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DIFFERENCES BETWEEN GENOTYPIC AND PHENOTYPIC RESISTANCE IN MDR-TB STRAINS IN SOUTH AFRICA

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Dissertation submitted to the Faculty of Health Science, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree
of
Master of Science in Medicine in Virology

Johannesburg, 2012

DECLARATION

I, Mandira Sewpersadh declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

_____
Mandira Sewpersadh

_____day of _____, 2012

Dedication

I would like to express my thanks and gratitude to my supportive husband, son and family.

Publications and presentations arising from this study

1. Sewpersadh M, Erasmus L, Bapela N, van der Walt M. Phenotypic and genotypic discordant drug-resistant *Mycobacterium tuberculosis* isolates identified and characterised from South Africa. Presented, as a poster at the American Thoracic Society conference held in San Francisco, USA in May 2012.

Abstract

South Africa (SA) is burdened with one of the highest tuberculosis (TB) infection rates worldwide. The dual epidemic of HIV and MDR- and extensively drug-resistant (XDR) -TB outbreaks prompted the World Health Organisation to call for a new rapid molecular diagnostics tool(s). To curb the spread of drug-resistant TB, SA introduced genotypic Hain MTBDR*plus* line probe assays (Hain LPA) for the routine rapid diagnosis of MDR-TB. This study aimed to determine the frequency, geographic distribution and genetic basis for phenotypic and genotypic drug susceptibility testing (DST) discordant findings. The cultures used in this study were isolated during the period June 2007- July 2008 from Western Cape, Gauteng, KwaZulu-Natal, and Northern Cape. A total of 118 comparable MGIT and Hain LPA DST were obtained with 41 isolates verified to be discordant. The predominant families were the LAM, T, X and, S. Hain LPA failed to identify INH resistance (R) in 46.3% (19/41) of isolates with MIC's supporting the phenotypic resistance. Genotypic RIF-R was shown in 31.7% (13/41) of isolates which was not expressed phenotypically but interestingly the MIC's favour the LPA resistance. Sequencing analysis of the *rpoB* gene region identified new mutations; Leu458Pro, Leu436Pro, and Asp441Gly, associated with missing wildtypes. For INH, the *katG* and *inhA* gene regions were sequenced. Mutations at codon Gly213Val, Pro232Lys, Lys254Asn, and Ser259Asn were detected in *katG* and a variety found in *inhA*. These mutations have not been previously reported neither are they incorporated on the Hain LPA strips. Detailed knowledge of the frequency distribution of resistance-linked mutations and associated MICs in different regions of SA, could facilitate understanding of the limitations of current molecular tests and inform testing algorithms. These findings impact on the use of new molecular diagnostics as well as epidemiological monitoring of drug-resistant strains.

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List of Abbreviations

ºC	Degree
%	Percentage
µl	Microlitres
µmol	Micromoles
AFB	Acid fast bacilli
Asn	Asparagine
Asp	Aspartic acid
bp	Base pair
BSL	Biosafety laboratory
C	Cytosine
cfu	Colony forming units
CO_2	Carbon dioxide
CR	Complement receptors
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DR	Direct repeat
DST	Drug susceptibility testing
DVR	Direct variable repeat
EMB	Ethambutol
G	Guanine
Gly	Glycine
HIV	Human Immunodeficiency Virus
INH	Isoniazid

IS	Insertion sequence
KZN	KwaZulu-Natal
Leu	Leucine
LJ	Löwenstein-Jensen
LPA	Line probe assay
Lys	Lysine
MDR-TB	Multidrug-resistant tuberculosis
MIC	Minimal inhibitory concentration
min	Minute
MIRU-VNTR	Mycobacterial interspersed repetitive unit of variable-number tandem repeats
ml	Millilitres
mM	Micromoles
MRC	Medical research council
MTBC	Mycobacterium tuberculosis complex
NALC	*N*-acetyl-*L*-cysteine
NaOH	Sodium hydroxide
NHLS	National Health Laboratory Services
nm	Nanometre
NTCP	National Tuberculosis Control Programme
NTM	Non-tuberculous mycobacteria
OD	Optical density
PCR	Polymerase chain reaction
PG	Proline glutamine
PPG	Proline proline glutamine
Pro	Proline

PZA	Pyrazinamide
R	Resistant
RFLP	Restriction fragment length polymerase
RIF	Rifampicin
rRNA	Ribosomal ribonucleic acid
SA	South Africa
SG	Study group
STAG-TB	Strategic and technical advisory group-tuberculosis
STR	Streptomycin
TB	Tuberculosis
UV	Ultra violet
Val	Valine
WHO	World Health Organisation
WT	Wild type
XDR-TB	Extensively drug resistant tuberculosis
ZN	Ziehl-Neelsen

CHAPTER 1

1 LITERATURE REVIEW

1.1 Introduction

Tuberculosis (TB) is a chronic infectious airborne disease caused by the tubercle bacillus *M. tuberculosis* (Cole et al, 1998). TB infections are characterised by the growth of rod-shaped bundles of the *M. tuberculosis* bacteria which in susceptible animals, including humans produce microscopic "tubercles" consisting of chronic granulomas, some with caseous necrosis. Lung tissue is frequently infected, but other parts of the body can be involved (Knechel, 2009). In 2009, 9.4 million new TB cases, including the HIV infected population, were recorded.

Although one-third of the world's population is infected by *M. tuberculosis*, only approximately 5-10% of the infected population who are HIV uninfected will develop TB at some stage in their life. HIV and TB co-infected populations are more like to develop TB. (WHO, 2010). Factors that contribute to the development of tuberculosis disease are complex and not completely understood but suppression of cell-mediated immunity plays a key role (Caws et al, 2008).

1.2 Classification of the Genus *Mycobacterium*

Mycobacterium tuberculosis, the causative agent of TB, was first discovered by Robert Koch in 1882 (Grange, 2009). In 1896, Lehmann and Neumann classified the tubercle bacillus under the binomial genus plus species term *Mycobacterium tuberculosis* (Grange, 2009). The *Mycobacterium* genus includes more than 120 different species and is classified under the order of Actinomycetalis, with its own family, Mycobacteriaceae. Within the *Mycobacterium* genus, the pathogens known to cause serious diseases in mammals are those causing tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*Mycobacterium leprae*) (Ryan et al, 2010). The genus name is derived from the Greek word, '*myces*-' which means "fungus" and relates to the mould-like manner in which mycobacteria grow in liquid culture (Kerr, 2004). There are two distinct subgroups in the *Mycobacterium* genus: slow growers and rapid growers (Tortoli, 2003) with the latter being closely related to the *Nocardia* genus. The slow growing population include members of the *Mycobacterium tuberculosis* Complex (MTBC) which are the causative agents of tuberculosis in animals and humans (Ryan et al, 2010). Species included in the MTBC are; *M. tuberculosis* (the major cause of human tuberculosis), *M. bovis* (cattle, deer), *M. africanum* (human), *M. canetti* (human), *M. caprae* (goats), *M. microti* (vole), *M. pinnipedii* (seal) (Grange 2009). The MTBC species are grouped by their similarity at the nucleotide level and 16S rRNA sequences but differ largely in their host tropisms, phenotypes and pathogenicity (Brosch et al, 2002). Rapid growers usually have two copies of 16S rRNA, while slow growers have only one copy (Tortoli, 2003).

The clinical significance of *M. tuberculosis* resulted in it being selected as the type species of the *Mycobacterium* genus and, with the exception of *M. leprae* and *Mycobacterium ulcerans*

the causative agent of Buruli ulcer all- other *Mycobacterium* species are grouped together and called the 'non-tuberculous mycobacteria' (NTM) (Grange, 2009).

1.3 Structure of Mycobacteria

The mycobacteria are mostly straight or curved rods which are non-sporing (Knechel, 2009) and do not have flagellae. The morphology of mycobacteria in clinical specimens vary between species and are affected by exposure to antimicrobial therapy (Grange 2009). Mycobacteria are Gram-positive and acid-fast bacilli (AFB) (Brosch et al, 2002). The typical size of mycobacteria is 0.5µm by 3µm and they have a complex cell wall structure which is core to their survival (Knechel, 2009). The cell wall has a high lipid content which includes mycolic acids and a trehalose-mycolic acid component called cord factor which has been identified as an important virulence factor. Below the cell wall is the cell membrane which has two phospholipid layers (Cole et al, 1998). The innermost layer of the cell wall gives the bacterium its rigidity and is made up of a net-like macromolecule comprising peptidoglycan and long polysaccharide chains crossed linked with short four amino acid peptides (Dean et al, 2001). To the peptidoglycan or murein layer, mycolic acids (long chain fatty acids) are attached giving the thick cell wall its rigidity (Tortoli, 2003). This thick lipid wall provides a barrier responsible for the physiological properties of *M. tuberculosis* which enables it to be resistant to antibiotics and resistant to host defence mechanisms (Knechel, 2009). *M. tuberculosis* contains a guanine (G) + cytosine (C) - rich genome (Cole et al, 1998).

1.4 Growth and Metabolic Characteristics of Mycobacteria

Most mycobacterial species, both slow and rapid growers are able to grow on simple media due to their undemanding nutritional requirements. Clinical cultivation of mycobacteria in laboratories is usually done on Löwenstein-Jensen (LJ) medium (Ryan, 2004). *M. tuberculosis* grows in small buff-coloured colonies resembling small cauliflowers or bread crumbs. It is a slow grower and takes between 6-8 weeks to produce a full lawn of growth (Kent et al, 1985). The commercial liquid-based mycobacterial culture system such as Becton Dickson BACTEC MGIT 960 system is also used and is preferred as it takes 8-12 day for bacterial growth instead of weeks (Whitelaw et al, 2009).

1.5 Mycobacterial Antigens

Mycobacteria have a complex antigenic structure and their antigens can be divided into three main categories: actively secreted, cell-wall bound, and cytoplasmic. The immune system of infected hosts is initially exposed to the first two categories and these play important roles in the activation of host defence mechanisms and in pathogenesis (Grange, 2009). Soluble and cell-bound mycobacterial antigens have been functionally characterised and a few have been used for the development of diagnostic tests involving antibody or cell-mediated host immune responses.

1.6 The *M. tuberculosis* Genome

The type strain of *M. tuberculosis,* H37Rv, was isolated in 1905 and is referred to as the TB reference strain. It is used worldwide in biomedical research applications due to its virulence in animal models (Cole et al, 1998). Sequenced in 1998, H37Rv genome consists of $4.4x10^6$ base pairs (bp) with about 4000 genes (Smith, 2003). The large genome is made up of 65% G + C content which is relatively constant throughout indicating that it evolved with little or no other bacterial DNA inclusion from other genera (Grange, 2009). It is rich in repetitive DNA, especially with insertion sequences, new multigene families and duplicated housekeeping genes (Cole et al, 1998).

In comparison to other bacteria, the majority of the genes are involved in lipid synthesis and metabolism. There are approximately 250 enzymes associated with lipid metabolism of *M. tuberculosis* (Grange, 2009). An unusual feature of the *M .tuberculosis* genome is the coding of the Pro-Glu (PG) and Pro-Pro-Glu (PPG) glycine-rich proteins (Cole et al, 1998) whose functions are unknown but are found in the cell membrane and cell wall. It is hypothesised that these proteins contribute to the antigenic variance of *M. tuberculosis* during infection (Smith, 2003).

1.7 The Origins and evolution of *Mycobacterium tuberculosis*

Evidence suggests that tuberculosis would have already infected and caused disease in human ancestors several thousand years (roughly 15000-20000 years) ago (Sreevatsan et al, 1997). Based on evidence of tuberculosis found in early hominids in East Africa during that period, it is suggested that tubercle bacilli emerged in Africa (Gutierrez et al, 2005; Grange, 2009).

The genus *Mycobacterium* is thought to have originated like other aerobic actimomycetes from soil bacteria with some species (*M. tuberculosis* and *M. leprae*) evolving to live in mammals (Smith, 2003). Prior to sequencing, *M. tuberculosis* was thought to have resulted from a species jump from animals to humans (Grange, 2009; Brosch et al, 2002). Domestication of cattle was initially presumed to have allowed the mycobacterium pathogen found in livestock to evolve to humans (Cole, 1998; Smith et al, 2009). Already in 1898 small differences were observed between the tubercle bacilli isolated from humans and cattle. Strains recovered from cattle were referred to as bovine tubercle bacilli (*M. bovis*) (Grange, 2009) and was hypothesised to be the evolutionary precursor of *M. tuberculosis* (Smith et al, 2009). However, detailed genomic sequencing (Cole et al, 1998) shows a different picture. MTBC complex differ in phenotypic characteristics but display extreme genetic homogeneity, 99.95% sequence similarity (Brosch et al, 2002) with a high degree of conservation in their house keeping genes and no significant trace of genetic exchange among species. It is therefore believed that MTBC are the clonal progeny of a single ancestor (Gutierrez et al, 2005; Brosch et al, 2002). Research shows that the ancestral member of the complex, '*M. prototuberculosis*" is about 3 million years old. Recent studies have suggested that *M. bovis* and *M. tuberculosis* have evolved separately around the same time (Sreevatsan et al, 1997).

1.8 Transmission of *M. tuberculosis*

Although tuberculosis has afflicted humanity for centuries, it has only recently come to light that the disease is a result of a 'transmissible airborne infection' (Dharmadhikari et al, 2009). Scientists like Aristotle believed that TB was contagious but the mechanisms of how and what caused the disease were not understood (Smith, 2003). Opposing theories of TB

transmission were entertained historically. In the Northern countries in the second half of the 17th century (Smith, 2003) a predominant dogma was that TB was a hereditary disease (Smith, 2003). In the 1950s, William Firth Wells and engineers conceptualised how causative agents of certain infectious diseases, , such as TB, might become airborne and be transmitted from one person to another (Dharmadhikari et al, 2009).

The majority of tuberculosis infections are spread through the inhalation of small airborne droplet nuclei carrying the causative agent (Ryan et al, 2010). The airborne droplets are generated from coughing, sneezing, talking or singing of a person with pulmonary or laryngeal tuberculosis (Knechel, 2009). These droplets are expelled into the air (a single cough can produce 3000 droplet nuclei) and can remain airborne for several hours (Maher, 2009a). The transmission of the infectious particle, less than 5μm (Maher, 2009a) in diameter is influenced by the virulence of the bacilli, exposure of the bacilli to UV light, degree of ventilation and aerosolization (Knechel, 2009). Exposure of the lungs to the *M. tuberculosis* bacilli leads to respiratory infections, however tuberculosis disease can be found in other organs such as the lymphatics, pleura, bones/joints or meninges and cause extrapulmonary tuberculosis (Dharmadhikari et al, 2009; Knechel, 2009).

1.9 Epidemiology of tuberculosis

Despite effective treatment, TB remains a major public health threat, causing 2-3 million deaths annually of which 98% are from developing countries (Arnold, 2006). This situation is further exacerbated by the emergence of drug-resistant strains of *M.tuberculosis* requiring prolonged, more expensive, less effective therapy. In 2009, based on infection and disease

prevalence surveys, surveillance systems, and death registrations (Dye et al, 2008), an estimated 8.7 million new TB cases were recorded, with a global incidence rate of 125 cases per 100 000 population (WHO, 2011). Globally there were 440 000 new MDR-TB cases (defined as *Mycobacterium tuberculosis* resistant to both INH and RIF) reported with 150 000 deaths in 2008. In 2009, 3.3% of all new cases reported had MDR-TB, with XDR-TB (TB resistant to flouroquinolones and at least one injectable second-line drug) been confirmed in 58 countries (WHO, 2010). The bulk of the global TB burden is from South-East Asia, African and Western Pacific regions (Figure 1.1).

In 2009, an estimated 1.1 million (26%) new TB case arose in patients with HIV, with a 53% HIV prevalence among TB patients in the African region (WHO, 2010).

