasing problem of multi-drug-resistant (MDR) tuberculosis (TB) is becoming a global problem. Successful treatment outcome for MDR-TB depends on reliable drug susceptibility testing of first-line and second line-anti-TB drugs.

Method: Drug susceptibility tesing (DST) for ethambutol (EMB), streptomycin (STR), kanamycin (KAN) and ofloxacin (OFX) was done on consecutive *M. tuberculosis* isolates identified as MDR-TB during August 2007 to January 2008 at a routine diagnostic laboratory. The results of the routine DST using the BACTEC MGIT 960 were compared to gold standard agar proportion method.

Result: The agreement between the BACTEC MGIT 960 system and the agar proportion method was 44% for EMB, 61% for STR and 89% for both KAN and OFX. The sensitivity and specificity of the BACTEC MGIT 960 system using the agar proportion method as a gold standard was 19% and 97% for EMB, 95% and 37% for STR, 55% and 92% for KAN and 100% and 89% for OFX, respectively.

Conclusions: The BACTEC MGIT 960 system showed excellent sensitivity for OFX and STR, while the sensitivity of the BACTEC MGIT 960 system for the detection of resistance to EMB and KAN was considerably lower. These findings showed the unreliability of culture based methods for EMB testing, highlighting the need for new, accurate and reliable methods. The critical concentrations for KAN need to be critically re-examined.

Key words: BACTEC MGIT 960, MDR-TB, TB, XDR-TB

3.1 Background

Drug-resistance remains a serious threat to the tuberculosis (TB) control programmes worldwide. South Africa is one of the high-burden multi-drug-resistant (MDR) TB [ie resistant to at least isoniazid (INH) and rifampicin (RIF)] countries in the world [1]. The emergence of extensively drug-resistant (XDR) TB [ie MDR-TB with additional resistance to any fluoroquinolone (FLQ) and to at least one of the three injectable second-line drugs, amikacin (AMK), kanamycin (KAN) and/or capreomycin (CAP)] is worsening the drug-resistance problem [1;2]. The World Health Organization (WHO) estimates that 10.5% of MDR-TB cases in South Africa are XDR-TB [1].

Standardised, rapid and accurate drug susceptibility testing (DST) methods for first-line and second-line anti-TB drugs is important to determine an effective treatment regimen and to decrease transmission. Various susceptibility testing methods are currently available especially for first-line anti-TB drugs [3;4]. However, the accuracy of these methods reported to vary according to the anti-TB drug being tested [5]. Drug susceptibility testing for RIF and INH is most accurate, but less reliable and reproducible for streptomycin (STR), ethambutol (EMB) and pyrazinamide (PZA) [4;5;6]. Inconsistent results are a common occurrence [7] and resistance is often underreported, this being especially true for those drugs for which the methods have not yet been standardised [8]. This is particularly true in the case of EMB resistance as the diagnostic breakpoint (5 to 7.5 µg/ml) is close to the Minimum Inhibitory Concentration (MIC) of EMB and true resistance may therefore be missed [7].

Second-line DST is less standardised and not as simple as DST for first-line anti-TB drugs [5]. This is mainly due to *in-vitro* drug stability, varying drug potency and the critical concentration defining resistance is often close to the MIC [4;5]. These factors increase the probability for misclassification of susceptibility or resistance and leading to poor reproducibility of DST results [4;5].

Although for several years the recommended methods for DST were conventional methods on solid media, these methods are slow and take a minimum of three to eight weeks before results are available [8,9]. Susceptibility results need to be reported within four weeks of specimen receipt [10]. The WHO recommends the use of liquid media for culture and DST in middle- and low-income countries to improve diagnosis of MDR- and XDR-TB [11,12]. The BACTEC MGIT 960 system (Becton Dickinson Microbiology System, Sparks, NV, USA) is

an automated, continuously monitored system, based on the detection of bacterial growth in drug-containing media which is compared with a drug-free control tube [13]. Drug susceptibility kits are available for the testing of INH, RIF, EMB, STR and PZA [13]. The BACTEC MGIT 960 system has been evaluated for the detection of resistance to first-line anti-TB drugs against the standard proportion method and the BACTEC 460 TB system and has shown a sensitivity of 100% for RIF and INH and a specificity ranging from 89% to 100% [14;15;16;17].

The BACTEC MGIT 960 system has been evaluated for DST of second-line anti-TB drugs and critical concentrations for second-line drugs has been established [18;19;20;21]. The BACTEC MGIT 960 system has shown good concordance with the standard proportion method [15;16;17;18]. Currently, there is no commercially available kit for performing DST of second-line drugs using the BACTEC MGIT 960 system [20;21]. This is inconvenient because the working solutions of each drug should be prepared by the users, making these tests more error-prone due to procedural inaccuracies. Testing for second-line anti-TB drugs should be precise and quality controlled using the reference *M. tuberculosis* H37RV strain and resistant *M. tuberculosis* strains.

The aim of the study was to determine the performance BACTEC MGIT 960 system for the routine susceptibility testing of EMB, STR, KAN and OFX at a high-throughput diagnostic laboratory in a high-burden setting. The susceptibility results using the BACTEC MGIT 960 system was compared against the agar proportion method.

3.2 Results

Routine DST results for STR and EMB using the BACTEC MGIT 960 system were available for all the MDR-TB isolates, however; only 205 MDR-TB isolates had results for second-line anti-TB drugs (OFX and KAN). The results of the routine DST using the BACTEC MGIT 960 system were compared to the results obtained by the agar proportion method. The results are summarized in Table 3.1. The mean time to obtain susceptibility testing results with the BACTEC MGIT 960 system was 6 days, with a range of 5 to 14 days, whereas the agar proportion method was 11 days, with a range of 6 to 21 days.

Detection of resistance by the BACTEC MGIT 960 system and the agar proportion method was as follows: 76% (262/343) and 42% (145/343) for STR, 67% (229/343) and 14%

(47/343) for EMB, 2% (5/205) and 13% (27/205) for OFX and 5% (11/205) and 11% (22/205) for KAN, respectively. A total of 3% (6/205) MDR-TB isolates met the criteria for classification as XDR-TB by the BACTEC MGIT 960 system and 7% (15/205) according to the agar proportion method.

The agreement between the BACTEC MGIT 960 system and the agar proportion method was 61% for STR and 44% for EMB and 89% for OFX as well as for KAN (Table 3.1). The κ values are shown in Table 3.2. The sensitivity and specificity of the BACTEC MGIT 960 system was 95% and 37% for STR, 19% and 97% for EMB, 100% and 89% for OFX, 55% and 92% for KAN, respectively (Table 3.2).

3.3 Discussion

The study describes the performance of BACTEC MGIT 960 system for testing of MDR-TB isolates against EMB, STR, KAN and OFX in a routine diagnostic laboratory. According to WHO recommendations, additional testing for the remaining first-line anti-TB drugs and second-line anti-TB drugs should be done once MDR-TB has been confirmed [22]. Reliable and accurate susceptibility testing of first and second-line anti-TB drugs is essential in order to determine an effective treatment regimen for MDR-TB and to avoid further development of resistance [1;23]. The accuracy of susceptibility testing results varies with the drug tested as well as with the method of drug susceptibility testing used. In this study the performance of the BACTEC MGIT 960 system for routine DST of MDR-TB isolates was compared against the agar proportion method using Middlebrook medium.

The sensitivity of BACTEC MGIT 960 system was comparable to the agar proportion method for OFX and STR (100% and 95%; specificity, 100% and 37%). A low specificity was found for STR and requires further investigation. However, the sensitivities in detecting resistance to EMB and KAN were lower (19% and 55%, respectively), whereas the specificities (ability to find true drug susceptibility) was excellent (97%) for EMB but slightly lower (92%) for KAN. The results for OFX susceptibility were similar to those obtained previously by Devasia *et al* [24] using the BACTEC MGIT 960 system. The specificity, however, was higher (100%) as compared to this study.

The susceptibility of *M. tuberculosis* to EMB, STR and PZA is less reliable and reproducible using solid medium [25;26;27]. Ethambutol susceptibility testing has long been a challenge

for diagnostic laboratories. Discrepancies have been reported when comparing results obtained in liquid versus solid media [27;28;29;30]. In this study, 54% (186/343) of the *M. tuberculosis* isolates were resistant according to the BACTEC MGIT 960 system but susceptible with the agar proportion method. It has been reported that the agar proportion method was not easy to standardise for EMB testing [26;27;31]. Johnson *et al*. [7] reported that 90% (43/47) of EMB resistance was not detected using the agar proportion method when compared with a liquid culture medium. These results were confirmed by DNA sequencing, which identified mutations in the *emb*B gene (associated with EMB resistance), in all of the isolates [7]. These findings raise concerns regarding routine testing for EMB, since EMB is used for the treatment of MDR-TB if susceptibility is demonstrated.

In this study, the proportion of XDR-TB was high (7%) with the agar proportion method when compared to the BACTEC MGIT 960 system (3%). This indicates the poor standardisation of second-line anti-TB DST. The discrepancies between the BACTEC MGIT 960 system and the agar proportion method could be due to the presence of borderline resistant *M. tuberculosis* strains, mainly in relation to the agar proportion method where the final results depend on an accurate count of colonies [22]. The discordance between the BACTEC MGIT 960 system and the agar proportion method might possibly be overcome by adjusting the critical drug concentrations used. In addition, the use of two concentrations (low and high) for these drugs may reduce false resistance.

The agar proportion method was used as a gold standard in this study for both first and second-line anti-TB drug testing. However, the findings from this study did indicate that the agar proportion method was a more accurate method than the BACTEC MGIT 960 system. The liquid culture DST was reported to be more sensitive, reliable and reproducible method than solid culture DST. Promising results were reported with an automated liquid culture-based system for second-line anti-TB testing, especially for aminoglycosides, polypeptides and fluoroquinolones [21;32]. The automated liquid culture system (BACTEC MGIT 960 system) is currently recommended by the WHO as the gold standard for second-line DST [30]. In case of first-line anti-TB drugs, the WHO recommends the use of both the BACTEC MGIT 960 system and the line-probe assay for testing [33]. However, confirmation of MDR-TB by conventional DST is still regarded as the gold standard for first-line anti-TB drugs [33]. The limitation of the study was that the discrepant results between the BACTEC MGIT 960 system and the agar proportion were not tested using DNA sequencing to resolve the discrepancies.

In conclusion, the BACTEC MGIT 960 system provided excellent sensitivity for rapid detection of resistance to OFX and STR when compared to the agar proportion method. However, the sensitivity of the BACTEC MGIT 960 system for the detection of resistance to EMB and KAN was considerably lower. The low sensitivity of BACTEC MGIT 960 system for EMB testing is similar to previous studies. This highlights the need for new, accurate and reliable methods. Further standardisation of the critical concentrations is needed for KAN, to generate reliable DST results.

3.4 Methods

3.4.1 Study design

This study was a descriptive study comparing the performance of the BACTEC MGIT 960 system with the standard agar proportion method for susceptibility of first and second-line anti-TB drugs. The diagnostic performances: such as sensitivity, specificity and predictive values were calculated using the agar proportion method as gold standard.

3.4.2 Specimens

The study was conducted using consecutive *M. tuberculosis* isolates identified as MDR-TB during August 2007 and January 2008 by routine DST using the BACTEC MGIT 960 system (Becton Dickinson, Sparks, Md, USA) at the National Health Laboratory Service (NHLS) laboratory, University of Limpopo (MEDUNSA campus). The NHLS laboratory is a high-throughput routine diagnostic microbiology laboratory that receives specimens for culture and DST from the attached Dr George Mukhari Hospital and the surrounding clinics and hospitals in Gauteng as well as the referring provinces of Limpopo, Mpumalanga and North West. The isolates were identified as *M. tuberculosis* using Ziehl–Neelsen (ZN) staining and were confirmed with the Accuprobe method (Gen-Probe, Inc, San Diego, California).

3.4.3 Drug susceptibility testing using BACTEC MGIT 960 system

Routine drug susceptibility testing for EMB, STR, KAN and OFX was done by NHLS laboratory using the BACTEC MGIT 960 system. The drug susceptibility testing results were collected from the NHLS database. The critical concentrations used are showed in Table 3.1. Isolation of the recovered mycobacterial isolates from the clinical specimens was performed using the BACTEC MGIT 960 system.

3.4.4 Drug susceptibility testing using the agar proportion method

All MDR-TB isolates were sub-cultured on Middlebrook medium prior to testing. Drug susceptibility testing of MDR-TB isolates using the agar proportion method was done for two first-line (EMB and STR) and two second-line drugs (KAN and OFX). The agar proportion method was performed on Middlebrook 7H11 medium according to the Clinical and Laboratory Standards Institute (CLSI) procedures and recommended critical concentrations (Table 1) [34]. Briefly, six-welled Petri plates of Middlebrook 7H11 medium (TB Diagnostic Services, South Africa) was used. Two quadrants in each plate contained drug-free medium, one was used as the proportional control and the other was used as a quality control. Fully susceptible *M. tuberculosis* H37Rv reference strain and a known MDR *M. tuberculosis* isolate were used as quality controls. The other four quadrants contained the drug concentrations (Table 3.3). Each quadrant was inoculated with a standard inoculum of 0.1 ml of mycobacterial suspension and the inoculum was distributed by tilting the plate. An aliquot of 0.1 ml of the 1:100 dilutions was used to inoculate the proportional control. The plates were sealed in a plastic bag and incubated at 37°C. The plates were examined 7, 10, 14 and 21 days of incubation. An isolate was classified as resistant when the colonies on the drug-containing quadrant were more than 1% compared to the colonies present on the drug-free control quadrant. An XDR-TB was defined as MDR-TB with additional resistance to KAN and OFX.

3.4.5 Analysis

The results were expressed as percentages. The agreement, sensitivity, specificity, positive and negative predictive values of the BACTEC MGIT 960 system compared to the agar proportion method, the gold standard, were calculated for EMB, STR, KAN and OFX. The agreement between the two methods was determined by the kappa (κ) statistic. The κ value, a measure of test reliability, was interpreted as follows: < 0.2, poor; 0.21 to 0.4, fair; 0.41 to 0.6, moderate; 0.61 to 0.8, good; ≥ 0.81, excellent [35].

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Table 3.1: Susceptibility testing results obtained with the BACTEC MGIT 960 system and the agar proportion method

Drug	No of isolates	Both S	Both R	MGIT 960 R, proportion S	MGIT 960 S, proportion R	Agreement (%)
STR	343	74	138	124	7	61
EMB	343	110	43	186	4	44
OFX	205	178	5	-	22	89
KAN	205	178	6	5	16	89

S=Susceptible; R=Resistant, STR=Streptomycin, EMB=Ethambutol, OFX=Ofloxacin, KAN=Kanamycin

Table 3.2: Performance of the BACTEC MGIT 960 system when compared to the agar proportion method

Drug	No of isolates	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	κ -Value
STR	343	95	37	52.7	91	0.29
EMB	343	19	97	92	37	0.11
OFX	205	100	89	19	100	0.282
KAN	205	55	92	27	97	0.314

STR=Streptomycin, EMB=Ethambutol, OFX=Ofloxacin, KAN=Kanamycin

Table 3.3: Drug concentrations used for the BACTEC MGIT 960 system and the agar proportion method

Drug	BACTEC MGIT 960 system (µg/ml)	Agar proportion method (µg/ml)
STR	1	2
EMB	5	7.5
OFX	1	2
KAN	5	5

STR=Streptomycin, EMB=Ethambutol, OFX=Ofloxacin, KAN=Kanamycin

CHAPTER 4

EVALUATION OF THE GENOTYPE® MTBDR*SL* ASSAY FOR SUSCEPTIBILITY TESTING OF SECOND-LINE ANTI-TUBERCULOSIS DRUGS

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Abstract

Background: The GenoType® MTBDR*sl* assay (Hain Lifesciene, Germany) is a new rapid assay for the detection of resistance to second-line anti-TB drugs.

Method: The MTBDR*sl* assay was evaluated on 342 MDR-TB isolates for ofloxacin (OFX), kanamycin (KAN), capreomycin (CAP) and ethambutol (EMB) resistance and results were compared to the agar proportion method. Discrepant results were tested by DNA sequencing.

Result: The sensitivity and specificity of the MTBDR*sl* assay was 70.3% and 97.7% for OFX, 25.0% and 98.7% for KAN, 21.2% and 98.7% for CAP and 56.3% and 56.0% for EMB, respectively. DNA sequencing identified mutations that were not detected by the MTBDR*sl* assay including: 8/11 phenotypically OFX-resistant isolates had mutations in *gyr*A (2/8 had additional mutations in the *gyr*B gene), 1/11 had mutations only in the *gyr*B gene; 6/21 phenotypically KAN-resistant isolates had mutations in the *rrs* gene; 7/26 and 20/26 phenotypically CAP-resistant isolates had mutations in the *rrs* and *tly*A genes, respectively.

Conclusion: The MTBDR*sl* assay showed a lower sensitivity when compared to previous studies. The assay performed favourably for OFX; however, the assay was less sensitive for the detection of KAN/CAP resistance and demonstrated low sensitivity and specificity for EMB resistance. It is recommended that the MTBDR*sl* assay should include additional genes to achieve a better sensitivity for all the drugs tested.

4.1 INTRODUCTION

The increasing problem of multidrug-resistant tuberculosis (MDR-TB) caused by *M. tuberculosis* strains that are resistant to isoniazid (INH) and rifampicin (RIF) and the emergence of extensively drug-resistant TB (XDR-TB), defined as 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)] has become a global health problem, threatening the success of TB control programmes (1,2). The WHO developed guidelines for drug susceptibility testing (DST) for first and second-line anti-TB drugs on Löwenstein-Jensen (LJ) medium or Middlebrook agar using the proportion method. However, DST of *M. tuberculosis* to second-line drugs is difficult, expensive, and not well standardised. In addition results are only available within 3 to 6 weeks. Therefore, there is a major interest in rapid molecular detection methods for resistance to these drugs.

The GenoType® MTBDR*sl* assay (Hain Lifescience, Germany) was developed for the rapid detection of resistance against second-line drugs (3). The assay uses multiplex PCR and reverse hybridisation to identify *M. tuberculosis* and relevant mutations in genes: *gyr*A, *rrs*, and *emb*B that confer resistance to FLQ, CAP/AMK/KAN/viomycin (VIO) and ethambutol (EMB), respectively (3). Previous studies reported that the majority of FLQ-resistant *M. tuberculosis* isolates had mutations in the quinolone resistance determining region (QRDR) in the *gyr*A gene (mutations mostly in codon A90, 91 and D94 and, more rarely, G88 and S91) and less frequently in the *gyr*B gene (4,5,6). Resistance to CAP, KAN and AMK has been shown to be associated with mutations at positions 1401 and 1402 and position 1484 in the *rrs* gene (7,8). Mutations 1401 and 1484 were found to cause high-level resistance to all drugs, whereas 1402 causes resistance to only CAP and KAN. In addition, the *tly*A gene, which encodes a putative rRNA methyltransferase, was reported to confer resistance to CAP (9). Ethambutol resistance is reported to be most frequently associated with mutations in the *emb*CAB operon. However, 50% to 70% of EMB resistant *M. tuberculosis* isolates contain missense mutations in the *emb*B gene with the majority (47% to 60%) of the *M. tuberculosis* strains carrying mutations at codon 306 (10,11).

The aim of the study was to evaluate the performance of the MTBDR*sl* assay for the detection of ofloxacin (OFX), KAN, CAP and EMB resistance in a high-TB burden area. The results obtained were compared with those of the standard agar proportion method, which was performed in parallel with the MTBDR*sl* assay.

4.2 MATERIALS AND METHODS

4.2.1 Study setting and clinical isolates

The study was conducted at the Diagnostic Microbiology Laboratory at the National Health Laboratory Service (NHLS) Tshwane Academic Division located in Pretoria, South Africa. A total of 342 consecutive MDR-TB isolates were collected from the National Health Laboratory Service (NHLS) Diagnostic Microbiology laboratory at the Medical University of Southern Africa. The laboratory received specimens from the surrounding clinics and hospitals in the referring provinces of Limpopo, North-West and Mpumalanga, a geographic area with high incidence of TB. All isolates were freshly sub-cultured on Middlebrook agar before being tested by the different methods.

4.2.2 Drug susceptibility testing

Drug susceptibility testing was done for OFX, KAN, CAP and EMB using the agar proportion method (Appendix A). An MDR-TB isolate was classified as resistant when the colonies on the drug-containing quadrant appeared 1% compared to the drug-free control quadrant.

4.2.3 GenoType® MTBDR*sl* assay

The DNA of the MDR-TB isolates was extracted according to a method described previously (12). The MTBDR*sl* assay was performed as described by the manufacturer (Appendix A). Either the absence of a wild-type band or the presence of a mutant band was indicative of a resistant isolate.

4.2.4 DNA sequencing of discrepant results

All discrepant isolates were sequenced to evaluate discrepancies between the MTBDR*sl* assay and the agar proportion method. The primers used for amplification and sequencing are shown in Table 4.1.

4.2.5 Statistical analysis

The sensitivity, specificity, positive and negative predictive values were calculated for each drug compared to the gold standard the agar proportion method. The agreement between the two methods was determined by the Kappa (κ) statistic. The κ value, a measure of test

reliability, was interpreted as follows: < 0.2, poor; 0.21 to 0.4, fair; 0.41 to 0.6, moderate; 0.61 to 0.8, good; ≥ 0.81, excellent (13).

4.3 RESULTS

Three-hundred-thirty-six (98.2%) of the total 342 isolates tested by the MTBDR*sl* assay, gave interpretable results. In six isolates, no TUB band was observed and these isolates were excluded from the analysis. No contamination was observed in the negative controls. A total of 6.3% (21/336) isolates met the criteria for the classification as XDR-TB by the agar proportion method and 2.4% (8/336) by the MTBDR*sl* assay. Turnaround times for DST ranged from 6 to 21 days (median, 11 days) for the agar proportion method and from 2 to 3 days (median, 2 days) for the MTBDR*sl* assay. The DST results of the MTBDR*sl* assay as compared to the agar proportion method are shown in Table 4.2.

Ofloxacin resistance: The sensitivity and specificity of the MTBDR*sl* assay for OFX resistance was 70.3% and 97.7%, respectively (Table 4.3). Out of the total 336 isolates tested, 299 (89.0%) were phenotypically susceptible to OFX. Two-hundred and ninety-two (97.7%) of these, showed wild-type patterns with the MTBDR*sl* assay and the remaining seven (2.3%) isolates had mutations. The DNA sequencing confirmed the presence of mutations in the *gyr*A gene in all seven isolates, with one isolate showing additional mutations in the *gyr*B. The phenotypic method identified 11.0% (37/336) resistant isolates to OFX, of these 26/37 (70.3%) had mutations with the MTBDR*sl* assay and the remaining 29.7% (11/37) showed wild-type patterns. Analysis of the discrepant results by DNA sequencing showed that 8/11 had mutations in the *gyr*A gene, with 2/8 showing additional mutations in the *gyr*B gene. One of the 11 isolates (phenotypically an XDR-TB isolate) had a mutation only in the *gyr*B gene (at position 1491 A→G) and was not detected by the MTBDR*sl* assay as the *gyr*B gene is not included in the assay (Table 4.4).

Among the 33/336 (9.8%) OFX-resistant isolates by the MTBDR*sl* assay, the *gyr*A MUT 3C/D94G was the most prevalent mutation occurring in 42.4% (14/33) isolates followed by the *gyr*A MUT 3D/D94H (30.3%;10/33), *gyr*A MUT 1/A90V mutation (3.0%; 1/33) and *gyr*A MUT 3B band/D94Y (3.0%;1/33). In 15.2% (5/33) isolates, both *gyr*A MUT 3C/D94G and *gyr*A MUT 1/A90V were present. Two isolates (6.1%) were resistant by omission of the wild-type pattern.

Kanamycin and/or capreomycin resistance: In 1.2% (4/336) isolates, the *rrs* gene control band was absent. Similar results were obtained after repeating the assay and hence, these isolates were excluded from the analysis. The sensitivity and specificity of the MTBDR*sl* assay was 25.0% and 98.7% for KAN, 21.2% and 98.7% for CAP, respectively (Table 4.3).

A total of 91.6% (304/332) were phenotypically susceptible to KAN and of these 98.7% (300/304) showed wild-type patterns with the MTBDR*sl* assay and the remaining 1.3% (4/304) had mutations in the *rrs* gene. The DNA sequencing confirmed the presence of mutations in all four isolates. Out of the 332 isolates, 28 (8.4%) were phenotypically resistant to KAN and only 25.0% (7/336) of these isolates had mutations by the MTBDR*sl* assay, while the remaining 75.0% (21) had wild-type patterns. Among the 21 discrepant isolates, six isolates (including three phenotypically XDR-TB isolates) had mutations in the *rrs* gene by DNA sequencing and were not detected by the MTBDR*sl* assay (Table 4.4). These isolates had various nucleotide changes at different positions in the *rrs* gene (between 87 to 1431 bp).

Two-hundred and thirty-two (90.1%) of the 332 isolates were phenotypically susceptible to CAP and of these 98.7% (295/299) showed wild-type patterns with the MTBDR*sl* assay and the remaining 4/299 (1.3%) had mutations in the *rrs* gene. DNA sequencing of the *rrs* gene showed mutations in all four discrepant isolates. Thirty-three of the 332 (9.9%) isolates were phenotypically resistant to CAP, of these only seven (21.2%) had mutations in the *rrs* gene with the MTBDR*sl* assay and the remaining 78.8% (26/33) showed wild-type patterns. The DNA sequencing identified mutations in 7/26 discrepant isolates (including three phenotypically XDR-TB isolate), which were not detected by the MTBDR*sl* assay. These isolates had various a nucleotide changes at different positions in the *rrs* gene (between 87 to 1431 bp). In addition, the *tly*A gene was sequenced for all the discrepant isolates. The DNA sequencing identified nucleotide change at position 33 (A→G) of the *tly*A gene in 20/30 isolates phenotypically resistant to CAP and 3/30 CAP-resistant isolates by the MTBDR*sl* assay (Table 4.4). This mutation was not detected by MTBDR*sl* assay as the *tly*A gene is not included in the assay.

Out of the 11 KAN/CAP resistant isolates by MTBDR*sl* assay, 9.1% (1/11) had the *rrs* MUT 1/A1401G and the other 9.1% (1/11) had the *rrs* MUT 2/A1484T mutation. The remaining 81.8% (9/11) were resistant by absence of wild-type patterns.

Ethambutol resistance: In 5.9% (20/336) of the isolates tested for EMB, both the wild-type and mutant EMB bands were observed, suggesting heteroresistance and thus were not included in the analysis. The sensitivity and specificity of the MTBDR*sl* assay for detection of EMB resistance was 56.3% and 56.0%, respectively (Table 4.3). Two-hundred and six-eight (84.8%) of the 316 isolates were phenotypically susceptible to EMB and of these 56% (150/268) showed wild-type patterns and the remaining 44 % (118/264) showed mutations with the MTBDR*sl* assay. Forty-eight of the 316 isolates (15.2%) were phenotypically resistant to EMB. However, only 56.3% (27/48) showed mutations with the MTBDR*sl* assay while the remaining 44.0% (21/48) showed wild-type patterns. The discrepant isolates were sequenced targeting the *emb*B gene to evaluate discrepancies and only 56.8% (67/118) isolates showed mutations with the DNA sequencing, while the remaining 43.2% (51/118) isolates had no mutation (Table 4.4).

Out of the 21 isolates susceptible to EMB by MTBDR*sl* assay, mutations was detected in nine (42.9%) isolates by DNA sequencing. The remaining 57.1% (12) isolates had wild-type patterns. The most prevalent mutation of the 145 EMB-resistant isolates with MTBDR*sl* assay was *emb*B MUT 1B/M306V (69.7%; 101/145), followed by *emb*B MUT 1A/M3061 (24.8%; 36/145) and the remaining isolates (5.5%; 8/145) were resistant by absence of wild-type patterns.

