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Genotypic and phenotypic heterogeneity of Mycobacterium tuberculosis recovered from patients with pulmonary disease involving drug-resistant tuberculosis

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GENOTYPIC AND PHENOTYPIC HETEROGENEITY OF MYCOBACTERIUM TUBERCULOSIS RECOVERED FROM PATIENTS WITH PULMONARY DISEASE INVOLVING DRUG-RESISTANT TUBERCULOSIS

Amanda Axcell

Degree of Master of Science in Medicine by Research Only

Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine by Research.

Johannesburg, 2012

DECLARATION

I, Amanda Axcell, declare that this dissertation is my own work. It is being submitted for the degree Master of Science in Medicine (by research) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

_____

Amanda Axcell

21 May 2012

BSc, BSc (Hons)

DEDICATION

To my parents, Barry and Estelle and my loving fiancé, Steven

I love you.

AWARDS

PHRI-AURUM-Global Infectious Diseases Research Training Program

A three month pre-doctoral continuous fellowship based at the Public Health Research Institute in the United States of America was awarded to Amanda Axcell in 2010. This fellowship was taken up from the 14th of February 2011 to the 20 May 2011.

ABSTRACT

Genetic heterogeneity of *Mycobacterium tuberculosis* demonstrating mixed infections or affecting single strains has been previously described. A single sputum culture from five patients with drug-resistant tuberculosis treated at Sizwe Hospital was analysed in-depth for genotypic and phenotypic heterogeneity. IS*6110*-based restriction fragment length polymorphism (RFLP) was performed on 20 colonies from each sputum for detection of mixed infections and clonal heterogeneity. No mixed infections were found, but IS*6110*-RFLP-linked clonal heterogeneity was observed in one patient. Drug susceptibility testing (DST) and sequencing of nine drug-resistance-associated genes performed on a total of 99 colonies from the five patients failed to show genotypic hetero-resistance. On DST, however, discordant rifampicin resistance findings were encountered in one patient. Minimal inhibitory concentrations performed on these colonies were close to the rifampicin critical concentration used for resistance determination, suggesting failure of the BACTEC MGIT 960 assay to reliably determine rifampicin susceptibility in strains with borderline resistance.

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LIST OF ABBREVIATIONS AND ACRONYMS

µg	Micogram
µl	Microlitre
AFB	Acid-fast bacilli
AIDS	Acquired Immune Deficiency Syndrome
AMK	Amikacin
ART	Antiretroviral therapy
BCG	Bacillus Calmette-Guérin
BL	Biosafety Laboratory
BSC	Biological Safety Cabinet
BTBRL	Braamfontein TB Referral Laboratory
CBTRL	DST/NRF Centre of Excellence for Biomedical TB Research
CDC	Centers for Disease Control
CDW	Corporate Data Warehouse
CFU	Colony Forming Unit
CAP	Capreomycin

CTAB	Hexadecyltrimethyl Ammonium Bromide
CTB	Centre for Tuberculosis
DMP	Diagnostic Media Products
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOTS	Directly Observed Therapy Short-Course
DST	Drug Susceptibility Testing
EDTA	Ethylenediaminetraacetic acid
EMB	Ethambutol
ETH	Ethionamide
FQ	Fluoroquinolone
HCL	Hydrochloric acid
HCW	Health Care Worker
HIV	Human Immunodeficiency Virus
IGRA	Inteferon Gamma Release Assay
INH	Isoniazid
IRIS	Immune reconstitution inflammatory syndrome

IS	Insertion sequence
KAN	Kanamycin
KZN	Kwa-Zulu Natal
LJ	Lowenstein Jensen
LSP	Large Sequence Polymorphism
MAC	*Mycobacterium avium* complex
MDR	Multi-drug resistant
mg	Milligram
MIC	Minimal Inhibitory Concentration
MIRU	Mycobacterial Interspersed Repetitive Unit
MGIT	Mycobacterial Growth Indicator Tube
ml	Millilitre
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NaCl	Sodium chloride
NALC	N-acetyl-L-cysteine

NaOH	Sodium hydroxide
NHLS	National Health Laboratory Service
nsSNP	Non Synonymous Single Nucleotide Polymorphism
NTBRL	National Tuberculosis Reference Laboratory
OADC	Oleic acid, Albumin, Dextrose, Catalase
OFX	Ofloxacin
PANTA	Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin
PAPR	Powered Air Purifying Respirator
PBP2	Penicillin Binding Protein
PBS	Phosphate buffer solution
PCR	Polymerase Chain Reaction
PGG	Principle Genetic Group
PGRS	Polymorphic GC Rich Repetitive Sequence Analysis
PHRI	Public Health Research Institute
PMN	Polymorphonuclear
PPD	Purified Protein Derivative
PPE	Personal Protective Equipment

Primer-F	Forward Primer
Primer-R	Reverse Primer
PZA	Pyrazinamide
REP-PCR	Repetitive Extragenic Palindrome-Based Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
RPM	Revolutions per minute
RSA	Republic of South Africa
SDS	Sodium dodecyl sulphate
SNP	Single Nucleotide Polymorphism
SOP	Standard Operating Procedure
sSNP	Synonymous Single Nucleotide Polymorphism
TB	Tuberculosis
TBE	Tris, boric acid, EDTA
TE	Tris-EDTA buffer
TNF-α	Tumour Necrosis Factor Alpha
Tris	Alpha, alpha, alpha-Tris-(hydroxymethyl)-methylamin
TST	Tuberculin Skin Test

UK	United Kingdom
USA	United States of America
VNTR	Variable Number of Tandem Repeats
WHO	World Health Organization
XDR	Extremely drug resistant

CHAPTER 1

1 INTRODUCTION

Now listen Julie baby,

It ain't natural for you to cry in the midnight,

Ain't natural for you to cry way into midnight through,

Until the wee small hours long 'fore the break of dawn,

Oh Lord.

Now Julie, an 'there ain't nothin' on my mind

More further away than what you're lookin' for

I see the way they jump at me, Lord, from behind the door

And look into my eyes

Your little star struck innuendos,

Inadequacies an' foreign bodies.

And the sunlight shining through the crack in the window pane,

Numbs my brain

And the sunlight shining through the crack in the window pane,

Numbs my brain, Oh Lord.

So open up the window and let me breathe,

I said, open up the window and let me breathe

I'm looking down the street below, Lord, I cried for you,

I cried, I cried for you, Oh Lord.

The cool room, Lord is a fool's room,

The cool room, Lord is a fool's room,

And I can almost smell your T.B. sheets

And I can almost smell your T.B. sheets

On your sick bed.

I gotta go, I gotta go

And you said, "Please stay. I wanna, I wanna

I wanna a drink of water, I wanna drink of water

Go into the kitchen and get me a drink of water"

I said "I gotta go, baby"

I said " I'll send somebody around later,

You know we got John comin' around here later

With a bottle of wine for you, baby, but I gotta go."

The cool room, Lord is a fool's room

The cool room, Lord, Lord is a fools room, a fools room

And I can almost smell your T.B. sheets

I can almost smell your T.B. sheets, T.B.

I gotta go.

I'll send ' round, send around one that grumbles later on, babe

See what I can pick up for ya, you know that

Yeah, I got a few things gotta do,

Don't worry about it, don't worry about it, don't worry,

Go, go, go, I've gotta go, gotta go, gotta, gotta go

Gotta go, gotta go, all right, all right,

I turned on the radio

If you wanna hear a few tunes, I'll turn on the radio for you

There you go, there you go, there you go baby, there you go.

You'll be alright too, yeah

I know it ain't funny, funny at all baby

To land in a cool room man

To land in the cool room, in the cool room

"T.B. Sheets"

Van Morrison, 1967

1.1 TUBERCULOSIS IN PERSPECTIVE: HISTORY, ART AND NATURE.

Tuberculosis (TB) has plagued humanity throughout history and refuses to relinquish its grip on mankind. TB has been given many intriguing names in the past, ranging from Consumption to King's Evil as well as Lupus vulgaris (skin TB), Phthisis, The White Plague and Captain of all Men of Death. TB has long been recognised as a scourge of humankind affecting all ages and walks of life and has been poignantly depicted in contemporary art. Examples abound, including painting, (The sick child, 1886 – Edvard Munch), music (T.B. sheets by Van Morrison, 1967 and TB blues by Jimmie Rodgers, circa 1931), opera (La Boheme by Puccini), theatre and film (Moulin Rouge! – Baz Luhrman), while many artists have been known to be afflicted by TB themselves: John Keats, the Bronte sisters, Robert Louis Stevenson (literature) and Frederic Chopin (music).

TB infection in humans has been documented as far back as to the Ancient Egyptian civilization living around 4000 B.C. Skeletal abnormalities, including characteristic Pott's deformities (TB infection of the spine) are seen in Egyptian mummies and are depicted in early Egyptian art (Cave, 1939, Daniel, 2006). In addition, amplified DNA from the tissues of mummified human remains has left no doubt that the cause of these skeletal deformities was infection with the MTB pathogen (Daniel, 2006). Palaeontologists have also used molecular tools to demonstrate that DNA extracts from bone and soft tissue samples from the ancient Egyptians bear a *Mycobacterium africanum*-specific spoligotyping signature (Zinc et al., 2003), a finding which is in keeping with current concepts on the evolution of *Mycobacterium tuberculosis* (MTB) as a human pathogen (Comas and Gagneux, 2011, de Jong et al., 2009, Hershberg et al., 2008).