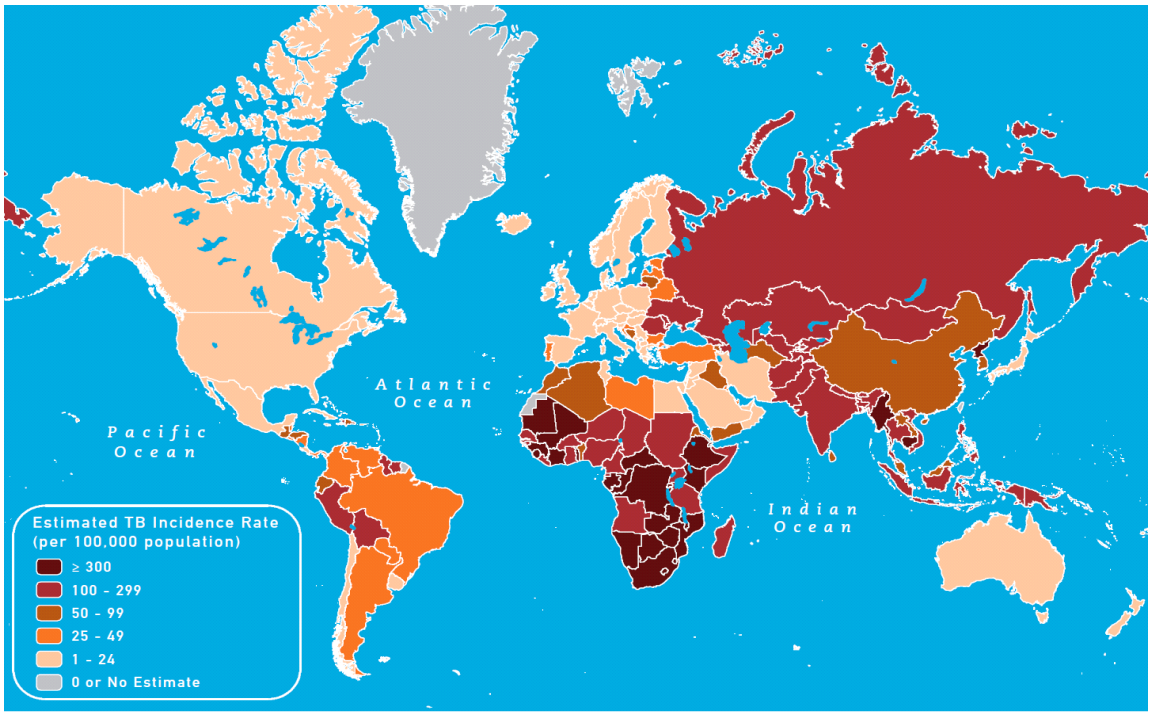


Figure 1.1 Global TB incidence rates (WHO, Global Tuberculosis report, 2010).

In SA, TB is a notifiable disease, with steadily increasing incidence rates recorded in the 20th century. In 2002, WHO recognised South Africa as one of the 22 high burden countries who

collectively contribute 80% of the epidemiological TB burden worldwide (WHO, 2010). In 2006, the TB crisis in SA was highlighted by an outbreak in a rural hospital in KwaZulu-Natal province with 39% (72/185) of culture-confirmed patients had MDR-TB, with 53 of them with XDR-TB (Gandhi et al, 2006). In 2007, TB was declared a national emergency (SA Department of Health, 2007). In 2008 WHO Strategic and Technical Advisory Group for Tuberculosis (STAG-TB) recognised the need to use molecular rapid molecular diagnostic tools and requested evidence for the use of LPA in low and middle income countries.

Currently, WHO estimates the TB incidence rates to be 971 per 100 000 population in SA with the total of 405 982 cases being notified (WHO, 2011a). In addition SA has a high burden of drug resistant TB. MDR-TB is found in 1.8% of all new TB cases and in 6.7% of re-treatment cases in SA with just over 9 000 confirmed MDR-TB cases reported in SA in 2010. (WHO, 2011a).

Drug resistance develops either through acquisition or transmission. Acquired resistance develops spontaneously through poor adherence, inappropriate treatment or poor absorption of drugs. In contrast, primary resistance develops when individuals are infected with an already drug-resistant strain (van Rie et al, 2005). It is important to understand the relative contribution of primary versus acquired drug resistance to the drug resistant TB burden in different communities. A central question is whether MDR-TB strains are transmissible or whether mutations conferring drug resistance affect the fitness of the strain by impairing reproductive function (Mathema et al, 2008). Several MDR and XDR-TB outbreaks have been reported, suggesting that drug resistant TB strains do have the potential for transmission (Sacks et al, 1999; Gandhi et al, 2006).

1.10 Molecular epidemiology of *Mycobacterium tuberculosis*

Molecular epidemiology is comparatively a young science aimed at addressing the epidemiological relationships between patients infected with TB through the association of the genotypes of the infecting bacteria causing disease (van der Spuy et al, 2008). It complements classical epidemiology by using molecular tools to track and confirm suspected outbreaks and transmission of different disease-causing stains over space and time (Bifani et al, 2002; McEvoy et al, 2009).

The introduction of various molecular genotyping methods has facilitated the development of a new field of scientific research, known as molecular epidemiology which has greatly enhanced our understanding of the disease dynamics of the TB epidemic. Using genotyping methods such as spoligotyping or IS*6110* –based restriction fragment length polymorphisms (RFLPs), genetically similar strains of *M. tuberculosis* can be classified into strain families or lineages such as W-Beijing or Haarlem (Mathema et al, 2008).

It is strategically important for the National TB Control Programme (DoH, 2007) to know the relative proportion of acquired and transmitted drug resistance in clinical isolates. Clustered cases of TB are defined as those who have identical or closely related genotypes and reflect on-going transmission. In contrast epidemiologically unrelated TB cases demonstrate broad genotypic variability and are interpreted to reflect acquisition of drug resistance. Drug resistance mutations can also provide genetic markers to demonstrate clonality (transmission). Genotypic data only provides information about the pathogen and therefore

should be interpreted in conjunction with additional and epidemiologic information to inform the TB control program (Barnes et al, 2003).

Other than from the Western Cape, there is relatively little genotypic information available regarding circulating drug susceptible or resistant *M. tuberculosis* strains in South Africa. Genotyping of drug-resistant *M. tuberculosis* isolates collected from clinics in the Boland-Overburg and Southern Cape-Karoo regions between January 2001 to February 2002 showed that four strain families were responsible for more than 70% of the drug-resistant TB in that area. This included the Beijing/W-like family, the IS*6110* low-copy-number clade and the F11 and F28 families (Streicher et al, 2004). Temporal analysis indicated that the Beijing clade had increased significantly in comparison to other clades primarily due to R220 strain (Johnson et al, 2010). This correlated with a previous study that showed the Beijing clade was increasing significantly in the Western Cape over this time period (van der Spuy et al, 2009). A hospital based prospective study of children with culture-confirmed tuberculosis in the Western Cape showed Beijing was the most prevalent genotype family, followed by LAM. The presence of both Beijing and Harlem genotype families was significantly associated with drug resistance (Marais et al, 2006). In KwaZulu-Natal (KZN) XDR-TB is reported to be was caused primarily by a single strain genotype with a distinct genotype by spoligotyping (F15/LAM4/KZN) (Pillay et al, 2007; Loerger et al, 2009).

However, spoligotyping of 41 XDR-TB isolates from 7 provinces, showed high genotypic diversity including seven internationally recognised genotype families (Beijing family, Latino-American-Mediterranean family, East-African-Indian family, the T, H, S and X3

families). The Beijing genotype family formed the largest group (Mlambo et al, 2008). A recent paper by Chihota et al, reporting on genotyping of MDR-TB strains from four provinces in SA showed distinct strain population structures in each province with specific strain families showing localisation to a particular geographical region (Chihota et al, 2012). In addition to recognising primary transmission versus acquired drug resistance, molecular epidemiology and comparative genomics can explore the evolutionary origins of *M. tuberculosis* and phylogenetic relationships between different strain families and distinguish exogenous reinfection from endogenous reactivation and cases of recurrent TB, thus enhancing our understanding of the epidemic (Mathema et al, 2008).

1.11 Pathogenesis and Virulence of Tuberculosis

The majority of tuberculous infections result from inhalation of airborne droplet nuclei carrying the causative bacteria (Ryan et al, 2010). Infection is established by interactions between *M. tuberculosis* and the host macrophages (Eley et al, 2009). The survival success of MTB, an intracellular pathogen, relies on avoiding the killing action of the host's phagocytes (Ryan et al, 2010).

There are four distinct stages in pulmonary tuberculosis; inhalation of the tubercle bacilli, activation of blood monocytes and other inflammatory cells, cessation of early logarithmic bacillary growth , and dormancy of the infection.

1.11.1 Primary infection

The initial encounter referred to as primary tuberculosis infection typically occurs in the macrophages (Eley et al, 2009). The inhaled pathogenic organism (stage one) deposited in the middle and lower lobes of the alveoli which are recognised by the alveolar macrophage complement receptors (CR1, CR3, CR4). The host's cell-mediated innate immune response is activated and the pathogen is phagocytosed (Ryan et al, 2010). Following phagocytosis by the macrophages, *M. tuberculosis*-specific adaptive cell mediated immunity develops (Eley et al, 2009).

The destruction of mycobacteria is dependent on the host phagocytes' intrinsic microbicidal capacity and the ingested mycobacterial virulence factors. Immediate killing of the mycobacteria leads to no infection setting in, shown in Figure 1.2. Those mycobacteria that escape the initial intracellular killing (Smith, 2003) by interfering with the acidification of the phagosome and affecting lysosomal enzyme activity, making it less effective (Eley et al, 2009), will multiply and be released from the macrophages. This process will attract blood monocytes and other inflammatory cells to the lung, referred to as stage two of pulmonary tuberculosis (van Crevel et al, 2002). We used to assume that tuberculosis results from a single infection with single *M. tuberculosis* strain. However there is evidence to suggest that mixed infections occur (Warren et al, 2004; van Rie et al, 2005; Cohen et al, 2011).

1.11.2 Reactivation

In tuberculosis, organisms that are not killed during primary infection enter a dormant or stationary phase as a result of oxygen and nutrient deprivation. Latent tuberculosis is present in one third of the world's population with less than 10% developing active disease (Eley et al, 2009; Bezuidenhout et al, 2009). Reactivation of the dormant infection occurs once the host experiences immunosuppression for a variety of reasons (Knechel, 2009) and may lead to the transmission of disease. *M. tuberculosis* reactivates at aerobic sites which favours growth of the aerobe MTB. Even though mycobacterial resuscitation-promoting factors have been identified, the mechanism of reactivation of these dormant foci is still poorly understood (Ryan et al, 2010). In developing countries with large immunosuppressed populations and/or settings of high TB incidence, the risk of re-exposure within the population is high and it is therefore difficult to determine whether recurrent TB disease can be attributed to reactivation or re-infection (Horsburgh, 2008). Charalambous et al have shown that HIV-infected gold miners are at higher risk of TB recurrence with a substantial portion resulting from re-infection (Charalambous et al, 2008).

In another report from a high tuberculosis incidence area in Cape Town, the age adjusted incidence rate of TB attributable to reinfection following successful treatment was four times that of new TB. These authors challenged the traditional belief that infection with one strain of TB in immunocompetent individuals protects against development of disease due to subsequent infection with a different strain. They suggest that patients who have been successfully treated for TB are at higher risk of developing disease from reinfection than the general population and that certain individuals are inherently vulnerable to TB disease (Verver et al, 2005).

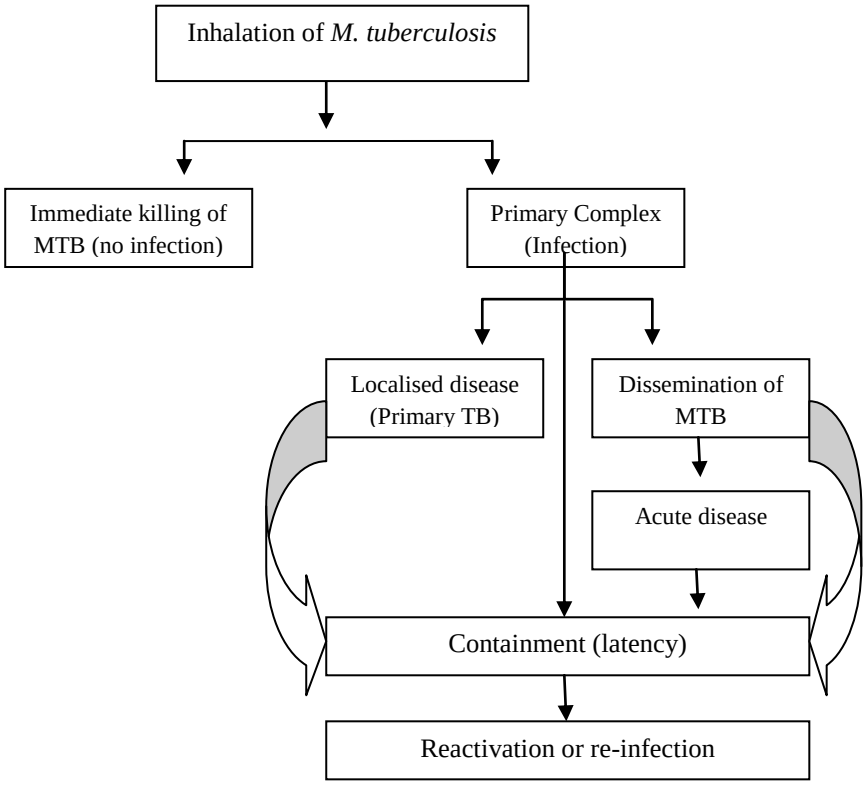


Figure 1.2 Key pathogenic stages following MTB inhalation from droplet nuclei (Adapted from: van Crevel et al, 2002)

1.12 Clinical Manifestations

The clinical features of TB vary widely depending on the anatomical site or sites involved, as well as the bacillary population (Hopewell, 2008). The development of TB differs in each patient depending on the individual's immune system. The different stages are; latency, primary disease, primary progressive disease and extra-pulmonary disease. Different clinical features of each stage are described in figure 1.3 (Knechel, 2009).

Cough is the universal indicator of tuberculosis with radiographs showing mid-lung infiltrates, and hilar lymphadenopathy in primary tuberculosis. The characteristic Ghon complex seen in radiographs are the result when lymph nodes fibrose and sometimes calcify (Ryan, 2010). In reactivated tuberculosis disease, radiography shows infiltrates in the apices of the lung coalesce to form cavities with progressive lung tissue destruction (Ryan, 2010). The clinical manifestations of tuberculosis are the same whether the patient is exposed in a high or low TB incident setting (Maher, 2009b). Growing evidence suggest that the genetic lineage of *M. tuberculosis* effects the clinical features of the disease (Malik et al, 2005).

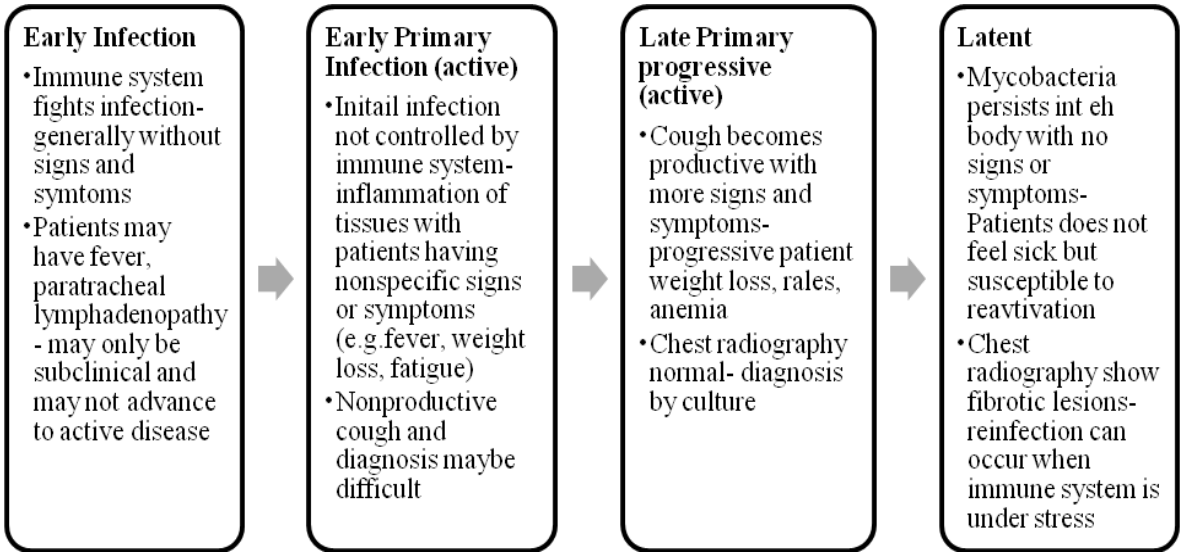


Figure 1.3 The different phases in tuberculosis disease progression (Adapted from; Knechel, 2009).

1.12.1 Signs and symptoms

The clinical signs and symptoms of TB are non-specific (Hopewell, 2008). Systemic symptoms, regardless of site of tuberculosis include fever, night sweats, tiredness, loss of appetite, weight loss, and secondary amenorrhoea (Maher, 2009b).

1.12.2 Pulmonary tuberculosis

Primary pulmonary TB maybe asymptomatic or it may present with clinical systemic symptoms (Ryan, 2010). The principle site of TB is the lungs with presenting clinical features of pulmonary TB being cough, sputum, chest pain, haemoptysis, and uncommonly breathlessness (Hopewell, 2008). Pulmonary TB patients co-infected with HIV experience weight loss and fever more than those patients who are HIV-negative. On the other hand, cough and haemoptysis are less common in HIV-positive patients. Unexplained productive coughing in patients for 2-3 weeks should be screened for TB since the sensitivity of cough as a predictor for TB is very high (Maher, 2009b).