4.5 DISCUSSION

In this study a large number of isolates (342 MDR-TB) were used to evaluate the MTBDR*sl* assay (3,14,15). A good agreement (κ=0.73) was found between the MTBDR*sl* assay and the agar proportion method for OFX, with a sensitivity of 70.3%. Previous studies have reported similar sensitivity values for the MTBDR*sl* assay for detecting FLQ-resistance, ranging from 70% to 87% (3,14,15,16). A significant finding of this study was that DNA sequencing identified mutations in 8/11 isolates phenotypically resistant to OFX in the *gyr*A gene (2/8 had additional mutations in *gyr*B), while 1/11 (phenotypically XDR-TB isolate) only had a mutation only in the *gyr*B gene. Similarly, Mokrousov *et al*. (6) found OFX-resistant isolates with *gyr*B mutations and one of these isolates was an XDR-TB strain. Based on these findings, genotypic testing for OFX-resistance should not be limited to the analysis of only the *gyr*A gene but should also include the *gyr*B gene. Furthermore, inclusion of a bigger region of the *gyr*A gene could have picked up an additional 72.7% (8/11) isolates, with a resultant increase in sensitivity to 92.0%.

In three phenotypically OFX-resistant isolates, no mutation was found in either the *gyr*A or *gyr*B genes. These findings suggest mutations in another target gene or the use of other mechanisms such as decreased cell-wall permeability to drug, drug efflux pump, or even drug inactivation (17).

Although the specificity of the MTBDR*sl* assay for the detection of KAN/CAP resistance was excellent (98.7% and 98.7%), the sensitivity was low (25.0% and 21.2%). Previous studies reported a higher sensitivity (77% to 100% for KAN and 80.0% to 86.7% for CAP) (3,14,15). However, these studies tested small numbers of isolates (5 to 13 isolates) and larger number of isolates are needed to be tested to assess sensitivity accurately. Huang *et al*. (16) tested 234 MDR isolates using the MTBDR*sl* assay and found a low sensitivity of 43.2% for the detection of KAN resistance.

The low sensitivity of KAN/CAP in this study could be due to other mutations associated with resistance to these drugs. The MTBDR*sl* assay uses only one gene (*rrs*) for the detection of KAN and CAP resistance. The *tly*A gene is reported to cause CAP resistance (9) and the promoter region of the *eis* gene, which encodes an aminoglycoside acetyltransferase, which is associated with low-level KAN resistance (18). It has been reported that *tly*A mutations are mainly associated with *in vitro* selected CAP-resistant mutants and are rare in clinical isolates of *M. tuberculosis* (9,19,20). However, this was not the case in this study, as 20/26 of the discrepant results which were pheonotypically resistant to CAP and 3/4 CAP-resistant isolates by the MTBDR*sl* assay had a mutation in the *tly*A gene. Therefore, the role of the *tly*A gene in CAP resistant clinical isolates should be further investigated.

In 15 phenotypically KAN-resistant isolates and six phenotypically CAP-resistant isolates, no mutation was detected in either the *rrs* or *tly*A genes. Unidentified mutations associated with CAP and KAN resistance could explain this finding. Further work is needed to determine the mechanism of the unexplained KAN and CAP resistance as resistance to one of these drugs is a marker for XDR-TB.

The sensitivity of the MTBDR*sl* assay for the detection of EMB resistance in this study was low (56.3%). Similarly, previous studies reported a sensitivity ranging from 56% to 69% (3,14,15,16). Ethambutol is known to be a problematic drug to be tested and often yields less reproducible results (21,22). DNA sequencing identified mutations in the *emb*B gene in only 56.8% (67/118) isolates. The discrepancy could be due to the shorter region of the *emb*B

gene analysed by sequencing. Thus, some of the mutations could lie outside the region analysed in this study. A limitation of the study is that the discrepant results were not repeated by the MTBDR*sl* assay to rule out non-specific hybridisation, however, no contamination was observed in the negative controls.

In this study, 5.9% (20/336) isolates showed heteroresistance for the *emb*B gene. Heteroresistance of *M. tuberculosis* is considered a precursor to development of full resistance (23). This highlights the important advantage of genotypic over the phenotypic methods, as genotypic methods are able to detect mixed strains.

The poor performance of the MTBDR*sl* assay in this study was not unique, since molecular methods detect only mutations that are screened for, while phenotypic tests detect resistance independent of the underlying mechanism and not all mutations conferring resistance to second-line anti-TB drugs are known, especially for KAN, CAP and EMB. In addition the prevalence of certain strains with specific mutations may vary in different geographical areas and thus will affect the test performance. On the other hand significant challenges exist in phenotypic susceptibility testing to second-line drugs. Although standard protocols exist, phenotypic susceptibility testing to second-line drugs is often unreliable and inaccurate, with poor clinical predictive values (24).

In conclusion, the MTBDR*sl* assay showed a lower sensitivity in this study compared to previous studies. The sensitivity of the assay was variable among the drugs tested. The MTBDR*sl* assay performed favourably compared to the agar proportion method for OFX; however, the assay was less sensitive for the detection of KAN/CAP resistance and demonstrated a low sensitivity and specificity for EMB resistance. The resolution of discrepant isolates with DNA sequencing has shown that the inclusion of the *gyr*B gene and covering a larger region of the *gyr*A gene may improve the sensitivity of the assay for the detection of FLQ resistance. Inclusion of other genes such as the *tly*A for the detection of CAP resistance and other regions of the *emb*B gene as well as other targets associated with EMB resistance could improve the performance of the assay.

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Table 4.1: Primer sequences for amplification and DNA sequencing of discrepant *M. tuberculosis* isolates

Drug	Gene	Sequence (5'-3')	Amplicon size (bp)	References
OFX	*gyr*A	F YGGTGGRTCRTTRCCYGGCGA R CGCCGCGTGCTSTATGCRATG	250	Dauendorffer *et al*., 2003
	*gyr*B	F GAGTTGGTGCGGCGTAAGAGC R CGGCCATCAAGCACGATCTTG	250	
KAN/CAP	16S (rrs)	F AGAGTTTGATCCTGGCTCAG R ACGGCTACCTTGTTACGACTT	1500	Weisburg et al., 1991
CAP	*tly*A	F GGCATCGCACGTCGTCGTCTTTCCGAGG R GGACGACCAGCAGAACACTGCGATG	820	Designed in this study
EMB	*emb*B	F CCGACCACGCTGAAACTG R GTAATACCAGCCGAAGGGATCCT	400	Jain *et al*., 2008

OFX=ofloxacin, KAN=kanamycin, CAP=capreomycin, EMB=ethambutol, bp=base pairs

Table 4.2: Drug susceptibility results of MDR-TB isolates using the GenoType® MTBDR*sl* assay and the agar proportion method

Drug	Total no. of isolates	S by both methods	R by both methods	S by agar R by MTBDRsl	R by agar S by MTBDRsl	Correctly identified as S by MTBDRsl (%)	Correctly identified as R by MTBDRsl (%)	Agreement (%)	κ value
OFX	336	292	26	7	11	292 (97.7)	26 (70.3)	94.6	0.73
KAN	332	300	7	4	21	300 (98.7)	7 (25.0)	92.5	0.327
CAP	332	295	7	4	26	295 (98.7)	7 (21.2)	91.0	0.283
EMB	316	150	27	118	21	150 (56.0)	27 (56.3)	56.0	0.067

OFX=ofloxacin, KAN=kanamycin, CAP=capreomycin, EMB=ethambutol, S=susceptible, R=resistant

Table 4.3: Diagnostic efficiency of the GenoType® MTBDR*sl* compared to the agar proportion method

Drug	No of isolates	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
OFX	336	70.3	97.7	78.8	96.4
KAN	332	25.0	98.7	63.6	93.5
CAP	332	21.2	98.7	63.6	91.9
EMB	316	56.3	56.0	18.6	87.7

OFX=ofloxacin, KAN=kanamycin, CAP=capreomycin, EMB=ethambutol, S=susceptible, R=resistant

Table 4.4: DNA sequencing results for the discrepant isolates between GenoType® MTBDR*sl* assay and the agar proportion method

Drugs	Locus	DNA sequencing results	R by MTBDR*sl* S by agar	R by agar S by MTBDR*sl*
OFX n=18			**7**	**11**
	*gyr*A	Mutation present (n=15)	7	8
		Mutation absent (n=3)	0	3
	*gyr*B	Mutation present (n=4)	1	3
		Mutation absent (n=14)	6	8
KAN n=25			**4**	**21**
	16S (*rrs*)	Mutation present(n=10)	4	6
		Mutation absent (n=15)	0	15
CAP n=30			**4**	**26**
	16S (*rrs*)	Mutation present (n=11)	4	7
		Mutation absent (n=19)	0	19
	*tly*A	Mutation present (n=23)	3	20
		Mutation absent (n=7)	1	6
EMB n=139			**118**	**21**
	*emb*B (M306)	Mutation present (n=76)	67	9
		Mutation absent (n=63)	51	12

OFX=ofloxacin, KAN=kanamycin, CAP=capreomycin, EMB=ethambutol, S=susceptible, R=resistant

CHAPTER 5

MOLECULAR CHARACTERIZATION AND SECOND-LINE ANTI-TB DRUG-RESISTANCE PATTERNS OF MULTIDRUG RESISTANT TUBERCULOSIS ISOLATES FROM THE NORTHERN REGION OF SOUTH AFRICA

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Abstract

Despite South Africa being one of the high-burden multidrug-resistant tuberculosis (MDR-TB) countries, information regarding the population structure of drug-resistant *Mycobacterium tuberculosis* strains is limited from many regions of South Africa. This study investigated the population structure and transmission patterns of drug-resistant *M. tuberculosis* isolates in a high-burden setting of South Africa as well as the possible association of genotypes with drug-resistance and demographic characteristics. A total of 336 consecutive MDR-TB isolates from four provinces of South Africa were genotyped using spoligotyping and mycobacterial interspersed repetitive units of variable number of tandem repeat typing. Drug susceptibility testing for ofloxacin, kanamycin and capreomycin was performed using the agar proportion method. Results showed 4.8% of MDR-TB isolates were resistant to ofloxacin, 2.7% to kanamycin, 4.5% to capreomycin, while 7.1% were XDR and the remaining 83.6% were susceptible to all the second-line drugs tested. Spoligotyping grouped 90.8% of the isolates into 25 clusters; while 9.2% isolates were unclustered. Ninety one percent of the 336 isolates were assigned to 21 previously described shared types, with the Beijing family being the predominant genotype in the North-West and Limpopo provinces, while the EAI1_SOM family was the predominant genotype in the Gauteng and Mpumalanga provinces. No association was found between genotypes and specific drug-resistance patterns or demographic information. The high-level of diversity and the geographical distribution of the drug-resistant *M. tuberculosis* isolates in this study suggest that the transmission of TB in the study settings is not caused by the clonal spread of a specific *M. tuberculosis* strain.

5.1 Introduction

Tuberculosis (TB) remains a major public health challenge worldwide. South Africa is third of the world's 22 high-burden TB countries responsible for 80% of the world's TB burden with the second-highest prevalence of TB per capita in the world, at 998 cases per 100 000 of the population (1). The TB problem in South Africa is compounded by the high prevalence of Human Immunodeficiency Virus (HIV) and the emergence and spread of drug-resistant *Mycobacterium tuberculosis* and especially, multidrug-resistant (MDR) [resistant to at least isoniazid (INH) and rifampicin (RIF)] *M. tuberculosis* and extensively drug-resistant strains (XDR) TB [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)] (2,3). In a nationwide survey in South Africa in 2008, 20.2% of all notified TB cases showed resistance to INH and nearly half of these (9.6% of all cases) were MDR-TB (4). Since the outbreak of XDR-TB in Tugela Ferry in KwaZulu-Natal, XDR-TB has been identified in all provinces of South Africa (3). According to the data from the National Health Laboratory Service (NHLS) in 2010, 6.3% of the diagnosed MDR-TB cases were XDR-TB (5).

The design of strategies for the management of MDR and XDR-TB depends on an understanding of the population structure, prevalence and spread of drug-resistant strains that drives the epidemic. Genotyping is an important tool for understanding the origin and transmission patterns of drug-resistant strains. The IS*6110* restriction fragment length polymorphism (RFLP) typing is the reference technique for genotyping of *M. tuberculosis* strains because of its high discriminatory power (6,7). However, the method is laborious, requires large amounts (2 µg) of DNA and has poor discriminatory power when applied to *M. tuberculosis* isolates with a low IS*6110* copy number (7,8). A number of PCR based methods, such as spoligotyping (9) and mycobacterial interspersed repetitive units of variable number of tandem repeats (MIRU-VNTRs) typing (10), have been developed to compensate for the limitations of IS*6110*-RFLP typing. Spoligotyping is based on the amplification and detection of the presence or absence of non-repetitive sequences called spacers found between direct repeat elements of the *M. tuberculosis* genome (9). This assay is simple, rapid, highly reproducible and results are expressed in a simple digital pattern, readily named and databased (8,9). The MIRU-VNTR typing is based on amplification of multiple loci (either 12, 15 or 24) using primers specific to each repeat locus and on the determination of the sizes

of the amplicons, which reflects the number of the targeted MIRU-VNTR copies (8,10,11,12). The MIRU-VNTR profiles are presented as multi-digit numerical codes, each digit represents the copy number in a locus (10,11,12). The discriminatory power of either spoligotyping or MIRU-VNTR typing alone is lower than IS*6110*-RFLP typing (8). However, if MIRU-VNTR typing is combined with spoligotyping, the discriminatory power approximates that of IS*6110*-RFLP analysis (13,14,15).

Despite the high prevalence of MDR and XDR-TB in South Africa, nationwide data regarding the circulating drug-resistant strains are limited. Most of the earlier studies were from the Western Cape province and were thus not representative of the epidemiology of drug-resistant strains in South Africa. Studies have shown that the global TB epidemiology is caused by different genotypes (16,17,18) and these genotypes occur at different frequencies, which differ between districts, cities, countries and continents (6,16,19). In South Africa the population structure of drug-resistant strains differs among the provinces. However, insufficient data are available from most of the provinces of South Africa, especially provinces in the northern region. Therefore, studies on the characterisation of drug-resistant strains are needed in order to make accurate assessments regarding the population structure of drug-resistant strains, to estimate the transmission dynamics and to determine prevalent families of circulating drug-resistant *M. tuberculosis* strains.

In this study, drug-resistant *M. tuberculosis* isolates from four provinces of South Africa (Gauteng, Limpopo, Mpumalanga and North-West) were characterised using spoligotyping and MIRU-VNTR (12 loci) typing in order to understand the population structure and transmission patterns. Furthermore, the study explored the possible association of genotypes (cluster) with demographic information (age, sex) and drug-resistance patterns.

5.2 Methods

5.2.1 Study population

In this study a convenience sample was used, including 336 consecutive *M. tuberculosis* isolates identified as MDR-TB isolates from June 2007 to January 2008 at the NHLS tertiary laboratory, University of Limpopo. The NHLS laboratory is a high-throughput routine diagnostic laboratory that receives specimens for culture and drug susceptibility testing (DST) from the attached Dr George Mukhari Hospital and the surrounding clinics and hospitals in

the referring provinces of Gauteng, Limpopo, Mpumalanga and North-West. The number of isolates from each province was as follows: 69.9% (235/336) isolates from Mpumalanga, 14.9% (50/336) isolates from the Gauteng, 9.2% (31/336) isolates from the North-West and 5.9% (20/336) isolates from Limpopo. The isolates were collected from 22 hospitals and 53 clinics in Mpumalanga, two hospitals and 11 clinics in Gauteng, five hospitals and 16 clinics in North West and two hospitals and nine clinics in Limpopo. During the study period the confirmed MDR and XDR-TB cases was 275 and three respectively, for Mpumalanga, 532 and 20 respectively, for Gauteng, 186 and two respectively, for North West and 53 and 2 respectively, for Limpopo province (20).

Demographic information including sex, age and clinic/hospital of specimen origin were collected for all the patients. Approval for the study protocol (MCREC/P/07/2008) was obtained from the Ethics Committees of the Faculty of Health Sciences, University of Pretoria and University of Limpopo.

5.2.2 Routine culture and drug susceptibility testing

Isolation of the recovered mycobacterial isolates from the clinical specimens was performed using the BACTEC mycobacterial growth indicator tube (MGIT) 960 system (Becton Dickinson, Sparks, Md, USA) as described by the manufacturer. The isolates were identified as *M. tuberculosis* using Ziehl–Neelsen staining and confirmed with the Accuprobe method (GENE-probe, inc, San Diego, Calif). Following identification, all the *M. tuberculosis* isolates were tested for susceptibility to the first-line anti-TB drugs INH, RIF, EMB and streptomycin (STR) by MGIT AST SIRE kit (Becton Dickinson, Sparks, Md., USA).

5.2.3 Drug susceptibility testing using the agar proportion method

Second-line anti-TB drug susceptibility testing was performed for KAN, OFX and CAP using the agar proportion method in 7H11 medium containing 5 µg/mL, 2 µg/mL and 10 µg/mL, respectively. The DST was done according to the Clinical Laboratory Standards Institute (CLSI) procedures and recommended critical concentrations (21) (Appendix A). Based on the DST profile, the *M. tuberculosis* isolates in this study were categorized into MDR-TB (susceptible to CAP, KAN and OFX), pre-XDR-TB (resistant to one of the second-line drugs: CAP, KAN and OFX) and XDR-TB (resistant to OFX and CAP and/or KAN).

5.3 Genotyping

Mycobacterial genomic DNA was extracted from 100 µl of 7H9 broth culture using the Amplicor Respiratory Specimen Preparation Kit (Roche diagnostics, Germany) as described by the manufacturer. Spoligotyping was performed using a commercial kit (Isogen Life Science BV, Utrecht, The Netherlands) as described previously (9) (Appendix A).

The MIRU-VNTR typing was performed using 12 MIRU-VNTR loci (2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39 and 40). Each locus was amplified individually for all 336 *M. tuberculosis* isolates according to the protocol described by Le-Flèche *et al*. (22) (Appendix A). To amplify the different locus specific primers were used as described by Supply *et al*. (12) (Appendix A). The amplicon size was determined by visual comparison with a 50 bp molecular marker (Fermentas, Vilnius, Lithuania). The number of alleles corresponding to the amplicon sizes was assigned using an allele calling table described in the Supply *et al*. (23) protocol. The results from each of the 12 loci were combined to create the 12 digit allelic profiles.

5.4 Data analysis

The spoligotyping results were entered in an excel sheet as a binary code representing either a positive or negative hybridisation result. Spoligotypes in binary format were entered in the SITVIT2 database (http://www.pasteur-guadeloupe.fr/tb/bd_myco.html Pasteur Institute of Guadeloupe), which is an updated version of the previously released SpolDB4 database. The MIRU-VNTR pattern was analysed using the MIRU-VNTR*plus* database (www.miru-vntrplus.org/). The results were entered into the database as numerical codes corresponding to the number of alleles at each locus. Dendrogrammes were constructed for spoligotyping, MIRU-VNTR and the combination of both methodologies. The categorical coefficient was used to calculate the distance matrix and the dendrogrammes were constructed using the Unweighted Pair Group Method with Arithmetic averages (UPGMA) algorithm. The evaluation of the discriminative power of each typing method alone as well as in combination was done using the Hunter-Gaston index (HGI), $D=1-1/N(N-1)\sum^{n}_{j}=1^{aj}$ where D is the index of discriminatory power, a_j is the number of strains in the population, which are indistinguishable from the jth strain and N is the number of strains in the population. Allelic diversity of each locus was classified as "highly discriminant" (HGI > 0.6), "moderately discriminant" (> 0.3) and "poorly discriminant" (HGI < 0.3) (24). A cluster was defined as two or more *M. tuberculosis* isolates from different patients with identical patterns. The

clustering rate was defined as $(n_c\text{-}c)/n$, where n is the total number of cases in the sample, c is the number of genotypes represented by at least two cases and n_c is the total number of cases in clusters of two or more patients (23).

The chi-square test or Fisher's exact test was performed to determine statistical association between genotypes and age, gender and drug-resistant patterns. P values < 0.05 were considered significant.

5.5 Results

A total of 336 MDR-TB isolates collected between June 2007 to January 2008 form the northern provinces of South Africa, including: Gauteng, Limpopo, Mpumalanga and North-West, were genotyped by spoligotyping and MIRU-VNTR typing. The study population included 51% (171/336) males and 44% (146/336) females, while the genders of 5% (19/336) of the patients were not available. The patients' median age was 34.4 years (SD 10.6), ranging from 6 to 69 years. Susceptibility testing results for second-line anti-TB drugs showed 4.8% (16/336) were resistant to OFX, 2.7% (9/336) to KAN, 4.5% (15/336) to CAP and 7.1% (24/336) were XDR-TB isolates and the remaining 83.6% (281/336) were susceptible to all the second-line anti-Tb drugs tested.

Spoligotyping results for the 336 MDR-TB isolates produced 56 distinct patterns, including 25 clustered patterns and 31 unclustered (unique) patterns. The number of *M. tuberculosis* isolates per cluster ranged from 2 to 69 isolates. Comparison of spoligotyping results with the SpolDB4 database showed that 90.8% (305/336) isolates belonged to 21 previously described shared types (ST); while 9.2% (31/336) were not found in the SpolDB4 database and were considered as orphans. The clustering rate of spoligotyping was 86.3% (Table 5.1).

Based on the spoligotypes, six major distinct families of TB were identified including Beijing, East African Indian (EAI), Haarlem (H), Latin American and Mediterranean (LAM), T family, S family as well as Manu 1 and Manu 2 families. The Beijing family was the predominant genotype detected in the North-West and Limpopo provinces, while the EAI1_SOM family was the predominant genotype in the Gauteng and Mpumalanga provinces. Stratification of spoligotyping data by drug resistance is shown in Table 5.2. There was no significant association (> 0.05) between the genotypes and age, gender or

specific drug susceptibility patterns. The distribution of genotypes in MDR, pre-XDR and XDR isolates is shown in Table 5.2.

A high diversity of MIRU-VNTR patterns was obtained among the *M. tuberculosis* isolates. A total of 324 distinct patterns were obtained from the 336 MDR-TB isolates, which included 11 clustered patterns and 313 unclustered patterns. One cluster comprised of three *M. tuberculosis* isolates, while the remaining 10 clusters comprised of only two *M. tuberculosis* isolates each. The clustering rate of the MIRU-VNTR typing was 3.6% (Table 5.1). Analysis of the allelic diversity of the 12 MIRU-VNTR loci revealed that MIRU 26 was the most discriminatory locus with nine alleles followed by the MIRU 31, 40 and 16 loci. The MIRU loci 4, 10, 23, 24, 27 and 39 were moderately discriminative. Among the 12 MIRU loci, MIRU 2 was less polymorphic with only three alleles, while MIRU 20 showed only a single copy in all 336 *M. tuberculosis* isolates analysed (Table 5.3).

The combination of spoligotyping and the MIRU-VNTR typing results provided the highest discriminatory power, with 327 distinct patterns, which included eight cluster patterns and 319 unclustered patterns. One cluster comprised of three *M. tuberculosis* isolates and the remaining seven only had two *M. tuberculosis* isolates each (Table 5.1). The dendrogramme for the combined typing of spoligotyping and the MIRU-VNTR typing is shown in Figure 5.1.

In order to determine possible transmission between the clustered isolates, the genotyping data were compared to the geographical origin of each isolate. The 336 isolates were from 119 hospitals/clinics in four provinces. The number of isolates received varied between the hospitals/clinics, where 65/119 (28 hospitals and 37 clinics) provided two to 27 and 55/119 (7 hospitals and 48 clinics) providing only one each. A possible epidemiological link was found in only eight clusters, each with two to six isolates, implying possible transmission. Six Beijing strains were isolated from patients attending the same clinic (Witbank Santa centre), while four strains were obtained from patients attending the Themba hospital in Mpumalanga. In the Gauteng province, four patients from the same hospital (Ga-Rankuwa) had isolates belonging to Beijing spoligotype. Another possible case of transmission was noted, where spoligotype families including EAI1_SOM, S, H3, LAM_ZWE, LAM3, LAM4 and LAM9 were isolated from two or more patients attending the same hospital/clinic in Mpumalanga, Gauteng and Limpopo provinces. With MIRU-VNTR typing, 23 isolates were clustered into 11 clusters, but all the clustered *M. tuberculosis* isolates were from different geographical settings. Using the combined typing results of spoligotyping and MIRU-VNTR typing, 17

M. tuberculosis isolates were clustered into 8 clustes; however, only two isolates were from the same clinic, while the remaining isolates were from different geographical settings.

5.6 Discussion

Despite the high incidence of TB and drug-resistance in South Africa, knowledge regarding the population structure of drug-resistant *M. tuberculosis* strains still remains limited in many regions of South Africa. This study is the first to include MDR-TB, pre-XDR and XDR-TB isolates to determine the population structure of drug-resistant strains in the northern region of South Africa (Gauteng, Limpopo, Mpumalanga and North-West). In this study the prevalence of XDR-TB was 7.1% (24/336). The reported percentage of XDR-TB patients among the MDR-TB patients varies between countries, ranging between 3% and 19% (25,26). A significant proportion of MDR-TB isolates (9.5%) in this study were resistant to a single marker for XDR-TB (pre-XDR), which raises concern that these pre-XDR-TB strains subsequently will become XDR-TB strains. Appropriate management of patients with pre-XDR is, therefore, important to minimize subsequent development into XDR-TB strains.

The Beijing family was the predominant genotype in the North-West and Limpopo provinces in this study. In contrast to this study, Stavrum *et al*. (27) found the LAM and X genotypes as the predominant genotypes in the North-West and Limpopo provinces, respectively, from strains isolated during 2001 to 2002. However, in the Western Cape region, the Beijing genotype is highly prevalent, where it represents 36.5% of the drug-resistant cases (28). The Beijing genotype is one of the most successful families, which was initially found in China but have disseminated around the world (16,29,30).

It was interesting to find the EAI1_SOM (ST 48) as the predominant genotype in the Gauteng and Mpumalanga provinces and as the second predominant strain in Limpopo and North-West provinces. Contrary, Stavrum *et al*. (27) found T1 and LAM to be the most predominant genotypes in Gauteng and Mpumalanga provinces, respectively, from strains isolated during 2001 to 2002. This EAI1_SOM family has been first isolated in Somalia (16) and according to the SITVIT 2 database it has been reported in East, North and Southern Africa as well as Europe, Asia and Middle East. In a study by Chihota *et al*. (31), the frequency of EAI1_SOM was higher in Gauteng in comparison to the Western Cape, Eastern Cape and KwaZulu-Natal provinces. In this study, it should be noted that the isolates from the Gauteng province

included only one hospital and 12 clinics. Therefore, the isolates from this study could not be representative for the whole Gauteng province.

Other significant families identified in this study included the T (mainly T1), LAM and S families, which were amongst the major genotypes, while the X, H and the Manu families were among the minor families. The LAM family of strains in this study was represented by the LAM_ZWE, LAM3, LAM3 and S/convergent and LAM9 genotypes. The LAM3 member of the LAM family, which is also known as F11, has been shown to be as successful as the Beijing genotype in contributing to the TB problem in the Western Cape province of South Africa (32).

In spite of the predominance of the Beijing and EAI1_SOM families in this study, none of the associations with drug-resistance were statistically significant. The Beijing family is generally considered to be associated with drug-resistance; however, this is not true for all geographical settings (30). In the Western Cape province alone, the Beijing family represents as high as 25% of the MDR-TB isolates (28). In this study the EAI1_SOM family was predominant among MDR-TB isolates in the Mpumalanga and Gauteng provinces, while the Beijing family dominated in North-West and Limpopo provinces.