Before the discovery of its infectious nature, TB was viewed as a disease of poverty, malnutrition and low social standing. In 1882 at a Berlin Physiological Society meeting, Robert Koch announced his breakthrough discovery that TB was in fact caused by the infectious agent now known as MTB (Koch, 1882). After the discovery of its bacterial cause, new public health measures to combat the spread of TB were implemented into various societies.

TB is a highly contagious bacterial disease and is primarily a pulmonary infection which ranges from mild infiltration to chronic, cavitary, and severely destructive disease (van Crevel et al., 2002). It can affect other organs and tissues of the body as well, such as the meninges, lymph nodes, and tissues of the spine (Jagirdar et al., 1996). The disease in humans is caused by members of the *Mycobacterium tuberculosis* complex (MTBC). TB is a curable disease, yet 2 billion people, equivalent to almost one third of the world's population, are infected with tubercle bacilli. Most of these cases are asymptomatic and said to have latent infection; however, ~ 10% of these individuals will go on to develop active disease. Individuals known to be immune-compromised are at increased risk of developing active disease at a rate of seven to 10% per year, compared to "immune-competent" individuals who have a 10% life time risk of developing active disease (Selwyn et al., 1989, Selwyn et al., 1992). In 2009 there were approximately 1.7 million deaths due to TB worldwide – this is comparable to ~ 4700 deaths a day (WHO, 2010a). In 1993, the World Health Organization (WHO) declared TB a "global emergency", the first disease to ever be designated so. The WHO has put into action various control programs such as the Stop TB Strategy and the Directly Observed Treatment Short Course (DOTS) Expansion in attempts to put a halt and reverse the global progression of TB (WHO, 2002).

1.2 AETIOLOGY OF TUBERCULOSIS

Bacteria in the genus *Mycobacterium* consist of aerobic, non-motile, non-sporulating acid-alcohol fast rods that are assigned to the suprageneric group of actinomycetes (Palomino et al., 2007). These bacteria have an unusually high content of guanine and cytosine in their genomic DNA as well as high lipid content in their cell walls (about 60% by weight). Most mycobacteria exist naturally in the environment and replicate freely in their natural ecosystems. The majority of these environmental bacteria rarely, if ever, cause disease. There are however, three important non-environmental mycobacterial species that are highly pathogenic to humans, viz MTB, *Mycobacterium leprae* and *Mycobacterium ulcerans*. Other mycobacteria are opportunistic and only cause disease in humans when the immune system of the host is compromised. A classic example is *Mycobacterium avium* of the *M. avium* complex (MAC) which is often associated with pulmonary or much less commonly with focal disease involving intestines or lymph nodes in HIV-positive patients, and disseminated disease with bacteraemia in AIDS patients with very low CD4 counts.

MTB is a member of MTBC which comprises eight closely related Mycobacterium species and subspecies. These are MTB, *Mycobacterium bovis, M. bovis* BCG, *M. bovis subsp caprae, Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium pinnipedii*, and *Mycobacterium canettii*. Although these bacteria exhibit distinct phenotypic characteristics and differ in their mammalian host ranges, they represent one of the most extreme examples of genetic homogeneity (Aranaz et al., 1999, Brosch et al., 2002, Cousins et al., 2003, Frothingham and Wilson, 1994). It is also highly likely that members of the MTBC evolved from a common ancestor known as *Mycobacterium prototuberculosis* (Brosch et al., 2002, Gutierrez et al., 2005, Sreevatsan et al., 1997).

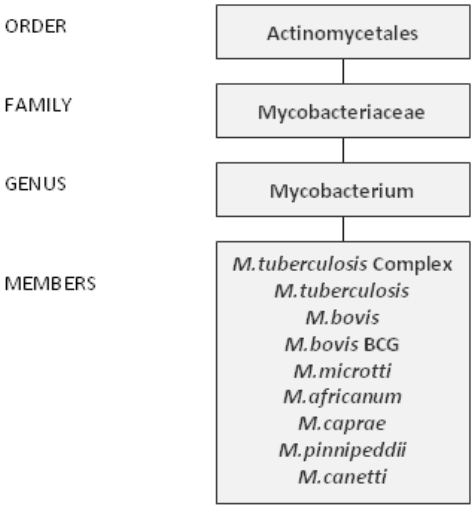

Figure 1.1 Taxonomic tree for mycobacteria culminating in species of *Mycobacterium tuberculosis* complex

1.3 PATHOGENESIS OF TUBERCULOSIS

1.3.1 Initiation of infection

TB is spread by the production of aerosols from individuals with pulmonary cavitary or laryngeal disease through high-velocity exhalation actions such as coughing or sneezing. The aerosols produced undergo rapid dehydration to become droplet nuclei (1-5µm in diameter) which contain MTB particles that can remain suspended in the air where they may be inhaled by nearby individuals (Frieden et al., 2003, Riley et al., 1959, Wells, 1934). After inhalation into the lungs, these particles are subsequently taken up by resident dendritic cells and monocyte-derived alveolar macrophages (van Crevel et al., 2002). The bacilli replicate

intracellularly and spread via the lymphatic system to the hilar lymph nodes (Frieden et al., 2003).

1.3.2 Immune-mediated pathology

The intense mycobacterium-macrophage interaction leads to a vigorous host cellular immune response involving cytokines and chemokines (Henderson et al., 1997, Manca et al., 1999, Raupach and Kaufmann, 2001, Roach et al., 2002). TNF-α is a dominant cytokine in TB pathogenesis and plays a key role in granuloma formation (Kindler et al., 1989) and macrophage activation and possesses immunoregulatory properties (Orme and Cooper, 1999, Raupach and Kaufmann, 2001). The production of TNF-α and other inflammatory cytokines (e.g. IL-1β, IL-2, IL-6, IL-12, IL-15, IL-17, IL-18, IFN-γ [pro-inflammatory Th1 profile], anti-inflammatory cytokines (IL-4, IL-10, TGF-β [Th2 profile]) and chemokines (e.g. IL-8) from the infected macrophage drives the recruitment of neutrophils, natural killer T cells, CD4 cells and CD8 cells to the infected area (Orme et al., 1993, Street and Mosmann, 1991). These cells in turn produce their own set of cytokines and chemokines which further amplifies the cellular recruitment and remodelling of the infection site (Russell, 2007). As a result of this response, infection is contained at the primary site of invasion i.e. the lung parenchyma and draining lymph nodes (Ghon complex), in 90% of infected individuals (Bloom and Murray, 1992, Ghon, 1923). The progression of infection is determined at the site of infection itself through the interaction between pro-inflammatory and anti-inflammatory cytokine-mediated responses to MTB. The host responds to infection through the remodelling of the infection site into a cellular mass known as a granuloma or ‘tubercle’ which consists mainly of infected

macrophages and activated T-cells. In the majority of MTB-infected persons the immune responses will contain the infection at the granuloma sites and will remain asymptomatic resulting in a state of latent TB. However, in about 10% of MTB-infected individuals over time, when their immune status becomes compromised, bacterial replication will accelerate in granulomas at latency sites (doubling time 25 to 32 hours) and result in active disease with clinical symptoms and immune-modulated pathology, including tissue necrosis and caseation (Medlar, 1955).

1.3.3. Pathogenesis of latency and reactivation

Latency is defined as evidence of MTB infection as a result of a positive immunological test, such as the tuberculin skin test (TST) or IFN-γ release assays (IGRA) in the absence of clinical signs and symptoms of disease, including a normal chest radiograph. In approximately 10% of cases, reactivation to active, infectious disease can occur within a few months to many decades following initial infection and sub-clinical persistence, and will often occur due to impaired immunity (for example, HIV infection). An interesting molecular-based study conducted in Denmark indicated the occurrence of extremely long periods of latency in untreated humans. In this study, IS*6110* fingerprinting revealed that a MTB strain isolated from a Danish man with TB in 1990 matched a fingerprint from a strain in the national strain collection dating back to 1958. This strain was isolated from the patient's father (Lillebaek et al., 2002).

It was initially thought that MTB infection existed only as a bimodal entity comprising either latent or active disease and latent infection occurred in a stable environment (due to continual immunological control of bacterial replication) at the level of the granuloma (Barry et al., 2009). More recently it has been proposed that latent TB infection be viewed as a continuous spectrum, extending from sterilizing immunity to active infection and clinical disease (Barry et al., 2009). It was recently pointed out that serial IGRA testing is able to identify individuals showing stable IGRA conversion, as well as those with unstable conversion, and that the latter group may have a low likelihood of progression to active disease but no single test was available to identify such phenotypes (Pai, 2010). However, a recent study using microarray and flow cytometry technology has suggested that in the near future biomarkers based on blood transcriptional profiling which included a distinct 393-transcript signature in patients with active TB may be able to identify individuals with latent TB that are most likely to progress to active TB and in the process facilitate targeted preventative therapy (Berry et al., 2010).