1.12.3 Extra-pulmonary tuberculosis

The predominant location of tuberculosis is the pulmonary system but an increased risk of extra-pulmonary tuberculosis disease occurs in 20% of immunocompromised patients (Knechel, 2009). The high frequency of extra-pulmonary TB among HIV-infected population results from failure of the immune system to contain the infection, thereby assisting haematogenous spread and infection of one or multiple non-pulmonary sites (Hopewell,

2008). Patients with extra-pulmonary tuberculosis present with the same systemic features (Hopewell, 2008) with clinical signs and symptoms related to the pathology at the local site of disease (Maher, 2009b).

The location of extra-pulmonary tuberculosis with the most severe disease is the central nervous system which results in meningitis or space occupying tuberculomas. Tubercular meningitis is fatal in most cases if untreated (Knechel, 2009). The acute dissemination of *M. tuberculosis* into the blood stream is referred to as miliary tuberculosis and the infection can spread throughout the body, leading to multi-organ involvement (Wang et al, 2007). Miliary TB progresses rapidly and can be fatal if not treated early. It is difficult to diagnose due to its systemic and nonspecific signs and symptoms (Knechel, 2009). Tuberculosis lymphadenopathy is the most common extra-pulmonary form of tuberculosis and most commonly presents with cervical lymph node enlargement (Maher, 2009b). Other sites for TB infections include the bone joints, pleura and genitourinary systems (Knechel, 2009).

1.13 Diagnosis of tuberculosis

Infectious TB disease with the high mortality rate worldwide spreads successfully due to the inability of health systems to detect sufficient number of cases prior to transmission to the uninfected population (Whitelaw et al, 2009). One of the key reasons for poor case detection is that TB is difficult to diagnose. Clinical and radiological findings provide only presumptive evidence in the diagnosis of TB (Somoskövi et al, 1999). The diagnosis of TB relies on the detection of TB bacteria in sputum, aspirates, biopsies or blood (Doherty, 2008). The bacteria can be detected by means of microscopy or culture but the sensitivity of these methods are

reliant on the quality of the specimen, concentration of bacteria and the volume of the specimen received (Whitelaw et al, 2009). In addition, MTB grows slowly resulting in longer turnaround times for culture, therefore the need for rapid new molecular diagnostic tests (Afanas'ev et al, 2007).

1.13.1 Specimen collection, storage and transport

Microbiological diagnostic tests performances are dependent on the specimen quality which is required to be representative of the site of infection. Therefore specimens should be collected aseptically, and stored and transported to the laboratory with minimal delay (WHO, 1998a) to prevent contaminating organisms from multiplying (Whitelaw et al, 2009).

1.13.2 Smear microscopy

Microscopic detection of *M. tuberculosis* is the most common method used worldwide because it is simple and inexpensive to perform (Doherty, 2008). Mycobacteria are difficult to stain with basic dyes (including those used in gram stain) because of the high lipid content of their cell wall. These acid-fast organisms resist decolorisation by acidified alcohol following prolonged application of a basic fuchsin dye. This property is referred to as acid-fastness and is used in smear microscopy. The sensitivity of smear microscopy is reliant on the quality of the sputum collected (Urbanczik, 2010) and the underlying pulmonary pathology. There are currently two staining methods used: Ziehl-Neelsen (ZN) for light microscopy and Auramine-O fluorochrome staining for fluorescence microscopy shown in Figure 1.4 (WHO, 1998a). The Auramine method is preferred over ZN as it has an improved

sensitivity (by about 10%) and it is easily read, requiring less time to perform (Whitelaw et al, 2009). Overall microscopy only has 50-60% sensitivity (and even lower in HIV-positive population) hence a more effective method of diagnosing TB is required (Doherty, 2008).

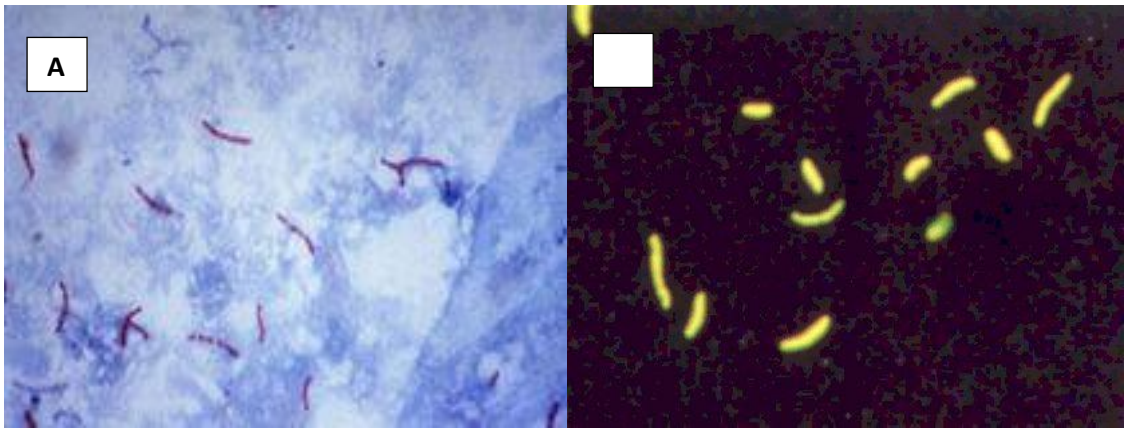


Figure 1.4 Microscopic images of *M. tuberculosis* (A- Ziehl-Neelsen B-Auramine-O)

1.13.3 Culture

Bacterial culture on solid or liquid media is the gold standard for diagnosis of *M. tuberculosis*. Cultures are also used to distinguish *M. tuberculosis* from other mycobacteria (Doherty, 2008) and to perform drug sensitivity testing (Wood, 2007). Prior to growing the bacteria on culture media, the specimen needs to be digested and decontaminated to remove other faster growing contaminating bacteria or fungi. The most commonly used decontamination and digestion methods are the sodium hydroxide (NaOH) method and the *N*-acetyl-*L*-cysteine-sodium hydroxide (NALC-NaOH) method (Whitelaw et al, 2009). During the decontamination process, some mycobacteria may be killed (WHO, 1998b). The bacterium in the specimen is concentrated by centrifugation to optimise yield. Once the

specimen is decontaminated, it is inoculated onto one or more culture media to achieve growth (See Figure 1.5).

The different types of media used are (Whitelaw et al, 2009);

Solid media used for culturing tubercle bacilli:

- Lowenstein-Jensen (LJ) - an egg-based medum
- Middlebrook 7H10 or 7H11 - agar-based medium, Dubos agar-based medium

Liquid media used for culturing tubercle bacilli:

- Middlebrook 7H9 broth
- Dubos Tween albumin broth

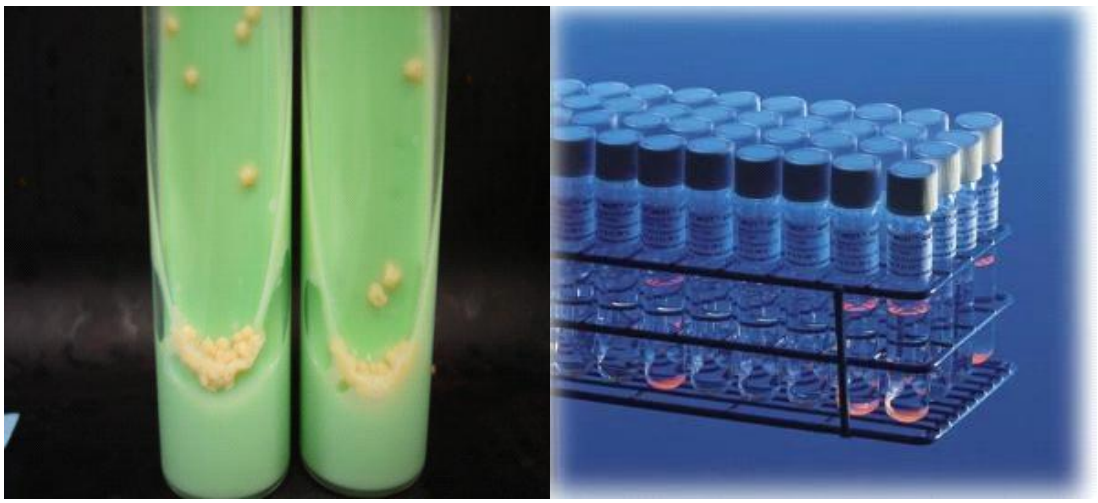


Figure 1.5 *M .tuberculosis* growing on solid (left) and liquid (right) culture media

Although solid media are used widely due to the low cost, there are advantages in using liquid media for culturing TB (Doherty, 2008). One of the key advantages is the rapid growth of the bacteria in liquid media (8-12 days to give a positive growth), compared with solid

media (takes 6-8 weeks) (Wood, 2007). The isolation rates of the bacteria from liquid culture are higher especially when using the commercial liquid-based mycobacterial culture system such as Becton Dickson BACTEC MGIT 960 system (Whitelaw et al, 2009). The MGIT 960 tubes contain 7ml of modified 7H9 both and the system is based on the consumption of oxygen by the growth of MTB which produces fluorescence when illuminated by a UV lamp, Fig.1.5 (Wood, 2007). The other two methods used for automated detection are the MB/BacT system which uses colorimetric CO_2 detection and Trek ESP II system based on pressure changes in the headspace above the liquid culture in a sealed tube. The key disadvantage of the commercial liquid-culture systems is the high costs of the reagents and the equipment (Whitelaw et al, 2009) and higher contamination rates than with solid media.

1.13.4 *M. tuberculosis* identification from culture

Once growth is obtained on either solid or liquid media, microscopic examination (ZN staining) is performed to confirm the presence of acid fast bacilli and not contaminants (WHO, 1998b). Identification is the confirmed by one of several techniques:

There are various techniques used to identify acid-fast bacilli from culture (Whitelaw et al, 2009).

1. Antigen detection: these lateral flow assays detect *M. tuberculosis* antigen specific to members of the MTB complex (examples include Capilia TB Rapid test that detects MPB64 antigen and SD Bioline Antigen Detection Test MPT 64).

2. Phenotypic methods determine the growth rates, pigmentation and growth at different temperatures of mycobacteria which differentiate them from NTM. Biochemical tests are also used but they are labour intensive, expensive, and results may be difficult to interpret.

3. Genotypic methods are DNA- or RNA-based assays which rapidly identifies MTB with easy interpretation. A large number of nucleic-acid-based tests are available for the detection of MTBC; however, they do not differentiate between the different members of the complex. Specifically the Hain LPA has been endorsed by WHO for the rapid detection of MDR-TB (WHO, 2008).

4. Mycolic acid analysis can be used to identify mycobacteria and is usually used to describe new mycobacterial species. High-performance liquid chromatography is used for these analyses in research settings only.

1.13.5 Drug susceptibility testing

The resistance to anti-tuberculosis drugs occurs naturally by the process of selection of drug-resistant mutants by the treatment agent(s) (Whitelaw et al, 2009) resulting in some bacilli acquiring resistance to drugs used for treatment when they are poorly managed (Campbell et al, 2011). MDR-TB, is spreading worldwide requiring more complex and costly treatment regimens with worse outcomes (Whitelaw et al, 2009). The emergence of XDR-TB has highlighted the need for more DST as well as rapid diagnosis of the type of resistance (Campbell et al, 2011).

1.13.5.1 Phenotypic (conventional) drug susceptibility testing

Phenotypic or conventional DST determines if the organism is resistant to an anti-tuberculosis drug by evaluating growth of the organism in the presence of the drug (Whitelaw et al, 2009) compared with a control. There are different phenotypic methods employed in various laboratory settings including; absolute concentration or resistance ratio method, agar proportion method and MGIT 960 (Becton-Dickenson Diagnostics, Sparks, MD, USA) (van Deun et al, 2010). The down side to these methods is the time delay as solid media takes up to 4-6 weeks to completion and the liquid MGIT 960 system only gives a DST result 1-2 weeks after the MGIT system flags positive for culture which itself takes 1-6 weeks (Al-Mutairi et al, 2011). Mycobacteriophage-based methods, like the commercial *FASTplaque*assay (Biotech labs Ltd, Iswich, UK) have been introduced incorporating DST which is based on the inhibition of replication of the bacteria in the presence of the drug giving a visual result within 2 days (van Deun et al, 2010).

1.13.5.2 Genotypic (molecular) methods

The molecular mechanisms conferring drug resistance have been described for several of the major first and second line anti-tuberculosis drugs. This understanding has facilitated the recent development of rapid molecular diagnostic tests for identification of drug resistance. Molecular methods or genotypic DST are DNA-based tests which identify mutations associated with drug resistance (Campbell et al, 2011). The major limitation to molecular methods is that for some anti-tuberculosis drugs not all resistance-conferring mutations have been well characterised for some anti-tuberculosis drugs (van Deun et al, 2010). Various in-house molecular tests have been developed and used for the detection of resistance-

determining mutations (Whitelaw et al, 2009). In 2008, the World health Organisation (WHO) endorsed the use of line probe assays (LPAs) for the rapid detection of MDR-TB (WHO, 2008). LPAs are based on multiplex polymerase chain reaction (PCR) amplification followed by reverse hybridisation for the identification of MTBC and the detection of resistance in the genes associated with the anti-tuberculosis drugs RIF and INH (Albert et al, 2010). The commercial assay, INNO-LiPa (INNO-LiPa, Innogenetics, Ghent, Belgium) detects RIF resistance in the *rpoB* gene only (Al-Mutairi et al, 2011) while the MTBDR*plus* (Hain Lifescience, GmbH) LPA, detects mutations conferring resistance in the *rpoB*, *katG* and *inhA* genes and produces a result within 1 day (Barnard et al, 2007). More recently, in 2010, WHO STAG-TB endorsed the use of the GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA), a molecular test that detects *Mycobacterium tuberculosis* as well as rifampicin resistance. WHO recommended GeneXpert MTB/RIF as the initial diagnostic test for TB in individuals suspected of MDR-TB or TB/ HIV-co-infection (WHO, 2011b).

DNA sequencing is considered the gold standard in which the nucleotide sequence of the amplified DNA is visible giving the most accurate result while DNA microarray (DNA biochip) assays have been developed for the detection of TB drug resistance but they have as yet not been fully evaluated.(van Deun et al, 2010).

1.13.6 Genotyping methods used in TB Epidemiology

Molecular methods are also used for epidemiological studies of MTB with many tools available, each with its own positive and negative characteristics (McEvoy et al, 2009). These

molecular tools have been used to give some insight into the genomic evolutionary changes at the DNA level (van der Spuy et al, 2008).

1.13.6.1 IS*6110* Restriction fragment length polymorphism (RFLP)

The *M. tuberculosis* genome contains/includes many insertion sequences (IS) which are able to replicate and 'jump' or transpose themselves from one region to another thereby displaying polymorphism in the number of elements per genome (McEvoy et al, 2009). IS*6110* is TB specific and is the most widely used genetic marker with IS*6110* RFLP considered as the 'gold standard' for MTB genotyping (Stavrum et al, 2009).

1.13.6.2 Spoligotyping

All MTBC members have the direct repeat (DR) chromosomal region which is composed of multiple 36-bp directly repeat sequences interspersed by non-repetitive unique spacer regions ranging from 35 to 41 bp in length (van der Spuy et al, 2008; McEvoy et al, 2009).One DR sequence and its spacer is referred to as direct variable repeat (DVR) where polymorphisms occur between MTB isolates (McEvoy et al, 2009). Spoliogotyping or spacer oligonucleotide typing is designed to determine the presence or absence of 43 spacer regions in *M.tuberculosis* isolates (van der Spuy et al, 2008). Spoligotyping has less discriminatory power to distinguish *M. tuberculosis* strains than IS*6110* (RFLP) (Barnes et al, 2003).

1.13.6.3 MIRU/VNTR analysis

This genotyping method is based on PCR amplification of mycobacterial interspersed repetitive units of variable-number tandem repeats (MIRU-VNTR) and has a higher discriminatory power than IS*6110* RFLP (Stavrum et al, 2009). MIRU-VNTR can also be used to determine mixed subpopulations from the same sputum specimen (Allix et al, 2004).

1.14 Treatment of Tuberculosis

Many antimicrobials have been shown to be ineffective against mycobacteria due their unusually impermeable lipid-rich cell wall. There are a few groups of antimicrobials which are active and are used in the treatment of MTB (Ryan et al, 2010). The primary drugs of choice recommended by the WHO are referred to as the first-line anti-tuberculosis drugs which are administered as a 6-month short course. Pyrazinamide and ethambutol together with INH and RIF are given for the first 2 months during the intensive phase of treatment followed by INH and RIF for the next 4 months (WHO, 2009). The injectable streptomycin may also be used in the initial phase of treatment but its usefulness has decreased over time due to increasing resistance to the drug (Donald et al, 2009). Second-line drugs are reserved for use when there is resistance to first-line drugs, drug intolerance or toxicity (WHO, 2009).