The MIRU-VNTR genotyping results showed a high diversity among the *M. tuberculosis* isolates in this study. Genotyping with spoligotyping alone was the least discriminatory (HDI) compared to MIRU-VNTR typing (HDI) and combined typing (HDI) with spoligotyping and MIRU-VNTR (HDI). The highest allelic diversity was observed for MIRU 26, 31, 40 and 16. It has been reported that loci 10, 16, 23, 26 and 40 were introduced as loci with the most allele polymorphisms and loci 4, 20, 24 and 27 as the most poorly discriminated loci (33). However, in this study MIRU locus 10 and 23 were moderately discriminative.

The *M. tuberculosis* isolates analysed in this study were collected from patients attending hospitals and clinics that are located in different geographical settings. It is, therefore, unlikely that patients attending the different hospitals and clinics were epidemiologically linked. A possible epidemiological link which may suggest transmission was found in only 6.9% (21/305) of the clustered *M. tuberculosis* using spoligotyping. This implies that there was no apparent epidemiological link among the remaining 84.5% (284/336) *M. tuberculosis* isolates shown to be clustered together with spoligotyping. However, when these isolates were typed by MIRU-VNTR, they showed different patterns. The MIRU-VNTR typing

differentiated clustered *M. tuberculosis* isolates by spoligotyping and significantly reduced the number of epidemiological links among the *M. tuberculosis* isolates analysed (Table 5.1). The combined typing results of spoligotyping and MIRU-VNTR typing further differentiated *M. tuberculosis* isolates, which were clustered by MIRU-VNTR typing (Table 5.1). The low clustering rate of the isolates and the lack of geographical links between most of the *M. tuberculosis* isolates suggest that active transmission of MDR-and XDR-TB strains may be limited in this region. Development of resistance could be through acquisition. The reason for the acquisition could be due to the high number of MDR-TB patients who default from treatment in South Africa. Nevertheless, population-based studies are needed to confirm this findings. In contrary, clonal transmission of the Beijing family (cluster R220) was reported as a major driver of the drug-resistant TB epidemic in the Western Cape province (28). One of the limitations of this study was the lack of information regarding the TB treatment history of the patients. The study also did not include *M. tuberculosis* pan-susceptible or mono-resistant isolates for comparison of the population structure in these groups.

5.7 Conclusions

The present study provided new and relevant information regarding the population structure of MDR and XDR-TB strains in four provinces of South Africa (Gauteng, Limpopo, Mpumalanga and North-West). The study demonstrated that the drug-resistant epidemic in this region is caused by a wide diversity of genotypes, with predominance of Beijing and EAI1_SOM families. The high genetic diversity among the drug-resistant *M. tuberculosis* isolates indicated that the MDR and XDR-TB epidemic in this region is not caused by the clonal spread of a specific *M. tuberculosis* strain. Comparison of genotypes with the geographical origin of each isolate showed less epidemiological links among the isolates, suggesting a low level of active transmission during the time period. Nevertheless, population-based studies over longer time periods are needed to fully understand the epidemiology and spread of TB in this region. Extensively drug-resistant TB and pre-XDR-TB cases comprised a substantial fraction of the MDR-TB isolates investigated in this study, indicating the need for interventions to improve surveillance as well as rapid drug susceptibility testing. Based on the data obtained from this study, the following recommendations were made for the national TB control programmes: greater vigilance, provision of rapid diagnostic assays, proper management of anti-TB drugs and the reduction

of the occurrence of acquisition by providing support to patients to maximize adherence to prescribed regimens.

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Table 5.1: Discriminatory power of spoligotyping and MIRU-VNTR typing, alone and in combination for the *M. tuberculosis* isolates

Methodology	No of distinct pattern	No of clusters	No of clustered isolates	No of unique isolates	Clustering rate (%)	HGI
Spoligotyping	56	25	305	31	86.3	0.9029
MIRU-VNTR	324	11	23	313	3.6	0.9977
Spoligotyping + MIRU-VNTR	327	8	17	319	2.7	0.9998

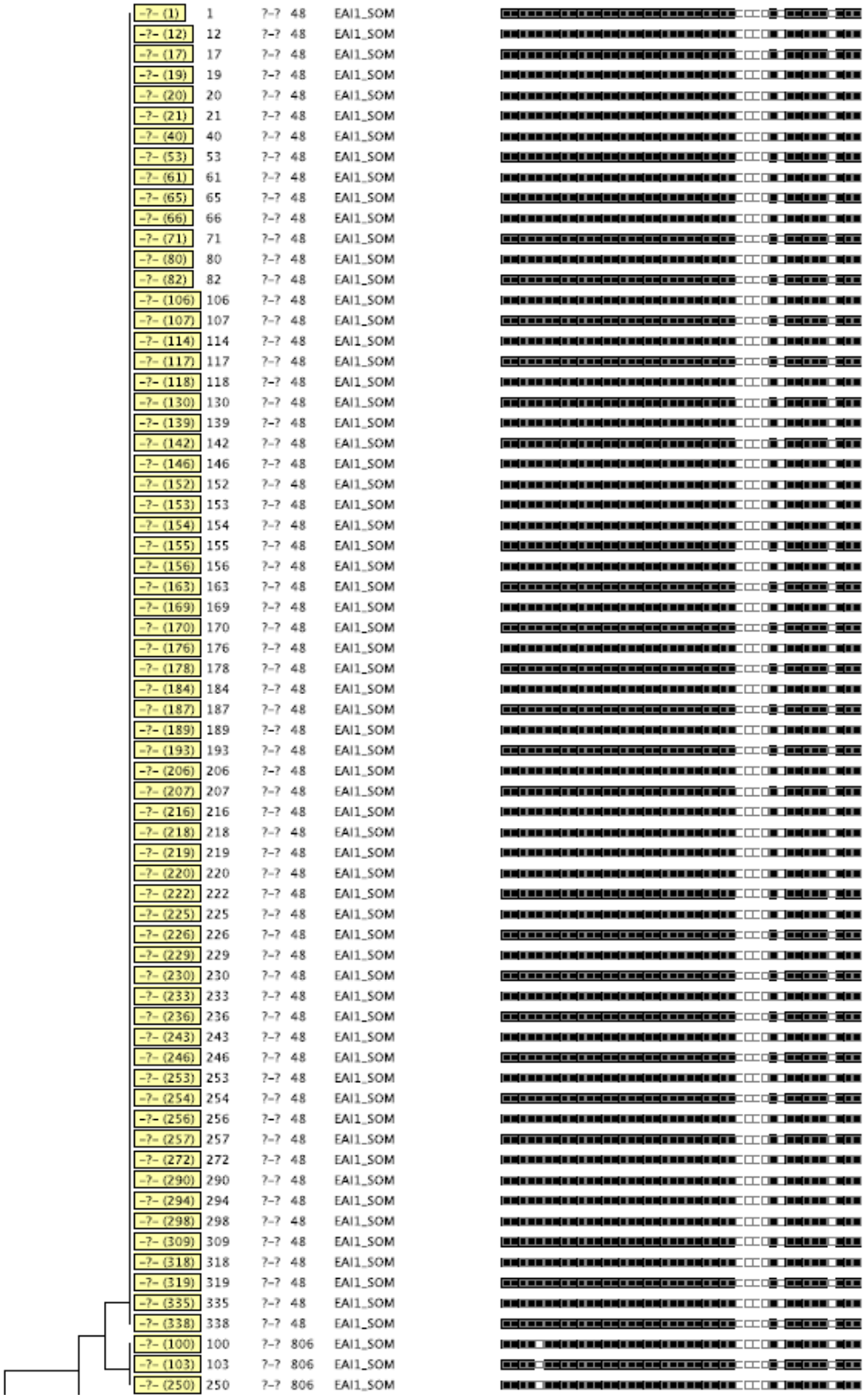
HGI: Hunter-Gaston index

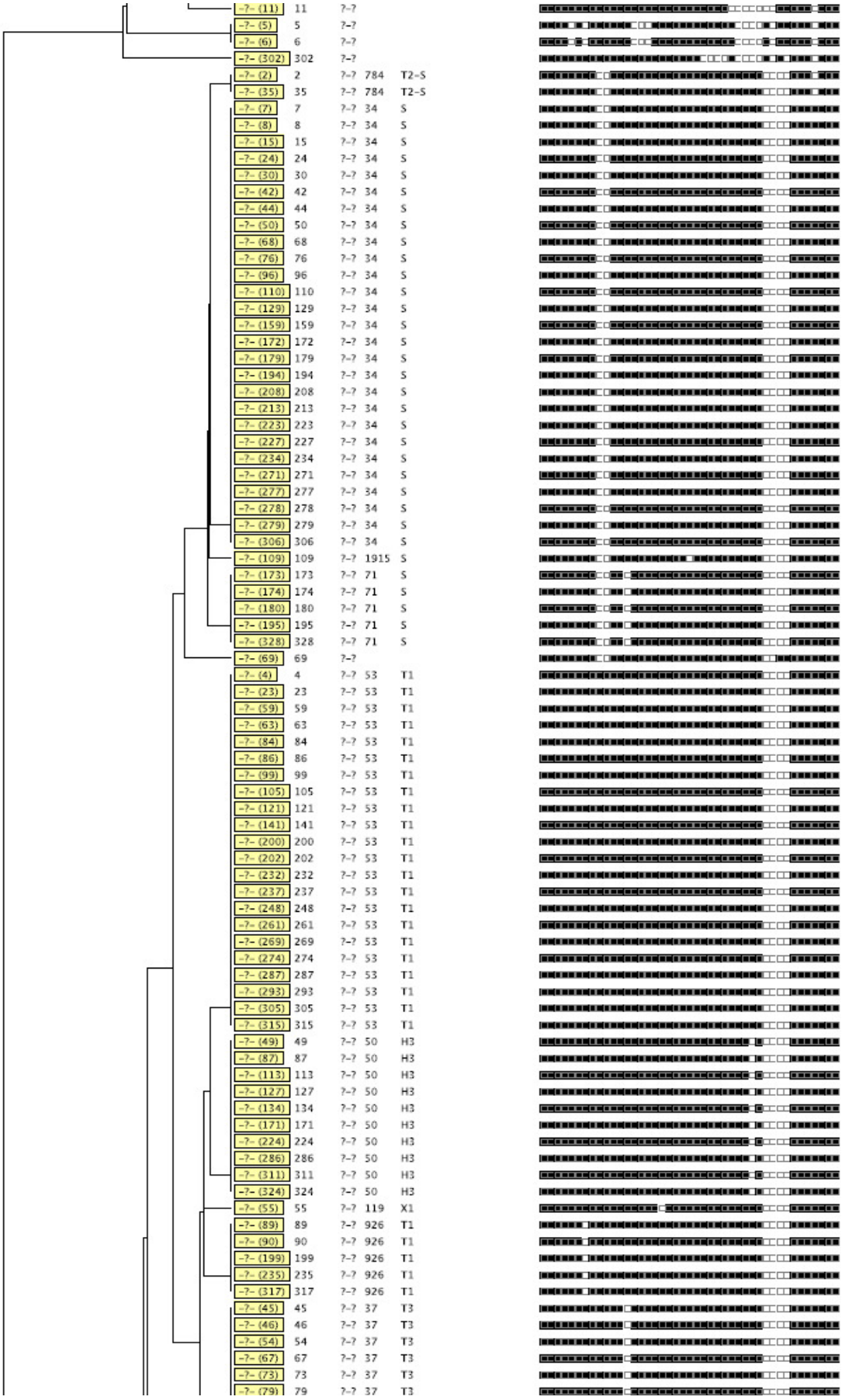
Table 5.3: Allelic polymorphism of the 12 MIRU-VNTR loci

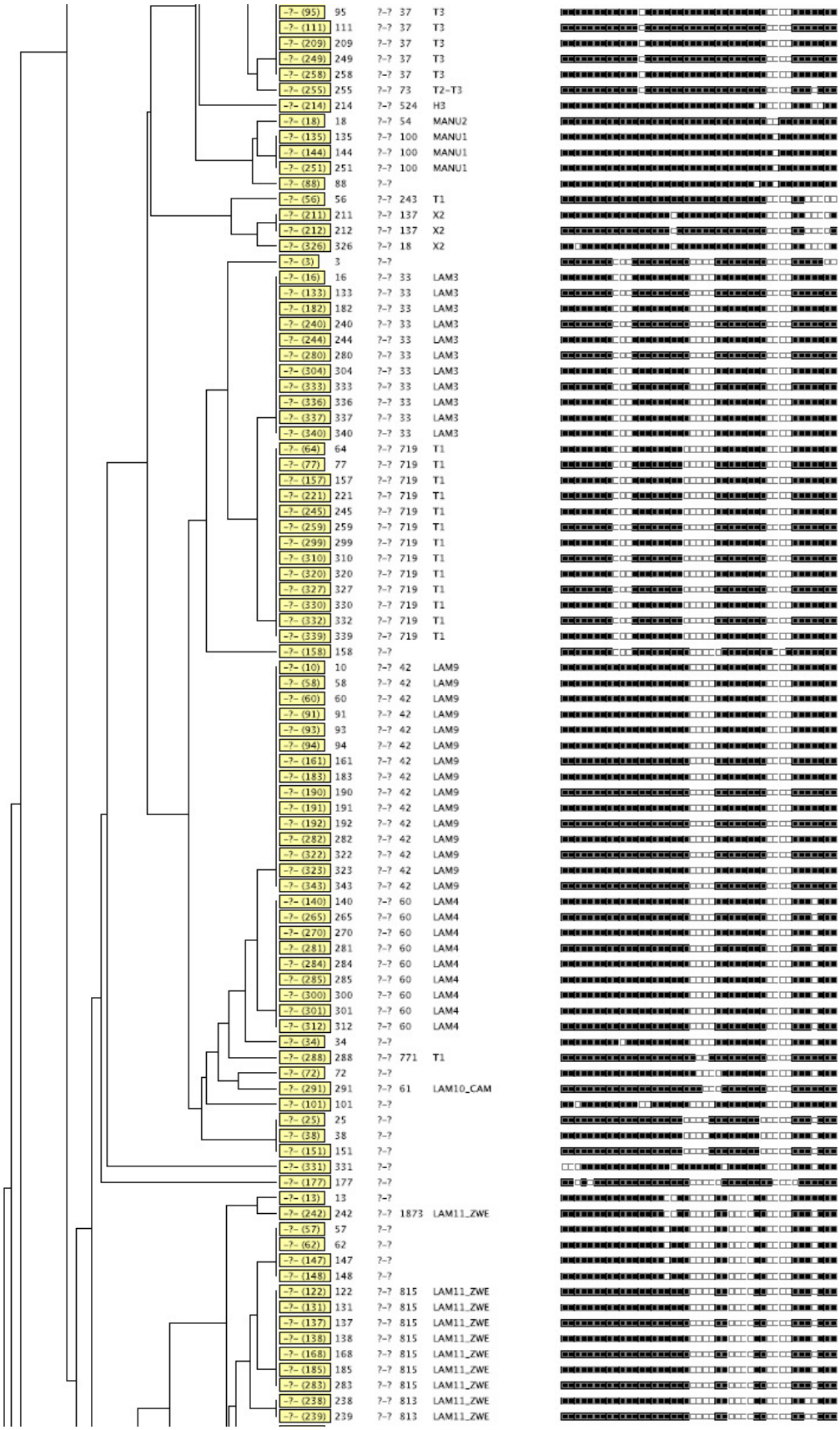
	No of strains with the specified MIRU copy number										
Allel locus	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**HGI**	**Conclusion**
2		17	307	12							0.162	Poorly discriminatory
4			227	35	1	74					0.534	Moderately discriminatory
40		61	12	152	89	13	10				0.684	Highly discriminatory
10	1	1	9	154	150	18	1	1	1		0.589	Moderately discriminatory
16		44	112	160	16	4					0.644	Highly discriminatory
20			337								0	Poorly discriminatory
23				2		172	158	4			0.519	Moderately discriminatory
24		177	123	23	7	7	1			2	0.585	Moderately discriminatory
26		7	71	25	17	132	21	52	9	3	0.766	Highly discriminatory
27		4	19	189	120	4					0.542	Moderately discriminatory
31			16	158	91	43	22	4	2		0.685	Highly discriminatory
39	2	78	196	55	5						0.5805	Moderately discriminatory

HGI: Hunter-Gaston index

UPGMA-Tree, Spoligo: Categorical







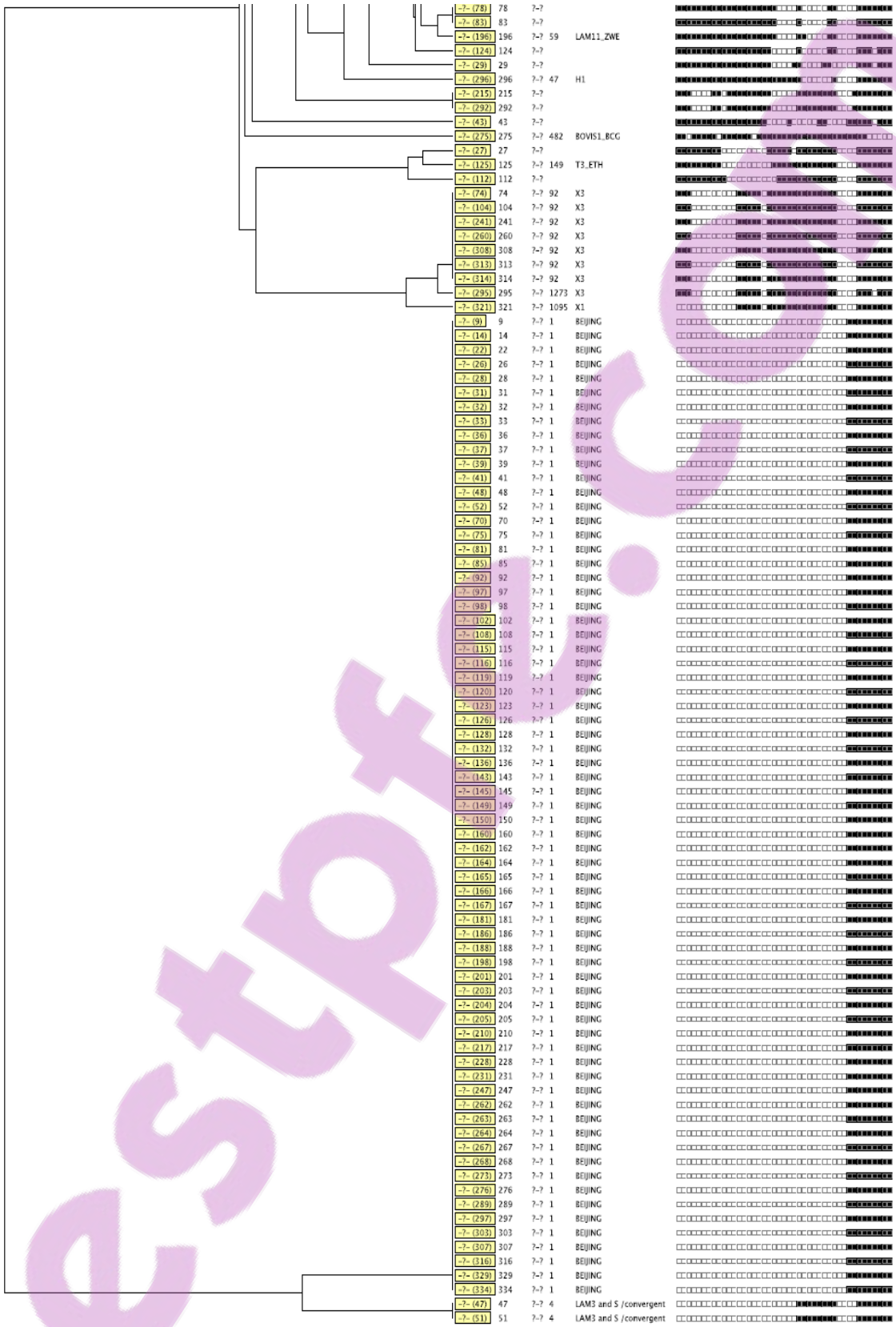
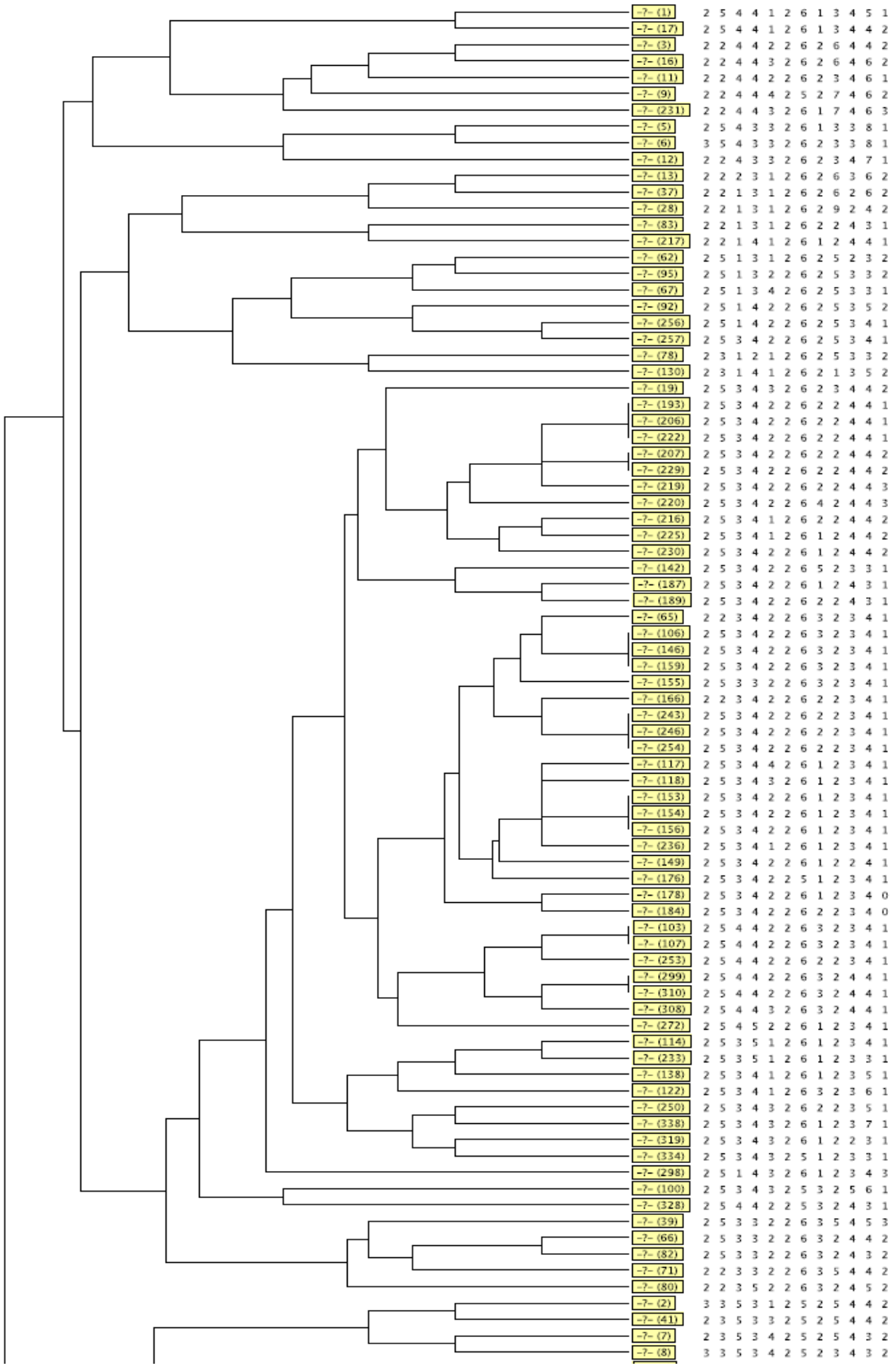
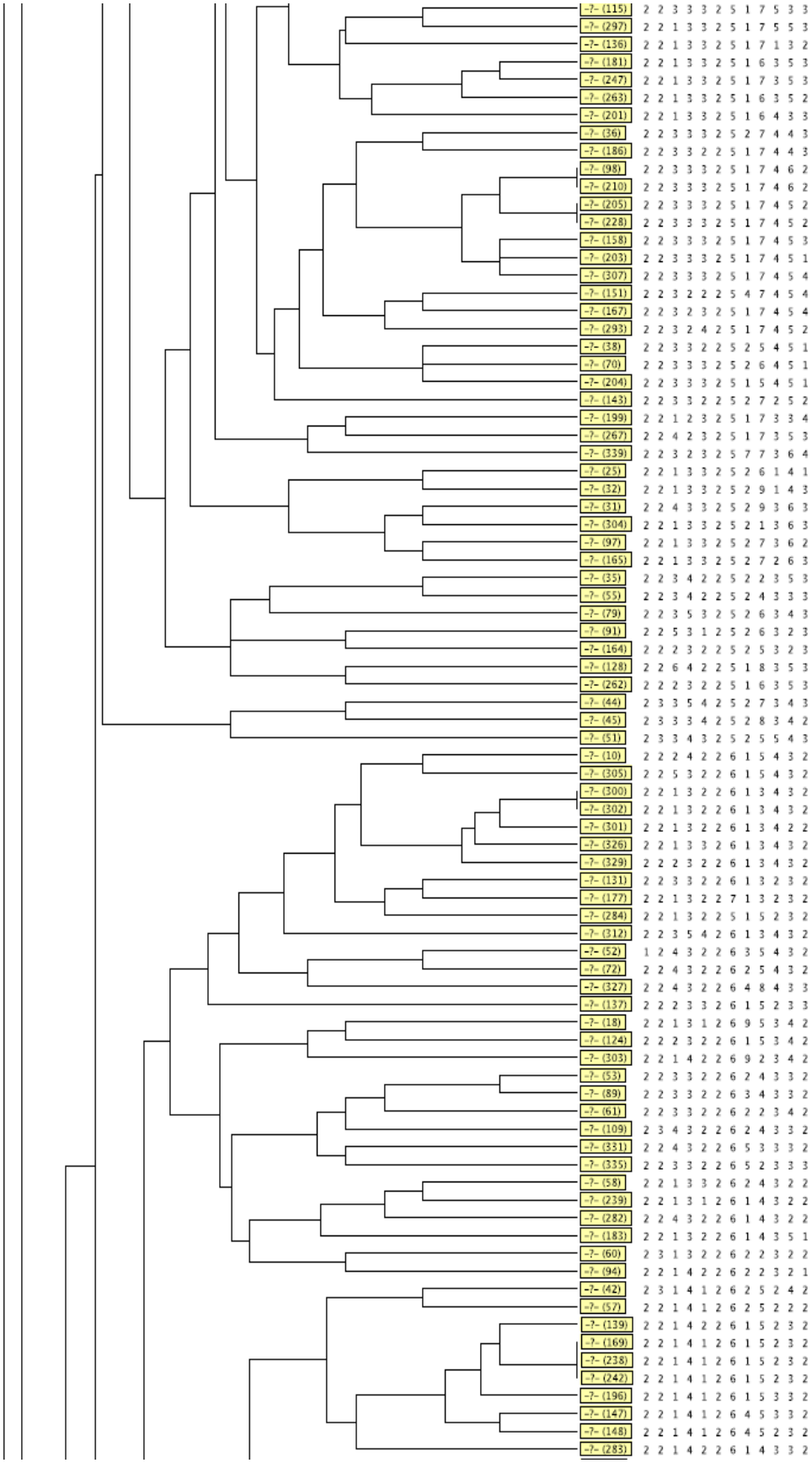


Figure 5.1 Dendrogramme of the 336 drug-resistant *M. tuberculosis* isolates analysed using spoligotyping