1.3.4 Virulence determinants of MTB

Various studies have recently indicated that infection with different strains of MTB can result in different host immune responses, i.e. variable degrees of pathogenicity and virulence have been noted in animal models (Dormans et al., 2004, Lopez et al., 2003, Manabe et al., 2003, Manca et al., 1999, Manca et al., 2001, Valway et al., 1998). Unlike other bacterial organisms, such as *Corynebacterium diphtheriae*, *Clostridium tetani*, *Escherchia coli* O157:H7 and *Vibrio cholerae* which produce toxins to cause disease, virulence in MTB is described in terms of mortality, morbidity as well as bacterial load or burden (Smith, 2003). Studies conducted in

the 1960's on guinea pigs showed that MTB strains isolated from patients with TB in India were less virulent than MTB strains isolated from TB patients in the United Kingdom (UK) (Collins and Smith, 1969, Mitchison et al., 1961, Mitchison et al., 1960, Singh, 1964).

Virulence of MTB can be measured in animal as well as macrophage models (Keane et al., 2000, Smith, 2003). In the latter model MTB's ability to inhibit phagosome-lysozome fusion and escape macrophage-mediated mechanisms of killing has been studied extensively (Gordon et al., 1980, Goren et al., 1976, Schluger and Rom, 1998). Once phagocytosed, MTB can be killed through several mechanisms and complicated interactions mediated by cytokines between lymphocyes and macrophages (van Crevel et al., 2002). In addition, various strategies have been developed to construct mutations in MTB genes thus allowing researchers to recognize genes that play a role in MTB pathogenicity and virulence (Parish et al., 1999). An excellent review of MTB pathogenesis and molecular determinants of virulence is available (Smith, 2003).

1.4 EPIDEMIOLOGY

1.4.1 The global burden of tuberculosis

Effective anti-TB drugs have been used for more than 50 years and globally BCG (Bacillus Calmette-Guérin) is one of the most widely used vaccines, yet TB remains a global health problem and the leading cause of death amongst individuals infected with HIV (Dye et al., 1999). The WHO estimates that one third of the world's population is infected with TB. In 2010, there was an estimated 8.8 million incident cases worldwide, equivalent to 128 cases

per 100 000 population (WHO, 2011) . In 2009, the total number of deaths due to TB worldwide was estimated at 1.7 million (WHO, 2010b). Over the past century, the proportion of TB cases in first-world nations has decreased substantially with resource-poor nations accounting for 90% of the global TB burden. Most of the estimated cases were recorded in Asia (59%) and Africa (26%), and South Africa was amongst the top five countries with the largest number of incident cases in 2010 (0.40 million-0.59 million) (WHO, 2011). Although case numbers of TB have fallen in countries from North and South America, Western and Central Europe and the Middle East, there has been a significant increase in the number of TB cases in sub-Saharan Africa and countries of the former Soviet Union (WHO, 2006). With regards to sub-Saharan Africa, the increase in the number of TB cases is strongly associated with the high prevalence of HIV infection, whereas the increase of TB in the countries of the former Soviet Union is associated with the socio-economic decline and poor healthcare infrastructure. A high incidence of TB infection has a negative impact on the socio-economic development of a nation. In addition to the direct costs involved in the treatment of TB (such as treatment and medication expenses), the indirect costs have an even bigger impact on the economy of developing nations. The indirect costs include aspects such as work absenteeism, loss of employment, travel expenses to care facilities, as well as loss of productivity due to illness and premature death.

1.4.2 HIV/TB Co-infection

Africa is the epicentre for the world's AIDS pandemic, with South Africa (RSA) having one of the highest incidences of HIV infection in the world. In addition to increasing the risk of reactivating latent MTB infection, HIV infection also poses the threat of rapid TB progression

soon after an individual has been infected or re-infected with MTB (Daley et al., 1992, Shafer et al., 1995). In addition, MTB enhances HIV viral replication (Daley et al., 1992, Shafer et al., 1996). It was found that TB-infected cases of HIV-positive patients were responsible for an estimated 7% of all TB transmissions and suggested that the spread of HIV across sub-Saharan Africa is the driving force behind the increasing number of TB cases each year (Corbett et al., 2003).

Generally, TB is difficult to diagnose in patients that are HIV positive. Screening for, and making a laboratory diagnosis of TB are normally achieved using smear microscopy, (fluorochrome or Ziehl-Neelsen method) but HIV-positive individuals tend to be smear negative due to a modified pathogenesis of the disease. In HIV negative patients, the T-cell-mediated responses leads to granuloma production, tissue necrosis and cavity formation. Some of these lesions communicate with the bronchial tree and contain numerous TB bacilli which are coughed-up in sputum. This process often fails in immune compromised HIV-positive patients, in whom extensive diffuse lesions in the absence of necrosis occur, which do not communicate with bronchi. With the introduction of antiretroviral therapy (ART) for HIV positive individuals, the risk of progression from latent TB to active TB is significantly reduced (Badri et al., 2002).

When both active TB and HIV infection with a low CD4 count (i.e. severe immune deficiency or advanced AIDS disease) is present, the initiation of combined ART and anti-TB treatment may result in the development of immune reconstitution inflammatory syndrome (IRIS) which manifests with new, worsening, or recurrent symptoms, signs and/or radiological

manifestations of TB. It has been recommended to first treat TB disease in such patients before commencing ART (Abdool Karim et al., 2011).

Although ART is available in RSA, many HIV positive individuals may not have easy access to these facilities. In addition, many individuals may not even be aware that they are infected with HIV. The problems relating to HIV/AIDS and TB in RSA are mainly public health associated and include poverty, social inequities, lack of infrastructure, lack of education and difficulty in accessing basic public health care facilities (particularly for individuals living in rural settlements which accounts for the majority of the population in RSA). These factors are hampering conventional internationally-recommended efforts to control these devastating diseases.

1.4.3 Transmission of tuberculosis.

Globally, there is a large reservoir of latent TB cases. When these latent cases activate into active cases, the latter become infectious, especially adults with cavitary disease. The mechanism whereby TB is spread is via aerosol formation by forced expiration such as coughing, sneezing, shouting etc. (Wells, 1934). The aerosolized droplets that are released into the environment can remain suspended in the air for prolonged periods. These MTB bearing particles which have little inertial mass are subsequently inhaled by exposed contacts. When inhaled, the larger droplets are entrapped and removed by the pulmonary muco-ciliary defence system of individuals while smaller droplets having floated in the air and dried out to become droplet nuclei, pass through the bronchial tree including small bronchioles and alveolar ducts to be lodged in alveoli where infection is initiated. Since infection is readily

spread via aerosolisation, overcrowded conditions such as homeless shelters and crowded living conditions (shacks and shanty towns) promote the transmission of MTB. In high incidence settings such as RSA, incident cases comprise a combination of both recent transmission events associated with short latency, as well as reactivation of a historic infection. Recent infection is often associated with outbreaks and requires public health intervention. For this reason alone, the importance of the need for TB surveillance and monitoring in RSA is highlighted.

1.4.4 Molecular epidemiology of tuberculosis

Molecular epidemiology is a relatively new concept in biology and is an amalgamation of various scientific fields including molecular biology, clinical medicine, statistics and epidemiology (Mathema et al., 2006). The aim of molecular epidemiology is to study the role of genetic and/or environmental risk factors at the molecular/cellular or biochemical level in disease aetiology and apply this knowledge to the general population and in the process guide the development of future TB control programs and strategies for healthcare improvement worldwide (Mathema et al., 2006). The various DNA fingerprinting techniques, along with IS*6110*-based RFLP (Kremer et al., 1999), applied in current molecular epidemiology research are described in detail in Chapter 2.

1.4.5 Prevention and control

There are four general strategies for the prevention and control of TB (CDC, 1988). These comprise 1) early identification and treatment of individuals with infectious TB (active case

finding), 2) identification and treatment of persons who are non-infectious, 3) creation of a safe environment in settings where the risk of TB transmission is high and 4) vaccination with BCG. Early identification and treatment of infectious individuals will most often render the patient non-infectious (clearance of bacilli from tissues and sputum) and ultimately result in clinical cure. Examples of patients that are non-infectious include: patients with extra-pulmonary disease, primary pulmonary disease in children, bacteriologically unconfirmed cases of pulmonary disease and infection with MTB not causing disease (latency i.e. a positive TST or IGRA assay such as the Quantiferon Gold test [Cellestis Limited, Carnegie, Victoria, Australia] with a normal chest x-ray and no symptoms of disease).

1.4.5.1 General public health measures

Case finding followed by early treatment is one of the most important measures taken to stop the spread of TB in the community (Palomino et al., 2007). In low prevalence settings such as the USA, high importance is placed on contact tracing since the greatest risk of infection is being in close contact with a TB patient. In high prevalence settings this is not the case. Case finding and contact tracing are very difficult tasks to perform in high burden countries such as RSA. Other general public health measures to combat the spread of TB in the community would involve drastic socio-economic changes such as trying to alleviate poverty and overcrowding as well as to promote good nutrition and respiratory hygiene. Knowledge regarding the signs and symptoms of TB may result in infected individuals seeking treatment in the early phases of their disease thus reducing the number of contacts that they may infect. Too often TB is misdiagnosed and goes untreated. The sooner infected individuals receive

treatment, the less likely they would be to spread the disease to other individuals in the community.