1.15 Molecular Basis and Mechanisms of Resistance to First-line Drugs

Resistance of *M. tuberculosis* to anti-tuberculous drugs results from a spontaneous genetic event, occurring at a frequency of 10^{-5} to 10^{-8}. Resistance to various drugs arises

independently (Pfyffer, 2000). Resistance in *M. tuberculosis* appears to be confined to chromosomal DNA and does not involve transfer of mobile genetic elements (such as plasmids) which occurs in other bacteria and mycobacteria (Pfyffer, 2000).

Resistance to the first-line anti-TB drugs occurs through sequential accumulation of mutations in the genes targeted by the drugs (Johnson et al, 2007). These mutations occur at the drug target region or at the enzymes that are involved in the activation of the drug (Somoskovi et al, 2001). These drug-resistant strains develop when chemotherapy is disrupted or otherwise inadequate (Ramaswamy et al, 1998).

The molecular mechanisms conferring drug resistance have been described for several of the major first and second line anti-tuberculosis drugs. This understanding has facilitated the recent development of rapid molecular diagnostic tests for identification of drug resistance (Zhang et al, 2009). While about 96% of rifampicin resistant isolates have mutations in a defined region of the *rpoB* gene, resistance to isoniazid is more complicated, involving at least two gene *katG* and *inhA* which together account for approximately 65-75% of resistant strains (Ramaswamy et al, 1998). For other drugs, known drug resistance mutations occur in a smaller proportion of isolates suggesting other mechanisms of resistance. Genes commonly associated with resistance to anti-tuberculous drugs are summarised in Table 1.1 below (Zhang et al, 2009; Ramaswamy et al, 1998). Several studies have shown that the frequency distribution of mutations among drug resistant isolates shows geographic variability both between and within countries (Barnard et al, 2008; Ohno et al, 1996; Miotto et al, 2006; Green et al, 2008).

Table 1.1 Summary of genes commonly associated with resistance to anti-tuberculosis drugs

Drug	**Gene(s) involved in resistance**	**Mutation frequency %**
Isonaizid	*katG*, *inhA*	50-95 8- 43
Rifampicin	*rpoB*	95
Pyrazinamide	*pncA*	72-97
Ethambutol	*embB*	47-65
Streptomycin	*rpsL*, *rrs*, *gidB*	52-59 8-21 Unknown
Amikacin/kanamycin	*rrs*	76
Capreomycin	*tlyA*	Unknown
Quinolones	*gyrA, gyrB*	75-94
Ethionamide	*etaA/athA*, *inhA*	37 56
PAS	*thyA*	36

1.15.1 Isoniazid

INH is a pro-drug with a simple structure containing a pyridine ring and a hydrazide group requiring activation (Somoskovi et al, 2001; da Silva et al, 2011). Almost all mycobacteria and prokaryotes other than *M. tuberculosis* are resistant to INH (Ramaswamy et al, 1998). INH is activated by the catalase/peroxidase enzyme encoded by *katG* which prevents the synthesis of mycobacterial mycolic acids by inhibiting the NADH-dependent enoyl-ACP reductase coded by *inhA* (da Silva et al, 2011). The basis of resistance to INH is more complex and is based on a genetic system involving a large number of gene regions such as *katG*, *inhA*, *kasA*, *aphC*, *oxyR* and less common *iniA*, *iniB* and *iniC* genes (Afanas'ev et al, 2007; Wei-Wei et al, 2007). Studies have shown that *katG* covers a wide range of 50-95% of INH resistance in isolates while *inhA* resistance is present in 20-35% of isolates (Wei-Wei et al, 2007; Aslan et al, 2008).

Molecular genetic studies have correlated INH resistance with loss of catalase-peroxidase activity which is controlled by the *katG* gene (Ramaswamy et al, 1998). Mutations in the catalase-peroxidase gene, usually at codon 315, prevent the conversion of the INH pro-drug into its active form (Wei-Wei et al, 2007). The promoter region of the *inhA* operon comprises two genes, *fabG1 (mabA)* and *inhA* (Afanas'ev et al, 2007; Ramaswamy et al, 1998). The metabolic activity of the operon is to code for β-ketoacyl reductase and the fatty-acid enoyl-acyl carrier protein reductase which participate in mycolic acid biosynthesis (Afanas'ev et al, 2007). Missense mutations in the promoter region (position -15T or -8C) result in over expression of the target for the active INH radical leading to INH resistance (Afanas'ev et al, 2007; Evans et al, 2009). Mutations in the *katG* gene code predominantly for high-level resistance to INH while low-level resistance is determined mainly by mutations present in the

inhA gene (Barnard et al, 2008; van Deun et al, 2010). Resistance to the anti-tuberculosis drug ethionamide is also cause by mutations in the *inhA* gene. Further research is required to better understand the mechanisms responsible for INH resistance in the other genes (Afanas'ev et al, 2007).

1.15.2 Rifampicin

RIF is a lipophylic ansamycin with efficient antimicrobial activity and is considered a key drug in the treatment of tuberculosis (da Silva et al, 2011). The activity of RIF involves binding to the β-subunit of ribonucleic (RNA) polymerase resulting in inhibition of transcription (Wei-Wei et al, 2007). Resistance to RIF is caused by amino acid substitutions as a result of missense mutations, or deletions or insertions in this region encoding the β-subunit of the RNA polymerase enzyme (Aslan et al, 2008; Ramaswamy et al, 1998). Resistance to RIF is largely confined to mutations present in an 81 base pair hotspot region on the *rpoB* gene (Aslan et al, 2008; Ramaswamy et al, 1998). This drug resistance region is known as the RIF resistance-determining region (RRDR) (Afanas'ev et al, 2007; Evans et al, 2009; WHO, 2007). The RRDR spans from codon 507 to 533 of the *rpoB* gene and more than 95% of resistant strains harbour mutations in this region (Afanas'ev et al, 2007; Evans et al, 2009; Wei-Wei et al, 2007).

The characterisation of the *M. tuberculosis rpoB* gene showed a wide variety of distinct alterations in the RRDR in epidemiologically unrelated patient isolates (Ramaswamy et al, 1998). Studies have commonly documented missense mutations at codon 531 (Ser), 526 (His) resulting in amino acid replacements. Amino acid substitutions Ser513Leu and His526Tyr

were most common (Afanas'ev et al, 2007). Several research groups have investigated the relationship between RIF MIC levels and structural changes found in the *rpoB* gene (Ramaswamy et al, 1998). They had found a strong correlation between *rpoB* variants and RIF MICs. High-level drug resistance to rifampicin, rifabutin and rifapentine had amino acid substitutions at codons 513, 526 and 531, whereas, amino acid substitution at positions 514, 521 or 533 conferred low-level resistance to RIF. In RIF resistant isolates that lacked changes in the RRDR, the molecular mechanisms involved are unknown (Ramaswamy et al, 1998).

1.15.3 Streptomycin

Streptomycin (STR) is aminocyclitol glycoside antibiotic and was the first antibiotic used in TB treatment (da Silva et al, 2011). STR resistance is associated with the 16s rRNA gene, *rrs* and *rpsL* which encodes for the ribosomal protein S12 (Ramaswamy et al, 1998).

1.15.4 Ethambutol

Ethambutol (EMB) ([S,S']-2,2'-[ethylenediimino])di-1-butanol has bactericidal activity and was first used against TB in 1966 (da Silva et al, 2011). It interferes with the biosynthesis of the cell wall arabinogalactan. Mutations in the *embB* operon are responsible for EMB resistance (Ramaswamy et al, 1998).

1.15.5 Pyrazinamide

Pyrazinamide (PZA) is a pro-drug introduced in the TB treatment regimen in the 1950s with the ability to inhibit semi-dormant bacilli (da Silva et al, 2011). PZA is an analogue of nicotinamide and in its active form, pyrazinoic acid encoded by the mycobacterial enzyme pyrazinamidase (PZase), prevents fatty acid synthesis (Somoskovi et al, 2001). Mutations in the *pncA* gene which codes for the PZase enzyme is the main target for resistance (da Silva et al, 2011).

1.16 Immunity and TB vaccines

Each year millions of infants are vaccinated with the BCG (BacilleCalmette-Guerin) vaccine to prevent severe disease such as meningitis and miliary TB (Dye et al, 2008). BCG is safe and may elicit long-term protective immunity but the escalating TB epidemic worldwide has occurred even with the widespread use of BCG vaccines. The global increase of TB related mortality shows that the BCG vaccine has failed and the development for new more effective vaccines is required (Hussey et al, 2009).

1.17 Experimental proposal

1.17.1 Study Background Problem

The HIV epidemic and emergence of drug-resistant TB threaten global control of TB. MDR-TB has escalated worldwide due to inadequate detection, diagnostic delays and low TB cure

rates (Barnard et al, 2008). The conventional methods of drug susceptibility testing (DST) require a minimum of 14 days for completion as these phenotypic methods are still dependent on the growth of the *M. tuberculosis* bacteria in culture (Afanas'ev et al, 2007). The growing burden of drug resistance together with outbreaks of XDR-TB prompted international calls for new rapid molecular diagnostics tools for early diagnosis of drug resistant TB (WHO, 2008). Improved understanding of the molecular mechanisms conferring TB drug resistance facilitated the recent development of such tests.

SA is a low to middle income country with a high incidence of TB including large numbers of drug resistant TB and high rates of HIV co-infection. A severe outbreak of XDR-TB in KwaZulu-Natal, where 52 out of 53 patients died within two weeks of diagnosis (Gandhi et al, 2006) highlighted the public health crisis. In 2007 South Africa participated in a large-scale demonstration project funded by the Foundation for Innovative New Diagnostics (FIND) to assess implementation and benefits of the use of new molecular diagnostic tests for the rapid detection of MDR-TB (WHO, 2008). Improved case finding and early implementation of appropriate treatment could reduce transmission, improve individual outcomes and prevent progression to XDR-TB.

The GenoType MTBDR*plus* (Hain Lifescience, GmbH) line probe assay (LPA) is a molecular diagnostic test based on the polymerase chain reaction (PCR) and reverse hybridization and is designed to detect *M. tuberculosis* and drug resistance to INH and RIF (Evans et al, 2009). The probes present on the LPA are specific for the detection of mutations present in the *rpoB*, *katG* and the promoter region of the *inhA* genes (Barnard et al, 2008; Matsoso et al, 2010). The distribution of mutations resulting in drug resistance in *M. tuberculosis* varies geographically, from region to region (Aslan et al, 2008; Evans et al,

2009). This highlights a limitation of the use of genotypic LPA in that it only covers the most frequent mutations conferring resistance (Bolotin et al, 2009) to the anti-tuberculosis drugs. Resistance due to mutations not represented in the molecular assay will result in discrepancies between genotypic composition on LPA and phenotypic expression of resistance with genotypic resistance in a strain being falsely labelled susceptible by the LPA. Also, silent mutations detected by the LPA will be associated with phenotypic susceptibility.

The wide use of genotypic assays for the detection of drug resistance would require constant monitoring of local drug resistance mutations within a country to prevent emergent clonal groups of *M. tuberculosis* from not being appropriately detected and classified (Evans et al, 2009). The high rate of HIV co-infection within the population of SA has contributed to the large *M. tuberculosis* pool from which new mutations in genes concerned with the action of anti-TB drugs can be selected, potentially resulting in cases where the LPA fails to detect resistance to the drugs.

Globally the TB epidemic consists of multiple genotype-specific sub-epidemics and over time the most 'successful' genotypes become dominant in different geographical locations. In developed and developing countries like SA, the bacterial population structure of resistant isolates is not well documented. The genotype profiles for the MDR-TB population causing disease across the country are largely unknown.

1.17.2 Study Objectives

1.17.2.1 Hypothesis

In SA, there is regional variation in the mutations encoding INH and RIF resistance in *M. tuberculosis* isolates. Some of these mutations are not represented on probes used in the LPA for the detection of MDR-TB. This results in discrepancies between genotypic and phenotypic resistance.

1.17.2.2 Aim

To determine the frequency, distribution and genetic basis of LPA/MGIT DST discordant findings in four provinces of South Africa that participated in a demonstration project to assess the effectiveness of a rapid line probe assay for presumptive MDR-TB diagnosis in smear-positive specimens from patients in high TB burden countries.

1.17.2.3 Specific objectives

1. To determine the frequency and distribution of genotypic/phenotypic discrepancies in four provinces in South Africa.
2. To repeat the LPA and phenotypic testing of all discordant samples included in study.

3. To determine Minimum Inhibitory Concentrations (MIC) of RIF and INH on isolates demonstrating phenotypic-genotypic discrepancies in resistance to these agents.
4. To use the LPA as a screening tool on all discrepant samples to determine the genes involved in resistance.
5. To perform sequencing on a selective basis to determine mutations that may be important for LPA testing based on objectives 1, 2, 3 and 4.
6. To characterize the isolates by spoligotyping to determine geographical distribution of multidrug-resistant *M. tuberculosis* families.

1.17.3 Study Outcomes

Selective sequencing of relevant genes in *M. tuberculosis* isolates showing discordant genotypic/phenotypic resistance results may help identify novel mechanisms of resistance. The results from MICs will assist in evaluating the need and role of these anti-tuberculosis drugs in the current TB control program in SA. From further genotype investigations, we would like to address the geographical distribution of *M. tuberculosis* genotypes from one province to another as well as to gain a better understanding of the molecular epidemic and genetics involved in drug resistance.

Chapter 2

2 Materials and Methods

2.1 Source of isolates for the present study

In 2007, four provinces in South Africa participated in a country-wide demonstration project in which smear microscopy-positive patients at high risk of drug-resistant-TB were enrolled to assess the use of the GenoType® MTBDR*plus* (Hain Lifescience GmbH, Nehren, Germany) line probe assay (Hain LPA) for the diagnosis of MDR-TB under routine National Tuberculosis Control Program (NTBCP) conditions. (ML van der Walt, G Coetzee, M Sewpersadh, M Barnard, L Matsotso, R O'Brien, R Odendaal, H Albert, publication in preparation).

During this demonstration study a number of *M. tuberculosis* isolates were identified that showed discordant drug susceptibility results when tested with the Hain LPA and conventional phenotypic DST. These isolates from Western Cape, KwaZulu-Natal, Gauteng and Northern Cape were included in the present study. Discordant results were defined as, differences between genotypic findings by the Hain LPA and conventional DST results for the anti-tuberculosis drugs RIF and INH. Based on this definition, cultures from 171 genotypic/phenotypic discordant results from the demonstration study were available for further study.

2.2 Laboratory methods for characterization of discordant cultures

All available isolates identified as discordant in the original demonstration study were re-tested using Hain LPA and broth-based phenotypic DST for INH and RIF. Isolates confirmed to have discordant results on this repeat testing were further characterised by determination of minimum inhibitory concentrations (MIC) and by spoligotyping. Finally, sequencing of RIF and INH resistance-related gene regions was performed when phenotypic resistance was reported but no genetic changes were identified on the LPA.

2.2.1 Phenotypic drug susceptibility testing

The original MGIT culture specimens from Gauteng and KwaZulu-Natal were collected from the NHLS laboratories while aliquots of the MGIT culture from the Cape Town NHLS laboratory were transferred into 2 ml screw-capped tubes and transported to MRC. All these cultures were stored in the MRC Biosaftey Level 3 Laboratory (BSL 3) at room temperature. Prior to this study, the Northern Cape MGIT cultures were collected and stored at -20ºC at the Sandringham NHLS Laboratory. Following retrieval, the frozen stock of the culture was left to thaw at room temperature and then 1 ml was inoculated into a MGIT 960 tube and incubated in the MGIT 960 machine until positive growth was attained. The sub cultures were then sent to MRC and stored with the rest of the culture collection.

The specimens were cultured using the MGIT 960 method (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) according to the manufacturer's instructions. MGIT tubes

were incubated until a positive growth signal on the MGIT 960 machine was obtained (day 0). The purity of the culture was tested by streaking the culture onto blood agar plates which were then incubated for 48 hours at 37°C for the detection of rapidly growing contaminants. If no growth appeared on the plates, drug susceptibility tests were set up. Prior to setting up the DST's the Hain LPA was done on the positive cultures to confirm the presence of *M .tuberculosis*. The MGIT tubes were incubated at 37°C and on day 2, DST to first-line drugs, RIF and INH, was performed on pure cultures using critical concentrations of 1.0 µg/ml for RIF and 0.1µg/ml for INH. Three MGIT 960 tubes were labelled and used for each test culture (one for RIF, one for INH and one for the growth control).

Aseptically 0.8 ml of BACTEC 960 SIRE supplement (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) was added to each of the tubes. 100 µl of the reconstituted drug was added to the corresponding drug labelled MGIT tubes but no drug was added to the growth control tube. 500 µl of the well-mixed culture suspension was added into the MGIT tubes containing the drugs. In the growth control tube, the culture suspension was diluted to 1: 100 (100 µl of the culture added to 10 ml of sterile saline) and 500 µl of the diluted suspension was added to the growth control tube. All tubes were mixed well by gently inverting each tube a few times, after which they were placed into the MGIT machine set carrier in the correct drug sequence for incubation. The instrument generated the results within 5 to 13 days.