UPGMA-Tree, MIRU-VNTR [12]: Categorical





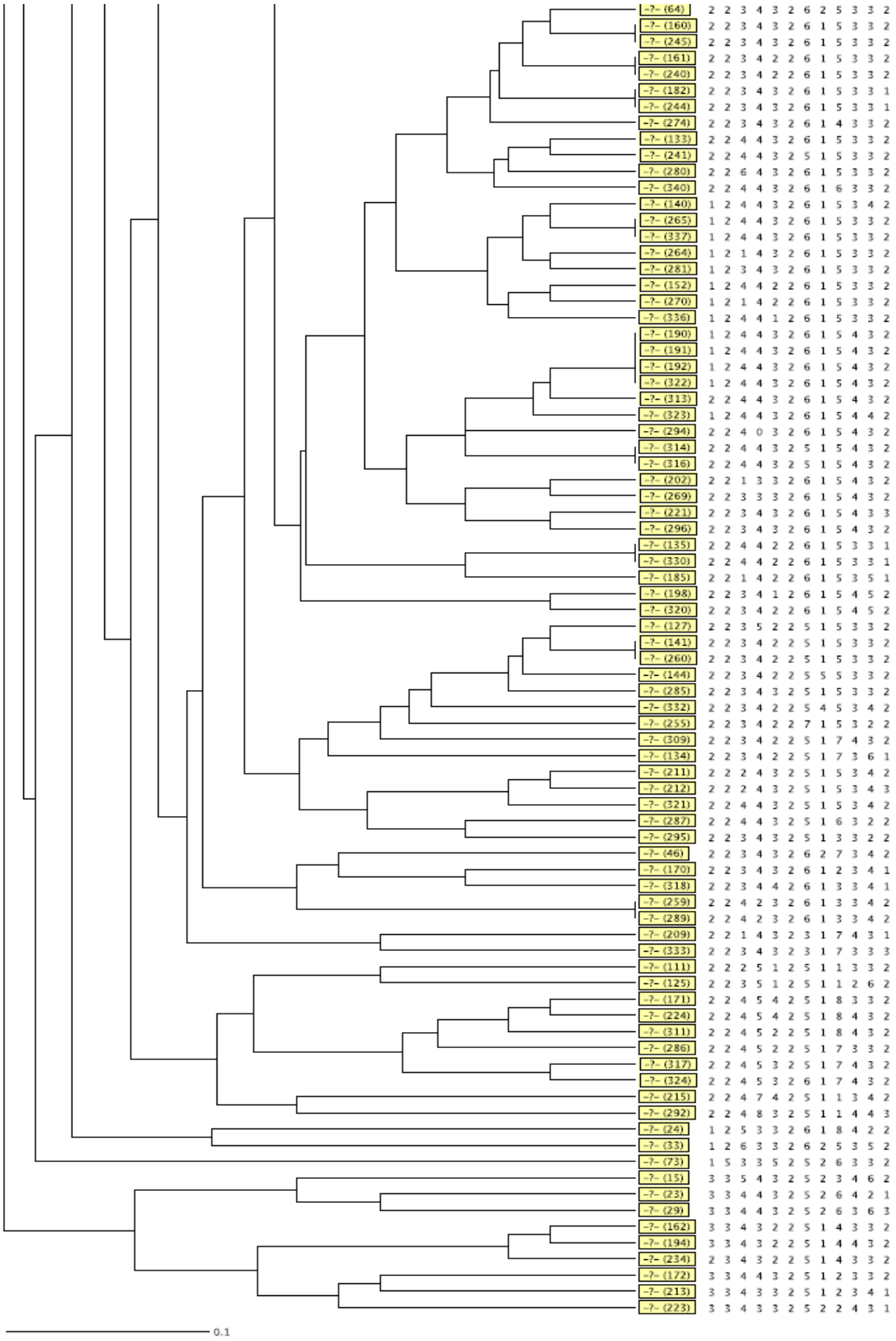
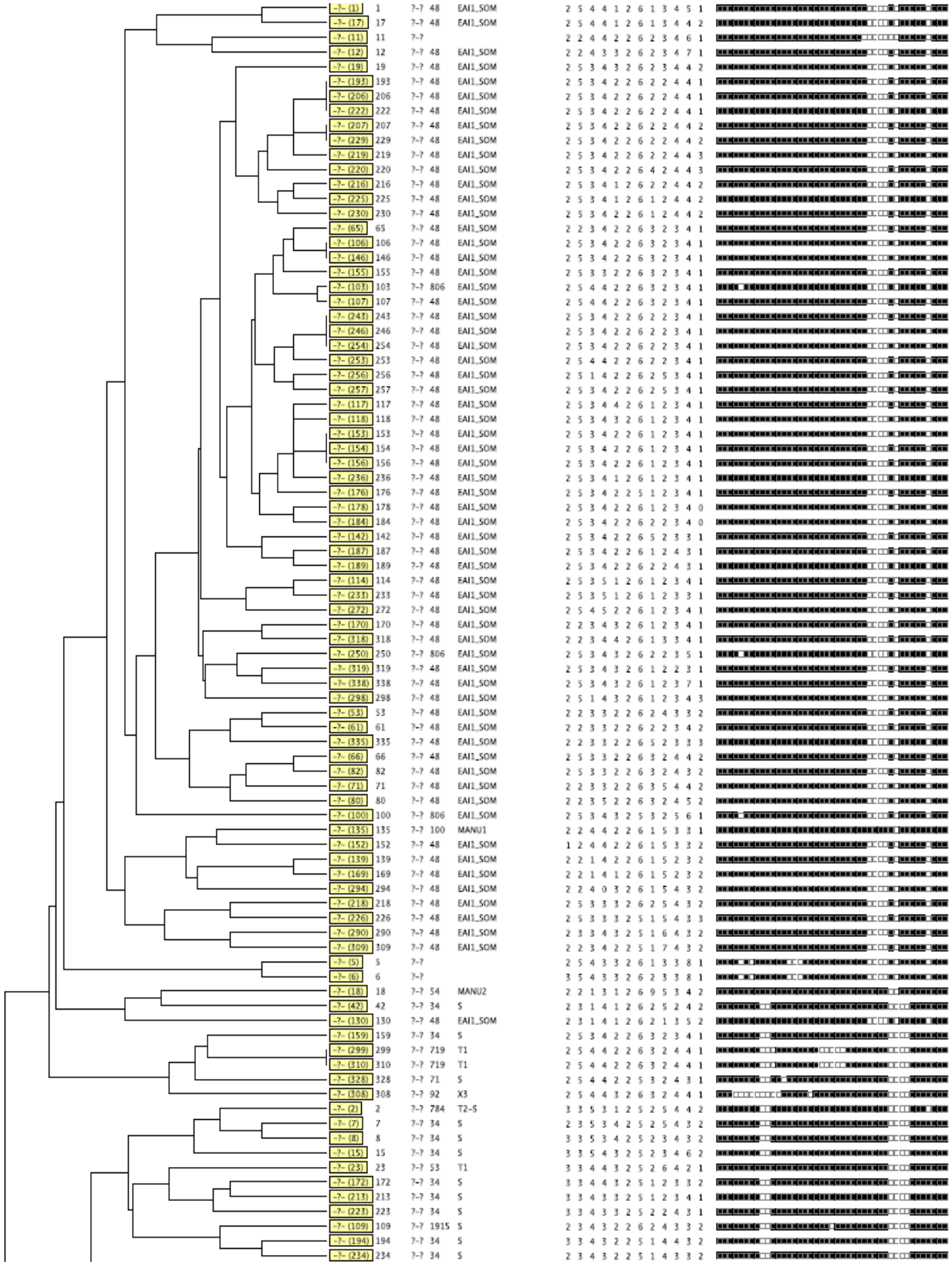
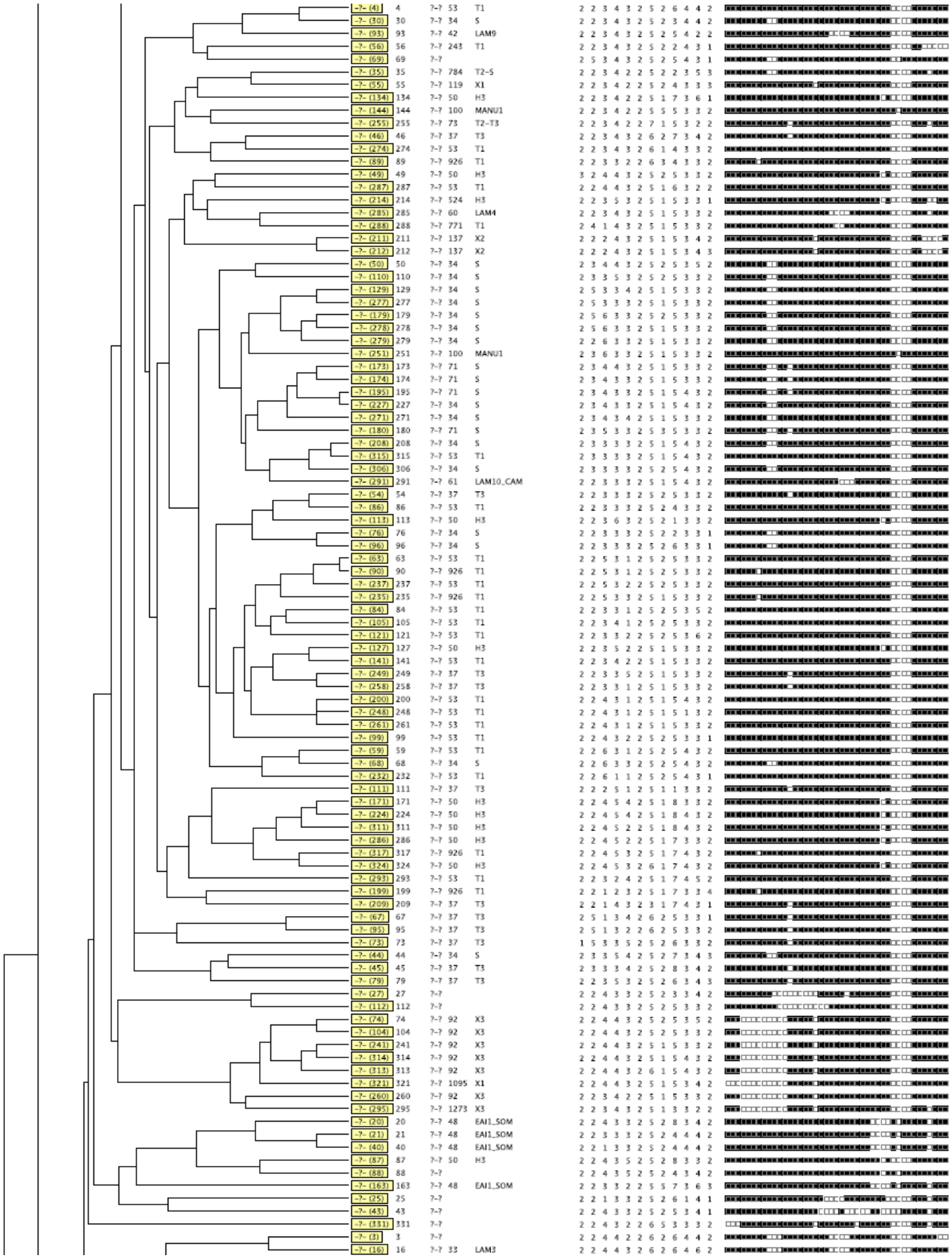
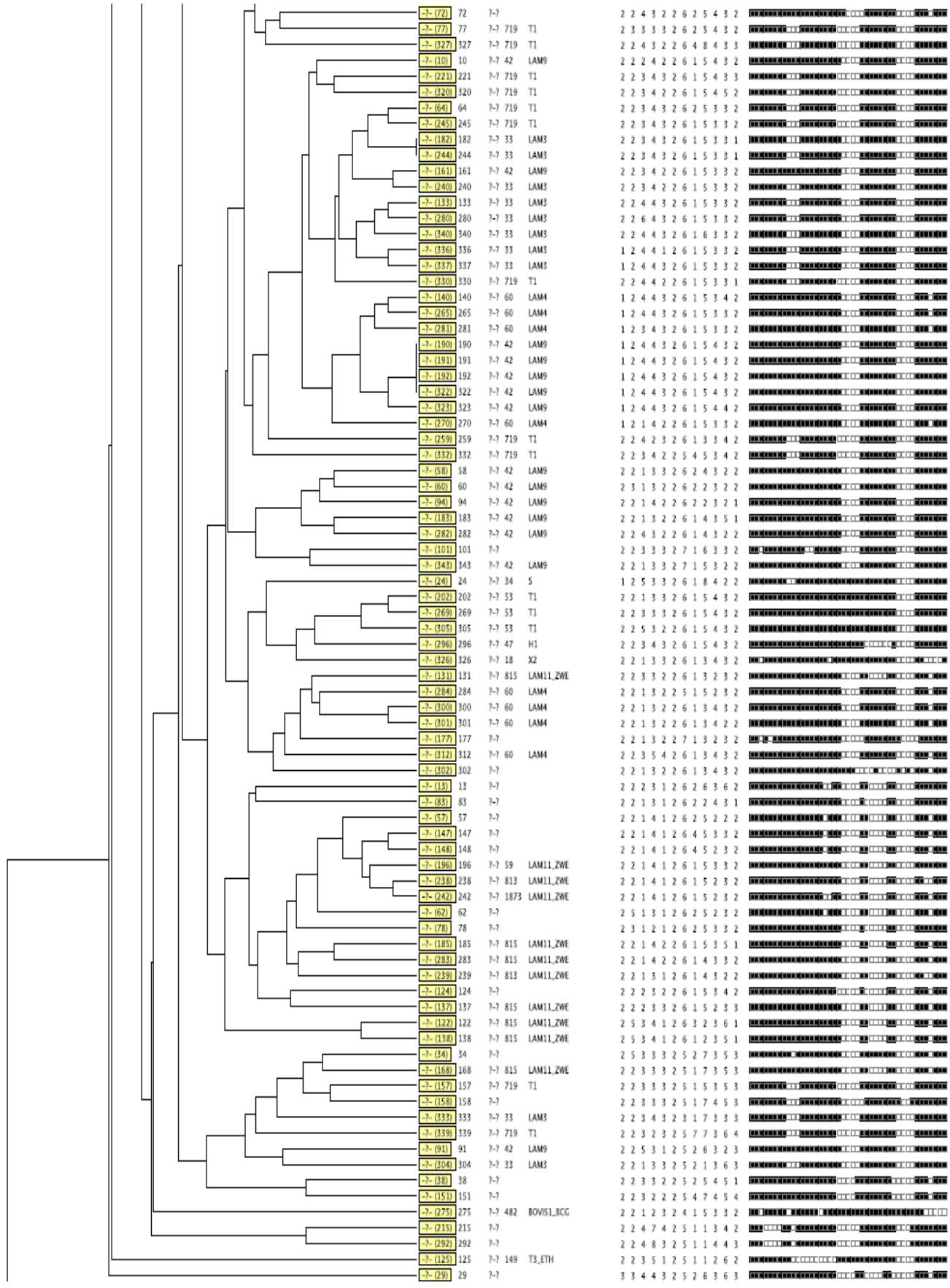


Figure 5.2 **Dendrogramme of the 336 drug-resistant *M. tuberculosis* isolates analysed using mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) typing**

UPGMA-Tree, MIRU-VNTR [12]: Categorical (1), Spoligo: Categorical (1)







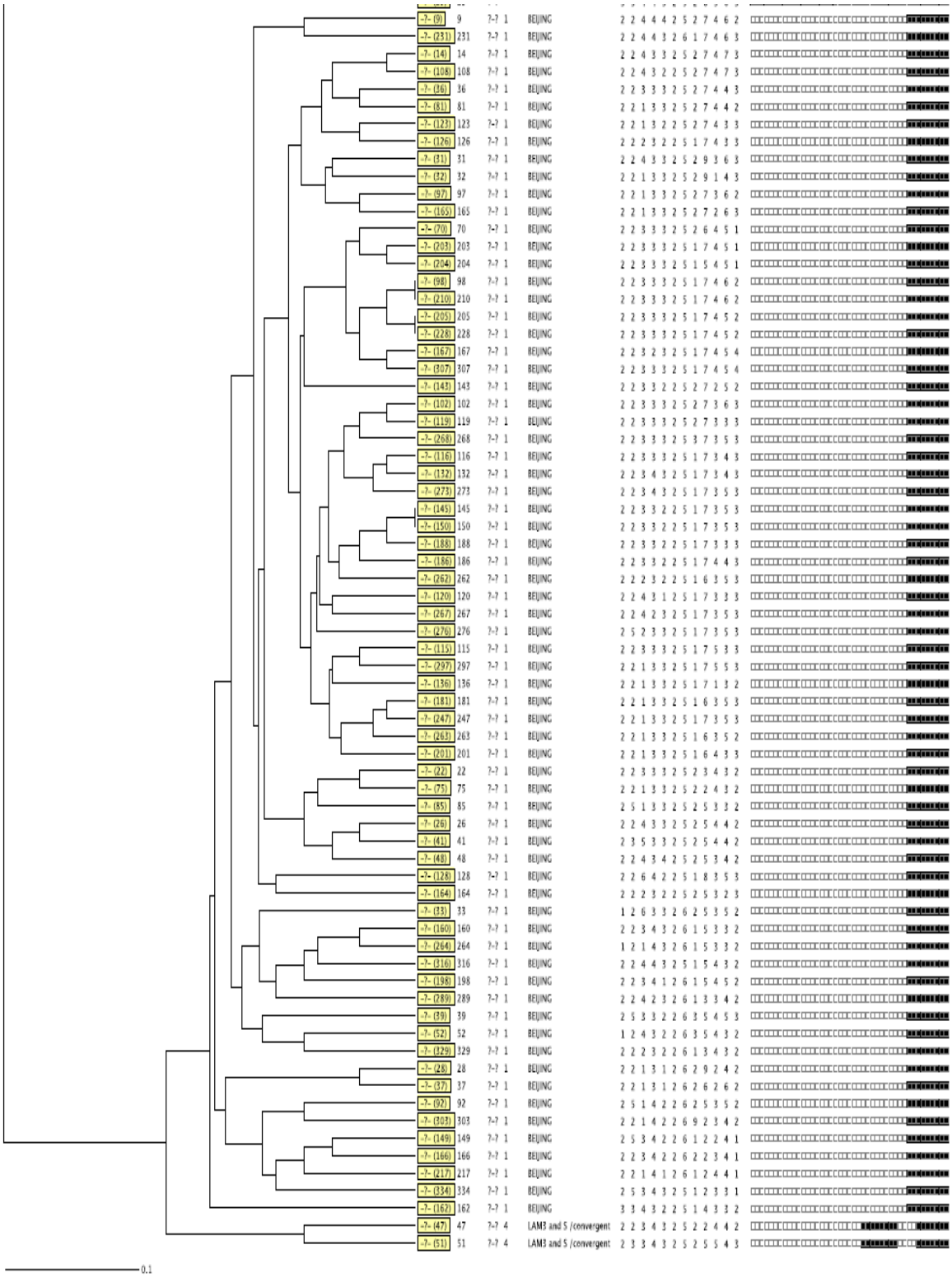


Figure 5.3 Combined dendrogramme of the 336 drug-resistant *M. tuberculosis* isolates analysed using spoligotyping and mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) typing

CHAPTER 6

CONCLUDING REMARKS

Tuberculosis (TB) remains a major public health challenge worldwide. The control of TB is hampered by the high incidence of multidrug-resistance (MDR) and the emergence of extensively drug-resistant (XDR) TB strains. South Africa is ranked as one of the high-burden MDR-TB countries (WHO, 2010a). Standardised and accurate identification of drug-resistance in *M. tuberculosis* is increasingly important for the appropriate management of patients, especially in settings with a high-burden of TB. Furthermore, molecular analysis of *M. tuberculosis* strains for epidemiological studies is critical elements in battling the MDR and XDR-TB epidemic.

Theme I: To evaluate phenotypic susceptibility testing for first- and second-line anti-TB drugs

It is well-known that the accuracy of drug susceptibility testing (DST) varies between the drugs tested. In first-line anti-TB drugs, it is most accurate for RIF and INH and less reliable and reproducible for ethambutol (EMB) and streptomycin (STR) (Woodley, 1986; Laszlo, 1999; Johnson *et al*., 2006; O'Grady *et al*., 2011). Second-line DST is complex and is not well standardised (Shah *et al*., 2007; Richter *et al*., 2009; O'Grady *et al*., 2011). Therefore, the aim of this study was to compare the performance of the BACTEC MGIT 960 system with the agar proportion method (gold standard) for routine testing of MDR-TB isolates against EMB, STR and two second-line drugs: kanamycin (KAN) and ofloxacin (OFX).

The sensitivity of the BACTEC MGIT 960 system was excellent for OFX (100%) and STR (95%) when compared to the agar proportion method. The specificity of the BACTEC MGIT 960 system was lower for STR than expected and requires further evaluation. However, the sensitivity of the BACTEC MGIT 960 system was considerably lower for KAN (54%) and for EMB (18.8%). The WHO recommends the use of the BACTEC MGIT 960 system and the line probe assays for first-line anti-TB drug susceptibility testing. The BACTEC MGIT 960 system is the gold standard by the WHO for second-line anti-TB drugs (WHO, 2010b). As some liquid culture DST was reported to be more sensitive and reliable than solid culture

DST for second-line anti-TB testing, especially for aminoglycosides, polypeptides and fluoroquinolones (Martin *et al*., 2008; Lin *et al*., 2009).

The high discrepancy rate between the BACTEC MGIT 960 system and the agar proportion method for EMB testing is not unique for this study. Phenotypic drug susceptibility testing for EMB is difficult and results are not always reliable (Laszlo, 1999; Johnson *et al*., 2006). This could have a significant impact on the TB control programme as EMB is used for treating MDR-TB (WHO, 2010b). Accurate and standardised testing of EMB is therefore, critical in deciding its use in the MDR-TB treatment regimen. New, reliable testing methods are needed.

Theme 2: **To evaluate a molecular assay for the detection of resistance to second- line anti-TB drugs**

With the global rise in MDR- and XDR-TB strains, there is an increasing need to determine susceptibility of *M. tuberculosis* to first- and second-line anti-TB drugs. In this study the GenoType® MTBDR*sl* assay was evaluated against the agar proportion method for the detection of resistance to OFX, KAN, CAP and EMB. It is critical to evaluate the performance of a newly developed assay in a specific setting prior to the implementation of a test especially for molecular assays. Differences in the prevalence of mutations in drug-resistant isolates in diverse geographical regions can pose challenges, especially if the assay relies on detecting a limited number of mutations.

The GenoType® MTBDR*sl* assay is based on a multiplex PCR assay followed by reverse hybridisation with specific DNA probes for the rapid detection of fluoroquinolone (FLQ), CAP/amikacin (AMK)/KAN/viomycin (VIO) and EMB resistance by targeting the *gyr*A, *rrs* and *emb*B genes, respectively (Hillemann *et al*., 2009). The assay is rapid and results were available within an average of two days, which is significantly shorter than the agar proportion method, which took 21 days. The results of the GenoType® MTBDR*sl* assay for the detection of OFX resistance was promising (70%) and was comparable with other studies (Hillemann *et al*., 2009; Brossier *et al*., 2010; Huang *et al*., 2010; Kiet *et al*., 2010). However, the assay was less sensitive for the detection of KAN/CAP resistance and demonstrated a low sensitivity and specificity for EMB resistance. Previous studies have reported a sensitivity and specificity ranging between 70% to 90% for the detection of resistance to OFX, 77% to 100% for KAN, 80.0% to 86.7% for CAP and 56% to 69% for

EMB (Hillemann *et al*., 2009; Brossier *et al*., 2010; Huang *et al*., 2010; Kiet *et al*., 2010). Most of the studies; however, included a limited number of drug-resistant isolates (53 to 64) (Hillemann *et al*., 2009; Brossier *et al*., 2010; Kiet *et al*., 2010). In this study 342 of MDR-TB isolates have been used to evaluate the GenoType® MTBDR*sl* assay.

To resolve the discrepant results between the agar proportion method and the GenoType® MTBDR*sl* assay, DNA sequencing of the *gyr*A and *gyr*B (OFX) in the quinolone resistance determining region (QRDR), *rrs* and *tly*A (CAP), *rrs* (KAN) and *emb*B (EMB) genes was performed. A total of 18 MDR-TB isolates showed discrepant results for OFX. Seven of these isolates were resistant to OFX according to the GenoType® MTBDR*sl* assay but susceptible by the agar proportion method while the remaining 11 isolates were resistant by the agar proportion method, but susceptible according to the GenoType® MTBDR*sl* assay (Table 4.1). Resolution of the discrepant results based on DNA sequencing showed mutations in the *gyr*A gene in all seven discrepant isolates found to be resistant by the GenoType® MTBDR*sl* assay, with one isolate showing additional mutations in the *gyr*B gene. However, the GenoType® MTBDR*sl* assay failed to detect 8/11 phenotypically OFX-resistant isolates shown to have mutations in the *gyr*A gene (two of the eight had additional mutations in the *gyr*B gene) by DNA sequencing. In addition 1/11 (phenotypically an XDR-TB isolate) had a mutation only in the *gyr*B gene and was not detected by the GenoType® MTBDR*sl* assay as the *gyr*B gene is not included in the assay.

Discrepant results for KAN included four resistant isolates according to the GenoType® MTBDR*sl* assay but susceptible by the agar proportion method. Twenty-one isolates, however, were phenotypically resistant to KAN but susceptible by the GenoType® MTBDR*sl* assay. The results of DNA sequencing agreed with the GenoType® MTBDR*sl* assay, since mutations were found in all four discrepant isolates. However, among the 21 discrepant isolates, six isolates (including three phenotypically XDR-TB isolates) had mutations in the *rrs* gene according to DNA sequencing and were not detected by the GenoType® MTBDR*sl* assay (Table 4.4).

In the case of CAP, four isolates were resistant to the CAP according to the GenoType® MTBDR*sl* assay but susceptible by the agar proportion method. The DNA sequencing of the *rrs* gene showed mutations in all four discrepant isolates found to be resistant by the GenoType® MTBDR*sl* assay. Twenty-six isolates were phenotypically resistant to CAP but susceptible by the GenoType® MTBDR*sl* assay. The DNA sequencing identified mutations

in 7/26 discrepant isolates (including three phenotypically XDR-TB isolate), which were not detected by the GenoType® MTBDR*sl* assay. An additional gene, *tly*A, associated with CAP resistance was sequenced for all the discrepant isolates. The DNA sequencing of the *tly*A gene showed mutation in 20/26 isolates phenotypically resistant to CAP but susceptible by the GenoType® MTBDR*sl* assay. In addition, a *tly*A mutation was found in 3/4 CAP-resistant isolate by the GenoType® MTBDR*sl* assay but susceptible by the agar proportion method. Previous studies reported that mutations within the *tly*A gene are uncommon and are mainly associated with *in-vitro* selected CAP-resistant mutants and are rare in clinical isolates of *M. tuberculosis* (Brossier *et al*., 2010; Via *et al*., 2010). This could be due to the limited use of CAP to treat *M. tuberculosis* infections in South Africa (Brossier *et al*., 2010; Via *et al*., 2010). Therefore, the role of the *tly*A gene in CAP-resistant clinical *M. tuberculosis* isolates should be further investigated.

A high discrepancy was found between the GenoType® MTBDR*sl* assay and the agar proportion method for EMB. In the case of EMB, most of the discrepant results were susceptible by the agar proportion method and resistant according to the GenoType® MTBDR*sl* assay. A total of 84.8% (268/316) isolates was phenotypically susceptible to EMB and 56.0% (150/316) showed wild-type patterns and the remaining 44.0% (118/316) showed mutations with the GenoType® MTBDR*sl* assay. Forty-eight of the 316 isolates (15.2%) were phenotypically resistant to EMB. However, only 56.3% (27/48) showed mutations with the GenoType® MTBDR*sl* assay while the remaining 43.7% (21/48) showed wild-type patterns. The DNA sequencing identified mutations in only 56.8% (67/118) discrepant isolates resistant by GenoType® MTBDR*sl* assay, while the remaining 43.2% (51/118) isolates showed no mutation (Table 4.4). The discrepancy could be due to the difference in the target gene analysed by DNA sequencing. It should be noted that, this study as well as previous studies, which evaluated the GenoType® MTBDR*sl* assay reported a low sensitivity for EMB (Hillemann *et al*., 2009; Brossier *et al*., 2010; Huang *et al*., 2010). This finding indicates that the *emb*B gene is not an accurate marker for the detection of EMB resistance. This finding may also reflect the shortcomings of the breakpoints and different performances of conventional drug susceptibility testing for EMB (Parsons *et al*., 2004; Parsons *et al*., 2011).