1.4.5.2 Infection control and prevention in health care facilities

An important aspect in the control and prevention of TB is to create a safe environment in situations where the risk of transmitting infection is high. In this regard, health care facilities are definitely top of the list. Within the healthcare sector, hazardous areas include autopsy suites, sputum induction cubicles, chest clinic/TB hospital waiting areas and mycobacteriology laboratories to name a few. It is important that the engineers designing these buildings have a special knowledge on the characteristics of TB transmission and take into account the design of these buildings to promote natural ventilation (i.e. open windows). Rooms housing infectious patients, as well as potentially infectious patients and specimens, should be handled under negative pressure. In the laboratory, universal precautions should be implemented when handling specimens and/or cultures. All specimens and cultures should be handled and processed within a class II biological safety cabinet (BSC) and health care personnel should be trained and wear personal protective equipment (PPE) and an appropriate respirator to filter out particles that are 1-5 μm in diameter (e.g. N95 respirator). The current protection for health care workers (HCWs) in the USA is adequate surveillance which includes periodic TST and Quantiferon Gold testing. Isoniazid (INH) preventative therapy is recommended for individuals who convert from TST-negative to – positive.

In RSA there has also been the establishment of specialised hospitals for the management of multidrug-resistant (MDR) and extremely drug-resistant (XDR) patients. Patients are hospitalised for as long as their routine laboratory tests yield a positive culture to prevent the transmission of drug-resistant strains in the community.

1.4.5.3 Preventive treatment

The aim of preventive treatment is to eradicate latent infection with MTB before reactivation disease develops. Many studies on preventative treatment were done in the 50's and 60's on presumably HIV negative individuals showing benefit (Ferebee, 1969, Ferebee and Palmer, 1956). A recent study demonstrated that treatment of latent TB infection (especially in individuals with a positive TST) reduces the risk of active TB in HIV positive patients (Akolo et al., 2010). Industrialised nations tend to favour the use of preventative therapy when appropriate over mass BCG vaccination of new-born infants, as is practiced in RSA. Treatment of latent infection is regarded as an important strategy for TB control in regions with low TB prevalence, as it preserves the health of the infected individual by reducing the possibility of the disease reactivating. It also protects the greater population by reducing the pool of latently infected persons with the potential to reactivate from the latent state. INH preventative therapy should be avoided in patients with active TB because of the danger of selecting resistant mutants during mono-therapy. Active TB should therefore be excluded before administering preventative therapy. This is especially important in countries where TB is common. Because of the high prevalence of latent TB in RSA, treatment of all individuals with latent infection would be unrealistic and is therefore not practiced.

1.4.5.4 Immunoprophylaxis with BCG

In addition to the urgent need for new anti-TB drugs and diagnostic methods, better vaccines against TB are also critically needed. Due to the existence of a large reservoir of latently infected individuals worldwide, prevention of TB infection and disease through vaccination may be the only realistic way of controlling the global incidence of TB (Comas and Gagneux, 2011). TB was one of the first diseases for which a vaccine was developed. Albert Calmette and Camille Guérin of the Pasteur Institute attenuated a mycobacterium related to MTB (*Mycobacterium bovis* bacillus Calmette-Guérin, aka BCG) by growing it serially on culture medium for 13 years and monitoring the decrease in its virulence throughout this period (Calmette A, 1929). Randomised and case control trials have shown BCG to be effective and provide high protection against the most detrimental forms of TB (including TB meningitis and military TB disease) in children under the age of five years (Colditz et al., 1994). BCG has demonstrated variable efficacy in different trials (Comstock and Palmer, 1966, Fine, 1995, Hart and Sutherland, 1977). New vaccines are desperately needed to win the battle against TB. An excellent review article regarding vaccines for TB is available (Doherty and Andersen, 2005).

1.5 CLINICAL PRESENTATION

The most common clinical manifestation of TB is pulmonary disease. Extra-pulmonary disease accounts for about 20% of disease in HIV negative individuals but is more common amongst individuals co-infected with HIV (Frieden et al., 2003). With regards to pulmonary TB, patients with a persistent cough (i.e. a productive cough lasting for more than two weeks)

should be assessed for TB. Other common symptoms for TB include fever, night sweats, weight loss (wasting), shortness of breath, appetite suppression, pallor, fatigue, haemoptysis and chest pain. On physical examination of the chest, there are minimal adventitious sounds in the lungs until the disease is quite advanced.

1.6 LABORATORY DIAGNOSIS OF TUBERCULOSIS

Laboratories serve major roles in the diagnosis and management of TB. Microscopy, culture-based methods and new 'cutting edge' molecular techniques that are currently used in the diagnosis and management of TB are discussed below.

1.6.1 Microscopy and culture-based methods

Until quite recently, diagnosis of TB was made almost exclusively based on smear microscopy and MTB culture results. Smear microscopy has low sensitivity in HIV positive patients due to disseminated disease and lower bacillary load in sputum samples obtained from these patients. Sputum smear microscopy, however, is often the only laboratory test that is available for TB testing in many parts of the world, particularly in resource-poor countries that do not have access to other forms of laboratory testing. The main advantage of smear microscopy is that it is inexpensive, simple to perform, has fast turnaround time and can be performed in the most basic laboratory setups. In resource-poor countries where there is a high incidence of TB, smear microscopy was and in many countries still is used for case detection in the national TB control programs, but with the high burden of HIV/AIDS a large proportion of such cases will

be missed. Also, smear microscopy gives no indication of the drug susceptibility status of the MTB strain. Ideally, if sputum samples are smear positive, they need to be cultured either on solid or liquid media to confirm the diagnosis which can take up to several weeks. In addition, samples need to be cultured if drug susceptibility testing (DST) is to be performed. Phenotypic drug resistance is normally determined by the proportion method, whereby an MTB isolate is considered to be resistant if more than 1% of the colonies grow on culture media containing a critical concentration of that drug. The 'gold standard' DST method that is currently used in TB laboratories relies on the detection of more than 1% growth of the inoculum on a culture medium containing the critical concentration of a particular anti-TB drug (Canetti et al., 1969). DST relies on a culture-based method that is slow to yield results due to the slow growth rate of MTB. The fact that culture takes up to several weeks to provide results often delays the initiation of appropriate treatment, which in turn can lead to the ongoing transmission of drug-resistant strains and the prescription of inappropriate treatments which contributes to poorer treatment outcome and amplification of resistance.

1.6.2 Molecular diagnosis of tuberculosis

The lengthy time required to diagnose TB has exacerbated the need for the development of new molecular diagnostic assays to reduce the time to diagnosis and in addition to include the identification of mutations in genes that ultimately result in phenotypic resistance. In 2008, the WHO approved the Hain MTBDR*plus*® line probe assay (Hain Lifescience, Nehren, Germany) for the rapid identification of MTBC strains as well as their resistance to rifampicin (RIF) and INH through the detection of mutations in the *rpoB* gene and the *inhA* promoter and

katG genes respectively. The Hain MTBDR*plus*® assay has been rolled out to numerous laboratories in RSA for a more rapid diagnosis of TB and its resistance to RIF and INH. Furthermore, the Hain Genotype MTBDR*sl*® test has recently been developed for the recognition of high frequency mutations occurring in the *gyrA* gene for ofloxacin (OFX) resistance, *rrs* gene for aminoglycoside resistance and the *embB* gene for resistance to ethambutol (EMB). Evaluation of the performance of this test suggested that the sensitivity for detecting fluoroquinolone (FQ) resistance was between 75.6% and 90.6% and the sensitivity for kanamycin (KAN) was between 77% and 100% (Hillemann et al., 2009, Kiet et al., 2010). The Hain line probe assay has been shown to reduce the time to diagnosis significantly and a result can be obtained from smear-positive samples within eight hours (Tukvadze et al., 2012). The first generation MTBDR*plus*® assay however, is only used on smear-positive samples.

The new GeneXpert (Cepheid, Sunnyvale, CA) was developed in 2004 and is currently being rolled out to the various provinces in RSA. This Xpert MTB/RIF assay is a PCR-based test that integrates DNA extraction, genomic amplification as well as the semi-quantitative detection of MTBC and its resistance to RIF. The test is designed to be performed in a single commercial cartridge. The use of the cartridge to carry out the entire test procedure greatly reduces the risk of cross contamination. In addition, the Xpert MTB/RIF assay was designed as a point of care test that is easy to perform and can yield results within two hours. Another advantage that the Xpert MTB/RIF assay has over the Hain MTBDR*plus*® assay is that it has been validated for smear-negative respiratory samples. The effectiveness of the Xpert has been evaluated for the direct detection of MTBC and RIF resistance in smear-negative clinical respiratory and non-respiratory samples (Moure et al., 2011). The performance of the Xpert

MTB/RIF assay has also been compared against other molecular diagnostic tests including the MTBDR*plus*®, Lightcycler Mycobacterium Detection (LCTB) (Roche) as well as with AFB smear microscopy and liquid culture on a single sputum specimen (Scott et al., 2011). A significant percentage of smear-negative cases are responsible for the ongoing transmission of the MTB bacillus, making the Xpert MTB/RIF assay an attractive test to advance TB diagnostics particularly in the HIV negative population due to its increased sensitivity and ability to detect MTB in more than 70% of smear-negative culture-positive pulmonary cases (Boehme et al., 2011). The GeneXpert seems to offer promising advances in TB diagnosis in that it is fast, reliable, highly sensitive and easy to use. The cost of running the GeneXpert is still very expensive and this factor needs to be taken into account when considering the implementation of this assay. It is suggested that its clinical and epidemiological advantages should be weighed against the resources available in each setting.