2.2.2 Genotypic drug susceptibility testing

Hain LPA was performed in three separate rooms, according to WHO recommendations (WHO, 2008). DNA extraction was performed in a BSL3 cabinet, the master mix preparation was performed in the second room and the amplification by PCR and hybridisation for amplicon identification were performed in the third laboratory. One thousand microlitres of liquid culture was used to perform the GenoType® MTBDR*plus* assay according to manufacturer's instructions (Hain Lifescience, GenoType MTBDR*plus* user's manual).

DNA extraction was performed by centrifuging the liquid culture for 5 minutes at 10 000 X g, then discarding the supernatant and re-suspending the pellet in 100 µl of sterile distilled water. The bacterial suspension was boiled at 95°C for 20 minutes and the cells were disrupted in an ultra-sonic water bath for 15 minutes. The mixture was centrifuged at 13 000 X g for 5 minutes, then 50 µl of the supernatant was placed into a clean tube and used as the template DNA for amplification.

Each PCR mixture contained 35 µl of PNM mix supplied by manufacturer (Hain Lifescience GmbH), 5 µl of 10x buffer (Qiagen, Benelux B.V), 2 µl 25 mM $MgCl_2$ (Qiagen, Benelux B.V), 0.2 µl (1 unit) HotStarTaq (Qiagen, Benelux B.V), 3 µl molecular grade water (Qiagen, Benelux B.V), and 5 µl template DNA. The mixture was subjected to the following cycling conditions; 1 cycle at 95°C for 15 minutes (min), 10 cycles each of, 95°C for 30 seconds

(sec), 58°C for 2 min, 20 cycles each of 95°C for 25 sec, 53°C for 40 sec, 70°C for 40 sec and 1 cycle at 70°C for 8 min.

Reverse hybridisation was performed on the automated GT Blot system (Hain Lifescience GmbH) and the LPA strips were read manually.

2.2.3 Determination of the minimal inhibitory concentrations (MICs)

MICs were determined using the Alamar Blue assay as previously described (Franzblau et al, 1998).

2.2.3.1 Preparation of inoculum stock

The optical density (OD) readings of each bacterial culture were measured using a spectrophotometer (Beckman, CA) at a wavelength of 600 nanometres (nm). The bacterial suspension was then diluted in 7H9 medium (Sigma Chemical Co, St Louis, Mo) containing 15% glycerol and OADC supplement (Becton Dickinson Microbiology System, Cockeysville, MD, USA) to obtain a bacterial inoculum of ~1 X 10^5- 1 X 10^7colony-forming units (cfu) /ml.

2.2.3.2 Preparation of drugs

RIF and INH (Sigma Chemical Co, St Louis, Mo) drugs were freshly prepared on the day of the experiments. INH antibiotic powder was dissolved in sterile distilled water and RIF powder was dissolved in 100% dimethyl sulfoxide (DMSO) to a final concentration of 10 mg/ml. A 1:2 serial dilution of each drug was made in a 96-well plate. 2 µl of each drug dilution was transferred to its appropriate well in the assay plates.

2.2.3.3 Alamar Blue assay

In the sterile 96 well plate (Cellstar, Greiner Bio-one, GmbH), 200 µl of sterile distilled water was added to the outer-perimeter wells to minimise evaporation in the test wells during incubation. To the remaining wells (wells in rows B-E and in columns 2-10), 98 µl of 7H9 medium (Sigma, Chemical Co, St Louis, Mo) containing 15% glycerol and OADC supplement (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) were added. Serial dilutions of RIF (in DMSO) and INH (in deionised water) with final drug concentrations ranging from 0.03 µg/ml to10 µg/ml for each of INH and RIF, were prepared. 2 µl of the drug dilutions were added in columns 2-10, and column 11 was drug free as it served as a control (100 µl of 7H9 was added in the control column 11). 100 µl of the culture inoculum (~1 X 10^5-1 X 10^7 cfu/ml) were added to columns 2-11. The plate was incubated at 37°C for 6 days. After incubation 50 µl of 1:1 mixture of 10X Alamar blue reagent (Separations, Johannesburg, South Africa) and 10% Tween 80 (Sigma, Chemical Co, St Louis, Mo) to column 11 (drug free – inoculums only). The plate was re-incubated at 37°C in for 24 hours. If column 11 turned pink (indicating growth) the reagent mixture was added to

all wells in the microplate. If the control remained blue, the reagent mixture was added to another control well and was read on the following day. The microplates were incubated for 24 hours at 37°C, and the colours were recorded. A blue colour in the well was interpreted as no growth and a pink colour was an indication of growth. The MIC was defined at the lowest drug concentration which prevented growth i.e. prevented a colour change from blue to pink.

2.2.4 Spoligotyping

Spacer oligonucleotide genotyping (spoligotyping) was used to confirm the species and determine the genotype of *M. tuberculosis* complex cultures included in the study (Kamerbeek et al, 1997).

2.2.4.1 Amplification of spacer DNA by PCR

Amplification of spacers was accomplished by using the primes DRa and DRb, which enable to amplify the whole DR region. PCR products are labelled with biotin, because primer DRa is biotinylated. The genotype determination was done using a spoligotyping kit (Ocimum Biosolutions, Hyderabad, India) and was used as per manufacturer's instructions (Ocimum Biosolutions, spoligotyping kit user manual). The method included two positive controls, *M. tuberculosis* H37Rv and *M. bovis* BCG and molecular grade water was used as the negative control. Each 40 µl amplification mixture contained 2 µl template DNA, 3 µl primer DRa (0.2 µmol/µl) 3 µl primer DRb (0.2 µmol/µl), 20 µl 2xTaq MasterMix (Qiagen, Benelux B.V), and 12 µl molecular grade water (Qiagen, Benelux B.V). Amplification was performed

at the following temperature cycling conditions; 1 cycle of each, 3 min at 94°C, 1 min at 94°C, 1 min at 55°C, 25 cycles for 30 sec at 72°C, 1 cycle for 7 min at 72°C, and hold cycle at 4°C.

2.2.4.2 Hybridization of PCR product and detection

The hybridization of the biotin-labelled PCR products to the immobilized 43 spacer-oligonucleotides that represent spacers of known sequence was performed as per manufacturer's instructions (Ocimum Biosolutions, Hyderabad, India). The presence of spacers were visualised on film as black squares after incubation with streptavidin-peroxidase, conjugate, washing and rinsing, followed by detection of hybridizing DNA using chemiluminescent ECL-detection liquid (Amersham Biosciences, UK), and finally exposure to X-ray film (Hyperfilm ECL, Amersham Biosciences, UK).

2.2.5 Sequencing technology

Using PCR and sequencing technology, previously described (Campbell et al, 2011) genes associated with the mechanisms of action of, resistance to RIF and INH, (*rpoB, katG* and *inhA*), were targeted to determine the genetic basis of resistance in isolates where no genetic alteration was detected by the Hain LPA.

2.2.5.1 DNA preparation for sequencing

The crude DNA extract used for the Hain LPA was cleaned up using the QIAquick PCR purification kit (Qiagen, Benelux B.V) as per manufacturer's instructions. We had used 10 µl of template DNA and reduced the volumes accordingly for all other regents used in the kit.

2.2.5.2 Amplification of gene regions

The drug resistance-determining regions of *rpoB*, the promoters of *inhA* and regions with established resistance associated mutations of *katG* loci were amplified using locus-specific primers (see Table 2.1). Each 25 µl PCR mixture contained 12.5 µl HotStarTaq master mix (Qiagen, Benelux B.V), 1.5 µl of the forward and reverse 5 µM primers, 8.5 µl distilled H_2O, and 1 µl of genomic DNA. The amplification parameters included an initial denaturation step at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 30 sec, with a final elongation step at 72°C for 7 min. All primers were synthesised by Inqaba Biotechnical Industries, Pretoria, South Africa. PCR products were analysed on 1.5% agarose –Tris-EDTA (Sigma, Chemical Co, St Louis, Mo) gels and stained with ethidium bromide (Sigma, Chemical Co, St Louis, Mo). Verified PCR products were cleaned using the QIAquick PCR purification kit (Qiagen, Benelux B.V) and were used as template DNA for sequencing.

Table 2.1 PCR and Sequencing Primers (Campbell et al, 2011)

Locus	Primer	Sequence (5'-3')	Size (bp)	F or R	Accession #	Position	Product (bp)	Note
rpoB	rpoB-1f	CTTGCACGAGG GTCAGACCA	20	F	NC000962	1023-1042	543	RRDR*
	rpoB-2r	ATCTCGTCGCTA ACCACGCC	20	R	NC000962	1546-1565	543	RRDR
inhA	inhA-1f	TGCCCAGAAAG GGATCCGTCATG	23	F	NC000962	(-162)-(-140)	455	promoter
	inhA-2r	ATGAGGAATGC GTCCGCGGA	20	R	NC000962	436-455	455	promoter
katG	katG-1f	AACGACGTCGA AACAGCGGC	20	F	NC000962	1433-1452	455	codon 315
	katG-2r	GCGAACTCGTCG GCCAATTC	20	R	NC000962	998-1017	455	codon 315

*RRDR- rifampicin resistance-determining region

Sequencing reactions (20 µl reaction mixtures) were completed with an ABI BigDye Terminator (version 3.1) cycle sequencing kit and the reactions included 4 µl of BigDye Terminator (version 3.1) reagent (ABI), 2 µl of 1.6 µM primer (with the same sequences used for PCR), 2 µl of 5X reaction buffer (ABI), 11 µl of distilled H_2O, and 1 µl of PCR template. PCR products were sequenced with a forward and a reverse primer at each locus for maximum coverage and reproducibility of results. The reactions were cycled according to manufacturer's guidelines. The unincorporated terminators were removed from the completed reactions by treatment with BigDye Xterminator kit (ABI), using the manufacturer's protocol and were examined using 3130xl genetic analyzer with standard run conditions for electrophoresis and data collection.

2.3 Data Analysis

2.3.1 Statistical analysis

Descriptive statistical analysis was applied initially to calculate the frequency and identify the proportion of discordant findings in each province using the statistical software, STATA 10. Comparative analysis was done on the genotypic and phenotypic results as well as on the MICs using Microsoft Excel 2007.

Chapter 3

3 Results

3.1 Study group

During the period June 2007- August 2008, 30 800 TB suspects at high risk for drug resistant-TB were screened in a large-scale demonstration project assessing the implementation and benefits of a new molecular diagnostic test for the rapid detection of MDR-TB under National Tuberculosis Control Programme (NTCP) conditions. The demonstration project's database was reviewed using statistical analysis (STATA 10) to identify specimens originally classified as discordant in the project for use in this study. Discordant was defined as differences between genotypic findings by the Hain LPA and conventional DST results for the anti-tuberculosis drugs RIF and INH. A total of 171 discordant cultures were identified and collected from the provinces after completion of the demonstration project. Overall only 69.0% (118/171) of the stored culture isolates yielded pure isolates when sub-cultured in MGIT instruments and or on solid media as shown in Figure 3.1.

Initially 44 cultures were contaminated and were re-decontaminated using 4% sodium hydroxide (NaOH). Following re-decontamination, (Figure 3.1), 37 /44 failed to grow in MGIT 960 as well as in 7H9 medium. Seven samples grew in 7H9 media, which were transferred into MGIT 960 tubes and were included with the 'clean' group once positive growth was obtained and their identification was confirmed by Hain LPA for entry into the

study. The LJ cultures of the 37 samples gave poor growth, contamination or no growth and therefore had to be excluded. A further 16 samples were excluded from the positive growth group as they showed persistent contamination during DST and no results were available for RIF and INH. DST's were repeated three times on these samples prior to exclusion from the study.

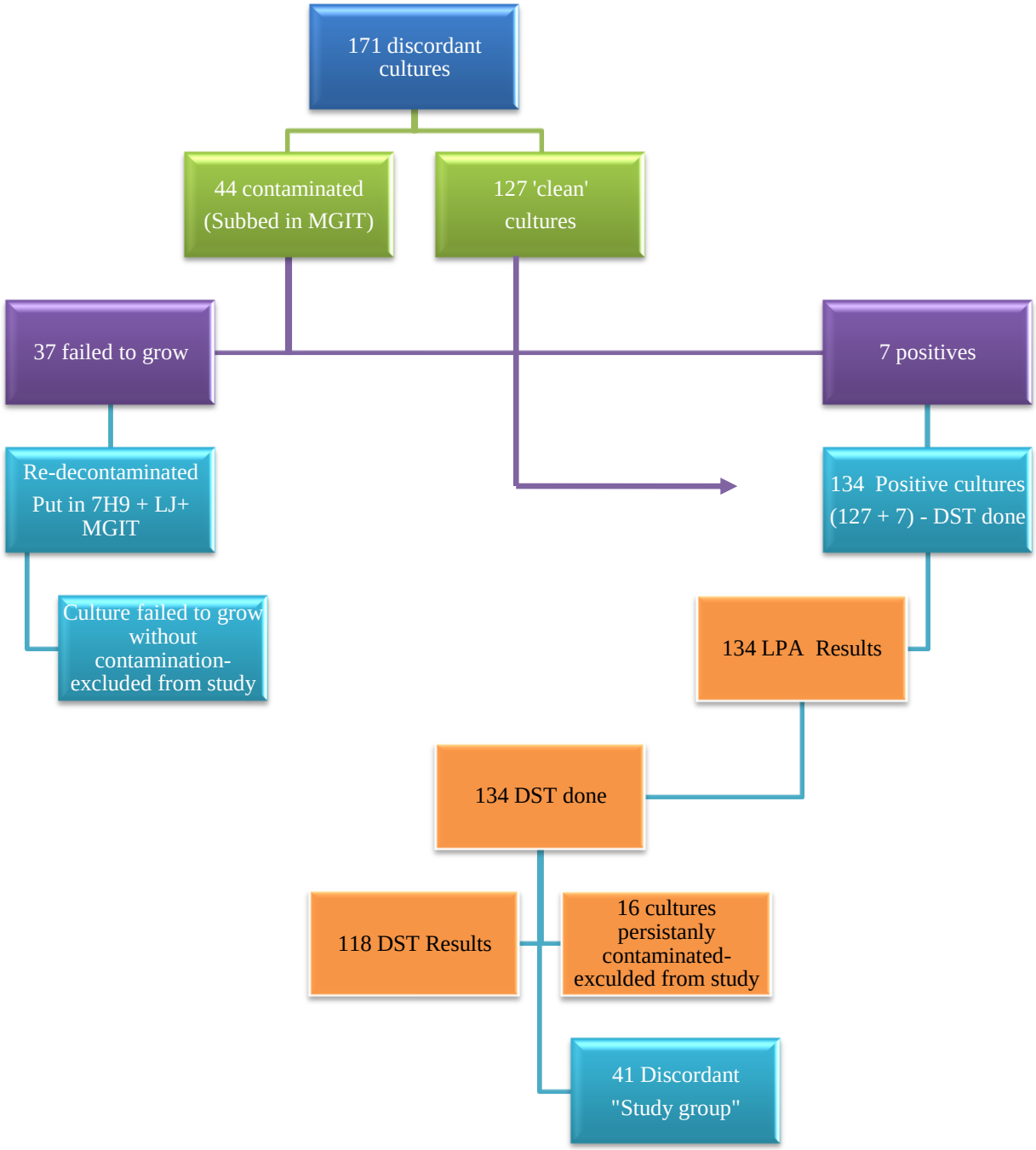


Figure 3.1 Flow diagram showing INH and RIF genotypic/phenotypic discordant study group selection.

3.1.1 The frequency and distribution of INH and RIF genotypic/phenotypic discordant isolates.

The samples were distributed across four of the nine provinces in SA; Western Cape, Northern Cape, KwaZulu-Natal and Gauteng. The distribution of the phenotypically and genotypically discordant samples for both the demonstration project and this study are shown in Table 3.1. In the original demonstration project, Gauteng had the lowest percentage of discordant samples at 2.6% (20/763) and the Western Cape the highest percentage, 9.2% (117/1266). Northern Cape and KwaZulu-Natal provinces had 4.7% (11/233) and 3.4% (23/666) discordant samples province respectively.

A large proportion of discordant samples were from Western Cape (68.4%), with KwaZulu-Natal and Gauteng contributing 13.5% and 11.7% respectively. Only 6.4% of the samples were from Northern Cape. Of the original 171 discordant samples, 118 were suitable for re-testing with Hain LPA and conventional DST, 77 gave concordant results for 65.3% (77/118) of samples which were previously recorded as discordant. These were excluded from the final study group.