The poor performance of the GenoType® MTBDR*sl* assay in this study could be due to differences in the frequency of mutations in drug-resistant *M. tuberculosis* isolates in this

region, which can affect the test performance. A better understanding of the geographical differences and the prevalence of mutations related to *M. tuberculosis* resistance is critical for the development of new diagnostic tools for drug-resistant *M. tuberculosis*.

Based on the finding of this study, the GenoType® MTBDR*sl* assay is not recommended for use for second-line anti-TB testing in its current format. The inclusion of additional better genetic markers associated with KAN, CAP and EMB resistance may improve the performance of the assay and would allow the implementation in routine diagnostic testing, especially where line-probe assays (GenoType® MTBDR*plus*) are established as part of the diagnostic algorithm. This might provide a means for rapid detection of XDR-TB.

Theme III: To genotype drug-resistant *M. tuberculosis* isolates and to determine the transmission patterns as well as the association with risk factors

Successful control of TB requires methods that will help in improving the current knowledge and understanding of the population structure, diversity and prevalence of predominant circulating drug-resistant *M. tuberculosis* strains that drives the epidemic. In this study MDR-TB isolates collected from Provinces were characterized Africa using spoligotyping and MIRU-VNTR typing.

A high diversity of strain families was found among the MDR-TB isolates studied; indicating the lack of clonal spread of a specific strain in the northern provinces. The Beijing and the EAI (EAI1_SOM) families were the most prevalent families identified. The predominance of the Beijing family is well recognized in the Western Cape province (Brudey *et al*., 2006; Johnson *et al*., 2010). This is a reflection of the Beijing family's ability to adapt to the human host-population in South Africa. The EAI1_SOM family is most prevalent in East Africa and was first identified in Somalia (Brudey *et al*., 2006). The high frequency of this genotype in this region could be due to the migration of people from East Africa toward Southern Africa.

The genotyping results of this study showed that spoligotyping and MIRU-VNTR typing can be used effectively for molecular epidemiological studies. The use of spoligotyping alone is insufficient to differentiate between clustered *M. tuberculosis* isolates. A secondary typing method is required to accurately define clustering strains. According to the literature, the use of combined typing methods, such as spoligotyping with IS*6110*-RFLP or MIRU-VNTR typing along with conventional epidemiological data can provide an improved overview of

TB transmission dynamics in a given setting. In this study spoligotyping overestimated the link between *M. tuberculosis* isolates; grouping 90.8% (305/336) isolates into 25 clusters. The MIRU-VNTR typing reduced the epidemiological link between the *M. tuberculosis* isolates. Only 6.8% (23/336) isolates were grouped in 11 clusters. The combined typing of spoligotyping and MIRU-VNTR typing further reduced the epidemiological link, grouping only 5% (17/336) isolates into eight clusters. Interestingly, the discriminatory power (0.9977) and clustering rate of MIRU-VNTR (MIRU-12) typing (3.6%) alone was approximately close to the discriminatory power (0.9998) and clustering rate (2.7%) of the combination typing with spoligotyping and MIRU-VNTR typing.

The integration of drug susceptibility testing with genotyping and epidemiological data represents a useful approach to study the epidemiology of drug-resistant TB in a specific region. Drug susceptibility testing was done for three second-line anti-TB drugs including CAP, KAN and OFX and a possible association with clustering were explored. Out of the 336 MDR-TB isolates tested; 4.8% (16/336) were resistant to OFX, 2.7% (9/336) to KAN, 4.5% (15/336) to CAP and 7.1% (24/336) were XDR and the remaining 83.6% (281/336) were susceptible to three the second-line anti-TB drugs tested. The prevalence of the XDR-TB (7.1%) in this study was within the global range (3% to 19%) of the reported prevalence among MDR-TB patients (CDC, 2006; CDC, 2007). However, the high proportion (9.5%) of MDR-TB isolates, which were resistant to a single marker (pre-XDR which is resistant to OFX, KAN or CAP) of XDR-TB raises concerns. Appropriate management of patients with pre-XDR is important to minimize subsequent development of resistance to XDR-TB. The association between genotypes and drug-resistance or demographic characteristics (age and gender) was not significant ($p>0.05$).

The comparison of a specific genotype (cluster) with the geographical origin of the isolates in this study has shown low transmission in the norther region of the country. Furthermore, the high diversity of drug-resistant genotypes indicates that transmission of drug-resistant strains in this setting is not related to the clonal spread of a specific *M. tuberculosis* strain. The drug-resistant epidemic could be through acquisition. Nevertheless, population-based studies are needed to assess this possibility more in detail. These findings of this study provide a baseline for future analysis. More studies on other risk factors, such as the impact of genotypes on the presentation of disease and the response to treatment as well as the association of genotypes with HIV status is also needed.

Genotyping studies are helpful in identifying shortcomings in the national TB control programmes in guiding the decision-making process for TB control and to develop appropriate strategies. It is recommended that greater vigilance is required to contain the drug-resistant epidemic and the national TB control programmes 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.

Future Research

The increasing incidence of MDR and XDR-TB around the world, necesitaties the need for accurate, standardised methods for susceptibility testing of first and second-line anti-TB drugs. Progress has been made over the past decade to improve TB diagnostics and develop new technologies. Some of the methods have been endorsed by the WHO such as the automated liquid culture systems (BACTEC MGIT 960 system) for culture and DST (WHO, 2007) and the use of line-probe assays for the rapid molecular detection of drug-resistance to first-line anti-TB drugs (WHO, 2008). However, these methods are more expensive, require costly equipment and are found to be technically demanding. Over 90% of the worldwide burden of TB is in low-income and middle-income countries. Therefore, there is a need for cheap point-of-care, easy to implement diagnostic tools that would support global TB control in this era of MDR and XDR-TB.

The ideal TB diagnostic test should be a simple, rapid, point-of-care test that could simultaneously identify drug-resistance. No existing test meets all of these specifications. The Xpert MTB/RIF system seems promising as it is easy to perform and a highly sensitive molecular assay (Parsons *et al*., 2011). It needs minimum laboratory expertise and results are available in less than 2 h, which permits a specific TB diagnosis and rapid detection of RIF resistance (Bonnet *et al*., 2007; Helb *et al*., 2010; Parsons *et al*., 2011). However, the Xpert MTB/RIF system is still expensive and therefore not suitable for resource poor countries (Parsons *et al*., 2011). If the price is reduced, it will represent a major breakthrough in rapid TB diagnostics and for RIF resistance screening.

Control of TB requires methods for tracing sources of infection, so that further transmission can be prevented. Molecular epidemiology studies have proven to be valuable tools to evaluate transmission dynamics and to provide more information on the distribution of the different *M. tuberculosis* genotypes. Although the IS*6110*-RFLP typing is still the gold

standard, alternative PCR-based methods, such as spoligotyping and MIRU-VNTR typing are commonly used for epidemiological studies. Initially, MIRU-VNTR involved a 12-loci set which was considered efficient for epidemiological purposes (Supply *et al*., 2001; Sun *et al*., 2004). Currently, the MIRU-VNTR typing based on 15 and 24 loci is recommended for population-based studies. The use of the 24 loci based MIRU-VNTR typing combined with spoligotyping can increase the discriminatory power to reach or exceed that of IS*6110*-RFLP typing (Supply *et al*., 2006; Oelemann *et al*., 2007; Allix-Beguec *et al*., 2008; Maes *et al*., 2008).

Molecular epidemiology techniques are moving toward real-time techniques, which will make it possible to take advantage of cluster data for epidemiological research. The initial design of MIRU-VNTR typing, which was based on simplex PCR has been converted to a high-throughput format using a multiplex fluorescence-labelled PCR and bioanalyzer system (Supply *et al*., 2001; Supply *et al*., 2006). New approaches to improve the standard spoligotyping method are currently under evaluation to replace the time-consuming membrane step. These include: i) the microsphere (bead)-based laser technology (Luminex, Austin, Texas, USA), which permits the identification and quantification of each PCR product as an alternative to reverse line-blot hybridisation (Zhang *et al*., 2010), ii) a novel automated MALDI-TOF mass spectrometry (MALDI-TOF MS) method for detection by replacing the hybridisation step with a multiplexed primer extension assay (Honisch *et al*., 2010). This assay is based on PCR and a multiplexed primer extension assay followed by MALDI-TOF MS detection on the MassARRAY® system (Sequenom, Inc.) and streamlines sample processing by avoiding extensive washing steps and microsphere conjugation (Honisch *et al*., 2010). It is also reported that the next-generation DNA sequencing will play an important role in improving the understanding of TB epidemiology (Mardis, 2008). Whole-genome sequencing could potentially become the new gold standard for strain typing in routine molecular epidemiology (MacLean *et al*., 2009).

The development and validation of new tools for rapid DST are needed, since there is no stand-alone (single) test for the diagnosis of MDR-TB and XDR-TB. Priority should be given to standardisation of second-line anti-TB drug suceptibility testing. New methods are needed for drugs, such as EMB, STR and PZA for which conventional DST is unreliable. More research is needed on the molecular basis of resistance especially regarding second-line anti-TB drugs and the role of molecular techniques in improving/replacing conventional DST

methods. There is still a need of alternative simple, fast and reliable genotyping methods, since the available genotyping methods are expensive and tecnichally demanding.

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APPENDIX A

DETAILED METHODOLOGY

1. Sub-culturing of MDR-TB isolates

All the MDR-TB isolates were sub-cultured in MGIT medium according to the standard procedures of the BACTEC MGIT 960 system (Becton Dickinson, Sparks, Md). In brief, the BACTEC MGIT 960 medium contains 7 ml Middlebrook 7H9 broth base and a fluorescent sensor that detects the concentration of oxygen in the culture medium. A 0.8 ml of BACTEC MGIT 960 supplement (oleic acid-albumin-dextrosecatalase, Becton Dickinson, Sparks, Md) and 0.5 ml of the suspension containing MDR-TB isolates was added to the MGIT culture tubes and were incubated in the BACTEC MGIT 960 system until a positive signal was flagged. The fluorescence due to bacterial growth in the MGIT tube is continuously monitored. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. Initially little fluorescence can be detected but actively growing and respiring mycobacteria consume the oxygen, which allows the compound to emit fluorescence. The relative growth ratio is determined by the system's software algorithm.

2. Drug susceptibility testing using the agar proportion method

Drug susceptibility testing for KAN, OFX, CAP and EMB using the agar proportion method was performed according to the Clinical and Laboratory Standards Institute (CLSI) (2003) procedures and recommended critical concentrations. Briefly, six welled Petri plates of Middlebrook 7H11 medium (TB Diagnostic Services, South Africa) was used. Two quadrants in each plate contained drug-free medium, one was used as proportional control and the other was used as a quality control. A standard strain of *M. tuberculosis* H37Ra was used as a quality control. The other four quadrants contained the following drug concentrations: KAN (5 µg/ml), OFX (2 µg/ml), CAP (10 µg/ml) and EMB (7.5 µg/ml). Each quadrant was inoculated with the standard inoculum of 0.1 ml of suspension and the inoculum was distributed by tilting the plate. A 0.1 ml of the 1:100 dilution was used to inoculate the proportional control. The plates were sealed in a plastic bag and were incubated at 37°C. An MDR-TB isolate was classified as resistant when the number of colonies (growth) on the drug-containing quadrant was ≥1% as compared to drug-free medium.

3. DNA extraction

Mycobacterial genomic DNA was extracted from cultured *M. tuberculosis* isolate using the Amplicor Respiratory Specimen Preparation Kit (Roche Molecular System, Germany) as described by the manufacturer. Briefly, 100 µl of the 7H9 broth culture was transferred into a 2 ml Eppendorf tube (LASEC, South Africa) and 500 µl wash solution was added and the mixture was vortexed for 5 sec. The suspension was centrifuged (Spectrafuge, Labnet International, Inc) at 12 500 *x g* for 20 min to pellet the cells. The supernatant was discarded and the pellet was re-suspended in 100 µl lysis buffer and was incubated at 60°C in a dry heating block (AccuBlock Digital Bath, Labnet International, Inc) for 45 minutes. Finally, 100 µl of neutralisation reagent was added to the suspension and the tubes were vortexed for 5 sec. The DNA sample was stored at -20°C until further analysis.

4. GenoType® MTBDR*sl* assay

The GenoType® MTBDR*sl* assay (Hain Lifescience, Germany) was performed as described by the manufacturer. The assay is based on a conventional multiplex PCR assay and reverse-hybridisation to probes immobilised on a plastic strip.

4.1 PCR amplification

Briefly, the PCR amplification reaction was performed in a 50 µl reaction volume composed of 35 µl of primer-nucleotide-mix (PNM) (Hain Lifescience, Germany), amplification buffer containing 2.5 mM $MgCl_2$, 1.25 U Hotstart *Taq* polymerase (Qiagen, Germany) and 5 µl of DNA template. Amplification was performed in a Perkin-Elmer thermocycler (Applied Biosystems, USA). The PCR amplification protocol consisted of a 15 min activation step at 95°C, followed by 10 cycles comprising of 30 sec at 95°C and 120 sec at 58°C, an additional 20 cycles comprising of 25 sec at 95°C, 40 sec at 53°C and 40 sec at 70°C and a final extension step at 70°C for 8 min.

4.2 Hybridisation of PCR products

Hybridisation was performed with an automated hybridisation GT Blot 48 system (Hain Lifescience GmbH, Nehren, Germany) according to the manufacturer's instruction. Briefly, 20 µl of PCR product were mixed with 20 µl of the denaturation solution (DEN) in the hybridisation well and incubated at room temperature (24°C) for 5 min. The GenoType®

MTBDR*sl* strips were introduced into the wells. The hybridisation tray was placed gently into the pre-heated automatic hybridisation GT Blot 48 system (Hain Lifescience GmbH, Nehren, Germany), which perfomed the hybridisation of the single-stranded biotin-labelled amplicons to the membrane-bound probes at 45°C for 30 min, followed by stringent washing and addition of a streptavidin-alkaline phosphatase (AP) conjugate at room temperature (24°C) for 30 min and an AP staining reaction to detect the colorimetric bands. Following the hybridiation step, the strips were removed and allowed to air dry and were fixed on paper for interpretation.

The GenoType® MTBDR*sl* strip contains 22 probes, including PCR amplification and hybridisation controls to verify the test procedures. In addition to the *M. tuberculosis* specific control (TUB) band, all targeted regions, *gyr*A, *rrs* and *emb*B have locus control bands. Three *gyr*A wild-type probes (WT1, WT2 and WT3) were included for the detection of FLQ-resistance which encompasses the region of the gene encoding amino acids 85 to 97. Additionally the strip have six probes, *gyr*A MUT1 (A90V), *gyr*A MUT2 (S91P), *gyr*A MUT3A (D94A), *gyr*A MUT3B (D94N/Y), *gyr*A MUT3C (D94G) and *gyr*A MUT3D (D94H) specifically targeting the most common mutations associated with resistance. Two wild-type probes covering the region of the *rrs* gene were included, while two probes *rrs* MUT1 (A1401G) and MUT2 (G1484T) were designed to assess the presence of mutations. The *emb*B wild-type probe WT1, which targets the *emb*B 306 codon were included for the detection of EMB-resistance while the *emb*B MUT1A and *emb*B MUT1B probes were designed to bind to the following mutations, ATG/ATA (M306I) and ATG/GTG (M306V). Either the omission of a wild type probe or the presence of a mutant probe was indicative of a resistant *M. tuberculosis* isolate.

5. PCR amplification of target genes for DNA sequencing

The following target genes in which mutations most frequently associated with resistance were amplified: *gyr*A and *gyr*B for OFX, *rrs* for KAN and CAP and *emb*B for EMB resistance. The primer sequences are shown in Table 5.1. Amplification was performed using the Maxim HotStart Green PCR Master Mix (Fermentas, Thermo Scientific, USA) according to the manufacturer's instructions. Briefly, the amplification reaction was performed in a 25 µl reaction volume composed of 12.5 µl of Maxim HotStart Green PCR Master Mix, 1.25 µl of each primer, 1 µl of template DNA and 9 µl of deionised water. Amplification was performed in a G-storm thermocycler (Somerton Biotechnology Centre,

United Kingdom) using the recommended cycle parameters: 4 min at 95°C, 40 cycles of 30 sec at 95°C, 30 sec at 55°C and 1 min at 72°C, followed by a final extension step at 72°C for 10 min.

Table 5.1: Primer sequences for amplification and DNA sequencing of target genes

Drug	Gene	Sequence (5'-3')	Amplicon size (bp)	References
OFX	*gyr*A	F YGGTGGRTCRTTRCCYGGCGA R CGCCGCGTGCTSTATGCRATG	250	Dauendorffer *et al*., 2003
	*gyr*B	F GAGTTGGTGCGGCGTAAGAGC R CGGCCATCAAGCACGATCTTG	250	
KAN/CAP	16S (rrs)	F AGAGTTTGATCCTGGCTCAG R ACGGCTACCTTGTTACGACTT	1500	Weisburg et al., 1991
CAP	*tly*A	F GGCATCGCACGTCGTCGTCTTTCCGAGG R GGACGACCAGCAGAACACTGCGATG	820	Designed in this study
EMB	*emb*B	F CCGACCACGCTGAAACTG R GTAATACCAGCCGAAGGGATCCT	400	Jain *et al*., 2008

OFX=ofloxacin, KAN=kanamycin, CAP=capreomycin, EMB=ethambutol, bp=base pairs

6. Spoligotyping of drug-resistant *M. tuberculosis*

Spoligotyping was performed using a commercial kit (Ocimum BioSolution, India) as described by the manufacturer. The method is based on PCR amplification of the direct repeats of *M. tuberculosis* isolates, followed by hybridisation to a membrane containing covalently linked oligonucleotides that correspond to the various spacer sequences.

6.1 Preparation of solutions used for spoligotyping

1. 0.5 M EDTA (pH 8.0): 186.12 g EDTA (Promega, Madison, USA) and 800 ml distilled water (pH adjusted to 8.0).
2. 20 mM EDTA, pH 8.0: Dilute 0.5 M EDTA 25 times.
3. 20xSSPE: 0.2 M $Na_2HPO_4*2H_2O$ (Merck Chemicals, Germany): 35.6 g/l
    - 3.6 M NaCl (Merck Chemicals, Germany): 210.24 g/l
    - 20 mM EDTA (Promega, Madison, USA): 7.4 g/l
    - The pH should be adjusted to 7.4 and autoclavd.
4. 2xSSPE: Dilute 20xSSPE, 10 times with demineralised water.
5. 10% SDS: 10 g SDS (Merck, Chemicals, Germany) and 100 ml distilled water
6. 2xSSPE/0.1% SDS: 100 ml 20xSSPE and 10 ml 10% SDS to 890 ml distilled water.
7. 2xSSPE/0.5% SDS: add 100 ml 20xSSPE and 50 ml 10% SDS to 850 ml distilled water.

6.2 Amplification of the DR region of drug-resistant *M. tuberculosis* strains

Amplification was performed using the HSTaq Master Mix (Qiagen, Hilden, Germany) as described previously (Kamerbeek *et al*., 1997). The direct-repeat region of the mycobacterial genome was amplified using primers DRa (5' biotinylated) and DRb (Kamerbeek *et al*., 1997) (Table 7.1). Briefly, 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. The Master Mix was distributed to a 96-well plate (Lasec, South Africa). *Mycobacterium tuberculosis* H37Rv DNA was used as a positive control while deionized water was used as negative controls. PCR plates were sealed and placed in a Eppendorf mastercycler (Hamburg, Germany) starting with a denaturing step of 15 min 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.

Table 6.1 Primers used for spoligotyping of drug resistance isolates (Kamerbeek *et al*., 1997).

Primer	Primer Sequence (5'–3')	Region
DRa	GGTTTTGGGTCTGACGAC (biotinylated)	DR region
DRb	CCGAGAGGGGACGGAAAC	DR region

6.3 Hybridisation and detection of PCR products

The biotin-labelled PCR products were used as probes to hybridise with 43 synthetic spacer oligonucleotides covalently bound to a membrane (Ocimum BioSolution, India). The presence of spacers is visualised on an X-ray film (Hyperfilm ECL, Amersham, UK) as black squares after incubation with streptavidin-peroxidase (Roche Molecular diagnostics, Germany) and enhanced chemiluminescence detection reagents (ECL-detection) (Amersham, UK). Hybridisation was performed in a 45-lane blotter (Isogen Life Science BV, Utrecht, The Netherlands). The hybridisation procedure was as follows:

1. A volume of 20 µl of the PCR product was added to 150 µl of 2xSSPE/0.1% SDS.
2. The diluted PCR products were heat-denatured at 99°C for 10 min in a heating block (AccuBlock Digital Bath, Labnet International, inc) and cooled on ice immediately.

3. Prior to hybridisation the membrane was washed in 250 ml of 2xSSPE/0.1% SDS for 5 min at 60°C.
4. The membrane and a support cushion were placed into the miniblotter, in such a way that the slots were perpendicular to the line pattern of the applied oligonucleotides.
5. Residual fluid was removed from the slots of the miniblotter by aspiration.
6. The slots of the miniblotter were filled with 150 µl of the diluted PCR products and was placed on the horizontal surface (without shaking) of a water bath (Labcon, South Africa) and was hybridised for 60 min at 60ºC.
7. Following hybridisation the samples were removed from the miniblotter by aspiration and the membrane was removed from the miniblotter using forceps.
8. The membrane was washed twice in 250 ml of 2xSSPE/0.5% SDS for 10 min at 60°C in a shaking water bath (Labcon, South Africa).
9. The membrane was placed in a rolling bottle and allowed to cool down to prevent inactivation of the peroxidase in the next step.
10. A 2.5 µl streptavidin-peroxidase conjugate (500 U/ml) was mixed with 10 ml of 2xSSPE/0.5% SDS and the mixture was added to the membrane in the rolling bottle (Lasec, South Africa).
11. The membrane was incubated for 60 min at 42°C in water bath (Lasec, South Africa).
12. The membrane was washed twice in 250 ml of 2xSSPE/0.5% SDS at 42°C in a shaking water bath for 10 min.
13. The membrane was rinsed twice with 250 ml of 2xSSPE for 5 min at room temperature (24°C).
14. The membrane was incubated for 1 min in 20 ml ECL detection mix (15 ml solution A and 15 ml solution B) for the chemiluminiscent detection of hybridised PCR products.
15. The membrane was covered with a transparent plastic sheet and was exposed to a X-ray film for 20 to 30 min in a Hypercassette Autoradiography Cassettes (Amersham, UK).
16. In case the signal was too weak or too strong the membrane was used again directly to expose another film for a shorter or longer period.
17. The X-ray film was developed and fixed using developer and fixer solutions (AGFA Health care, South Africa) for 10 min. The presence of spacers was visualised on film as black squares.

6.4 Re-use of the membrane

A membrane was re-used for about 10 times after re-generation of the membrane as follows:

7. The membrane was washed twice by incubating in 1% SDS at 80°C for 30 min.
7. The membrane was washed in 20 mM EDTA pH 8, for 15 min at room temperature (24°C).
7. The membrane was stored at 4°C until use (sealed in plastic, to avoid dehydration of the membrane).

7. MIRU-VNTR typing of drug-resistant *M. tuberculosis*

MIRU-VNTR typing was performed using 12 MIRU-VNTR loci (2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39 and 40). The method is based on the amplification of each locus, followed by separation of PCR products using agarose electrophoresis.

PCR amplification of the MIRU-VNTR loci

Each locus was amplified individually for each of the isolates using specific primers as described previously by Supply *et al.* (2001) (Table 8.1) using a protocol described by Le-Fleche *et al*. (2002). Each of the PCR reaction mixes contained 0.5 µl of the specific primers, 10 µl of HSTaq Master Mix (Qiagen, Hilden, Germany) and 7 µl de-iononized water for a final 20 µl reaction volume. The Master Mix was distributed to 96-well plates (LASEC, South Africa). Two microlitre of mycobacterial DNA was added for each sample. The DNA of *M. tuberculosis* H37Rv was used as a positive control while de-ionized water was used as the negative control. The PCR plate was sealed and placed in a Eppendorf mastercycler (Hamburg, Germany) starting with a denaturation step of 1 sec at 95°C, followed by 40 cycles of 5 min at 94°C, 1 min at 62°C and 1 min 50 s at 72°C and a final extension step at 72°C for 10 min.

Preparation of the buffer used for MIRU-VNTR typing

1. Stock solution: 50 X TAE buffer
   - 242 g Tris [2 M] (Sigma Chemical co, USA)
   - 57.1 ml glacial acid [2 M] (Merck, Germany)
   - 100 ml 0.5 M EDTA [0.05 M] (pH 8.0) (Promega, Madison, USA)
2. Working solution: 1X TAE add 100 ml of 50 X TAE to 900 ml distilled water

Separation of PCR product by gel electrophoresis

The PCR products were separated by gel electrophoresis using a 2% agarose (White head Scientific, South Africa) gel. The gels were prepared using 2 g of agarose powder in 100 ml 1 X TAE buffer and were stained with ethidium bromide (10 mg.ml^{-1}) (White head Scientific, South Africa) and were electrophoresed at 85 V/cm (Eilte-300 Power supply, Wealtec Corp., Kennesaw, GA) for 2 h in 1 X TAE buffer (pH 8.0 to 8.5). A 50 bp DNA ladder (Fermentas, Thermo Scientific, USA) was used as a molecular size marker. The gels were visualized under UV illumination (TFM-26 Ultra Transilluminator, UVP, Upland, CA) and the image was captured using a digital gel documentation system (DigiDoc-It Imaging System, UVP, Upland). The PCR fragment size was determined by visual comparison with the molecular marker. The allele calling table in the Supply *et al.* (2006) protocol was used to assign the number of alleles corresponding to the amplicon size. The results from each of the 12 loci were combined to create the 12 digit allelic profile.

Table 7.1: Primers used for MIRU-VNTR typing of drug-resistance isolates (Supply *et al.*, 2001)

MIRU locus	MIRU-VNTR length (bp)	Primer sequence (5'–3')
4	77	GCGCGAGAGCCCGAACTGC
		GCGCAGCAGAAACGTCAGC
26	51	TAGGTCTACCGTCGAAATCTGTGAC
		CATAGGCGACCAGGCGAATAG
40	54	GGGTTGCTGGATGACAACGTGT
		GGGTGATCTCGGCGAAATCAGATA
10	53	GTTCTTGACCAACTGCAGTCGTCC
		GCCACCTTGGTGATCAGCTACCT
16	53	TCGGTGATCGGGTCCAGTCCAAGTA
		CCCGTCGTGCAGCCCTGGTAC
31	53	ACTGATTGGCTTCATACGGCTTTA
		GTGCCGACGTGGTCTTGAT
2	53	TGGACTTGCAGCAATGGACCAACT
		TACTCGGACGCCGGCTCAAAAT
23	53	CTGTCGATGGCCGCAACAAAACG
		AGCTCAACGGGTTCGCCCTTTTGTC
39	53	CGCATCGACAAACTGGAGCCAAAC
		CGGAAACGTCTACGCCCCACACAT
20	77	TCGGAGAGATGCCCTTCGAGTTAG
		GGAGACCGCGACCAGGTACTTGTA
24	54	CGACCAAGATGTGCAGGAATACAT
		GGGCGAGTTGAGCTCACAGAA
27	53	TCGAAAGCCTCTGCGTGCCAGTAA
		GCGATGTGAGCGTGCCACTCAA

bp=base pairs

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Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rüsch-Gerdes S, Willery E, Savine E, de Haas P, Van Deutekom H, Roring S, Bifani P, Kurepina N, Kreiswirth B, Sola C, Rastogi N, Vatin V, Gutierrez MC, Fauville M, Niemann S, Skuce R, Kremer K, Locht C and Van Soolingen D (2006) Proposal for standardsation of optimised mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology* 44:4498–510.