1.7 TREATMENT

1.7.1 Treatment of drug-susceptible tuberculosis

The current standard treatment regimen for drug-susceptible TB relies on a six-month, four-drug combination therapy. This treatment regimen consists of two months of treatment with INH, RIF, pyrazinamide (PZA) and EMB (initial phase/early bactericidal phase), followed by four months of treatment with INH and RIF (continuation phase/sterilisation phase).

There are a few biological features that account for the reasoning behind the treatment of TB with a combination of drugs. One important factor is the fact that treatment with a single

antibiotic may inevitably result in the selection of drug-resistant populations ultimately leading to treatment failure and, even more concerning, the transmission of drug-resistant strains within the population. Secondly, distinct populations of both drug-susceptible and drug-resistant bacilli may co-exist in the lung (Kaplan et al., 2003, Post et al., 2004, Shamputa et al., 2006). Furthermore the bacterial populations present will also possess different metabolic activities in that some bacteria will be actively dividing whereas others will remain "persistent" and/or dormant within the lung. Bacilli that are actively dividing are more responsive to anti-TB drugs, most of which were selected for their abilities to kill actively dividing organisms rather than dormant persisters. Treatment of TB has two goals: the first is to rapidly target the actively dividing bacteria that exist within the extra cellular lung cavities to achieve a negative microscopy smear and negative sputum culture thus reducing transmissibility. This is achieved during the initial phase of treatment. The second goal is to achieve complete sterilization of the infection, including elimination of the bacteria that are less metabolically active residing in acidic environments such as the phago-lysozomes of macrophages where the pH is almost certainly low (Mitchison, 1979). This is mainly achieved through the continuation phase of treatment.

The DOTS strategy is an important strategy for TB control and was developed in the mid 1900's. DOTS is an internationally recommended approach to TB control and relies on the supervision of patient treatment. DOTS has now been expanded worldwide.

1.7.2 Treatment of drug-resistant tuberculosis

The current approach for MDR-TB treatment in RSA (and other resource poor countries) is a standardised approach (DOTS-plus) which is in line with WHO recommendations, as opposed to individualised treatment in first-world countries. This standardised treatment regimen consists of treatment with at least five drugs for six months (intensive phase), followed by treatment with at least three drugs for 18 months (continuation phase). The basis for the standardised treatment strategy is thus based on resistance data obtained from DST surveillance either through the corporate data warehouse (CDW) or lab-based surveys. XDR-TB treatment on the other hand, relies on an individual approach whereby treatment is based on the patients' treatment history as well as DST results.

1.8 DRUG RESISTANCE: NATURE AND DETECTION

There are two mechanisms by which an individual may become infected with a drug-resistant strain of MTB, namely primary drug resistance and acquired/secondary drug resistance. Primary drug resistance refers to a drug-resistant strain that has been isolated from a patient who has not received any previous anti-TB treatment. Secondary/acquired drug resistance occurs when a patient is infected with a drug-susceptible strain that becomes drug-resistant during the course of treatment. In these cases inadequate treatment selects for the emergence of drug-resistant mutants. MTB isolates containing drug-resistant mutations were observed as soon as the first anti-TB drugs were developed and used to treat TB infection (Youmans et al., 1946). The development of drug-resistant TB generally occurs as a result of inappropriate treatment (e.g. choice of drugs, dosage and/or duration of treatment), program factors

(irregular drug supply) and/or patient factors (such as poor adherence or mal-absorption), whereby drug-resistant mutants emerge from point mutations that occur spontaneously in MTB organisms under the selective pressure of treatment. Once a high prevalence of drug-resistant TB is around there is a great risk of transmission of drug-resistant strains (i.e. primary drug resistance). Table 1.1 refers the various first- and second-line drugs involved the treatment of TB and refers to their targets, mechanisms of action and genes associated with resistance.

Table 1.1 Mechanisms of action of anti-TB drugs and the target genes associated with drug resistance *

Antibiotic	Mechanism of Action	Primary Target	Genes associated with resistance
Isoniazid (INH)	Inhibits mycolic acid synthesis	*inhA*, secondary targets include *katG*, *kasA* and *dfrA*	*katG* (required for drug activation) *inhA* (promotor mutations).
Rifampicin (RIF)	Inhibits transcription initiation	RNA polymerase β-subunit	*rpoB*
Ethambutol (EMB)	Inhibits arabinogalactan synthesis	Possibly *embB*	*embB*
Pyrazinamide (PZA)	Unknown	Unknown (possibly inhibits FAS-1 or alters membrane energetics)	*pncA* (required for drug activation)
Streptomycin (STREP)	Inhibits Protein Synthesis	30S ribosomal subunit	*rpsL* and *rrs*
Fluoroquinolones (FQs)	Inhibits DNA gyrase	Topoisomerase II (DNA gyrase), topoisomerase IV	*gyrA* and *gyrB*
Ethionamide (ETH)	Inhibits mycolic acid synthesis	*inhA*	*ethA* (required for drug activation) and *inhA* (promotor mutations)
Capreomycin (CAP)	Inhibits protein synthesis	Methylated nucleotides in both ribosomal subunits	*tlyA* and *rrs*
Kanamycin (KAN) Amikacin (AMK)	Inhibits protein synthesis		*rrs*

* Modified from Sacchettini et al., 2008

1.8.1 Acquired drug resistance

In 1943, Streptomycin (STREP) was discovered as an effective anti-TB agent and brought about much hope and enthusiasm to society that a cure for this disease was finally imminent. However, this optimism was short lived when the development of drug resistance was observed due to treatment with STREP mono-therapy. The observation that treatment with a single antibiotic resulted in drug resistance led research investigators to recommend treatment of TB with a combination of different drugs. The British Medical Journal published a paper in 1950 recommending the use of the two drugs, para-aminosalicyclic acid (PAS) and STREP in combination to treat patients with pulmonary disease (MRC, 1950). It was noticed in their study that a treatment regimen based on both STREP and PAS significantly prevented the development of STREP resistance in MTB isolates tested from patients receiving both drugs compared to patients that only received STREP.

Mutants that harbour resistance-conferring mutations are selected for and accumulate under selective (antibiotic) pressure. Unlike most bacteria, drug resistance conferring mutations in MTB do not take place by horizontal transfer of resistance containing genetic elements. Drug resistance mutations in MTB transpire as a result of various point mutations (i.e. nucleotide insertions, deletions or substitutions) that occur in the genes that are associated with drug resistance (Ramaswamy and Musser, 1998). When combination therapy is properly managed, drug supplies are adequate and patients adhere to their prescribed treatment, TB control is effective (Kochi et al., 1993). However, since TB treatment is a lengthy process and involves the use of multiple anti-TB drugs that have toxic side effects, this often leads to patient non-compliance and hence the acquisition of drug resistant mutants. Therefore, in addition to the

development of new drugs in the treatment of TB, research that focuses on the shortening of the duration of TB treatment will also play a vital role in clinical management and preventing the emergence of drug resistant TB.

1.8.2 Drug resistance and fitness

A common concept with regards to drug resistance suggests that drug resistance is associated with a fitness cost: that is, although drug resistance mutations may provide MTB strains with an advantage in the presence of the drug, in the absence of the drug these mutations are coupled with a fitness burden that may be detrimental to the strains survival (Bjorkman et al., 2000, Böttger and Springer, 2008, Böttger and Zurich, 2006). Fitness in the microbiological sense generally refers to the organism's ability to survive, replicate and be transmitted. Certain studies have demonstrated that compensatory mutations may occur to compensate for the fitness burden that is associated with certain drug-resistance mutations (Böttger and Springer, 2008, Casali et al., 2012). In addition, these compensatory mutations may facilitate the survival following transmission of drug-resistant strains in the population. For example, high-level INH resistance occurs as a result of deletion of *katG*. This results in complete loss of catalase-peroxidase activity which is associated with a significant fitness cost (Cohn et al., 1954). Recent studies have indicated that a complete loss of *katG* activity in clinical isolates is associated with a secondary mutation of alkyl-hydroperoxidase, *ahpC* (Sherman et al., 1996). The hypothesis concerning this phenomenon was that the loss of catalase peroxidase activity would ultimately result in the acquisition of compensatory mutation in *ahpC* which would enable more effective spread of the drug resistant strain in the population (Böttger and Springer, 2008). It was later noted that the *katG* S315T mutation that occurs in approximately

60% of clinical strains with INH resistance confers a medium level of drug resistance and does not appear to be associated with a fitness cost (Pym et al., 2002, van Sooligen et al., 1999, Van Sooligen et al., 2000).