Table 3.1 Distribution of INH and RIF genotypic/phenotypic discordant *M. tuberculosis* samples in the 4 provinces

Province	Demonstration Project			Excluded		Study	
	No of Specimens Enrolled	No of Discordant Samples	% of Study samples	No Growth	Not Discordant	Study Group	%
Western Cape	1266	117(9.2%)	68.4 (117/171)	14	69	34	82.9 (34/41)
Northern Cape	233	11(4.7%)	6.4 (11/171)	2	5	4	9.8 (4/41)
KwaZulu-Natal	666	23(3.4%)	13.5 (23/171)	21	1	1	2.4 (1/41)
Gauteng	763	20(2.6%)	11.7 (20/171)	16	2	2	4.9 (2/41)
Total	2928	171(5.8%)	100.0	53	77	41	100.0

The final distribution of the study group samples for each province is shown in Figure 3.2. The majority of the samples in this study were from Western Cape, 82.9% (117/171). Gauteng had the lowest number of discordant samples, contributing 2.6% (20/763) and the Western Cape with the highest number of discordants, 9.2% (117/1266). Northern Cape and KwaZulu-Natal provinces were represented by 4.7% (11/233) and 3.4% (23/666) of the samples for each province respectively.

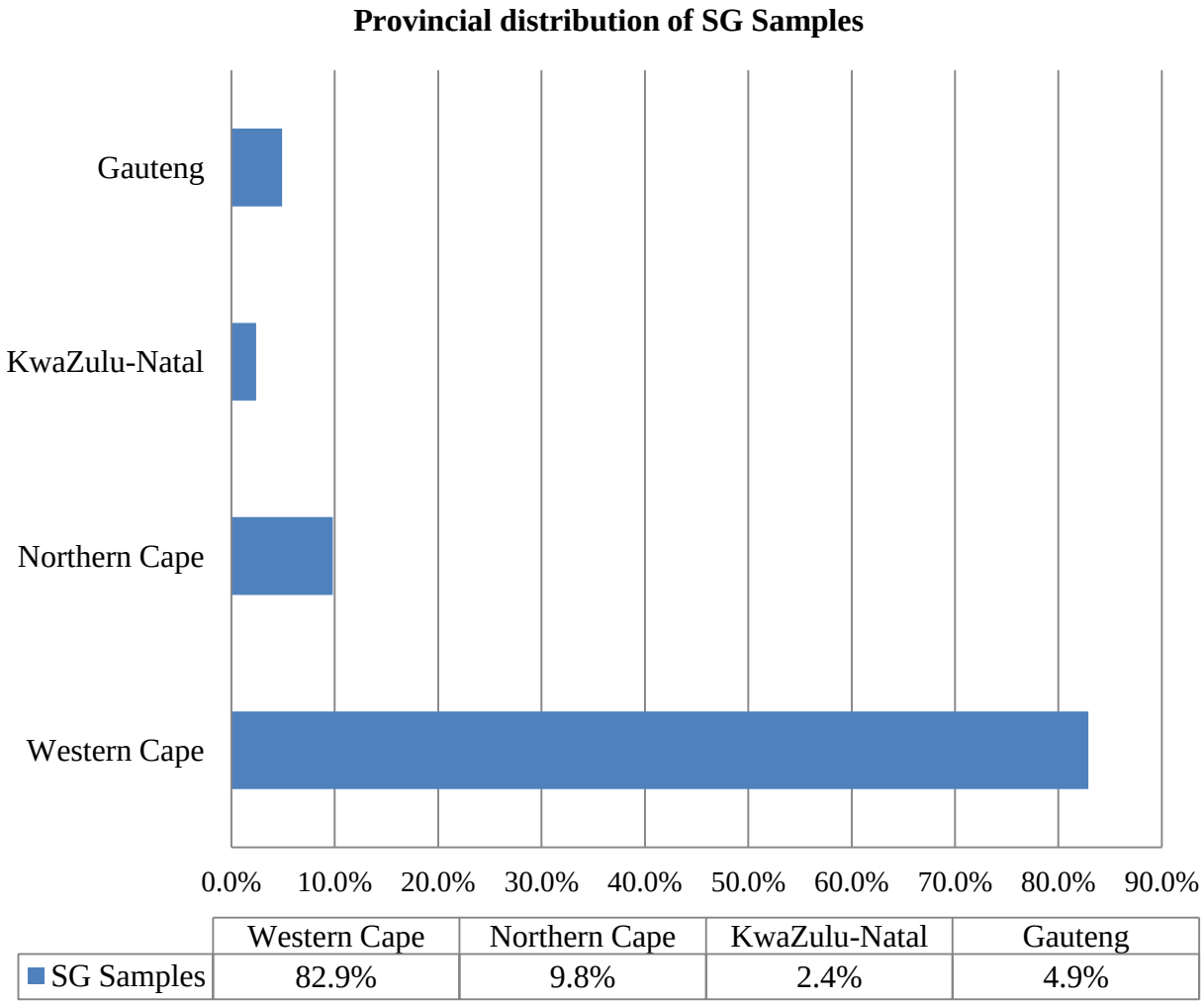


Figure 3.2 Provincial distribution of the INH and RIF discordant study group (SG) samples

3.1.2 Repeat LPA and phenotypic testing of all discordant isolates found in the demonstration project.

The conventional MGIT (phenotypic) DST for RIF and INH as well as the Hain LPAs (genotypic) were performed on the 118 cultures with 34.8% (41/118) of specimens confirming INH and/or RIF discordant results and entered into this study. The Hain LPA was interpreted in accordance with manufacturer's instructions i.e. isolates were classified as

resistant if they had a missing wild type band and corresponding mutation band or just missing wild type band.

The 41 specimens were grouped according to their discordant patterns shown in Table 3.2. Phenotypic INH and RIF resistance were expressed in 29.3% (12/41) of the discordant isolates classifying them as MDR-TB isolates. Genotypically, 17.1% (7/41) were grouped as MDR-TB isolates. Of the 12 specimens defined as MDR on phenotypic testing (Groups 2, 3 and 8), genotypic testing missed the INH resistance on 6 isolates, showed susceptibility to both RIF and INH on 1 isolate and missed RIF resistance on another 5 isolates. Seven isolates detected as MDR-TB on genotypic testing (group 7) were RIF susceptible on phenotypic DST.

Twenty-three of 41 (56.1%) isolates with phenotypic INH resistance were classified as susceptible to INH on genotypic testing (Groups 1-4). Seven isolates with phenotypic RIF resistance were susceptible to RIF on genotypic testing (groups 3, 5, 8) Genotypic resistance to RIF were identified in 36.6% (15/41) of isolates (Groups 4, 6, 7) which was not expressed phenotypically. All 12 isolates identified as INH resistant genotypically (group 7 and 8) were confirmed on phenotypic testing.

Table 3.2 Types of INH and RIF discordant groups between MGIT and Hain LPA DST in *M. tuberculosis* isolates

		MGIT		LPA		
Group	No. of Isolates	INH	RIF	INH	RIF	Type of Discordance
1	13	R	S	S	S	RS-SS
2	6	R	R	S	R	RR-SR
3	1	R	R	S	S	RR-SS
4	3	R	S	S	R	RS-SR
5	1	S	R	S	S	SR-SS
6	5	S	S	S	R	SS-SR
7	7	R	S	R	R	RS-RR
8	5	R	R	R	S	RR-RS

S, sensitive; R, resistance

3.1.3 Genes involved in resistance to INH and RIF as classified by the Hain LPA.

In 53.3% (8/15) of the RIF resistant strains identified by the Hain LPA, a missing WT 8 in the *rpoB* RRDR region covering base pairs 530-533 was responsible for the conferred resistance with only 50% (4/8) detecting the corresponding mutation 3. Missing WT 3 and 4 in 26.6% (4/15) of strains were detected without any corresponding mutations. Two strains with missing WT 2 and one strain missing WT 4 showed no corresponding mutations on the Hain LPA. In summary, 11/15 isolates determined as discordant RIF resistance on genotypic testing were due to a missing wildtype with no corresponding mutation. Of these 9/11 were

phenotypically susceptible while all 4 specimens that had a missing wildtype and a mutation were confirmed as phenotypically resistant.

INH resistance was detected mostly in the *katG* gene, 66.7% (8/12) of the strains. The omission of the WT and the presence of mutation 1 were seen in seven out of the eight strains with 1 strain having mutation 2 instead. There was one strain in which the WT was present as well as mutation 1 in the *katG* gene suggesting a mixed infection. In the *inhA* gene all four strains had WT 1 missing with mutation 1 present. None of the INH resistant strains had resistance detected simultaneously in the katG and inhA gene regions.

Eighty-five (6/7) percent of MDR-TB isolates (group 7) exhibited a *katG*315 mutation. The *katG*315 mutation is therefore strongly associated with MDR-TB strains. Of the 12 specimens (groups 2, 3 and 8) classified as MDR-TB on conventional DST 2 showed *katG* mutation, 3 showed *inhA* mutation and 7 failed to show INH resistance genotypically.

Table 3.3 Pattern of gene mutations detected by the Hain LPA in INH and RIF discordant *M. tuberculosis* strains

Group	#	katG missing WT	Mutation	inhA missing WT	Mutation	rpoB missing WT	Mutation
1 (RS-SS) n=13							
2 (RR-SR) n=6	4					8 (530-533)	3 (S531L)
	1					8 (530-533)	
	1					2 (510-513)	
3 (RR-SS) n=1	1						
4 (RS-SR) n=3	2					2 (510-513)	
	1					3 (513-517) + 4 (516-519)	
5 (SR-SS) n=1	1						
6 (SS-SR) n=5	5					2 (510-513)	
7 (RS-RR) n=7	2	1 (315)	1 (S315T1)			3 (513-517) + 4 (516-519)	
	1	1 (315)	2 (S315T2)			3 (513-517) + 4 (516-519)	
	2	1 (315)	1 (S315T1)			8 (530-533)	
	1			1 (-15/-16)	1 (C15T)	8 (530-533)	
	1	1 (315)	1 (S315T1)			4 (516-519)	
8 (RR-RS) n=5	3			1 (-15/-16)	1 (C15T)		
	1	1 (315)	1 (S315T1)				
	1		1 (S315T1)				

3.1.4 MIC determination to the anti-tuberculosis drugs INH and RIF

Colorimetric MIC results for all 41 of the clinical isolates were available by the 9th day of incubation (Figure 3.3). The MIC result ranges of each discordant group obtained are shown

in Table 3.5. Cut off /Breakpoint for conventional phenotypic DST on MGIT is 1.25 µg/ml for RIF and 0.1 µg/ml for INH. The MIC drug dilution series used is shown in Table 3.4.

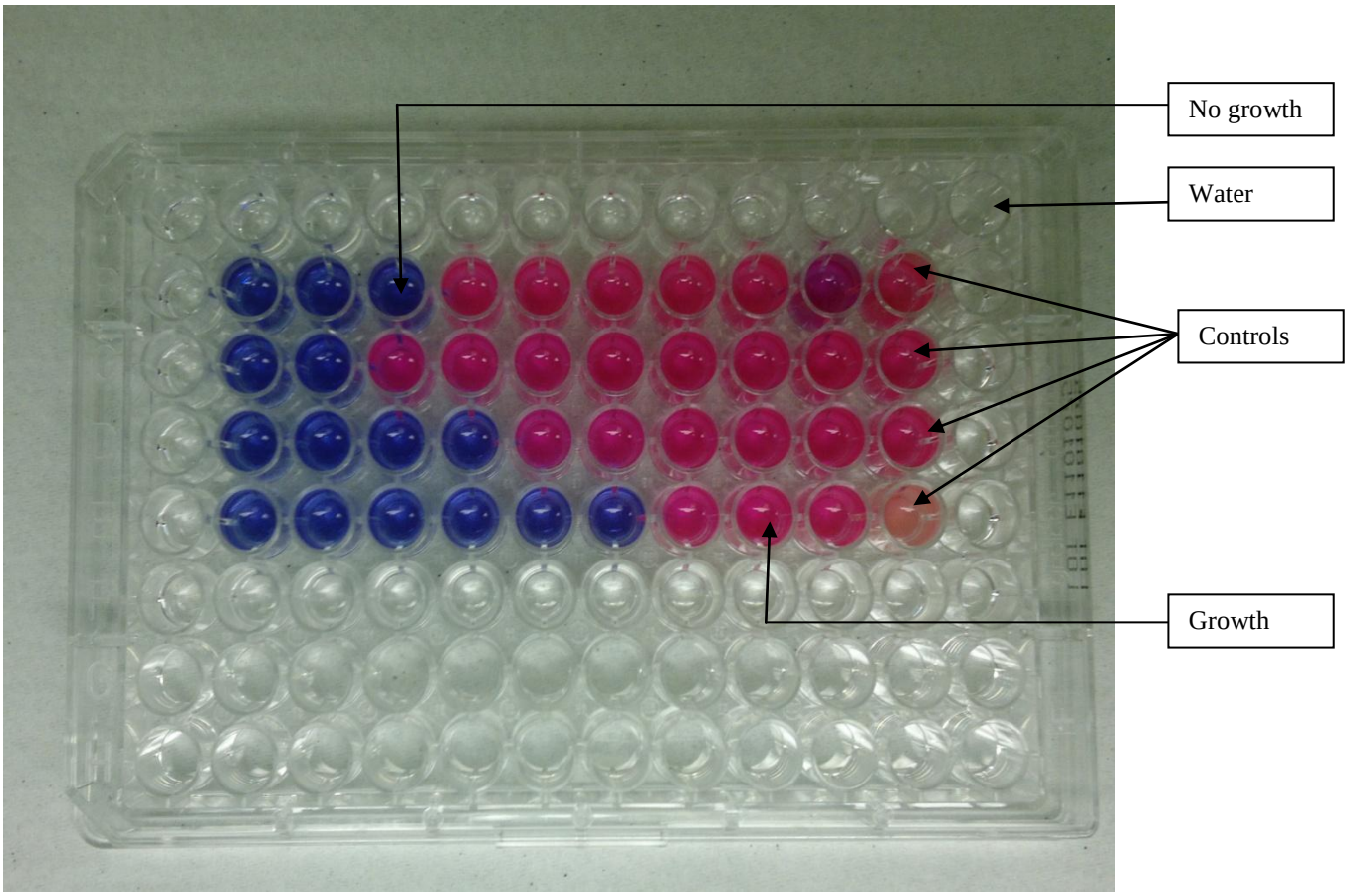


Figure 3.3 Microtitre plate showing results from Alamar blue assay

Table 3.4 MIC dilution series used for both RIF and INH concentrations

W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12
Water	10.0 µg/ml	5.0 µg/ml	2.5 µg/ml	1.25 µg/ml	0.6 µg/ml	0.3 µg/ml	0.15 µg/ml	0.07 µg/ml	0.03 µg/ml	Control	Water

Table 3.5 Ranges of MIC's to INH and RIF for the different discordant groups

		MGIT		LPA			
Group	No. of Specimens	INH	RIF	INH	RIF	INH MIC (0.1µg/ml)	RIF MIC (1.25µg/ml)
1	13	R	S	S	S	0.07-1.25	0.03-1.25
2	6	R	R	S	R	0.6-1.25	1.25-2.5
3	1	R	R	S	S	1.25	2.5
4	3	R	S	S	R	0.15-0.6	0.6-1.25
5	1	S	R	S	S	0.6	1.25
6	5	S	S	S	R	0.03-0.6	0.03-2.5
7	7	R	S	R	R	0.03-5	0.6-5
8	5	R	R	R	S	0.07-2.5	0.03-1.25

Twenty three of 41 isolates with phenotypic INH resistance were classified as susceptible to INH on genotypic testing (Groups 1-4). The MIC's for 22/23 of these isolates supports the phenotypic resistant profile with one susceptible isolate having a MIC concentration one dilution away from breakpoint. Seven of 13 isolates with phenotypic RIF resistance were susceptible to RIF on genotypic testing (groups 3, 5, 8) with four isolates having MIC's as per phenotypic result, two with MICs one dilution away from breakpoint and one isolate with a susceptible MIC 5 dilutions below breakpoint. Genotypic resistance to RIF were identified in 15/41 of isolates (Groups 4, 6, 7) which was not expressed phenotypically with 11/15 isolates having MIC values as per genotypic resistance, 3/15 isolates with MIC values one dilution from breakpoint and, only 1/15 isolate having a MIC value 5 dilutions away from

breakpoint. All 12 isolates identified as INH resistant genotypically (group 7 and 8) were confirmed on phenotypic testing with corresponding MIC's. Interestingly, in group 7 which is MDR genotypically, with genotypic RIF resistance expressed as phenotypically susceptible, had MIC values in keeping with the genotypic result.

The MIC ranges for group 1, classified as genotypically susceptible to INH and RIF by the Hain LPA, phenotypic INH resistance was concordant to the MIC values for 12/13 with 1/13 isolate expressing susceptibility 5 dilutions away from breakpoint Table 3.6. For RIF in this group, the phenotypic and genotypic results were concordant with MIC values expressing susceptibility in 10/13 isolates with 3/13 isolates resistant to RIF by MIC testing. In group 2, the INH MIC's correlate to the phenotypic result of resistant in all isolates while 4 of the RIF resistance findings shown on the LPA were confirmed by MIC determination with 2 isolates showing borderline resistance of 1.25µg/ml (Table 3.6). Group 3, MIC results for both INH and RIF are associated with the resistance results observed phenotypically. In group 7 (Hain LPA MDR), the MIC for RIF confirm the genotypic resistance expressed on the LPA (6/7) with only 1 isolate having a susceptible MIC to RIF in the group (Table 3.6).