APPENDIX B

DATA AND DETAILED RESULT

Table 1: Demographic and geographical information of patients included in this study

No.	Age	Gender	Location		Region
1	31	F	STA	Standerton Hospital	Mpumalanga
2	33	F	BON	Bongani Hospital, Hazyview	Mpumalanga
3	18	F	KWAGC	Kwaggafontein C Clinic	Mpumalanga
4	24	M	VOL	Amajuba Memorial Hospital	Mpumalanga
5	20	M	GAR	Ga-Rankuwa	Gauteng
6	20	M	GAR	Ga-Rankuwa	Gauteng
7	29	M	CAL	Carolina Hospital	Mpumalanga
8	4m	F	WIT	Witbank Hospital	Mpumalanga
9	42	M	MOK	Mokopane Hospital	Limpopo
10	26	M	GAR	Ga-Rankuwa	Gauteng
11	42	M	RUSHD	Rustenburg Municipal Clinic	North West
12	23	F	SHO	Shongwe Hospital	Mpumalanga
13	56	M	BAR	Barbeton Hospital	Mpumalanga
14	0	M	MAFRI	M'africa PHC Clinic	Mpumalanga
15	28	F	RENEE	Renee PHC Clinic	Gauteng
16	30	M	BOITC	Boitekong Clinic	North West
17	40	F	FIGTR	Fig Tree Clinic	Mpumalanga
18	18	M	THE	Themba Hospital	Mpumalanga
19	39	F	LYNVI	Lynville Clinic	Mpumalanga
20	41	M	KOS	Koster Hospital	North West
21	39	M	LANGL	Langeloop PHC Clinic	Mpumalanga
22	46	M	SOSH3	Soshanguve Clinic Iii	Gauteng
23	29	M	LEBOH	Lebohang/Leslie Clinic	North West
24	20	M	CALCU	Calcutta Clinic	Limpopo
25	35	M	GAR	Ga-Rankuwa	Gauteng
26	39	F	BOREL	Borelelo Clinic	North West
27	49	M	THE	Themba Hospital	Mpumalanga
28	33	F	ODI	Odi Community Hospital	North West
29	50	M	SECMU	Secunda Municipality	Mpumalanga
30	32	M	BRI	Brits Hospital	North West
31	22	F	THE	Themba Hospital	Mpumalanga
32	26	M	THE	Themba Hospital	Mpumalanga
33	39	F	GES	Moses Kotane Hospital	North West
34	N/A	N/A		Standerton Hospital	Mpumalanga
35	34	M	BARSA	Barberton TB Hospital	Mpumalanga
36	5	M	GAR	Ga-Rankuwa	Gauteng
37	64	M	RUS	Rustenburg Hospital	North West
38	25	F	LYD	Lydenburg Hospital	Mpumalanga
39	30	M	ERM	Ermelo Hospital	Mpumalanga
40	33	F	STA	Standerton Hospital	Mpumalanga
41	69	M	NEL	Rob Ferreira Hospital	Mpumalanga
42	30	F	THE	Themba Hospital	Mpumalanga
43	32	M	BARSA	Barberton TB Hospital	Mpumalanga

Table 1: Demographic and geographical information of patients included in this study (continued)

No.	Age	Gender	Location		Region
44	44	F	MHLUZ	Mhluzi Clinic	Mpumalanga
45	32	M	STAND	Standerton Clinic	Mpumalanga
46	31	M	PHI	Philadelphia Haspital	Limpopo
47	34	F	JEPPT	Jeppes Rust Clinic	Gauteng
48	32	F	STEEN	Steenbok Clinic	Limpopo
49	42	M	SIPHO	Siphosensimbi PHC Clinic	Mpumalanga
50	26	F	ACTPE	Aids Care, Training & Support	Mpumalanga
51	54	F	RAMOO	Ramokoka Clinic	North West
52	39	F	LANGL	Langeloop PHC Clinic	Mpumalanga
53	33	F	SES	Sesifuba Hospital	Mpumalanga
54	30	F	TWEEA	Tweefontein A Clinic	Mpumalanga
55	N/A	N/A	PHI	Philadelphia Haspital	Limpopo
56	28	M	RUSHD	Rustenburg Municipal Clinic	North West
57	26	F	BARSA	Barberton TBHospital	Mpumalanga
58	35	F	STA	Standerton Hospital	Mpumalanga
59	56	M	MHL	Kwamhalnga Hospital	Mpumalanga
60	37	F	SECMU	Secunda Municipality	Mpumalanga
61	27	M	KILDA	Kildare Clinic	Mpumalanga
62	31	M	NAASC	Naas PHC Clinic	Mpumalanga
63	25	M	PIR	Piet Retief Hospital	Mpumalanga
64	28	M	EERSS	Eersterus Clinic	Gauteng
65	41	M	WITSA	Witbank Santa Centre	Mpumalanga
66	38	M	NEL	Rob Ferreira Hospital	Mpumalanga
67	30	M	SIPHO	Siphosensimbi PHC Clinic	Mpumalanga
68	30	F	WITSA	Witbank Santa Centre	Mpumalanga
69	34	F	WIT	Witbank Hospital	Mpumalanga
70	50	M	WITSA	Witbank Santa Centre	Mpumalanga
71	40	M	WITSA	Witbank Santa Centre	Mpumalanga
72	23	F	WITSA	Witbank Santa Centre	Mpumalanga
73	0	M	WITSA	Witbank Santa Centre	Mpumalanga
74	21	F	WITSA	Witbank Santa Centre	Mpumalanga
75	18	M	THE	Themba Hospital	Mpumalanga
76	36	F	NEL	Rob Ferreira Hospital	Mpumalanga
77	52	F	SIPHO	Siphosensimbi PHC Clinic	Mpumalanga
78	26	F	MOK	Mokopane Hospital	Limpopo
79	37	M	LEBOH	Lebohang/Leslie Clinic	North West
80	0	F	SHO	Shongwe Hospital	Mpumalanga
81	44	M	SECMU	Secunda Municipality	Mpumalanga
82	34	M	MAFRI	M'africa PHC Clinic	Mpumalanga
83	34	F	JUSTI	Justicia Clinic	Limpopo
84	49	M	BEH	Bethall Hospital	Mpumalanga
85	41	M	STA	Standerton Hospital	Mpumalanga
86	40	M	KOMHD	Komatipoort Municipal Clinic	Mpumalanga
87	0	F	NEL	Rob Ferreira Hospital	Mpumalanga
88	32	F	STEEN	Steenbok Clinic	Limpopo
89	0	M	EZIWE	Eziweni Clinic	Mpumalanga

Table 1: Demographic and geographical information of patients included in this study (continued)

No.	Age	Gender	Location		Region
90	0	F	BON	Bongani Hospital, Hazyview	Mpumalanga
91	51	M	THE	Themba Hospital	Mpumalanga
92	25	M	BARSA	Barberton TB Hospital	Mpumalanga
93	0	F	WITSA	Witbank Santa Centre	Mpumalanga
94	37	F	WITSA	Witbank Santa Centre	Mpumalanga
95	N/A	N/A	WITSA	Witbank Santa Centre	Mpumalanga
96	32	M	WITSA	Witbank Santa Centre	Mpumalanga
97	25	M	WITSA	Witbank Santa Centre	Mpumalanga
98	N/A	N/A	WITSA	Witbank Santa Centre	Mpumalanga
99	30	F	PIR	Piet Retief Hospital	Mpumalanga
100	25	M	JUB	Jubilee Hospital	Gauteng
101	32	M	WITSA	Witbank Santa Centre	Mpumalanga
102	35	F	BETHD	Bethal Municipal Clinic	Mpumalanga
103	44	M	FAIMI	Fairview Mine	Mpumalanga
104	20	F	STASA	Standerton TB Hospital	Mpumalanga
105	30	F	STASA	Standerton TB Hospital	Mpumalanga
106	36	M	BAR	Barbeton Hospital	Mpumalanga
107	51	F	TEMHA	Dr Ha Tempelman	Limpopo
108	20	M	KANYA	Kanyamazane Health Centre	Mpumalanga
109	26	M	BEATT	Beatty Avenue Clinic	Mpumalanga
110	30	M	KLIPF	Klipfontein Clinic	Limpopo
111	65	M	BON	Bongani Hospital, Hazyview	Mpumalanga
112	22	M	KANYA	Kanyamazane Health Centre	Mpumalanga
113	21	F	STASA	Standerton TB Hospital	Mpumalanga
114	38	F	ERM	Ermelo Hospital	Mpumalanga
115	36	M	SHO	Shongwe Hospital	Mpumalanga
116	54	F	SHO	Shongwe Hospital	Mpumalanga
117	18	F	THE	Themba Hospital	Mpumalanga
118	18	F	THE	Themba Hospital	Mpumalanga
119	29	M	KANYA	Kanyamazane Health Centre	Mpumalanga
120	29	F	KWARR	Kwarrielaagte Clinic	Limpopo
121	30	F	PIR	Piet Retief Hospital	Mpumalanga
122	18	F	BON	Bongani Hospital, Hazyview	Mpumalanga
123	46	M	THE	Themba Hospital	Mpumalanga
124	18	M	BROOK	Brooklyn Clinic	Mpumalanga
125	22	M	EZIWE	Eziweni Clinic	Mpumalanga
126	49	M	WITSA	Witbank Santa Centre	Mpumalanga
127	47	F	WITSA	Witbank Santa Centre	Mpumalanga
128	N/A	N/A	GAR	Ga-Rankuwa	Gauteng
129	20	F	MOHOD	Mohodi Clinic	Mpumalanga
130	29	F	BARSA	Barberton TB Hospital	Mpumalanga
131	34	F	TIN	Tintswalo Hospital	Mpumalanga
132	13	M	BON	Bongani Hospital, Hazyview	Mpumalanga
133	43	M	MSOGW	Msogwaba Clinic	Mpumalanga
134	49	F	STASA	Standerton TB Hospital	Mpumalanga

Table 1: Demographic and geographical information of patients included in this study (continued)

No.	Age	Gender	Location		Region
135	37	M	BARSA	Barberton TB Hospital	Mpumalanga
136	9	M	MAP	Mapulaneng Hospital	Mpumalanga
137	36	M	TIN	Tintswalo Hospital	Mpumalanga
138	41	F	IKHUS	Ikhutseng Clinic	North West
139	35	F	WIT	Witbank Hospital	Mpumalanga
140	34	F	GAR	Ga-Rankuwa	Gauteng
141	11m	M	JUB	Jubilee Hospital	Gauteng
142	22	F	NEL	Rob Ferreira Hospital	Mpumalanga
143	39	M	KWAGC	Kwaggafontein C Clinic	Mpumalanga
144	45	F	NAACC	Naas Clinic Block C	Mpumalanga
145	37	M	BARSA	Barberton TB Hospital	Mpumalanga
146	49	M	AMC	Anglocoal Highveld Hospital	Mpumalanga
147	36	M	BROED	Broederstroom PHC Clinic	North west
148	28	M	NEL	Rob Ferreira Hospital	Mpumalanga
149	29	F	WITSA	Witbank Santa Centre	Mpumalanga
150	31	F	PHED1	Phedisong 1 Clinic	North West
151	40	M	DELHD	Delmas Municipal Clinic	Mpumalanga
152	33	M	BARPF	Barberton Prison Farm	Mpumalanga
153	27	F	EZIWE	Eziweni Clinic	Mpumalanga
154	33	M	BAR	Barbeton Hospital	Mpumalanga
155	44	F	GAR	Ga-Rankuwa	Gauteng
156	33	M	MSOGW	Msogwaba Clinic	Mpumalanga
157	34	F	BARSA	Barberton TB Hospital	Mpumalanga
158	35	F	ODI	Odi Community Hospital	North West
159	5m	M	GAR	Ga-Rankuwa	Gauteng
160	37	M	DAVEL	Davel PHC Clinic	Mpumalanga
161	0	M	AMC	Anglocoal Highveld Hospital	Mpumalanga
162	27	F	BAKUB	Bakubung Clinic	North West
163	32	M	GAR	Ga-Rankuwa	Gauteng
164	45	F	KGABO	Kgabo Health Centre Clinic	Limpopo
165	28	F	BARSA	Barberton TB Hospital	Mpumalanga
166	39	F	ODI	Odi Community Hospital	North West
167	41	M	STA	Standerton Hospital	Mpumalanga
168	35	M	GAR	Ga-Rankuwa	Gauteng
169	34	F	NEL	Rob Ferreira Hospital	Mpumalanga
170	35	M	SES	Sesifuba Hospital	Mpumalanga
171	25	M	LYNVI	Lynville Clinic	Mpumalanga
172	29	M	MARIK	Marikana Clinic	North West
173	23	F	BAFOK	Bafokeng PHC Clinic	North West
174	25	F	RENEE	Renee PHC Clinic	Gauteng
175	45	M	BARSA	Barberton TB Hospital	Mpumalanga
176	29	F	KEKAS	Kekana Stad Clinic	Gauteng
177	30	M	PCWIT	Poly Clinic Witbank	Mpumalanga
178	31	F	HAZYV	Hazyview Fixed Clinic	Mpumalanga
179	31	F	NEL	Rob Ferreira Hospital	Mpumalanga
180	21	F	WITSA	Witbank Santa Centre	Mpumalanga
181	45	M	WITSA	Witbank Santa Centre	Mpumalanga

Table 1: Demographic and geographical information of patients included in this study (continued)

No.	Age	Gender	Location		Region
182	26	F	WITSA	Witbank Santa Centre	Mpumalanga
183	39	M	BARSA	Barberton TB Hospital	Mpumalanga
184	22	F	BETHN	Bethanie PHC Clinic Rustenburg	North West
185	36	F	KAMHL	Kamhlushwa Clinic	Mpumalanga
186	26	F	BOULD	Boulders Clinic	Mpumalanga
187	27	F	BAKUB	Bakubung Clinic	North West
188	34	F	MISPE	Mispel Clinic	Mpumalanga
189	35	M	SOSH3	Soshanguve Clinic III	Gauteng
190	35	M	SOSH3	Soshanguve Clinic III	Gauteng
191	54	M	SOSH3	Soshanguve Clinic III	Gauteng
192	30	F	STASA	Standerton TB Hospital	Mpumalanga
193	38	M	SOSH3	Soshanguve Clinic III	Gauteng
194	36	M	WIT	Witbank Hospital	Mpumalanga
195	57	M	BEATT	Beatty Avenue Clinic	Mpumalanga
196	40	M	WONDE	Wonderkop PHC Clinic	North West
198	23	M	CAL	Carolina Hospital	Mpumalanga
199	47	M	STASA	Standerton TB Hospital	Mpumalanga
200	28	F	STA	Standerton Hospital	Mpumalanga
201	29	F	GAR	Ga-Rankuwa	Gauteng
202	32	M	FIGTR	Fig Tree Clinic	Mpumalanga
203	40	F	GAR	Ga-Rankuwa	Gauteng
204	31	F	EMZIN	Emzinoni Clinic	Mpumalanga
205	46	M	LYNVI	Lynville Clinic	Mpumalanga
206	30	M	WITSA	Witbank Santa Centre	Mpumalanga
207	19	F	KANYA	Kanyamazane Health Centre	Mpumalanga
208	39	M	NEL	Rob Ferreira Hospital	Mpumalanga
209	26	F	WIT	Witbank Hospital	Mpumalanga
210	22	F	GATEM	Gateway Clinic Moretele	Mpumalanga
211	22	F	GATEM	Gateway Clinic Moretele	Mpumalanga
212	26	F	WIT	Witbank Hospital	Mpumalanga
213	N/A	N/A		Matikwana Hospital	Mpumalanga
214	26	F	BARSA	Barberton TB Hospital	Mpumalanga
215	22	F	BARSA	Barberton TB Hospital	Mpumalanga
216	45	M	STA	Standerton Hospital	Mpumalanga
217	40	M	LEGOG	Legogote Clinic	Mpumalanga
218	23	F	KAAPM	Kaapmuiden Clinic	Mpumalanga
219	38	M	BARSA	Barberton TB Hospital	Mpumalanga
220	29	M	TEMHA	Dr Ha Tempelman	Limpopo
221	40	F	KHUMB	Khumbula Clinic	Mpumalanga
222	27	M	BETHD	Bethal Municipal Clinic	Mpumalanga
223	29	F	BARSA	Barberton TB Hospital	Mpumalanga
224	34	F	BARSA	Barberton TB Hospital	Mpumalanga
225	31	M	WINTE	Winterveld Clinic (Dube)	Gauteng
226	23	F	BOITC	Boitekong Clinic	North West
227	0	F	MIDDP	Middleplaas Clinic	Mpumalanga
228	12	M	WIT	Witbank Hospital	Mpumalanga
229	40	F	KHUMB	Khumbula Clinic	Mpumalanga

Table 1: Demographic and geographical information of patients included in this study (continued)

No.	Age	Gender	Location		Region
230	46	F	LYD	Lydenburg Hospital	Mpumalanga
231	29	F	MATSU	Matsulu Health Centre	Mpumalanga
232	39	F	WITSA	Witbank Santa Centre	Mpumalanga
233	50	F	PCWIT	Poly Clinic Witbank	Mpumalanga
234	33	F	WITSA	Witbank Santa Centre	Mpumalanga
235	25	F	HAZYV	Hazyview Fixed Clinic	Mpumalanga
236	43	F	SIYAM	Siyathemba Community Health	Mpumalanga
237	33	F	TIN	Tintswalo Hospital	Mpumalanga
238	47	M	WITSA	Witbank Santa Centre	Mpumalanga
239	32	F	BON	Bongani Hospital, Hazyview	Mpumalanga
240	57	M	BON	Bongani Hospital, Hazyview	Mpumalanga
241	47	M	NEL	Rob Ferreira Hospital	Mpumalanga
242	29	F	BARSA	Barberton TB Hospital	Mpumalanga
243	53	F	BEATT	Beatty Avenue Clinic	Mpumalanga
244	N/A	N/A		Phola PHC Clinic	Limpopo
245	31	F	ODI	Odi Community Hospital	North West
246	33	M	VEREN	Verena Clinic	Mpumalanga
247	39	F	SOSH2	Soshanguve Clinic II	Gauteng
248	25	F	BAR	Barberton Hospital	Mpumalanga
249	28	F	TIN	Tintswalo Hospital	Mpumalanga
250	38	M	SHEMI	Sheba Mine	Mpumalanga
251	32	M	LEGOG	Legogote Clinic	Mpumalanga
253	22	M	LANGL	Langeloop PHC Clinic	Mpumalanga
254	34	F	EERSS	Eersterus Clinic	Gauteng
255	22	F	MSOGW	Msogwaba Clinic	Mpumalanga
256	56	F	SOSHX	Soshanguve Clinic Block X	Gauteng
257	34	M	MAT	Matikwana Hospital	Mpumalanga
258	32	M	NELSB	Area Military Health Unit	Mpumalanga
259	6	F	LEBOT	Lebotloane Clinic	Mpumalanga
260	34	F	WATER	Waterval Clinic	Mpumalanga
261	34	F	WATER	Waterval Clinic	Mpumalanga
262	41	M	DELHD	Delmas Municipal Clinic	Mpumalanga
263	40	F	SIPHO	Siphosensimbi PHC Clinic	Mpumalanga
264	43	M	PHED1	Phedisong 1 Clinic	North West
265	42	F	SOSH2	Soshanguve Clinic II	Gauteng
267	20	F	LYNVI	Lynville Clinic	Mpumalanga
268	45	M	SOSHJ	Block JJ Soshanguve Clinic	Gauteng
269	34	M	SOSH2	Soshanguve Clinic II	Gauteng
270	37	M	SOSHJ	Block JJ Soshanguve Clinic	Gauteng
271	49	M	MARAY	Marapyane PHC Clinic	Mpumalanga
272	54	F	GES	Moses Kotane Hospital	North West
273	0	M	MHL	Kwamhalnga Hospital	Mpumalanga
274	27	F	WINTE	Winterveld Clinic (Dube)	Gauteng
275	32	F	HAZYV	Hazyview Fixed Clinic	Mpumalanga
276	27	M	HAZYV	Hazyview Fixed Clinic	Mpumalanga

Table 1: Demographic and geographical information of patients included in this study (continued)

No.	Age	Gender	Location		Region
277	38	M	SIYAB	Siyabuswa Clinic	Mpumalanga
278	45	M	FAIMI	Fairview Mine	Mpumalanga
279	0		GAR	Ga-Rankuwa	Gauteng
280	26	F	GAR	Ga-Rankuwa	Gauteng
281	38	M	PHOLC	Phola PHC Clinic	Limpopo
282	31	F	WITSA	Witbank Santa Centre	Mpumalanga
283	34	F	MGOBO	Mgobodi Clinic	Gauteng
284	39	F	WITSA	Witbank Santa Centre	Mpumalanga
285	31	F	MOLOT	Moloto Clinic	Mpumalanga
286	28	F	BARSA	Barberton TB Hospital	Mpumalanga
287	45	M	NAACC	Naas Clinic Block C	Mpumalanga
288	48	M	SIPHO	Siphosensimbi Phc Clinic	Mpumalanga
289	22	F	CLASS	Classic House Clinic	North West
290	31	M	VEREN	Verena Clinic	Mpumalanga
291	47	F	KRAAL	Kraalhoek Clinic	Limpopo
292	54	F	BEATT	Beatty Avenue Clinic	Mpumalanga
293	38	M	HARTE	Hartbeespoort Clinic	North West
294	54	M	VLAK1	Vlaklaagte No1 Clinic	Mpumalanga
295	19	F	SOSHJ	Block JJ Soshanguve Clinic	Gauteng
296	48	M	SIHLA	Sihlangu 5 Clinic	Mpumalanga
297	31	F	BON	Bongani Hospital, Hazyview	Mpumalanga
298	0		GAR	Ga-Rankuwa	Gauteng
299	0		GAR	Ga-Rankuwa	Gauteng
300	0		GAR	Ga-Rankuwa	Gauteng
301	0		GAR	Ga-Rankuwa	Gauteng
302	N/A	N/A		Themba Hospital	Mpumalanga
303	44	M	SILVK	Silverkrans Clinic	North West
304	31	M	NEL	Rob Ferreira Hospital	Mpumalanga
305	15	F	NEL	Rob Ferreira Hospital	Mpumalanga
306	0	M	LOUWS	Low's Creek Clinic	Mpumalanga
307	30	F	PCWIT	Poly Clinic Witbank	Mpumalanga
308	28	F	TEMHA	Dr Ha Tempelman	Limpopo
309	34	F	JEPPT	Jeppes Rust Clinic	Gauteng
310	0	F	EVA	Evander Hospital	Mpumalanga
311	32	F	BARSA	Barberton TB Hospital	Mpumalanga
312	37	M	LOUIE	Louieville PHC Clinic	Mpumalanga
313	49	M	WITSA	Witbank Santa Centre	Mpumalanga
314	29	M	GAR	Ga-Rankuwa	Gauteng
315	53	M	BARSA	Barberton TB Hospital	Mpumalanga
316	35	F	STEEN	Steenbok Clinic	Limpopo
319	38	M	SOSH2	Soshanguve Clinic II	Gauteng
320	41	M	GAR	Ga-Rankuwa	Gauteng
321	40	M	GAR	Ga-Rankuwa	Gauteng
322	32	F	STEEN	Steenbok Clinic	Limpopo
323	27	F	MAT	Matikwana Hospital	Mpumalanga
324	29	M	GAR	Ga-Rankuwa	Gauteng
326	45	M	MATSU	Matsulu Health Centre	Mpumalanga

Table 1: Demographic and geographical information of patients included in this study (continued)

No.	Age	Gender	Location		Region
327	0		GAR	Ga-Rankuwa	Gauteng
328	N/A	N/A	MATSU	Matsulu Health Centre	Mpumalanga
329	28	F	MSOGW	Msogwaba Clinic	Mpumalanga
330	49	F	TIN	Tintswalo Hospital	Mpumalanga
331	34	M	NEL	Rob Ferreira Hospital	Mpumalanga
332	50	M	BON	Bongani Hospital, Hazyview	Mpumalanga
333	31	F	STA	Standerton Hospital	Mpumalanga
334	41	F	PHOLC	Phola PHC Clinic	Limpopo
335	38	M	BON	Bongani Hospital, Hazyview	Mpumalanga
336	53	M	RAMOS	Ramotse Clinic	Gauteng
337	47	F	GES	Moses Kotane Hospital	North West
338	28	M	PIR	Piet Retief Hospital	Mpumalanga
339	0	M	MHL	Kwamhalnga Hospital	Mpumalanga
340	22	F	MSOGW	Msogwaba Clinic	Mpumalanga
343	22	M	LANGL	Langeloop PHC Clinic	Mpumalanga

Table 2: Drug susceptibility testing results of the agar proportion method and the GenoType® MTBDR*sl* assay

	Proportion method								GenoType® MTBDRsl assay		
ID	**INH**	**SM**	**EMB**	**RMP**	**KAN**	**CAP**	**OFX**	**ID**	**FLQ**	**AMG/CAP**	**EMB**
1	R	S	S	R	S	S	S	1	S	S	R
2	S	S	S	R	S	S	S	2	S	S	S
3	R	S	S	R	S	S	S	3	S	S	S
4	S	S	S	R	S	S	S	4	S	S	S
5	R	S	S	R	S	S	S	5	S	S	R
6	R	S	S	R	S	S	S	6	S	S	S
7	R	S	S	R	S	S	S	7	S	S	S
8	R	R	S	R	S	S	S	8	S	S	S
9	R	S	S	R	S	S	S	9	S	S	S
10	R	S	R	R	S	S	S	10	S	S	R
11	R	S	S	R	S	S	S	11	S	S	S
12	R	S	S	R	S	S	S	12	S	S	S
13	R	S	S	R	S	S	S	13	S	S	S
14	R	S	S	R	S	S	S	14	S	S	SR HETRO
15	R	R	S	R	S	S	S	15	S	S	S
16	R	S	S	R	S	S	S	16	S	S	S
17	R	R	S	R	S	S	S	17	S	S	R
18	R	R	R	R	S	S	S	18	S	S	R
19	R	S	S	R	S	S	S	19	S	S	S
20	R	S	S	R	R	R	R	20	R	S	S
21	R	S	S	R	S	S	S	21	S	S	S
22	R	S	S	R	S	S	S	22	S	S	R
23	R	R	S	R	S	S	S	23	S	S	S
24	R	S	S	R	S	S	S	24	S	S	S
25	R	S	S	R	S	S	S	25	S	S	R
26	R	S	S	R	S	S	S	26	S	R	S
27	R	S	S	R	S	S	S	27	S	R	S
28	R	R	S	R	S	S	S	28	S	S	R
29	R	S	S	R	S	S	S	29	S	S	S
30	R	S	S	R	S	S	S	30	S	S	S
31	R	R	S	R	S	S	S	31	S	S	S
32	R	R	S	R	S	S	S	32	S	S	R
33	R	R	R	R	S	S	S	33	S	S	R
34	R	R	S	R	S	R	S	34	S	S	S
35	R	S	S	R	S	S	S	35	S	S	S
36	R	R	S	R	S	S	S	36	S	S	S
37	R	R	S	R	S	S	S	37	S	S	S
38	R	R	S	R	S	S	S	38	S	S	R
39	R	R	S	R	S	S	S	39	R	R	R
40	R	R	S	R	R	R	R	40	S	S	S
41	R	S	S	R	S	S	S	41	S	S	S
42	R	R	R	S	S	S	S	42	S	S	R
43	R	R	S	R	S	S	S	43	S	S	R
44	R	R	S	R	S	S	S	44	S	S	R
45	R	R	S	R	S	S	S	45	R	R	S
46	R	S	S	R	S	S	S	46	S	S	S