1.9 EMERGENCE OF MULTIDRUG-RESISTANT AND EXTREMELY DRUG-RESISTANT TUBERCULOSIS IN SOUTH AFRICA

One of the greatest concerns with regards to TB at present is an increase in the number of MDR and XDR strains worldwide. RSA has been identified as one of the highest burden countries for drug-resistant TB. Globally, there were an estimated 440 000 cases of MDR-TB in 2008 and RSA was amongst the top four countries that had the largest number of MDR-TB cases (13 000; range 10 000 to 16 0000) (WHO, 2010b). MDR-TB by definition implies resistance to both INH and RIF, the two most effective drugs used in the first-line treatment of TB. Previously, XDR-TB was defined as resistance to three of the six classes of second line drugs and was first identified in the late 1980's. XDR-TB was first reported in RSA in 2006 and was re-defined as the occurrence of TB in persons whose MTB isolates are resistant to INH and RIF, as well as to any FQ and at least one of three second-line injectable drugs (i.e. AMK, KAN and CAP) (Dye and Williams, 2010, Ghandi et al., 2006). MDR- and XDR-TB have become a massive public health problem worldwide, but are of particular concern in countries such as RSA because of the high prevalence of drug-resistant strains and also due to other various socio-economic factors such as HIV/Aids and poverty. In addition, drug-resistant TB is very difficult to treat because it involves the loss of the two most potent first-line anti-TB drugs (INH and RIF) and the inclusion of second-line drugs to the treatment regimen. Second-line drugs are more expensive, less effective and more toxic than first-line

drugs and successful treatment outcome of drug-resistant TB is significantly lower than that of drug-susceptible TB (Goble et al., 1993, Post et al., 2004).

1.9.1 Tugela Ferry Outbreak of extremely drug-resistant tuberculosis

On the 1st of September 2006, the WHO announced that a new deadly strain of XDR-TB had been discovered in Tugela Ferry, a rural settlement in Kwa-Zulu Natal (KZN), RSA. In 2005, a study was conducted to identify TB strains from a rural district hospital in KZN. Of the 544 patients enrolled in the study, 221 had MDR-TB, and of these 53 had XDR-TB (Gandhi et al., 2006). Forty four patients out of the 53 were tested for HIV infection and all were HIV positive. It is believed that patients were infected by means of nosocomial transmission (i.e. primary drug resistance). The outbreak of this XDR-strain was of great concern because the median time period from diagnosis to death was 16 days and 52 out of the 53 patients died.

Pillay and Sturm, (2007) noted that the majority of the patients involved in the Tugela Ferry XDR outbreak were infected with the same strain of MTB, namely the F15/LAM4/KZN strain. The investigator's searched their databases for isolates from studies performed between 1994 and 2005 that involved the resistance patterns of isolates of MTB with the F15/LAM4/KZN strain fingerprint. These isolates were used to study the F15/LAM4/KZNs strain development from MDR- to XDR-TB. It was noticed that drug resistance had developed in a local MTB strain to as many as seven drugs in just over a decade (Pillay and Sturm, 2007). It was suggested that the implementation of the DOTS-plus strategy in RSA in 2001 (which did not include routine DST of second-line drugs) may be partly responsible for the development of the resistance in the F15/LAM4/KZN strain due to a proportion of patients

receiving regimens containing too few active TB drugs. This event was a localized outbreak that occurred in the KZN region of RSA and many aspects of the epidemiology of MDR- and XDR-TB in RSA remains unknown.

A recent study conducted in RSA used a molecular approach to gain insight into the genotypic population structure of XDR-TB strains to provide insight as to whether XDR strains are acquired or transmitted within the community (Mlambo et al., 2008). It was concluded that the majority of the XDR-TB cases genotyped by spoligotyping (63%), developed XDR-TB through acquisition. The investigators also noted that a significant proportion of MDR-TB cases (17%) investigated displayed resistance to a single marker for XDR-TB, (either OFX or KAN). Since the development of drug resistance in MTB occurs via the accumulation of mutations in the genome, this pool of pre-XDR strains are very likely to develop into XDR strains if not managed appropriately. It was also observed that there were a higher number of patients with OFX resistance than KAN resistance. This is possibly due to the fact that the use of FQs is widespread to treat other ailments such as urinary tract infections or diarrhoea thus leading to the development of resistance to these drugs.

CHAPTER 2

2 CURRENT MOLECULAR METHODS USED FOR GENOTYPIC CHARACTERISATION OF MYCOBACTERIUM TUBERCULOSIS

Molecular technology has been employed for the past few decades to study the diversity and/or relatedness of various MTB isolates. Examples of these studies include the identification of transmission chains and outbreaks (Alland et al., 1994, Barnes et al., 1997, Braden et al., 1997, Glynn et al., 1999, Small et al., 1994, Weis et al., 2002, Yaganehdoost et al., 1999), classification of MTB strains into families and lineages (Alland et al., 2003, Baker et al., 2004, Filliol et al., 2003, Gutacker et al., 2002, Mostowy et al., 2002, Sola et al., 2001), identifying episodes of exogenous re-infection and/or relapse (Bandera et al., 2001, Caminero et al., 2001, van Rie et al., 1999) and more recently to identify the presence of heterogeneity involving drug-resistance mutations in patients' sputum isolates (Kaplan et al., 2003, Post et al., 2004, Shamputa et al., 2004), as well as the occurrence of mixed infections and bacterial subpopulations in individual patients (Cohen et al., 2011, Shamputa et al., 2006, Shamputa et al., 2004, van Rie et al., 2005, Warren et al., 2003).

Various DNA fingerprinting techniques have been employed the past few decades. These include mostly IS*6110*-based restriction fragment length polymorphism (IS*6110*-based RFLP) analysis, spacer oligonucleotide genotyping (spoligotyping), mycobacterial interspersed repetitive-unit-variable-number of tandem repeats (MIRU-VNTR), polymorphic GC rich repetitive sequence analysis (PGRS) and single nucleotide polymorphism (SNP) analysis. The

current "gold standard" for the typing of MTB isolates is IS*6110*-based RFLP and for this reason was the genotyping method of choice used in this investigation. IS*6110*-based RFLP, spoligotyping and MIRU-VNTR are the most popular genotyping tools currently used and are discussed below. PGRS and SNP analysis are also discussed briefly.

2.1 IS*6110*-BASED RESTRICTION FRAGMENT LENGTH POLYMORPHISM.

IS*6110*-based RFLP is the current standard approach that is used to genotype MTB isolates. RFLP analysis looks at the distribution and variability in chromosomal positions of the insertion sequence IS*6110* in different MTB strains. IS*6110* element sites are distributed more or less randomly throughout the genome and copy numbers vary between 0 to 26 copies per strain (Kurepina et al., 1998). Generally speaking, MTB strains that are epidemiologically linked will have identical IS*6110* RFLP banding patterns, whereas strains that are not epidemiologically linked will have different RFLP banding patterns i.e. patients with MTB strains with identical RFLP patterns are assumed to have had direct contact with each other or contact through a common source (Barnes and Cave, 2003). In addition, identical RFLP patterns are indicative of recent transmission which may signify an outbreak. Strains with unique RFLP patterns, however, are thought to signify activation of a latent infection (endogenous reactivation). MTB strains with fewer than six IS*6110* insertion sites ("low-copy-number" strains, clusters I, IIA, IV and V) have a limited degree of polymorphism and other genotyping methods are recommended in these circumstances (Bauer et al., 1999).

Insertion elements are small (0.7 to 2kb) mobile elements that are distributed throughout the bacterial chromosome and contain only genes that are involved in their transposition and

regulation (Blot, 1994). IS*6110* is an insertion element that is unique to MTBC members and was first described by Thierry et al., (Thierry et al., 1990). IS*6110* has an imperfect 28 base pair inverted repeat at its ends and it generates a 3- to 4-bp duplication on insertion. Although certain "hot-spots" have been identified for IS*6110* insertion, the distribution of IS*6110* throughout the chromosome appears to be more or less random (McHugh and Gillespie, 1998). In 1993 van Embden et al., published an article recommending a standardised method for the strain identification of MTB using IS*6110* as a tool for DNA fingerprinting. Briefly, this method consists of first extracting mycobacterial DNA from a purified bacterial culture. Thereafter, the MTB genomic DNA is digested with the restriction enzyme *Pvu*II (*Pvu*II cleaves IS*6110* at a single asymmetric site yielding reasonable size MTB fragments). The restriction fragments are then subjected to electrophoresis on a 1% agarose gel, vacuum blotted onto a nylon membrane and hybridised with a probe that is specific to the right side of IS*6110*. The labelled fragments are visualised using an enhanced chemiluminescence reaction initiated by the addition of two substrates. The membrane is wrapped in a plastic film and placed alongside a sheet of light sensitive film in a cassette that blocks out all light. Once the film is developed, RFLP banding patterns can be visualised and interpreted (van Embden et al., 1993). Molecular weight standards are included in the test to facilitate in accurate computer assisted analysis of each IS*6110* banding pattern.