Table 3.6 MIC results obtained for all 41 INH/RIF discordant isolates

Group	MGIT INH	MGIT RIF	LPA INH	LPA RIF	MIC INH	MIC RIF
1	R	S	S	S	0.15	0.03
	R	S	S	S	0.3	0.6
	R	S	S	S	1.25	0.3
	R	S	S	S	0.6	1.25
	R	S	S	S	1.25	0.6
	R	S	S	S	1.25	0.6
	R	S	S	S	1.25	0.15
	R	S	S	S	0.6	0.03
	R	S	S	S	1.25	0.6
	R	S	S	S	1.25	1.25
	R	S	S	S	0.07	0.15
	R	S	S	S	0.15	2.5
	R	S	S	S	1.25	0.15
2	R	R	S	R	1.25	2.5
	R	R	S	R	0.6	2.5
	R	R	S	R	1.25	2.5
	R	R	S	R	1.25	1.25
	R	R	S	R	0.6	1.25
	R	R	S	R	1.25	2.5
3	R	R	S	S	1.25	2.5
4	R	S	S	R	0.15	0.6
	R	S	S	R	0.6	0.6
	R	S	S	R	0.6	1.25
5	S	R	S	S	0.6	1.25
6	S	S	S	R	0.07	1.25
	S	S	S	R	0.07	2.5
	S	S	S	R	0.6	2.5
	S	S	S	R	0.15	1.25
	S	S	S	R	0.03	0.03
7	R	S	R	R	0.15	2.5
	R	S	R	R	1.25	0.6
	R	S	R	R	2.5	1.25
	R	S	R	R	1.25	5
	R	S	R	R	1.25	2.5
	R	S	R	R	0.3	1.25
	R	S	R	R	5	2.5
8	R	R	R	S	0.15	1.25
	R	R	R	S	1.25	0.6
	R	R	R	S	1.25	1.25
	R	R	R	S	2.5	0.6
	R	R	R	S	0.6	0.03

S, sensitive; R, resistance

3.1.5 Characterisation of the discordant isolates by spoligotyping

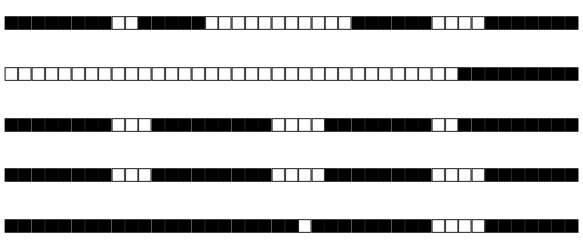
All 41 INH/RIF discordant isolates were characterised by spoligotyping and grouped into 17 previously identified strain families and 8 families which were not in the international Spoligotype database (SpolD4B), Table 3.7. The five major families amongst the phenotypic and genotypic discordant isolates were; East Asian Beijing, Latin America and Mediterranean (LAM), X, T and S. The other strain families in the international spoligotype database and the distribution of strains across provinces are shown in Table 3.7. There were 8 isolates from previously unreported families that were termed orphans (Figure 3.4). Of the 8 orphan isolates, 3 belonged to a mixed lineage, 2 belonged to a low copy number clade (LCC), and 1 belonged to the LAM lineage. Two orphan isolates were not assigned to any families (Figure 3.4).

The LAM lineage was the most frequent, making up 21.9% (9/41) of the isolates, whereas EAI was least frequent, making up 2.4% (1/41). Seven of the orphan isolates could not be assigned to any of the lineages previously described (Table 3.7). In the Hain LPA RIF resistant isolates, lineage X was predominant (7/21), whereas in the phenotypic RIF resistant isolates the LAM lineage was most frequently represented (3/13). In the INH phenotypic and genotypic concordant isolates, majority of the isolates were from the X and LAM lineages. In the discordant INH groups all lineages were present.

Table 3.7 The different lineages observed among the 41 discordant isolates spoligotyped across four South African provinces

Spoligo Family		Frequency (n)	Percent
BEIJING		7	17.07
	Western Cape	7	
LAM		9	21.95
	Western Cape	7	
	Gauteng	1	
	Northern Cape	1	
T		4	9.76
	Western Cape	3	
	Northern Cape	1	
EAI		1	2.44
	Gauteng	1	
S		3	7.32
	Western Cape	1	
	Gauteng	1	
	KwaZulu-Natal	1	
U		2	4.88
	Western Cape	2	
X		8	19.51
	Western Cape	7	
	Northern Cape	1	
Unknown spoligotype		7	17.07
	Western Cape	7	
		41	100.00

No.	Pattern	SIT*	Family	Octal value
1		33	LAM3	776177607760771
2		1	BEIJING	000000000003771
3		Orphan	*F9*	677777606060771
4		Orphan	*MIX*	776177607763771
5		1	BEIJING	000000000003771
6		280	T1	770000777760771
7		1241	U	777777607700771
8		237	U	777777777700000
9		1	BEIJING	000000000003771
10		33	LAM3	776177607760771
11		92	X3	700076777760771
12		92	X3	700076777760771
13		33	LAM3	776177607760771
14		Orphan	*Undesignated*	777774777760771
15		33	LAM3	776177607760771
16		159	T1	777740017760771
17		92	X3	700076777760771
18		92	X3	700076777760771
19		Orphan	*MIX*	776177607763771
20		Orphan	*Undesignated*	760721047700771
21		48	EAI1	777777777413731
22		1	BEIJING	000000000003771
23		789	S	676377777760771
24		1	BEIJING	000000000003771
25		92	X3	700076777760771
26		Orphan	*LCC*	077776775760731
27		1222	LAM9	770177607760771
28		34	S	776377777760771
29		92	X3	700076777760771
30		33	LAM3	776177607760771
31		1329	X1	777776777560771
32		92	X3	700076777760771
33		1	BEIJING	000000000003771
34		154	T1	757777777760771
35		33	LAM3	776177607760771
36		Orphan	*LCC*	300076777760771



37	1333	S	776370001760771
38	1	BEIJING	000000000003771
39	Orphan	*MIX*	776177607763771
40	33	LAM3	776177607760771
41	44	T5	777777757760771

*SIT: Standard international type

Figure 3.4 Spoligotype patterns of the 41 INH/RIF discordant *M.tuberculosis* isolates used in the study.

3.1.6 Sequencing analysis of the gene regions which confer resistance to the anti-tuberculosis drugs rifampicin and isoniazid

3.1.6.1 The *rpoB* gene region

Since the majority of strains resistant to RIF harbour mutations within the RRDR of *rpoB*, this region and flanking sequence were analysed in this study. Twenty-two phenotypic and genotypic rifampicin discordant isolates were sequenced for mutations associated with the *rpoB* gene region. Among the isolates analysed, 59.1% (13/22) had mutations in *rpoB* (Table 3.8). The most frequently observed mutation was found in codon 458 (CTG to CCG, Leu458Pro), observed in 7 of the 22 (31.8%) of isolates. Four isolates had a Leu436Pro (CTG to CCG) mutation with two isolates having mutations in codon 441 (GAC to GGC, Asp441Gly) (Table 3.8). None of these mutations are present on the Hain LPA. Twelve of the 13 isolates with novel mutations were classified as RIF resistant on Hain LPA based on missing WT (no mutation detected). All 12 were phenotypically susceptible. One isolate was classified as susceptible on Hain LPA and was phenotypically RIF resistant.

In the isolates which were phenotypically susceptible but genotypically resistant to rifampicin (Groups 4, 6, 7), 73.3% (11/15) displayed mutations correlating to the genotypic LPA resistance expressed. Although the other 4 isolates in this group had resistant MIC values, no mutations were observed in the *rpoB* gene region sequenced for RIF. In the 7 isolates which were phenotypically resistant but genotypically classified susceptible (Groups 3, 5, 8), 28.6% (2/7) showed a mutation present in codon 458 (CTG to CCG, Leu458Pro) which is not present on the Hain LPA and has not been previously reported (Table 3.9).

The two isolates which had a missing WT 8 without a corresponding mutation showed a mutation/mutations in codon 441 on sequencing which is not represented on the Hain LPA. The MIC of these two isolates' showed 1 resistant strain (2.5 µg/ml) and the other with borderline (1.25 µg/ml) resistance. In the isolates which displayed a missing WT 2 with no matching mutation on the LPA, mutation in codon 436 was observed in 57.1% (4/7) of the isolates with MICs confirming resistance in 3 isolates. Mutation in codon 458 was associated with 80% (4/5) of isolates with no WT 3 and/or WT 4 present (Table 3.9) without an associated mutation band. The MICs for isolates with a codon 458 mutation showed 2 resistant and 2 susceptible isolates. Of the 7 isolates with *rpoB* gene mutation 458, 6/7 were genotypically resistant but susceptible on phenotypic DST and one isolate was genotypically susceptible but phenotypically resistant confirmed with a borderline MIC value of 1.25 µg/ml.

Table 3.8 Frequency of mutations in the *rpoB* gene region detected

Location of mutations	Nucleotide changes	Amino acid changes	No. (%) of strains n = 22
Codon 436	CTG to CCG	Leu (L) to Pro (P)	4 (18.2)
Codon 441	GAC to GGC	Asp (D) to Gly (G)	2 (9.0)
Codon 458	CTG to CCG	Leu (L) to Pro (P)	7 (31.8)

Table 3.9 Sequencing results of the *rpoB* gene region in genotypic and phenotypic discordant samples

Group	MGIT RIF	LPA RIF	LPA *rpoB* profile	MIC RIF	Location of mutations	Nucleotide changes	Amino acid changes
3	R	S	NC	2.5			
4	S	R	WT2 MIS NO MUT	0.6	Codon 458	CTG to CCG	Leu (L) to Pro (P)
	S	R	WT3 + 4 MIS NO MUT	0.6	Codon 458	CTG to CCG	Leu (L) to Pro (P)
	S	R	WT2 MIS NO MUT	1.25	Codon 436	CTG to CCG	Leu (L) to Pro (P)
5	R	S	NC	1.25	Codon 458	CTG to CCG	Leu (L) to Pro (P)
6	S	R	WT2 MIS NO MUT	1.25	Codon 436	CTG to CCG	Leu (L) to Pro (P)
	S	R	WT2 MIS NO MUT	2.5	Codon 436	CTG to CCG	Leu (L) to Pro (P)
	S	R	WT2 MIS NO MUT	2.5			
	S	R	WT2 MIS NO MUT	1.25			
	S	R	WT2 MIS NO MUT	0.03	Codon 436	CTG to CCG	Leu (L) to Pro (P)
7	S	R	WT8 MIS NO MUT	2.5	Codon 441	GAC to GGC	Asp (D) to Gly (G)
	S	R	WT4 MIS NO MUT	0.6	Codon 458	CTG to CCG	Leu (L) to Pro (P)
	S	R	WT3 + 4 MIS NO MUT	1.25			

	S	R	WT8 MIS + NO MUT	5			
	S	R	WT3 + 4 MIS NO MUT	2.5	Codon 458	CTG to CCG	Leu (L) to Pro (P)
	S	R	WT8 MIS NO MUT	1.25	Codon 441	GAC to GGC	Asp (D) to Gly (G)
	S	R	WT3 + 4 MIS NO MUT	2.5	Codon 458	CTG to CCG	Leu (L) to Pro (P)
	R	S	NC	1.25			
	R	S	NC	0.6			
8	R	S	NC	1.25	Codon 458	CTG to CCG	Leu (L) to Pro (P)
	R	S	NC	0.6			
	R	S	NC	0.03			

NC- no change

3.1.6.2 The *katG* and *inhA* gene region

A total of twenty-three isolates were each sequenced for mutations in the *katG* and *inhA* loci. All isolates were phenotypically resistant to isoniazid and genotypically susceptible according to the Hain LPA. In the *katG* gene region only four mutations (Table 3.10) were detected in codon 213 (GGT to GTT, Gly213Val), codon 232 (CCC to AAA, Pro232Lys), codon 254 (AAG to AAC, Lys254Asn) and, in codon 259 (AGC to AAC, Ser359Asn). These mutations are not present on the Hain LPA and have not been previously reported to be associated with resistance. There were three isolates which had simultaneous mutations in *inhA* and *katG* in isolates (Table 3.12). The *katG*315 mutation which is strongly correlated with MDR-TB strains (Bolotin et al, 2009) was not observed in any of the discordant isolates. The remaining twenty isolates showed no mutations in the analysed region of the *katG* gene.

Table 3.10 Frequency of mutations in the *katG* gene locus

Location of mutations	Nucleotide changes	Amino acid changes	No. (%) of strains n = 23
Codon 213	GGT to GTT	Gly (G) to Val (V)	1 (4.3)
Codon 232	CCC to AAA	Pro(P) to Lys(K)	1 (4.3)
Codon 254	AAG to AAC	Lys (K) to Asn (N)	1 (4.3)
Codon 259	AGC to AAC	Ser (S) to Asn (N)	1 (4.3)

Among the twenty-three phenotypic isoniazid resistant isolates analysed for mutations in the *inhA* gene region, 47.8% (11/23) of the isolates had mutations in this region. There were 18 different locations with codon mutations (Table 3.11). The most frequent mutations detected in *inhA* locus was the amino acid change at positions; codon -2 (Thr to Ser), codon -23 (Gly to Stop codon), and codon -36 (Ala to Gly), each contributing 17.4% of these mutations. Two isolates had a maximum combination of 7 different amino acid changes at various codons (Table 3.11) both these isolates were resistant to INH at the 1.5 µg/ml level. Five other isolates had combination nucleotide substitutions at other codons. Interestingly mutation in codon -22 and -23, gave rise to "stop" codons while mutations in codon -12, -21, -35, -48 did not convert into interpretable amino acid changes. As expected none of these discordant isolates contained a cytosine-to-thymine transition at the nucleotide positioned 15 bases upstream from the start codon which has been reported as one of the most common mutations in the *inhA* promoter region and is represented on the Hain LPA strip. There were no mutations present in the *inhA* promoter region for the other 12 phenotypically resistant isolates which were classed as susceptible by the LPA (Table 3.12).

Table 3.11 Frequency of mutations in the *mabA-inhA* promoter gene locus detected

Location of mutations	Nucleotide changes	Amino acid changes	No. (%) of strains n = 23
Codon -2	ACT to AGT	Thr (T) to Ser (S)	4 (17.4)
Codon -5	GCC to TGG	Ala(A) to TGG (W)	2 (8.7)
Codon -12	CCA to CTG	Pro(P) to Leu (L)	1 (4.3)
Codon -12	CCA to CCG	none	1 (4.3)
Codon -21	ACC to ACT	none	3 (13.0)
Codon -22	GGA to TGA	Gly (G) to Stop codon	3 (13.0)
Codon -23	GGA to TGA	Gly (G) to Stop codon	4 (17.4)
Codon -24	AAC to AGC	Asn (N) to Ser (S)	1 (4.3)
Codon -30	GCG to GGG	Ala (A) to Gly (G)	2 (8.7)
Codon -31	ATC to TTC	Ile (I) to Phe (F)	1 (4.3)
Codon -33	CAG to TGG	Gln (Q) to Trp (W)	1 (4.3)
Codon -34	CGG to GGG	Arg (R) to Gly (G)	2 (8.7)
Codon -35	CTG to TTG	none	1 (4.3)
Codon -36	GCT to GGT	Ala (A) to Gly (G)	4 (17.4)
Codon -41	AAG to TGG	Lys (K) to Trp (W)	1 (4.3)
Codon -48	GGA to GGG	none	1 (4.3)
Codon -63	ACC to TCC	Thr (T) to Ser (S)	2 (8.7)
Codon -63	ACC to ATC	Thr (T) to Ile (I)	1 (4.3)

Table 3.12 Sequencing results of *inhA* and *katG* gene regions in INH genotypic and phenotypic discordant isolates

Group	MGIT INH	LPA INH	MIC INH	*inhA* promoter region			*katG* region		
				Location of mutations	Nucleotide changes	Amino acid changes	Location of mutations	Nucleotide changes	Amino acid changes
1	R	S	0.15	Codon -21	ACC to ACT	none			
				Codon -23	GGA to TGA	Gly (G) to Stop codon			
				Codon -48	GGA to GGG	none			
	R	S	0.3				Codon 232	CCC to AAA	Pro(P) to Lys(K)
	R	S	1.25						
	R	S	0.6						
	R	S	1.25	Codon -22	GGA to TGA	Gly (G) to Stop codon			
				Codon -23	GGA to TGA	Gly (G) to Stop codon			
				Codon -24	AAC to AGC	Asn (N) to Ser (S)			
				Codon -30	GCG to GGG	Ala (A) to Gly (G)			
				Codon -34	CGG to GGG	Arg (R) to Gly (G)			
				Codon -31	ATC to TTC	Ile (I) to Phe (F)			
				Codon -36	GCT to GGT	Ala (A) to Gly (G)			
	R	S	1.25	Codon -12	CCA to CTG	Pro(P) to Leu (L)			
				Codon -12	CCA to CCG	none			
				Codon -21	ACC to ACT	none			
				Codon -22	GGA to TGA	Gly (G) to Stop codon			
				Codon -23	GGA to TGA	Gly (G) to Stop codon			
				Codon -33	CAG to TGG	Gln (Q) to Trp (W)			
				Codon -36	GCT to GGT	Ala (A) to Gly (G)			
	R	S	1.25						
	R	S	0.6						

	R	S	1.25						
	R	S	1.25						
	R	S	0.07	Codon -2 Codon -5 Codon -36	ACT to AGT GCC to TGG GCT to GGT	Thr (T) to Ser (S) Ala(A) to TGG (W) Ala (A) to Gly (G)	Codon 213	GGT to GTT	Gly (G) to Val (V)
	R	S	0.15	Codon -63	ACC to TCC	Thr (T) to Ser (S)			
	R	S	1.25	Codon -2 Codon -5	ACT to AGT GCC to TGG	Thr (T) to Ser (S) Ala(A) to TGG (W)			
2	R	S	1.25						
	R	S	0.6	Codon -22 Codon -23 Codon -30 Codon -33 Codon -35 Codon -36	GGA to TGA GGA to TGA GCG to GGG CAG to TGG CTG to TTG GCT to GGT	Gly (G) to Stop codon Gly (G) to Stop codon Ala (A) to Gly (G) Gln (Q) to Trp (W) none Ala (A) to Gly (G)			
	R	S	0.6	Codon -2	ACT to AGT	Thr (T) to Ser (S)			
	R	S	1.25						
	R	S	0.6	Codon -63	ACC to ATC	Thr (T) to Ile (I)			
	R	S	1.25	Codon -21 Codon -41 Codon -63	ACC to ACT AAG to TGG ACC to TCC	none Lys (K) to Trp (W) Thr (T) to Ser (S)	Codon 254	AAG to AAC	Lys(K) to Asn (N)
3	R	S	1.25	Codon -2	ACT to AGT	Thr (T) to Ser (S)			
4	R	S	0.15						
	R	S	0.6				Codon 259	AGC to AAC	Ser (K) to Asn (N)
	R	S	0.6						

Sequencing analysis only detected mutations, either in *katG* and/or in *inhA* for 56.2% (13/23) of the phenotypically resistant discordant isolates. Interestingly neither *katG* nor *inhA* genetic alterations were detected in 10 (43.4%) clinical isolates which were phenotypically resistant with corresponding resistant MIC values.