Table 2: Drug susceptibility testing results of the agar proportion method and the GenoType® MTBDR*sl* assay (continued)

	Proportion method							GenoType® MTBDRsl assay			
ID	**INH**	**SM**	**EMB**	**RMP**	**KAN**	**CAP**	**OFX**	**ID**	**FLQ**	**AMG/CAP**	**EMB**
47	R	S	S	R	S	S	S	47	S	S	S
48	R	S	S	R	S	S	S	48	S	S	SR HETRO
49	R	R	S	S	S	R	S	49	S	S	S
50	R	S	S	R	S	S	S	50	S	S	R
51	R	R	S	R	S	S	S	51	S	S	R
52	R	S	S	R	S	S	S	52	S	S	S
53	R	S	S	R	S	S	S	53	S	S	R
54	R	R	S	R	S	S	S	54	S	S	R
55	R	S	S	R	R	R	R	55	S	S	S
56	R	S	S	R	S	S	S	56	S	S	S
57	R	S	S	R	S	S	S	57	S	S	R
58	R	S	S	R	S	S	S	58	S	S	R
59	R	R	S	R	S	S	S	59	S	S	R
60	R	S	S	R	S	S	S	60	S	S	R
61	R	S	S	R	R	R	R	61	S	S	S
62	R	S	S	R	S	S	S	62	S	S	R
63	R	R	S	R	S	S	S	63	S	S	S
64	R	S	S	R	S	S	S	64	S	S	R
65	R	S	S	R	S	R	S	65	S	S	R
66	R	S	S	R	S	S	S	66	R	S	S
67	R	S	S	R	S	S	S	67	-	-	-
68	R	S	S	R	S	S	R	68	S	S	SR HETRO
69	R	S	S	R	S	S	S	69	S	S	SR HETRO
70	R	R	S	R	S	S	S	70	S	S	R
71	R	S	S	R	S	S	S	71	S	S	R
72	R	S	S	R	S	S	S	72	R	S	R
73	R	S	S	R	S	S	S	73	R	R	R
74	R	S	S	R	R	R	R	74	S	S	R
75	R	R	S	R	S	S	S	75	S	S	S
76	R	S	S	R	S	S	S	76	S	S	S
77	R	R	S	R	S	S	S	77	S	S	S
78	R	S	S	R	S	S	S	78	S	S	R
79	R	S	S	R	S	S	S	79	S	S	R
80	R	S	S	R	S	S	S	80	R	R	R
81	R	S	S	R	R	R	R	81	S	S	R
82	R	S	S	R	R	R	S	82	S	S	S
83	R	S	S	R	S	S	S	83	R	S	SR HETRO
84	R	S	S	R	S	S	R	84	R	R	R
85	R	R	S	R	R	R	R	85	S	S	S
86	R	S	S	R	S	S	S	86	S	S	S
87	R	S	S	R	S	S	S	87	S	S	S
88	R	S	S	R	S	R	S	88	S	S	R
89	R	S	R	R	S	S	S	89	S	S	R
90	R	R	S	R	S	S	S	90	S	S	R
91	R	R	S	R	R	R	R	91	S	S	R
92	R	R	S	R	S	S	S	92	S	S	S
93	R	S	S	R	S	S	S	93	S	S	R

Table 2: Drug susceptibility testing results of the agar proportion method and the GenoType® MTBDR*sl* assay (continued)

Proportion method								GenoType® MTBDRsl assay			
ID	INH	SM	EMB	RMP	KAN	CAP	OFX	ID	FLQ	AMG/CAP	EMB
94	R	S	S	R	S	R	S	94	S	S	R
95	R	S	S	R	S	R	S	95	R	R	R
96	R	R	R	R	S	S	S	96	R	S	SR HETRO
97	R	R	S	R	R	R	R	97	R	S	SR HETRO
98	R	S	S	R	S	S	R	98	R	S	S
99	R	S	S	R	S	S	R	99	R	S	R
100	R	S	S	R	S	S	R	100	S	S	S
101	R	R	S	R	R	R	S	101	S	S	R
102	R	R	S	R	S	S	S	102	S	S	R
103	R	R	S	R	S	S	S	103	S	S	SR HETRO
104	R	R	R	R	S	S	S	104	S	S	R
105	R	R	R	R	R	R	S	105	S	S	R
106	R	R	R	R	S	S	S	106	S	S	R
107	R	S	S	R	S	S	S	107	S	S	SR HETRO
108	R	S	S	R	S	S	S	108	S	S	S
109	R	S	S	R	S	S	S	109	S	S	R
110	R	S	S	R	S	S	S	110	S	S	R
111	R	R	S	R	S	S	S	111	R	S	SR HETRO
112	R	S	S	R	S	S	S	112	R	S	R
113	R	S	S	R	R	R	R	113	S	S	R
114	R	R	R	R	S	S	R	114	R	R	S
115	R	R	S	R	S	S	S	115	S	S	R
116	R	R	S	R	S	S	R	116	S	S	R
117	R	R	S	R	S	S	S	117	S	S	S
118	R	S	S	R	S	S	S	118	S	S	S
119	R	R	S	R	S	S	S	119	R	S	SR HETRO
120	R	S	S	R	S	S	S	120	R	S	R
121	R	S	S	R	R	R	R	121	R	S	R
122	R	S	S	R	S	S	R	122	S	S	R
123	R	R	R	R	R	R	R	123	S	S	R
124	R	S	S	R	S	S	S	124	S	S	R
125	R	S	S	R	S	S	S	125	S	S	S
126	R	R	R	R	S	S	S	126	R	S	R
127	R	S	S	R	S	S	S	127	R	S	S
128	R	R	S	R	S	S	R	128	R	S	R
129	R	S	S	R	R	R	R	129	R	S	S
130	R	S	S	R	S	S	R	130	S	S	S
131	R	R	S	R	S	S	R	131	S	S	S
132	R	S	S	R	S	S	S	132	R	S	S
133	R	S	S	R	S	S	S	133	S	S	SR HETRO
134	R	R	R	R	S	S	R	134	S	S	S
135	R	S	S	R	S	S	S	135	S	S	S
136	R	R	R	R	S	S	S	136	S	S	R
137	R	S	R	R	S	S	S	137	S	S	SR HETRO
138	R	R	S	R	S	S	S	138	S	S	R
139	R	R	S	R	S	S	S	139	R	S	S
140	R	S	S	R	S	S	S	140	S	S	R

Table 2: Drug susceptibility testing results of the agar proportion method and the GenoType® MTBDR*sl* assay (continued)

	Proportion method							GenoType® MTBDRsl assay			
ID	**INH**	**SM**	**EMB**	**RMP**	**KAN**	**CAP**	**OFX**	**ID**	**FLQ**	**AMG/CAP**	**EMB**
141	R	S	R	R	S	S	S	141	S	S	S
142	R	R	S	R	S	S	S	142	S	S	S
143	R	S	S	R	S	S	S	143	S	S	S
144	R	S	S	R	S	S	S	144	S	S	R
145	R	S	R	R	R	R	R	145	S	S	S
146	R	S	R	R	S	S	S	146	S	S	S
147	R	S	S	R	S	R	S	147	S	S	R
148	R	R	S	R	S	S	S	148	S	S	S
149	R	S	S	R	S	S	S	149	S	S	R
150	R	R	S	R	S	S	S	150	S	S	S
151	R	S	S	R	S	S	S	151	S	S	R
152	R	R	S	R	S	S	S	152	S	S	R
153	R	R	S	R	S	S	S	153	R	S	R
154	R	R	S	R	S	S	S	154	S	S	R
155	R	R	S	R	S	S	R	155	S	S	S
156	R	S	S	R	S	S	S	156	S	S	R
157	R	S	S	R	S	S	S	157	S	S	R
158	R	R	R	R	S	S	S	158	S	S	S
159	S	S	S	S	S	S	S	159	R	S	SR HETRO
160	R	R	R	R	S	S	S	160	S	S	R
161	R	R	S	R	S	S	S	161	S	S	S
162	R	S	S	R	S	S	S	162	S	S	S
163	R	R	S	R	R	R	S	163	S	S	S
164	R	S	S	R	S	S	S	164	S	S	R
165	R	R	S	R	S	S	S	165	S	S	S
166	S	S	S	R	S	S	S	166	S	S	R
167	R	S	S	R	S	S	S	167	S	S	S
168	R	S	R	R	S	S	S	168	S	S	R
169	R	R	S	R	S	S	S	169	S	S	S
170	R	R	R	R	S	S	S	170	S	S	S
171	R	R	S	R	S	S	S	171	S	S	R
172	R	S	S	R	S	S	S	172	S	S	R
173	R	S	S	R	S	S	S	173	S	S	R
174	R	S	R	R	S	S	S	174	S	S	R
175	R	R	S	R	S	S	S	175	S	S	S
176	R	S	R	R	S	S	S	176	S	S	R
177	R	R	S	R	S	S	S	177	S	S	S
178	R	S	S	R	S	S	S	178	R	R	R
179	R	R	S	R	S	S	S	179	S	S	S
180	R	R	S	R	R	R	R	180	S	S	S
181	R	R	R	R	S	S	S	181	S	S	R
182	R	R	S	R	S	S	S	182	S	S	R
183	R	S	S	R	S	S	S	183	S	S	R
184	R	R	S	R	S	S	S	184	S	S	R
185	R	R	S	R	S	S	S	185	S	S	R
186	R	R	S	R	S	S	S	186	S	S	S
187	R	S	S	R	R	R	S	187	S	S	R

Table 2: Drug susceptibility testing results of the agar proportion method and the GenoType® MTBDR*sl* assay (continued)

	Proportion method							GenoType® MTBDRsl assay			
ID	INH	SM	EMB	RMP	KAN	CAP	OFX	ID	FLQ	AMG/CAP	EMB
188	R	R	S	R	S	S	S	188	S	S	R
189	R	S	S	R	S	S	S	189	S	S	R
190	R	S	S	R	S	S	S	190	S	S	R
191	R	S	S	R	S	S	S	191	S	S	R
192	R	R	S	R	S	S	S	192	S	S	S
193	S	S	S	S	S	S	S	193	S	S	S
194	R	S	S	R	S	S	S	194	S	S	S
195	R	S	S	R	S	S	S	195	S	S	S
196	R	R	S	R	S	S	S	196	S	S	S
197								197	S	S	R
198	R	S	S	R	S	S	S	198	R	R	R
199	R	R	S	R	S	S	S	199	S	S	S
200	R	R	R	R	R	R	R	200	S	S	R
201	R	S	S	R	S	S	S	201	S	S	S
202	R	S	S	R	S	S	S	202	S	S	S
203	R	S	S	R	S	S	S	203	S	S	S
204	S	S	S	R	S	S	S	204	S	S	R
205	R	R	S	R	S	S	S	205	S	S	R
206	R	S	S	R	S	S	S	206	S	S	S
207	R	S	S	R	S	S	S	207	S	S	S
208	R	S	R	R	S	S	S	208	S	S	S
209	R	R	S	R	S	S	S	209	S	S	S
210	R	R	S	R	S	S	S	210	S	S	S
211	R	S	S	R	S	S	S	211	S	S	S
212	R	R	S	R	S	R	R	212	S	S	S
213	R	R	S	R	S	S	S	213	S	S	S
214	R	R	S	R	S	S	S	214	S	S	S
215	R	R	S	S	S	S	S	215	S	S	S
216	R	S	S	R	S	S	S	216	S	S	R
217	R	R	R	R	S	S	S	217	S	S	R
218	R	S	S	R	S	S	S	218	S	S	S
219	R	R	S	R	S	S	S	219	S	S	S
220	R	S	R	R	S	S	S	220	S	S	R
221	R	R	S	R	S	S	S	221	S	S	S
222	R	R	S	R	S	S	S	222	S	S	S
222	R	R	S	R	S	S	S	223	S	S	R
223	R	S	S	R	S	S	S	224	S	S	R
225	R	S	S	R	S	S	S	225	S	S	S
226	R	S	R	R	S	S	S	226	S	S	S
227	R	R	S	R	S	S	S	227	S	S	R
228	R	R	R	R	S	S	S	228	S	S	R
229	R	R	S	R	S	S	S	229	S	S	S
230	R	R	S	R	R	R	R	230	S	S	S
231	R	R	S	R	S	S	S	231	S	S	R
232	R	S	S	R	S	S	S	232	S	S	R
233	R	R	S	R	R	R	R	233	S	S	R
234	R	R	S	R	S	S	S	234	S	S	R

Table 2: Drug susceptibility testing results of the agar proportion method and the GenoType® MTBDR*sl* assay (continued)

	Proportion method							GenoType® MTBDRsl assay			
ID	**INH**	**SM**	**EMB**	**RMP**	**KAN**	**CAP**	**OFX**	**ID**	**FLQ**	**AMG/CAP**	**EMB**
235	R	R	S	R	S	S	S	235	S	S	S
236	R	R	S	R	R	R	R	236	S	S	S
237	R	R	S	R	S	S	S	237	S	S	R
238	R	S	S	R	S	S	S	238	S	S	S
239	R	S	S	R	R	R	R	239	S	S	R
240	R	S	S	R	S	S	S	240	S	S	S
241	R	S	S	R	S	S	S	241	S	S	R
242	R	S	S	R	S	S	S	242	S	S	S
243	R	R	S	R	S	S	S	243	S	S	S
244	R	R	S	R	S	S	S	244	S	S	R
245	R	S	S	R	S	S	S	245	S	S	S
246	R	S	R	R	S	S	S	246	S	S	S
247	R	S	S	R	S	S	S	247	S	S	R
248	R	R	S	R	S	S	S	248	S	S	S
249	R	S	S	R	S	S	S	249	S	S	SR HETRO
250	R	R	S	R	S	S	S	250	S	S	R
251	R	R	S	R	S	S	S	251	S	S	R
252	R	R	S	R	S	S	S	252	S	S	R
253	R	S	S	R	S	S	S	253	S	S	R
254	R	S	S	R	S	S	S	254	S	S	R
255	R	S	S	R	S	S	S	255	S	S	R
256	R	S	S	R	S	S	S	256	S	S	R
257	R	R	S	R	S	S	S	257	S	S	R
258	R	R	R	R	S	S	S	258	S	S	S
259	R	S	S	R	S	S	S	259	R	S	S
260	R	R	S	R	S	S	S	260	R	S	S
261	R	R	S	R	S	S	S	261	S	S	S
262	R	S	S	R	S	S	S	262	S	S	R
263	R	S	S	R	S	S	S	263	S	S	S
264	R	R	S	R	S	S	S	264	S	S	S
265	R	S	S	R	S	S	S	265	-	-	-
266	R	S	S	R	S	S	S	266	S	S	R
267	R	S	S	R	R	R	R	267	S	S	R
268	R	S	S	R	S	S	S	268	S	S	S
269	R	S	S	R	S	S	S	269	S	S	R
270	R	R	R	R	S	S	S	270	S	S	S
271	R	S	S	R	S	S	S	271	S	S	S
272	R	R	S	R	S	S	R	272	S	S	S
273	R	R	R	R	S	S	S	273	S	S	S
274	R	R	S	R	S	S	S	274	S	S	S
275	R	R	S	R	S	S	S	275	S	S	S
276	R	S	S	R	R	R	S	276	S	S	S
277	R	S	S	R	S	S	S	277	S	S	S
278	R	S	S	R	S	S	S	278	S	S	S
279	R	S	R	R	S	S	S	279	S	S	R
280	R	S	S	R	S	S	S	280	S	S	R

Table 2: Drug susceptibility testing results of the agar proportion method and the GenoType® MTBDR*sl* assay (continued)

	Proportion method							GenoType® MTBDRsl assay			
ID	**INH**	**SM**	**EMB**	**RMP**	**KAN**	**CAP**	**OFX**	**ID**	**FLQ**	**AMG/CAP**	**EMB**
281	R	S	S	R	R	R	R	281	R	S	R
282	R	R	R	R	S	S	S	282	S	S	S
283	R	S	R	R	S	S	R	283	S	S	S
284	R	R	R	R	R	S	S	284	S	S	S
285	R	S	R	R	S	S	S	285	S	S	R
286	R	S	R	R	S	S	S	286	S	S	S
287	R	S	R	R	S	S	S	287	S	S	R
288	R	S	R	R	S	S	S	288	S	S	S
289	R	R	S	R	S	S	S	289	S	S	S
290	R	S	S	R	R	R	S	290	S	S	S
291	R	S	S	R	S	S	S	291	S	S	S
292	R	R	S	R	R	R	S	292	S	S	SR HETRO
293	R	S	S	R	S	S	S	293	S	S	R
294	R	S	R	R	S	S	S	294	S	S	R
295	R	S	S	R	S	S	S	295	S	S	R
296	R	S	S	R	S	S	S	296	S	S	S
297	R	R	S	R	S	S	S	297	S	S	S
298	R	S	S	R	S	S	S	298	S	S	S
299	R	S	S	R	S	S	S	299	S	S	S
300	R	S	S	R	S	S	S	300	S	S	S
301	R	R	S	R	S	S	S	301	S	S	S
302	R	S	S	R	S	S	S	302	S	S	S
303	R	S	S	R	S	S	S	303	S	S	S
304	R	S	S	R	S	S	S	304	S	S	R
305	R	S		R	S	S	S	305	S	S	R
306	R	S	S	R	S	S	S	306	S	S	R
307	R	R	R	R	S	S	S	307	-	-	-
308	R	S	R	R	S	S	S	308	S	S	S
309	R	S	S	R	S	S	S	309	R	S	S
310	R	R	S	R	S	S	R	310	S	S	S
311	R	S	S	R	S	S	S	311	S	S	S
312	R	S	S	R	S	S	S	312	S	S	R
313	R	S	S	R	S	S	S	313	S	S	R
314	R	S	S	R	S	S	S	314	S	S	S
315	R	S	S	R	S	S	S	315	S	S	S
316	R	R	R	R	S	S	S	316	S	S	R
317	R	R	R	R	R	R	R	317	S	S	S
318	R	S	S	R	S	S	S	318	-	-	-
319	R	R	R	R	S	S	S	319	S	R	S
320	R	S	S	R	S	S	S	320	S	S	R
321	R	S	S	R	S	S	S	321	S	S	R
322	R	S	S	R	S	S	S	322	S	S	S
323	R	S	S	R	S	S	S	323	S	S	S
324	R	S	S	R	S	S	S	324	S	S	S
326	R	R	R	R	S	S	S	326	S	S	SR HETRO
327	R	R	S	R	S	S	S	327	S	S	S

Table 2: Drug susceptibility testing results of the agar proportion method and the GenoType® MTBDR*sl* assay (continued)

	Proportion method								GenoType® MTBDRsl assay		
ID	**INH**	**SM**	**EMB**	**RMP**	**KAN**	**CAP**	**OFX**	**ID**	**FLQ**	**AMG/CAP**	**EMB**
328	R	S	S	R	S	S	S	328	S	S	S
329	R	S	S	R	S	S	S	329	S	S	S
330	S	S	S	S	S	S	S	330	S	S	S
331	R	S	S	R	S	S	S	331	S	S	S
332	R	R	S	R	S	S	S	332	S	S	SR HETRO
333	R	S	S	R	S	S	S	333	S	S	S
334	R	S	S	R	S	S	S	334	S	S	S
335	S	S	S	S	S	S	S	335	S	S	S
336	R	S	S	R	S	S	S	336	S	S	SR HETRO
337	R	S	S	R	S	S	S	337	S	S	S
338	R	R	S	R	S	S	S	338	S	S	S
339	R	R	S	R	S	S	S	339	S	S	S
340	R	S	S	R	S	S	S	340	S	S	S
343	R	S	S	R	S	S	S	343	S	S	R

Table 5.2: Distribution of the different *M. tuberculosis* families and drug susceptibility patterns in the four provinces of South Africa

Province	DST result	No of Isolates	Spoligotypes (n)																					
			Beijing	EAI1_SOM	T1	S	LAM3	LAM4	LAM9	LAM11_ZWE	LAM10_CAM	LAM and S/convergent	X1	X2	X3	H1	H3	T2-S	T2-T3	T3	T3_ETH	Manu1	Manu2	Unknown
Mpumalanga	MDR	191	37	49	31	24	6	3	11	7				3	5	1	10	2	1	9	1	3	1	21
	Pre-XDR	23	3	5	4	1		1	1	2					2		1			1				3
	XDR	21	6	5	3	2			1	1				1	1		1							
Gauteng	MDR	45	6	9	7	4	3	5	3	1		1	1	2										3
	Pre-XDR	5	1	3						1														
	XDR	-																						
North West	MDR	27	10	2	4	3	2			2		1								1				2
	Pre-XDR	3		2																				1
	XDR	1		1																				
Limpopo	MDR	17	6	2		2	1		1		1				1					1				2
	Pre-XDR	1																						1
	XDR	2						1					1											

Table 3 MIRU-VNTR typing and spoligotyping results of the MDR-TB isolates

ID	MIRU02	MIRU04	MIRU40	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	SpolDB4 ST	SpolDB4 Lineage	Octaspoligotype
1	2	5	4	4	1	2	6	1	3	4	5	1	48	EAI1_SOM	777777777413731
2	3	3	5	3	1	2	5	2	5	4	4	2	784	T2-S	776377777760731
3	2	2	4	4	2	2	6	2	6	4	4	2		Unknown	776177607760760
4	2	2	3	4	3	2	5	2	6	4	4	2	53	T1	777777777760771
5	2	5	4	3	3	2	6	1	3	3	8	1		Unknown	753743777413731
6	3	5	4	3	3	2	6	2	3	3	8	1		Unknown	753743777413731
7	2	3	5	3	4	2	5	2	5	4	3	2	34	S	776377777760771
8	3	3	5	3	4	2	5	2	3	4	3	2	34	S	776377777760771
9	2	2	4	4	4	2	5	2	7	4	6	2	1	Beijing	000000000003771
10	2	2	2	4	2	2	6	1	5	4	3	2	42	LAM9	777777607760771
11	2	2	4	4	2	2	6	2	3	4	6	1		Unknown	777777777003731
12	2	2	4	3	3	2	6	2	3	4	7	1	48	EAI1_SOM	777777777413731
13	2	2	2	3	1	2	6	2	6	3	6	2		Unknown	777774606060771
14	2	2	4	3	3	2	5	2	7	4	7	3	1	Beijing	000000000003771
15	3	3	5	4	3	2	5	2	3	4	6	2	34	S	776377777760771
16	2	2	4	4	3	2	6	2	6	4	6	2	33	LAM3	776177607760771
17	2	5	4	4	1	2	6	1	3	4	4	2	48	EAI1_SOM	777777777413731
18	2	2	1	3	1	2	6	9	5	3	4	2	54	MANU2	777777777763771
19	2	5	3	4	3	2	6	2	3	4	4	2	48	EAI1_SOM	777777777413731
20	2	2	4	3	3	2	5	2	8	3	4	2	48	EAI1_SOM	777777777413731
21	2	2	3	3	3	2	5	2	4	4	4	2	48	EAI1_SOM	777777777413731
22	2	2	3	3	3	2	5	2	3	4	3	2	1	Beijing	000000000003771

Table 3 MIRU-VNTR typing and spoligotyping results of the MDR-TB isolates (cont)

ID	MIRU02	MIRU04	MIRU40	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	SpolDB4 ST	SpolDB4 Lineage	Octaspoligotype
23	3	3	4	4	3	2	5	2	6	4	2	1	53	T1	777777777760771
24	1	2	5	3	3	2	6	1	8	4	2	2	34	S	776377777760771
25	2	2	1	3	3	2	5	2	6	1	4	1		Unknown	777777417740731
26	2	2	4	3	3	2	5	2	5	4	4	2	1	Beijing	000000000003771
27	2	2	4	3	3	2	5	2	3	3	4	2		Unknown	777000767760771
28	2	2	1	3	1	2	6	2	9	2	4	2	1	Beijing	000000000003771
29	3	3	4	4	3	2	5	2	6	3	6	3		Unknown	777777414140731
30	2	2	3	4	3	2	5	2	3	4	4	2	34	S	776377777760771
31	2	2	4	3	3	2	5	2	9	3	6	3	1	Beijing	000000000003771
32	2	2	1	3	3	2	5	2	9	1	4	3	1	Beijing	000000000003771
33	1	2	6	3	3	2	6	2	5	3	5	2	1	Beijing	000000000003771
34	2	5	3	3	3	2	5	2	7	3	5	3		Unknown	777377607760731
35	2	2	3	4	2	2	5	2	2	3	5	3	784	T2-S	776377777760731
36	2	2	3	3	3	2	5	2	7	4	4	3	1	Beijing	000000000003771
37	2	2	1	3	1	2	6	2	6	2	6	2	1	Beijing	000000000003771
38	2	2	3	3	2	2	5	2	5	4	5	1		Unknown	777777417740731
39	2	5	3	3	2	2	6	3	5	4	5	3	1	Beijing	000000000003771
40	2	2	1	3	3	2	5	2	4	4	4	2	48	EAI1_SOM	777777777413731
41	2	3	5	3	3	2	5	2	5	4	4	2	1	Beijing	000000000003771
42	2	3	1	4	1	2	6	2	5	2	4	2	34	S	776377777760771
43	2	2	4	3	3	2	5	2	5	3	4	1		Unknown	777777020303731
44	2	"2(+3)"	3	5	4	2	5	2	7	3	4	3	34	S	776377777760771
45	2	3	3	3	4	2	5	2	8	3	4	2	37	T3	777737777760771
46	2	2	3	4	3	2	6	2	7	3	4	2	37	T3	777737777760771

Table 3 MIRU-VNTR typing and spoligotyping results of the MDR-TB isolates (cont)

ID	MIRU02	MIRU04	MIRU40	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	SpolDB 4 ST	SpolDB4 Lineage	Octaspoligotype
47	2	2	3	4	3	2	5	2	2	4	4	2	4	LAM3 and S /convergent	000000007760771
48	2	2	4	3	4	2	5	2	5	3	4	2	1	Beijing	000000000003771
49	3	2	4	4	3	2	5	2	5	3	3	2	50	H3	777777777720771
50	2	3	4	4	3	2	5	2	5	3	5	2	34	S	776377777760771
51	2	3	3	4	3	2	5	2	5	5	4	3	4	LAM3 and S /convergent	000000007760771
52	1	2	4	3	2	2	6	3	5	4	3	2	1	Beijing	000000000003771
53	2	2	3	3	2	2	6	2	4	3	3	2	48	EAI1_SOM	777777777413731
54	2	2	3	3	3	2	5	2	5	3	3	2	37	T3	777737777760771
55	2	2	3	4	2	2	5	2	4	3	3	3	119	X1	777776777760771
56	2	2	3	4	3	2	5	2	2	4	3	1	243	T1	777777777760600
57	2	2	1	4	1	2	6	2	5	2	2	2		Unknown	777775606060731
58	2	2	1	3	3	2	6	2	4	3	2	2	42	LAM9	777777607760771
59	2	2	6	3	1	2	5	2	5	4	3	2	53	T1	777777777760771
60	2	3	1	3	2	2	6	2	2	3	2	2	42	LAM9	777777607760771
61	2	2	3	3	2	2	6	2	2	3	4	2	42	LAM9	777777777413731
62	2	5	1	3	1	2	6	2	5	2	3	2		Unknown	777775606060731
63	2	2	5	3	1	2	5	2	5	3	3	2	53	T1	777777777760771
64	2	2	3	4	3	2	6	2	5	3	3	2	719	T1	776177407760771
65	2	2	3	4	2	2	6	3	2	3	4	1	48	EAI1_SOM	777777777413731
66	2	5	3	3	2	2	6	3	2	4	4	2	48	EAI1_SOM	777777777413731
67	2	5	1	3	4	2	6	2	5	3	3	1	37	T3	777737777760771
68	2	2	6	3	3	2	5	2	5	4	3	2	34	S	776377777760771
69	2	5	3	4	3	2	5	2	5	4	3	1		Unknown	776377777763771
70	2	2	3	3	3	2	5	2	6	4	5	1	1	Beijing	000000000003771