IS*6110*-based RFLP analysis has various advantages and limitations when compared to other molecular typing techniques. In addition to being the "gold standard" genotyping method employed for MTBC strains, other advantages of this method include the fact that it is utilised in many laboratories throughout the world. This provides an opportunity to compare data within and between various laboratories and research facilities. The patterns can also be

computerised using specialised software. In addition IS*6110* has been proven to be a stable biological marker and thus adequate for the study of transmission. Cave et al., (1994) looked at the stability of IS*6110* by examining 41 MTB isolates obtained from 18 patients over a range of eight months to 4.5 years. Patients that were included in the study had a history of non-compliance to therapy, relapse of disease and acquisition of drug resistance. The authors noted that the IS*6110* fingerprint patterns for a given patient remained identical throughout the study period mentioned above. It was also noted that the acquisition of drug resistance did not alter the fingerprint patterns obtained from patient isolates (Cave et al., 1994). Warren et al., (2002) also investigated the stability of IS*6110* banding patterns from isolates obtained from patients residing in high incidence settings. The conclusion drawn by the authors was that IS*6110* possesses a half-life of 8.74 years when a constant rate of change is assumed. They also reported that a high rate of change is seen during the early phases of disease when replication is at its most active (t1/2 = 0.57 years) and the lower rate of change is seen in the late disease phase due to longer bacterial replication times (t1/2=10.69 years) when patients are on treatment (Warren et al., 2002). IS*6110* RFLP also provides a widespread diversity in patterns of MTB isolates that contain more than six bands. In addition the membranes can be re-hybridised (for example, with left sided IS*6110* probe). Mixed infections can also be readily detected utilising this method. For example, low-intensity bands (LIBs) present on IS*6110* RFLP patterns may represent mixed bacterial populations with slightly different RFLP patterns (de Boer et al., 2000). An important disadvantage of IS*6110*-based RFLP analysis is the slow turnaround time (30 to 40 days) due to the fact that a viable culture is required to generate an accurate genotype. IS*6110* is also technically more complicated than other fingerprinting methods such as spoligotyping. It also cannot be used to reliably type and discriminate between strains with less than six IS*6110* copies e.g. low-copy-number isolates

belonging to clusters I, IIA, IV and V of an international collection of 5069 clinical isolates from four population-based studies (Gutacker et al., 2006). Strains that have less than six IS*6110* bands that display identical banding patterns using IS*6110* have been shown to have unique fingerprinting patterns when using a secondary type of genotyping methods, indicating that the strains are genetically different (Bauer et al., 1999, Rhee et al., 2000).

2.2 SPOLIGOTYPING

Spacer oligonucleotide genotyping or "spoligotyping" is the most commonly used PCR-based method for genotyping and differentiating between MTBC strains (Kamerbeek et al., 1997). MTBC strains contain a chromosomal region that has 10 to 50 copies of a 36bp direct repeat (DR) sequence which is punctuated by spacer DNA sequences. These spacer sequences that occur in-between any two DRs are conserved among certain strains (van Embden et al., 1993). The composition of the DR locus in MTBC strains which contains a variable number of both direct repeats as well as the presence or absence of various spacer sequences, makes spoligotyping a suitable method for genotyping MTB strains. Spoligotyping is based on the visualization of 43 interspersed spacer sequences existing in the genomic DR locus of MTBC strains (Kamerbeek et al., 1997). The presence of these 43 spacers is detected by amplifying the entire DR genomic region of the unknown MTBC strain, resulting in a mixture of a large number of different size fragments. This is achieved by the use of two inversely oriented primers complementary to the sequences of the DRs thus amplifying the DNA sequences located in-between two adjacent DR sequences. The reverse primer is labelled with biotin so that all the reverse strands synthesised are biotin labelled. Commercial membranes spotted with the 43 synthetic oligonucleotides are hybridised with biotin-labelled amplicons and the

hybridised products are ultimately visualised using streptavidin peroxidase and enhanced chemiluminescence.

Advantages that spoligotyping has over IS*6110*-based RFLP include: the benefit that only small amounts of DNA are required for spoligotyping, and that the results of this typing method can be expressed as positive or negative for each spacer, in which case the results can be conveyed in a digital format. Spoligotyping in conjunction with other typing methods has proven to be a powerful tool in defining lineages of MTB prevalent in different geographical regions (Filliol et al., 2003) and the evolution of MTB over time (Flores et al., 2007). Although spoligotyping is a simple, robust and powerful genotyping method, it has been shown to be less discriminatory than IS*6110*-based RFLP analysis, thus limiting its application in molecular epidemiology.

2.3 MIRU-VNTR TYPING

The MTB genome contains numerous interspersed repeat units (MIRUs) which constitute a variable number of tandem repeat sequences (VNTR) that are scattered throughout the MTB genome. Of the 41 MIRUs previously identified, 12 MIRU loci were selected for the genotyping of clinical MTB isolates (Mazars et al., 2001, Supply et al., 2000). Subsequently a 15-locus set was proposed for epidemiological purposes and a 24-locus set for phylogenetic study purposes (Supply et al., 2006). MIRU-VNTR relies on the use of PCR and gel electrophoresis to categorise the number and sizes of the repeats in each of the 12 (or more) independent loci, each of which have a unique repeated sequence (Barnes and Cave, 2003). The results obtained from the assay can be captured in a twelve digit format giving MIRU a

big advantage over other genotyping methods in that the results can be catalogued onto a computer database thus allowing for intra- and inter-laboratory comparisons of strain types. The discriminatory power of MIRU-VNTR depends on the number of loci evaluated. Generally speaking, if 12 loci are used, MIRU is less discriminatory than IS*6110* genotyping for isolates with high-copy-numbers of IS*6110* insertions, but is more discriminatory than IS*6110* genotyping for isolates with low-copy-number of IS*6110* insertions (Mathema et al., 2006). MIRU used in conjunction with spoligotyping has also been shown to be as discriminatory as IS*6110* RFLP (Mathema et al., 2006). The advantages of MIRU-VNTR are that it is technically simpler to perform than IS*6110* and that it can be performed directly on the cell lysate (i.e. no DNA purification is required). MIRU analysis can be automated and therefore be used as a high throughput technique for MTB genotyping. MIRU-VNTR may replace IS*6110* RFLP in the future, particularly if more loci are added thus increasing the discriminatory power of this method.

2.4 POLYMORPHIC GC REPETITIVE GENOTYPING

PGRS is a Southern blot hybridisation technique that can be used to genotype MTB strains. This method was first described by Ross et al., in 1992 and uses a PGRS-specific probe cloned in plasmid pTBN12 (Ross et al., 1992). PGRS has been used to further discriminate strains that display identical IS*6110* RFLP patterns (Chaves et al., 1996), particularly strains with IS*6110* low-copy-numbers (Rhee et al., 2000, Yang et al., 1996). The main disadvantages of PGRS are that 1) it is resource intensive and 2) raw data is often too complex to be entered and standardised on a computer database.

2.5 SINGLE-NUCLEOTIDE POLYMORPHISMS

Both synonymous single-nucleotide polymorphisms (sSNP) and nonsynonymous single-nucleotide polymorphisms (nsSNP) are able to provide genetic information that can be used to differentiate different MTB strains. nsSNPs result in an amino acid change and are therefore subject to selective pressures such as antibiotics and can thus provide clues to the nature and spread of drug resistance strains within populations. sSNPs on the other hand are almost functionally neutral and do alter the encoding amino acid. sSNPs can therefore be used to study genetic drift and evolutionary relationships amongst different MTB strains (Mathema et al., 2006). For example, two sSNPs (codon 463 [Leu463Arg] of the catalase peroxidase encoding gene, *katG*, and codon 95 [Thr95Ser] of the A subunit of DNA gyrase gene, *gyrA*), were used to divide modern MTB strains into three principle genetic groups (PGGs) – PGG1, PGG2 and PGG3 (Sreevatsan et al., 1997). These three PGGs were further divided into nine clusters (I to VIII and IIA) using SNP analysis (Gutacker et al., 2006, Gutacker et al., 2002). These two studies demonstrate the application of SNP analysis in studying the phylogenetic relatedness of MTB strains.

CHAPTER 3

3 GENETIC HETEROGENEITY IN MYCOBACTERIUM TUBERCULOSIS: CLONAL HETEROGENEITY AND MIXED INFECTIONS

Genetic heterogeneity of MTB arises within a patient following infection with a single strain undergoing genetic change through mutation of genes, or through mixed infection with more than one strain following re-infection during the initial infection episode. The term hetero-resistance refers to the occurrence of a mixed population of both drug-resistant and drug-susceptible organisms that occur in a single clinical sample or isolate (Rinder, 2001). The presence of hetero-resistance in the infecting MTB strain, or hetero-resistance due to mixed infection, may compromise the treatment and clinical outcome of the disease. Conflicting DST results have not infrequently been observed in the TB laboratory and this may cause considerable confusion for clinicians when trying to monitor patient progress and consider therapeutic options for patients with such discordant laboratory reports (van Deun et al., 2009).

3.1. HETERO-RESISTANCE IN ORGANISMS

The occurrence of hetero-resistance has been reported in other micro-organisms including *Staphylococcus aureus* (McDougal and Thornsberry, 1984, Ryffel et al., 1994, Stranden et al., 1996), *Gardnerella vaginalis* (Altrichter and Heizmann, 1994), *Helicobacter pylori* (Weel et

al., 1999*), Pseudomonas aeruginosa* (Sheehan et al., 1982) and *Cryptococcus neoformans* (Mondon et al., 1999) to name a few.