Chapter 4

4.1 Discussion

The re-emergence of TB and drug-resistant isolates of *M. tuberculosis* poses a severe threat to the global control of TB (Evans et al, 2009), especially in Asia and Africa (Stavrum et al, 2009). Rapid, reliable and cost effective new drug susceptibility diagnostic tools are required urgently to control the TB and MDR-TB epidemics (Luo et al, 2010). Molecular methods can provide accurate and rapid drug susceptibility results which are critical to ensure that patients receive effective treatment and become non-infectious (Campbell et al, 2010). However, regional specific information on the type and frequency of drug resistance-conferring mutations is also required (Luo et al, 2010). Our study adds to the body of knowledge and specifically describes a novel set of mutations observed from phenotypic and genotypic discordant isolates.

The results from this study demonstrates the utility of detection of mutations associated with drug resistance to rapidly and accurately determine the drug susceptibility of *M. tuberculosis* complex isolates as compared to conventional phenotypic methods but also highlights some limitations of the methodology.

The isolates used in this study were distributed across Western Cape, Northern Cape, Gauteng and KwaZulu-Natal provinces of South Africa. The rate of discordant isolates identified in the larger Demonstration project from Western Cape, Northern Cape, Gauteng

and KwaZulu-Natal were 9.2%, 4.7%, 2.6% and 3.4% respectively. The proportion of INH and/or RIF phenotypic and genotypic discordant isolates varied with the majority of the isolates from the country coming from the Western Cape, 82.9% with the lowest number of isolates (2.4%) coming from KwaZulu-Natal.

Among the isolates, phenotypic MDR-TB was expressed in 29.3%, genotypically only 17.1% of the same study set was classified as MDR-TB isolate by the Hain LPA. While all INH resistance detected genotypically was confirmed phenotypically, phenotypic INH resistance was not detected by the Hain in 23 (56.1%) of isolates. In 22 of these, the MIC results confirmed resistance. Similarly, as discussed below, sequencing analysis only detected mutations, either in *katG* and/or in *inhA* for 56.2% (13/23) of the phenotypically resistant discordant isolates. These results reflect the complexity of the genetic basis of INH resistance compared with RIF.

Many laboratories would do phenotypic INH DST if RIF monoresistance is detected by the Hain LPA and in SA according to current NTCP guidelines both RIF monoresistance and MDR would be treated as MDR-TB. However, false classification of MDR-TB as RIF monoresistance could have implications for surveillance especially as the Western Cape have recently shown a trend of increasing RIF monoresistance. Accurate discrimination between MDR and RIF monoresistance is also important as utilisation of many molecular tests is based on the assumption that RIF resistance can be used as a proxy for MDR-TB.

Results for RIF were less clear cut. Seven of 13 isolates with phenotypic RIF resistance were susceptible to RIF on genotypic testing with four isolates having MIC's as per phenotypic result. Three of the 4 had MICs at breakpoint concentration and the other 1 dilution above breakpoint. These borderline resistance/susceptible observations in isolates have also been reported by van Deun et al, as the reason for discordant phenotypic and genotypic DST results (van Deun et al, 2009). Genotypic RIF resistance in 15 isolates (36.6%) was not expressed phenotypically and 11/15 MIC results confirmed genotypic resistance. This included 6/7 isolates classified as MDR-TB genotypically and INH-monoresistant on phenotypic DST. Furthermore 11/15 isolates determined as RIF resistance on genotypic testing demonstrated a missing wildtype with no corresponding mutation. Of these, 9/11 were phenotypically susceptible. However, all 4 specimens that had a missing wildtype and a mutation were confirmed as phenotypically resistant. This suggests that RIF resistance diagnosed on LPA due to missing WT only should be confirmed on phenotypic DST.

DNA sequencing is still the most sensitive molecular diagnostic tool for identification of mutations. The study shows the advantage of sequencing over DNA probe based assays to detect a limited set of specific mutations, since several mutations associated with drug resistance identified in this study have not been previously reported. Hain LPA probes of the RRDR do not cover these mutations and further research should be performed as these mutations may have important ramifications for new probe based genotypic diagnostic tools for the detection of RIF resistance. With the use of molecular diagnostics for the rapid detection of MDR-TB there is some concern regarding isolates which are not detected by the selection of probes on the Hain LPA. Patients infected with these strains will not receive correct treatment and may go on to develop further resistance conferring mutations,

contributing to the pool of resistance. For this reason in routine practice currently in SA, many laboratories will do phenotypic DST if Hain LPA shows monoresistance. A study by van Deun et al, 2009 has reported that conventional RIF DSTs can yield highly discordant results even among proficient laboratories and therefore the mutations found and reported in this study should further be investigated.

The molecular mechanism of resistance to RIF is well understood and documented in comparison to the resistance mechanisms of other anti-tuberculosis drugs. It has been well established that mutations within the RRDR region of the *rpoB* gene confer more than 95% of resistance to RIF. The commonest mutations within the RRDR region are Ser531Leu, His526Tyr, and Asp516Val (Campbell et al, 2011). Amongst RIF susceptibility discordant strains in a study previously reported, predominant mutations recorded in the *rpoB* gene were Leu511Pro, Asp516Tyr, His526leu/Ser, and Leu533Pro (van Deun et al, 2009).

On sequencing, 13 of the 22 (59.1%) RIF- resistant isolates demonstrated mutations in the *rpoB* region. However, none of the previously reported mutations from SA (Green et al, 2008; Evans et al, 2009) were detected among this discordant group. In this study, the sequenced strains harboured only 3 types of mutations, Leu458Pro, Leu436Pro, and Asp441Gly, in eleven genotypically (Hain LPA) RIF resistant and two genotypically RIF susceptible isolates. Despite the mutations resulting in an amino acid change, 10 of the 11 isolates harbouring these mutations were phenotypically susceptible. On MIC testing, 9/13 were resistant.

Mutation Asp441Gly in two isolates were associated with a missing WT8 on the Hain LPA with no corresponding mutation detected. One isolate with this mutation was resistant with the other isolate expressing borderline resistance to RIF. Mutation Leu436Pro was present in isolates with a missing WT2 and no mutation bands present with MIC's favouring the genotypic resistance. Resistant isolates missing WT3 and/or WT4 without one of the probe mutations had mutation Leu458Pro. Of these isolates according to the MIC's, 50% were resistant and the other 50% had MIC's of one dilution away from the breakpoint.

These mutations 441, 436 and, 458 are not represented on the Hain LPA strips and would be missed at diagnosis or detected on the basis of missing wild type only. Further studies are required to characterize the roles of these mutations in RIF resistance. With the increased use of genotypic methods to diagnose RIF resistance, these mutations may be more frequently observed, and subsequently, their clinical and epidemiological significance may be better understood.

Although INH has been a key component of tuberculosis chemotherapeutic regimens for several decades, the mode of action of INH though extensively investigated, remains incompletely understood (Rouse et al, 1995; Campbell et al, 2011). INH is classified as a prodrug which is activated by the catalase-peroxidase enzyme which is coded for by *katG*. Mutations within *katG*, particularly at codon 315, can result in the loss of catalase activity giving rise INH resistance. The target of activated INH is enoyl-acyl carrier protein reductase (InhA). The increased expression of inhA as a result of promoter mutations leads to the resistance to isoniazid through a titration mechanism. In this study we sequenced a region of

katG which includes codon 315 and the *inhA* promoter region because it has been well documented that most INH-resistant isolates which together account for approximately 65-75% of resistant strains (Ramaswamy et al, 1998), contain mutations in one or both of these regions.

There are several different loci that are known to be involved in resistance but the most frequent resistance-conferring mutations are detected in *katG* and less commonly in the *inhA* promoter region (Luo et al, 2010; Bolotin et al, 2009). Mutations in codon 315 of *katG* are known to only introduce a slight fitness cost to the bacterium (Luo et al, 2010; Bolotin et al, 2009) and are a proven mechanism of INH resistance (Campbell, 2011). In our study, 85.7% of phenotypic MDR-TB isolates and 40% of the isoniazid mono-resistant had mutations in codon 315 of the *katG* by Hain LPA but unfortunately this could not be confirmed by sequencing as these isolates were concordant and excluded from further study investigations. This is in agreement with previous studies in which mutations in codon 315 of *katG* is significantly more common in MDR isolates (van Doorn et al, 2006; Valvatne et al, 2009). Among the isolates sequenced in our study, only four of twenty three isolates had a mutation in *katG*, at codon Gly213Val, Pro232Lys, Lys254Asn, and Ser259Asn. None of the discordant isolates had the *katG*315 mutation which is strongly correlated with MDR-TB strains (Bolotin et al, 2009).

Eleven (47.8%) of twenty-three phenotypic isoniazid-resistant isolates sequenced had mutations in the *inhA* promoter region. There were eighteen different codon mutations and interestingly, 7 isolates had combinations of nucleotide changes at various codons. Mutations

at codon -22 and -23 strangely gave rise to ‘stop’ codons and nucleotide mutation at -12, -21, -35, and -48 did not translate into interpretable amino acid changes. None of the discordant isolates in this study had the common C-15T mutation associated with the *inhA* promoter region. Sequencing was only able to detect mutations in 56.2% of phenotypically resistant isoniazid discordant isolates with corresponding resistant MIC values. Our failure to identify mutations in the *katG* and *inhA* promoter regions sequenced in these discordant isolates confirms that additional mechanisms are responsible for INH resistance. Our study has shown that certain regulatory defects that affect *inhA* promoter region does exist with the interesting ‘stop’ codon transcription and the un-interpretable amino-acid changes, but further investigations are required to determine their role in INH resistance.

The distribution of *M. tuberculosis* genotypes in some areas of SA has been previously described (Chihota et al, 2012; Stavrum et al, 2009); however, apart from this study, there are, to our knowledge no studies which have mapped the distribution of RIF and/or INH phenotypic and genotypic discordant *M. tuberculosis* genotypes across SA.

In this study, 41 RIF and/or INH phenotypic and genotypic discordant isolates were genotyped by spoligotyping. Eighty-three percent of the spoligopatterns were reported in SpolDB4 (Brudey et al, 2006) and were assigned seven different lineages. Previous studies looking at the different genotypes in South Africa have reported the Beijing/W-like and the T-lineage to be the predominant lineages among drug-resistant isolates in the Western Cape (Stavrum et al, 2009; Streicher et al, 2003). Our findings show that the four predominant families found in South Africa amongst discordant isolates are the LAM, T, X and, S which

is in agreement with findings reported by Chihota et al. (Chihota et al, 2012). In this study, the most common lineage was the LAM lineage which made up 21.9% of the discordant isolates. The LAM lineage can be split into sublineages LAM1 to LAM11 with the sublineage LAM3 being the most frequently associated with phenotypic RIF resistant isolates (23%) in this study. Isolates from the Beijing lineage have been the cause of large outbreaks of TB worldwide and have shown an association with MDR-TB (Toungossova et al, 2002; Stavrum et al, 2009). The Beijing lineage, in our study, accounted for 17.0% of the total number of isolates tested and was the third most prevalent genotype after the LAM and X lineages. In our study, five out of seven genotypically INH susceptible isolates according to the Hain LPA and phenotypically resistant are of the X3 lineage which is in concordance with the findings reported from Western Cape which had suggested that the X3 lineage may represent dissemination of a dominant isoniazid-monoresistant clone with a unique isoniazid resistance determinant. (Evans et al, 2009). Furthermore, in the INH concordant group (genotypically and phenotypically INH resistant) the majority of the isolates were from the X3 and LAM lineages while among the discordant groups all lineages were present. This study shows that phenotypic and genotypic RIF and/or INH discordant isolates are not linked to a single strain lineage. The numerous spoligotype patterns observed in this study suggests there is a high diversity of circulating *M. tuberculosis* strains in South Africa (Chihota et al, 2012) with no one single genotype dominating the TB epidemic in this area.

4.2 Limitations

One of the challenges with this study was obtaining a good yield of pure cultures for the phenotypic DST, as the culture collection dated from 2007-2008 period. Even though isolates

were cultured on Lowenstein Jensen (L-J) solid media following NaOH-NALC decontamination as well as plating out contaminated isolates for single pure colonies on L-J plates, we were unable to obtain pure colonies from the contaminated isolates. Failure to grow and persistent contamination in isolates resulted in their exclusion, therefore decreasing the overall number of discordant isolates available for the study.

Another problem was the small number of discordant cultures available for the study which is not adequately representative of the overall discordance in the main national study. This was due to some laboratories not having storage space and discarded cultures from the culture collection and there was some loss of viability of the cultures when obtained from the various provinces.

Gene sequencing was only performed on the genes represented on the LPA (i.e. *rpoB, katG* and *inhA*). In this study, resistance resulting from mutations in other genes known to be associated with drug resistance were not investigated. The sequencing of the genes was limited because of budget availability. Meaningful analysis of the genotyping is limited by the small sample numbers in this study.

Chapter 5

5 Conclusion

While several studies have been published on the frequency of LPA mutations in different regions (Barnard et al, 2008; Green et al, 2008), this is the first study from South Africa that describes the character and distribution of mutations in genes conferring rifampicin and isoniazid resistance specifically in strains with discordant phenotypic and genotypic drug susceptibility patterns.

The results from this study illustrate that the geographical distribution of mutations resulting in drug resistance in *M.tuberculosis* in SA is different from that reported from other parts of the world. Although there is a high level of agreement between genotypic and phenotypic data reported, this study highlights the need for ongoing investigations in SA of the resistance mechanisms involving anti-tuberculosis drugs. In this study, three new previously unreported mutations in the *rpoB* gene region have been identified and their association with the Hain LPA described, further research into these mutations is required for a better understanding and their role in drug resistance. The limited detection of mutations in the *katG* and *inhA* genes and the failure to detect commonly associated and widely reported mutations in these gene regions in this study indicate that other known gene regions associated with INH resistance should be investigated to better understand the mechanisms responsible for the phenotypic resistance expressed with corresponding MICs. The large number of different spoligotype patterns observed in this study indicates that there is a high diversity of circulating *M. tuberculosis* strains in SA.

Detailed knowledge of the frequency distribution of resistance-associated mutations and associated MICs in different regions of SA could facilitate understanding of the limitations of current molecular tests and inform testing algorithms. In addition, this information, together with identification of novel resistance mechanisms, could facilitate the development of customised testing going forward. Better understanding of the MICs associated with molecular changes could assist with decisions on inclusion of drugs with pharmacokinetics falling within the therapeutic range, into treatment regimens. Lastly, the relationships between molecular resistance, strain fitness and virulence remain to be investigated.

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