Table 3 MIRU-VNTR typing and spoligotyping results of the MDR-TB isolates (cont)

ID	MIRU02	MIRU04	MIRU40	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	SpolDB4 ST	SpolDB4 Lineage	Octaspoligotype
71	2	2	3	3	2	2	6	3	5	4	4	2	48	EAI1_SOM	777777777413731
72	2	2	4	3	2	2	6	2	5	4	3	2		Unknown	777777703760731
73	1	5	3	3	5	2	5	2	6	3	3	2	37	T3	777737777760771
74	2	2	4	4	3	2	5	2	5	3	5	2	92	X3	700076777760771
75	2	2	1	3	3	2	5	2	2	4	3	2	1	Beijing	000000000003771
76	2	2	3	3	3	2	5	2	2	3	3	1	34	S	776377777760771
77	2	3	3	3	3	2	6	2	5	4	3	2	719	T1	776177407760771
78	2	3	1	2	1	2	6	2	5	3	3	2		Unknown	777777604060771
79	2	2	3	5	3	2	5	2	6	3	4	3	37	T3	777737777760771
80	2	2	3	5	2	2	6	3	2	4	5	2	48	EAI1_SOM	777777777413731
81	2	2	1	3	3	2	5	2	7	4	4	2	1	Beijing	000000000003771
82	2	5	3	3	2	2	6	3	2	4	3	2	48	EAI1_SOM	777777777413731
83	2	2	1	3	1	2	6	2	2	4	3	1		Unknown	777777604060771
84	2	2	3	3	1	2	5	2	5	3	5	2	53	T1	777777777760771
85	2	5	1	3	3	2	5	2	5	3	3	2	1	Beijing	000000000003771
86	2	2	3	3	3	2	5	2	4	3	3	2	53	T1	777777777760771
87	2	2	4	3	5	2	5	2	8	3	3	2	50	H3	777777777720771
88	2	2	4	3	5	2	5	2	4	3	4	2		Unknown	777777777733771
89	2	2	3	3	2	2	6	3	4	3	3	2	926	T1	773777777760771
90	2	2	5	3	1	2	5	2	5	3	3	2	926	T1	773777777760771
91	2	2	5	3	1	2	5	2	6	3	2	3	42	LAM9	777777607760771
92	2	5	1	4	2	2	6	2	5	3	5	2	1	Beijing	000000000003771
93	2	2	3	4	3	2	5	2	5	4	2	2	42	LAM9	777777607760771
94	2	2	1	4	2	2	6	2	2	3	2	1	42	LAM9	777777607760771

Table 3 MIRU-VNTR typing and spoligotyping results of the MDR-TB isolates (cont)

ID	MIRU02	MIRU04	MIRU40	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	SpolDB4 ST	SpolDB4 Lineage	Octaspoligotype
95	2	5	1	3	2	2	6	2	5	3	3	2	37	T3	777737777760771
96	2	2	3	3	3	2	5	2	6	3	3	1	34	S	776377777760771
97	2	2	1	3	3	2	5	2	7	3	6	2	1	Beijing	000000000003771
98	2	2	3	3	3	2	5	1	7	4	6	2	1	Beijing	000000000003771
99	2	2	4	3	2	2	5	2	5	3	3	1	53	T1	777777777760771
100	2	5	3	4	3	2	5	3	2	5	6	1	806	EAI1_SOM	757777777413731
101	2	2	3	3	3	2	7	1	6	3	3	2		Unknown	677717607760771
102	2	2	3	3	3	2	5	2	7	3	6	3	1	Beijing	000000000003771
103	2	5	4	4	2	2	6	3	2	3	4	1	806	EAI1_SOM	757777777413731
104	2	2	4	4	3	2	5	2	5	3	3	2	92	X3	700076777760771
105	2	2	3	4	1	2	5	2	5	3	3	2	53	T1	777777777760771
106	2	5	3	4	2	2	6	3	2	3	4	1	48	EAI1_SOM	777777777413731
107	2	5	4	4	2	2	6	3	2	3	4	1	48	EAI1_SOM	777777777413731
108	2	2	4	3	2	2	5	2	7	4	7	3	1	Beijing	000000000003771
109	2	3	4	3	2	2	6	2	4	3	3	2	1915	S	776377737760771
110	2	3	3	5	3	2	5	2	5	3	3	2	34	S	776377777760771
111	2	2	2	5	1	2	5	1	1	3	3	2	37	T3	777737777760771
112	2	2	4	3	3	2	5	2	5	3	3	2		Unknown	777400177760771
113	2	2	3	6	3	2	5	2	1	3	3	2	50	H3	777777777720771
114	2	5	3	5	1	2	6	1	2	3	4	1	48	EAI1_SOM	777777777413731
115	2	2	3	3	3	2	5	1	7	5	3	3	1	Beijing	000000000003771
116	2	2	3	3	3	2	5	1	7	3	4	3	1	Beijing	000000000003771
117	2	5	3	4	4	2	6	1	2	3	4	1	48	EAI1_SOM	777777777413731
118	2	5	3	4	3	2	6	1	2	3	4	1	48	EAI1_SOM	777777777413731
119	2	2	3	3	3	2	5	2	7	3	3	3	1	Beijing	000000000003771

Table 3 MIRU-VNTR typing and spoligotyping results of the MDR-TB isolates (cont)

ID	MIRU02	MIRU04	MIRU40	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	SpolDB4 ST	SpolDB4 Lineage	Octaspoligotype
120	2	2	4	3	1	2	5	1	7	3	3	3	1	Beijing	000000000003771
121	2	2	3	3	2	2	5	2	5	3	6	2	53	T1	777777777760771
122	2	5	3	4	1	2	6	3	2	3	6	1	815	LAM11_ZWE	777777606060731
123	2	2	1	3	2	2	5	2	7	4	3	3	1	Beijing	000000000003771
124	2	2	2	3	2	2	6	1	5	3	4	2		Unknown	777777404060731
125	2	2	3	5	1	2	5	1	1	2	6	2	149	T3_ETH	777000377760771
126	2	2	2	3	2	2	5	1	7	4	3	3	1	Beijing	000000000003771
127	2	2	3	5	2	2	5	1	5	3	3	2	50	H3	777777777720771
128	2	2	6	4	2	2	5	1	8	3	5	3	1	Beijing	000000000003771
129	2	5	3	3	4	2	5	1	5	3	3	2	34	S	776377777760771
130	2	3	1	4	1	2	6	2	1	3	5	2	48	EAI1_SOM	777777777413731
131	2	2	3	3	2	2	6	1	3	2	3	2	815	LAM11_ZWE	777777606060731
132	2	2	3	4	3	2	5	1	7	3	4	3	1	Beijing	000000000003771
133	2	2	4	4	3	2	6	1	5	3	3	2	33	LAM3	776177607760771
134	2	2	3	4	2	2	5	1	7	3	6	1	50	H3	777777777720771
135	2	2	4	4	2	2	6	1	5	3	3	1	100	MANU1	777777777773771
136	2	2	1	3	3	2	5	1	7	1	3	2	1	Beijing	000000000003771
137	2	2	2	3	3	2	6	1	5	2	3	3	815	LAM11_ZWE	777777606060731
138	2	5	3	4	1	2	6	1	2	3	5	1	815	LAM11_ZWE	777777606060731
139	2	2	1	4	2	2	6	1	5	2	3	2	48	EAI1_SOM	777777777413731
140	1	2	4	4	3	2	6	1	5	3	4	2	42	LAM9	777777607760731
141	2	2	3	4	2	2	5	1	5	3	3	2	53	T1	777777777760771
142	2	5	3	4	2	2	6	5	2	3	3	1	48	EAI1_SOM	777777777413731
143	2	2	3	3	2	2	5	"5(+2)"	7	2	5	2	1	Beijing	000000000003771
144	2	2	3	4	2	2	5	5	5	3	3	2	100	MANU1	777777777773771

Table 3 MIRU-VNTR typing and spoligotyping results of the MDR-TB isolates (cont)

ID	MIRU02	MIRU04	MIRU40	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	SpolDB4 ST	SpolDB4 Lineage	Octaspoligotype
145	2	2	3	3	2	2	5	"5(+1)"	7	3	5	3	1	Beijing	000000000003771
146	2	5	3	4	2	2	6	3	2	3	4	1	48	EAI1_SOM	777777777413731
147	2	2	1	4	1	2	6	4	5	3	3	2		Unknown	777775606060731
148	2	2	1	4	1	2	6	4	5	2	3	2		Unknown	777775606060731
149	2	5	3	4	2	2	6	"4(+1)"	2	2	4	1	1	Beijing	000000000003771
150	2	2	3	3	2	2	5	1	7	3	5	3	1	Beijing	000000000003771
151	2	2	3	2	2	2	5	4	7	4	5	4		Unknown	777777417740731
152	1	2	4	4	2	2	6	1	5	3	3	2	48	EAI1_SOM	777777777413731
153	2	5	3	4	2	2	6	1	2	3	4	1	48	EAI1_SOM	777777777413731
154	2	5	3	4	2	2	6	1	2	3	4	1	48	EAI1_SOM	777777777413731
155	2	5	3	3	2	2	6	3	2	3	4	1	48	EAI1_SOM	777777777413731
156	2	5	3	4	2	2	6	"3(+1)"	2	3	4	1	48	EAI1_SOM	777777777413731
157	2	2	3	3	3	2	5	1	5	3	5	3	719	T1	776177407760771
158	2	2	3	3	3	2	5	1	7	4	5	3		Unknown	776177603771771
159	2	5	3	4	2	2	6	3	2	3	4	1	34	S	776377777760771
160	2	2	3	4	3	2	6	1	5	3	3	2	1	Beijing	000000000003771
161	2	2	"3(+1)"	4	2	2	6	1	5	3	3	2	42	LAM9	777777607760771
162	3	3	4	3	2	2	5	1	4	3	3	2	1	Beijing	000000000003771
163	2	2	3	3	2	2	5	5	7	3	6	3	48	EAI1_SOM	777777777413731
164	2	2	2	3	2	2	5	2	5	3	2	3	1	Beijing	000000000003771
165	2	2	1	3	3	2	5	2	7	2	6	3	1	Beijing	000000000003771
166	2	2	3	4	2	2	6	2	2	3	4	1	1	Beijing	000000000003771
167	2	2	3	2	3	2	5	1	7	4	5	4	1	Beijing	000000000003771
168	2	2	3	3	3	2	5	1	7	3	5	3	815	LAM11_ZWE	777777606060731
169	2	2	1	4	1	2	6	1	5	2	3	2	48	EAI1_SOM	777777777413731

Table 3 MIRU-VNTR typing and spoligotyping results of the MDR-TB isolates (cont)

ID	MIRU02	MIRU04	MIRU40	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	SpolDB4 ST	SpolDB4 Lineage	Octaspoligotype
170	2	2	3	4	3	2	6	1	2	3	4	1	48	EAI1_SOM	777777777413732
171	2	2	4	5	4	2	5	1	8	3	3	2	50	H3	777777777720771
172	3	3	4	4	3	2	5	1	2	3	3	2	34	S	776377777760771
173	2	3	4	4	3	2	5	1	5	3	3	2	71	S	776337777760771
174	2	3	4	3	3	2	5	1	5	3	3	2	71	S	776337777760771
175	2	5	1	4	2	2	6	1	2	3	4	1	48	EAI1_SOM	777777777413731
176	2	5	3	4	2	2	5	1	2	3	4	1	48	EAI1_SOM	777777777413731
177	2	2	1	3	2	2	7	1	3	2	3	2		Unknown	657777603770371
178	2	5	3	4	2	2	6	1	2	3	4	0	48	EAI1_SOM	777777777413731
179	2	5	6	3	3	2	5	2	5	3	3	2	34	S	776377777760771
180	2	3	5	3	3	2	5	3	5	3	3	2	71	S	776337777760771
181	2	2	1	3	3	2	5	1	6	3	5	3	1	Beijing	000000000003771
182	2	2	3	4	3	2	6	1	5	3	3	1	33	LAM3	776177607760771
183	2	2	1	3	2	2	6	1	4	3	5	1	42	LAM9	777777607760771
184	2	5	3	4	2	2	6	2	2	3	4	0	48	EAI1_SOM	777777777413731
185	2	2	1	4	2	2	6	1	5	3	5	1	815	LAM11_ZWE	777777606060731
186	2	2	3	3	2	2	5	1	7	4	4	3	1	Beijing	000000000003771
187	2	5	3	4	2	2	6	1	2	4	3	1	48	EAI1_SOM	777777777413731
188	2	2	3	3	2	2	5	1	7	3	3	3	1	Beijing	000000000003771
189	2	5	3	4	2	2	6	2	2	4	3	1	48	EAI1_SOM	777777777413731
190	1	2	4	4	3	2	6	1	5	4	3	2	42	LAM9	777777607760771
191	1	2	4	4	3	2	6	1	5	4	3	2	42	LAM9	777777607760771
192	1	2	4	4	3	2	6	1	5	4	3	2	42	LAM9	777777607760771
193	2	5	3	4	2	2	6	2	2	4	4	1	48	EAI1_SOM	777777777413731

Table 3 MIRU-VNTR typing and spoligotyping results of the MDR-TB isolates (cont)

ID	MIRU02	MIRU04	MIRU40	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	SpolDB4 ST	SpolDB4 Lineage	Octaspoligotype
194	3	3	4	3	2	2	5	1	4	4	3	2	34	S	776377777760771
195	2	3	4	3	3	2	5	1	5	4	3	2	71	S	776337777760771
196	2	2	1	4	1	2	6	1	5	3	3	2	59	LAM11_ZWE	777777606060771
198	2	2	3	4	1	2	6	1	5	4	5	2	1	Beijing	000000000003771
199	2	2	1	2	3	2	5	1	7	3	3	4	926	T1	773777777760771
200	2	2	4	3	1	2	5	1	5	4	3	2	53	T1	777777777760771
201	2	2	1	3	3	2	5	1	6	4	3	3	1	Beijing	000000000003771
202	2	2	1	3	3	2	6	1	5	4	3	2	53	T1	777777777760771
203	2	2	3	3	3	2	5	1	7	4	5	1	1	Beijing	000000000003771
204	2	2	3	3	3	2	5	1	5	4	5	1	1	Beijing	000000000003771
205	2	2	3	3	3	2	5	1	7	4	5	2	1	Beijing	000000000003771
206	2	5	3	4	2	2	6	2	2	4	4	1	48	EAI1_SOM	777777777413731
207	2	5	3	4	2	2	6	2	2	4	4	2	48	EAI1_SOM	777777777413731
208	2	3	3	3	3	2	5	1	5	4	3	2	34	S	776377777760771
209	2	2	1	4	3	2	3	1	7	4	3	1	37	T3	777737777760771
210	2	2	3	3	3	2	5	1	7	4	6	2	1	Beijing	000000000003771
211	2	2	2	4	3	2	5	1	5	3	4	2	137	X2	777776777760601
212	2	2	2	4	3	2	5	1	5	3	4	3	137	X2	777776777760601
213	3	3	4	3	3	2	5	1	2	3	4	1	34	S	776377777760771
214	2	2	3	5	3	2	5	1	5	3	3	1	524	H3	777777777720711
215	2	2	4	7	4	2	5	1	1	3	4	2		Unknown	703377407761771
216	2	5	3	4	1	2	6	2	2	4	4	2	48	EAI1_SOM	777777777413731
217	2	2	1	4	1	2	6	1	2	4	4	1	1	Beijing	000000000003771
218	2	5	3	3	3	2	6	2	5	4	3	2	48	EAI1_SOM	777777777413731
219	2	5	3	4	2	2	6	2	2	4	4	3	48	EAI1_SOM	777777777413731

Table 3 MIRU-VNTR typing and spoligotyping results of the MDR-TB isolates (cont)

ID	MIRU02	MIRU04	MIRU40	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	SpolDB4 ST	SpolDB4 Lineage	Octaspoligotype
220	2	5	3	4	2	2	6	4	2	4	4	3	48	EAI1_SOM	777777777413731
221	2	2	3	4	3	2	6	1	5	4	3	3	719	T1	776177407760771
222	2	5	3	4	2	2	6	2	2	4	4	1	48	EAI1_SOM	777777777413731
223	3	3	4	3	3	2	5	2	2	4	3	1	34	S	776377777760771
224	2	2	4	5	4	2	5	1	8	4	3	2	50	H3	777777777720771
225	2	5	3	4	1	2	6	1	2	4	4	2	48	EAI1_SOM	777777777413731
226	2	5	3	3	3	2	5	1	5	4	3	3	48	EAI1_SOM	777777777413731
227	2	3	4	3	3	2	5	1	5	4	3	2	34	S	776377777760771
228	2	2	3	3	3	2	5	1	7	4	5	2	1	Beijing	000000000003771
229	2	5	3	4	2	2	6	2	2	4	4	2	48	EAI1_SOM	777777777413731
230	2	5	3	4	2	2	6	1	2	4	4	2	48	EAI1_SOM	777777777413731
231	2	2	4	4	3	2	6	1	7	4	6	3	1	Beijing	000000000003771
232	2	2	6	1	1	2	5	2	5	4	3	1	53	T1	777777777760771
233	2	5	3	5	1	2	6	1	2	3	3	1	48	EAI1_SOM	777777777413731
234	2	3	4	3	2	2	5	1	4	3	3	2	34	S	776377777760771
235	2	2	5	3	3	2	5	1	5	3	3	2	926	T1	773777777760771
236	2	5	3	4	1	2	6	1	2	3	4	1	48	EAI1_SOM	777777777413731
237	2	2	5	3	2	2	5	2	5	3	3	2	53	T1	777777777760771
238	2	2	1	4	1	2	6	1	5	2	3	2	813	LAM11_ZWE	777777606060631
239	2	2	1	3	1	2	6	1	4	3	2	2	814	LAM11_ZWE	777777606060631
240	2	2	3	4	2	2	6	1	5	3	3	2	33	LAM3	776177607760771
241	2	2	4	4	3	2	5	1	5	3	3	2	92	X3	700076777760771
242	2	2	1	4	1	2	6	1	5	2	3	2	1873	LAM11_ZWE	777774606060731
243	2	5	3	4	2	2	6	2	2	3	4	1	48	EAI1_SOM	777777777413731

Table 3 MIRU-VNTR typing and spoligotyping results of the MDR-TB isolates (cont)

ID	MIRU02	MIRU04	MIRU40	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	SpolDB4 ST	SpolDB4 Lineage	Octaspoligotype
244	2	2	3	4	3	2	6	1	5	3	3	1	33	LAM3	776177607760771
245	2	2	3	4	3	2	6	1	5	3	3	2	719	T1	776177407760771
246	2	5	3	4	2	2	6	2	2	3	4	1	48	EAI1_SOM	777777777413731
247	2	2	1	3	3	2	5	1	7	3	5	3	1	Beijing	000000000003771
248	2	2	4	3	1	2	5	1	5	1	3	2	53	T1	777777777760771
249	2	2	3	3	5	2	5	1	5	3	3	2	37	T3	777737777760771
250	2	5	3	4	3	2	6	2	2	3	5	1	806	EAI1_SOM	757777777413731
251	2	3	6	3	3	2	5	1	5	3	3	2	100	MANU1	777777777773771
253	2	5	4	4	2	2	6	2	2	3	4	1	48	EAI1_SOM	777777777413731
254	2	5	3	4	2	2	6	2	2	3	4	1	48	EAI1_SOM	777777777413731
255	2	2	3	4	2	2	7	1	5	3	2	2	73	T2-T3	777737777760731
256	2	5	1	4	2	2	6	2	5	3	4	1	48	EAI1_SOM	777777777413731
257	2	5	3	4	2	2	6	2	5	3	4	1	48	EAI1_SOM	777777777413731
258	2	2	3	3	1	2	5	1	5	3	3	2	37	T3	777737777760771
259	2	2	4	2	3	2	6	1	3	3	4	2	719	T1	776177407760771
260	2	2	3	4	2	2	5	1	5	3	3	2	92	X3	700076777760771
261	2	2	4	3	1	2	5	1	5	3	3	2	53	T1	777777777760771
262	2	2	2	3	2	2	5	1	6	3	5	3	1	Beijing	000000000003771
263	2	2	1	3	3	2	5	1	6	3	5	2	1	Beijing	000000000003771
264	1	2	1	4	3	2	6	1	5	3	3	2	1	Beijing	000000000003771
265	1	2	4	4	3	2	6	1	5	3	3	2	60	LAM4	777777607760731
267	2	2	4	2	3	2	5	1	7	3	5	3	1	Beijing	000000000003771
268	2	2	3	3	3	2	5	3	7	3	5	3	1	Beijing	000000000003771
269	2	2	3	3	3	2	6	1	5	4	3	2	53	T1	777777777760771
270	1	2	1	4	2	2	6	1	5	3	3	2	60	LAM4	777777607760731

Table 3 MIRU-VNTR typing and spoligotyping results of the MDR-TB isolates (cont)

ID	MIRU02	MIRU04	MIRU40	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	SpolDB4 ST	SpolDB4 Lineage	Octaspoligotype
271	2	3	4	3	4	2	5	1	5	3	3	2	34	S	776377777760771
272	2	5	4	5	2	2	6	1	2	3	4	1	48	EAI1_SOM	777777777413731
273	2	2	3	4	3	2	5	1	7	3	5	3	1	Beijing	000000000003771
274	2	2	3	4	3	2	6	1	4	3	3	2	53	T1	777777777760771
276	2	5	2	3	3	2	5	1	7	3	5	3	1	Beijing	000000000003771
277	2	5	3	3	3	2	5	1	5	3	3	2	34	S	776377777760771
278	2	5	6	3	3	2	5	1	5	3	3	2	34	S	776377777760771
279	2	2	6	3	3	2	5	1	5	3	3	2	34	S	776377777760771
280	2	2	6	4	3	2	6	1	5	3	3	2	33	LAM3	776177607760771
281	1	2	3	4	3	2	6	1	5	3	3	2	60	LAM4	777777607760731
282	2	2	4	3	2	2	6	1	4	3	2	2	42	LAM9	777777607760771
283	2	2	1	4	2	2	6	1	4	3	3	2	815	LAM11_ZWE	777777606060731
284	2	2	1	3	2	2	5	1	5	2	3	2	60	LAM4	777777607760731
285	2	2	3	4	3	2	5	1	5	3	3	2	60	LAM4	777777607760731
286	2	2	4	5	2	2	5	1	7	3	3	2	50	H3	777777777720771
287	2	2	4	4	3	2	5	1	6	3	2	2	53	T1	777777777760771
288	2	4	1	4	3	2	5	1	5	3	3	2	771	T1	777777717760771
289	2	2	4	2	3	2	6	1	3	3	4	2	1	Beijing	000000000003771
290	2	3	3	4	3	2	5	1	6	4	3	2	48	EAI1_SOM	777777777413731
291	2	2	3	3	3	2	5	1	5	4	3	2	61	LAM10_CAM	777777743760771
292	2	2	4	8	3	2	5	1	1	4	4	3		Unknown	703377407761771
293	2	2	3	2	4	2	5	1	7	4	5	2	53	T1	777777777760771
294	2	2	4	0	3	2	6	1	5	4	3	2	48	EAI1_SOM	777777777413731

Table 3 MIRU-VNTR typing and spoligotyping results of the MDR-TB isolates (cont)

ID	MIRU02	MIRU04	MIRU40	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	SpolDB4 ST	SpolDB4 Lineage	Octaspoligotype
295	2	2	3	4	3	2	5	1	3	3	2	2	1273	X3	700076777760731
296	2	2	3	4	3	2	6	1	5	4	3	2	47	H1	777777774020771
297	2	2	1	3	3	2	5	1	7	5	5	3	1	Beijing	000000000003771
298	2	5	1	4	3	2	6	1	2	3	4	3	48	EAI1_SOM	777777777413731
299	2	5	4	4	2	2	6	3	2	4	4	1	719	T1	776177407760771
300	2	2	1	3	2	2	6	1	3	4	3	2	60	LAM4	777777607760731
301	2	2	1	3	2	2	6	1	3	4	2	2	60	LAM4	777777607760731
302	2	2	1	3	2	2	6	1	3	4	3	2		Unknown	777777760412731
303	2	2	1	4	2	2	6	9	2	3	4	2	1	Beijing	000000000003771
304	2	2	1	3	3	2	5	2	1	3	6	3	33	LAM3	776177607760771
305	2	2	5	3	2	2	6	1	5	4	3	2	53	T1	777777777760771
306	2	3	3	3	3	2	5	2	5	4	3	2	34	S	776377777760771
307	2	2	3	3	3	2	5	1	7	4	5	4	1	Beijing	000000000003771
308	2	5	4	4	3	2	6	3	2	4	4	1	92	X3	700076777760771
309	2	2	3	4	2	2	5	1	7	4	3	2	48	EAI1_SOM	777777777413731
310	2	5	4	4	2	2	6	3	2	4	4	1	719	T1	776177407760771
311	2	2	4	5	2	2	5	1	8	4	3	2	50	H3	777777777720771
312	2	2	3	5	4	2	6	1	3	4	3	2	60	LAM4	777777607760731
313	2	2	4	4	3	2	6	1	5	4	3	2	92	X3	700076777760771
314	2	2	4	4	3	2	5	1	5	4	3	2	92	X3	700076777760771
315	2	3	3	3	3	2	5	1	5	4	3	2	53	T1	777777777760771
316	2	2	4	4	3	2	5	1	5	4	3	2	1	Beijing	000000000003771
317	2	2	4	5	3	2	5	1	7	4	3	2	926	T1	773777777760771
318	2	2	3	4	4	2	6	1	3	3	4	1	48	EAI1_SOM	777777777413732

Table 3 MIRU-VNTR typing and spoligotyping results of the MDR-TB isolates (cont)

ID	MIRU02	MIRU04	MIRU40	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	SpolDB4 ST	SpolDB4 Lineage	Octaspoligotype
319	2	5	3	4	3	2	6	1	2	2	3	1	48	EAI1_SOM	777777777413731
320	2	2	3	4	2	2	6	1	5	4	5	2	719	T1	776177407760771
321	2	2	4	4	3	2	5	1	5	3	4	2	1095	X1	000076777760771
322	1	2	4	4	3	2	6	1	5	4	3	2	42	LAM9	777777607760771
323	1	2	4	4	3	2	6	1	5	4	4	2	42	LAM9	777777607760771
324	2	"2(+3)"	4	5	3	2	6	1	7	4	3	2	50	H3	777777777720771
326	2	2	1	3	3	2	6	1	3	4	3	2	18	X2	677776777760601
327	2	2	4	3	2	2	6	4	8	4	3	3	719	T1	776177407760771
328	2	5	4	4	2	2	5	3	2	4	3	1	71	S	776337777760771
329	2	2	2	3	2	2	6	1	3	4	3	2	1	Beijing	000000000003771
330	2	2	4	4	2	2	6	1	"5(+2)"	3	3	1	719	T1	776177407760771
331	2	2	4	3	2	2	6	5	3	3	3	2		Unknown	077776775760731
332	2	2	3	4	2	2	5	4	5	3	4	2	719	T1	776177407760771
333	2	2	3	4	3	2	3	1	7	3	3	3	33	LAM3	776177607760771
334	2	5	3	4	3	2	5	1	2	3	3	1	1	Beijing	000000000003771
335	2	2	3	3	2	2	6	5	2	3	3	3	48	EAI1_SOM	777777777413731
336	1	2	4	4	1	2	6	1	5	3	3	2	33	LAM3	776177607760771
337	1	2	4	4	3	2	6	1	5	3	3	2	33	LAM3	776177607760771
338	2	5	3	4	3	2	6	1	2	3	7	1	48	EAI1_SOM	777777777413731
339	2	2	3	2	3	2	5	7	7	3	6	4	719	T1	776177407760771
340	2	2	4	4	3	2	6	1	6	3	3	2	33	LAM3	776177607760771
343	2	2	1	3	3	2	7	1	5	3	2	2	42	LAM9	777777607760771