3.1.1 Hetero-resistance in *Staphylococcus aureus*

Ryffel et al., (1994) demonstrated that methicillin-resistant *Staphylococcus aureus* (MRSA) display variable levels of resistance to methicillin, from borderline resistance to high basal resistance, depending on the genetic background of the strains. Methicillin resistance in *S. aureus* is dependent on *mecA*, the structural gene for low affinity penicillin binding protein (PBP2). Expression of this protein has been shown to lead to hetero-resistant populations of cells displaying variable levels of resistance to methicillin as well as other β-lactam antibiotics. Methicillin-resistant strains that demonstrate a basal resistance to low concentrations of the β-lactams produce a small proportion of sub-clones or mutants that are capable at growing at high concentrations of these antibiotics (Ryffel et al., 1994). The genetic basis responsible for this high level of resistance to the β-lactams has not been fully identified, possibly because other chromosomal mutations in the genome that are not related to *mecA*, are involved.

3.1.2 Hetero-resistance in *Helicobacter pylori*

The presence of mixed infections (Hirschl et al., 1994) as well as the occurrence of hetero-resistance with regards to metronidazole has been observed in *Helicobacter pylori* (Arents et al., 2001, Dore et al., 1998, Weel et al., 1999). *H. pylori* is a gram negative, microaerophilic organism that exists normally in the human gastric mucosa (Lee, 1994). *H. pylori* infection is

responsible for conditions such as gastritis and gastric ulcers and has also been linked to duodenal ulcers and stomach cancer. Dore et al., (1998) conducted an experiment to investigate whether or not specific genotypes may be associated with different patterns of susceptibility to metronidazole. The investigators used repetitive extragenic palindrome-based polymerase chain reaction (REP-PCR) to genotype isolates taken from 12 patients with duodenal ulcers residing in different regions of the USA. In addition, they performed antibiotic susceptibility testing with regards to amoxicillin, clarithromycin and metronidazole using multiple colony expansion and single colony expansion techniques (eight to ten colonies taken from primary cultures of each biopsy specimen). It was noted that 92% of isolates were indistinguishable by their REP-PCR fingerprint patterns; however in contrast a high degree of phenotypic heterogeneity to metronidazole was observed among the isolated single colonies. Argents et al., (2001) also demonstrated in their work that the occurrence of metronidazole-resistant and –susceptible bacteria from the same biopsy specimen does not necessarily imply the co-existence of a mixed strain infection with one resistant and one sensitive strain.

3.2 MIXED INFECTIONS AND CLONAL HETEROGENEITY IN MTB

Until recently TB was generally believed to occur as a consequence of infection with a single MTB strain and infection was thought to confer protection against additional infection with a second MTB strain (exogenous re-infection). Furthermore, it was thought that a recurrence of the disease was caused by endogenous reactivation of a dormant strain (latency) that caused the original infection, rather than exogenous re-infection (Stead, 1967).

Various studies have now demonstrated the occurrence of mixed infections (caused by clonally distinct strains of MTB) isolated from both single sputum samples (Richardson et al., 2002, Shamputa et al., 2004, Warren et al., 2004) and multiple sputum samples collected serially over time from MTB smear-positive individuals (Braden et al., 2001, Richardson et al., 2002, Shamputa et al., 2006).

Some of the first studies designed to investigate mixed infections were carried out using phage typing. More than thirty years ago such a study using phage typing to demonstrate mixed infections among colonies obtained from cultures from Eskimo patients showed that 14.1% of the patients were infected with more than one strain of MTB (Mankiewicz and Liivak, 1975). Other studies have used phage typing and IS*6110*-based RFLPs to demonstrate the occurrence of distinct MTB strains cultured from different anatomical sites in the same patients (Richardson et al., 2002). Warren et al., (2004) conducted a study that focused on the extent of multiple MTB infections in sputum specimens from patients residing in a Cape Town community with a high incidence setting of the disease. Using a novel PCR method based on comparative genomic data targeting Beijing and non-Beijing strains, the investigators demonstrated that both HIV-negative and HIV-positive individuals could be infected with more than one strain of MTB during the same disease episode or in a successive MTB episode (re-infection). 19% of all patients that were included in the study were simultaneously infected with strains belonging both to the Beijing and non-Beijing evolutionary lineages and 57% of patients infected with a Beijing strain were also infected with a non-Beijing strain. It was also noted that mixed infections were more likely to occur in re-treatment cases (23%) compared to new cases (17%), although this finding was not statistically significant. From the results obtained in this study it is apparent that mixed infections are common in high incidence

settings and the authors note that these findings may indicate a conservative estimate on the frequency of mixed infections in this particular setting (Warren et al., 2004). Van Rie et al., (2005) also concluded in their study that mixed infections are likely to occur in a setting where infection pressure is high. In this latter study the investigators looked at serially recovered sputum samples from patients residing in Cape Town with MDR-TB. IS*6110*-based RFLP and a novel strain specific PCR reaction indicated that re-infection, during or before active disease, may result in changing drug susceptibility profiles in patients with drug-resistant TB. The authors noted that there were three possible mechanisms whereby mixed infections can lead to changing drug susceptibility patterns during therapy. In the first scenario it was proposed that during the initial period of anti-TB chemotherapy the drug susceptible population would be wiped out due to treatment with first-line anti-TB drugs, thus favouring selection for the drug-resistant population. Patients initially presenting as a drug-susceptible case would now be converted into a drug-resistant case according to routine DST results. This scenario indicates selection via antibiotic pressure. The second scenario suggests that if the antibiotic pressure was removed by factors such as poor adherence or default, the drug-susceptible population could re-emerge as the dominant population suggesting selection in the absence of antibiotic pressure. Lastly, it was noted that the drug-susceptible population would also re-emerge during the introduction of second-line therapy into the treatment regimen. This is possibly due to the reduced bactericidal activity of second-line antibiotics showing selection due to reduced antibiotic pressure (van Rie et al., 2005).

Genetic diversity within the same MTB clone of an individual patient's sputum involves genetically distinct subpopulations of MTB defined as groups of organisms within a strain exhibiting genetic variation e.g. slight DNA fingerprint variations in a strain (clonal

heterogeneity) or resistant mutants co-existing with susceptible wild-type progeny of specific resistance-related genes such as *katG or rpoB* in the same MTB strain in a sputum specimen (hetero-resistance). Shamputa and co-workers, (2004) screened 10 individual MTB colonies isolated from 97 HIV-negative patients for clonal genetic heterogeneity using three fingerprinting techniques. In this study heterogeneity in bacterial subpopulations was defined as MTB strains with slight variations in their DNA fingerprinting patterns (i.e. less than three IS*6110* bands) due to evolutionary events occurring within an isolate from a patient originally infected with the same strain. Clonal genetic heterogeneity was identified in samples from eight of the 97 patients analysed (8.2%).

Hetero-resistance was identified by genetic analysis performed on MTB isolates from lesions in surgically removed lungs of patients who underwent pneumonectomy because they suffered from intractable TB or experienced complications related to their TB (Kaplan et al., 2003). The study showed that a single founder strain of MTB may undergo various genetic transitions during treatment leading to the development of drug resistance. It was proposed that under various selective pressures, these mutations take place and are selected for at discrete physical locales in the lung. These events result in a parallel evolution of heterogeneous populations of both drug-resistant and drug-susceptible bacilli in the lung. It was also observed that the acquisition of drug-resistant mutations occurred at the luminal surfaces of the cavities where there was more oxygen present (Kaplan et al., 2003). The results observed in this study are in agreement with those of a study by Post el al., (2004). In order to gain a better understanding of the evolution of MDR-TB in patients, serial isolates recovered from 13 HIV-negative patients were characterised using three different molecular methods comprising IS*6110*-based RFLPs, spoligotyping and sequencing of genes associated with drug resistance. The genes

sequenced were *rpoB, mabA-inhA* (including promotor), *katG, pncA, embB, rpsL, rrs and gyrA*. It was noted that 31% of the patients' isolates acquired additional drug resistance mutations during the study period and that heterogeneous populations of bacilli with different resistance mutations and different drug susceptibility profiles were present in the sputum samples at a single time point during the study. This led the investigators to suggest that under these circumstances, consideration be given for treatment regimens to be designed to include drugs that target both drug-susceptible and drug-resistant phenotypes (Post et al., 2004).

The evolution and development of drug resistance was studied in a strain of MTB that was isolated from a non-compliant patient over 12 years of active disease (Meacci et al., 2005). Ten sequential MTB isolates from this patient were characterised according to their phenotypic and genotypic drug resistance profiles. After 12 years of interrupted anti-TB treatment, the initial fully sensitive MTB isolate had evolved into an MDR strain. Molecular typing revealed that the single parental strain that infected the patient persisted throughout the disease. Molecular analysis of drug resistance related genes also demonstrated that discrete subpopulations of MTB with resistance-conferring mutations to INH, RIF and STREP had evolved over time.

3.3 AIM AND OBJECTIVES OF STUDY

3.3.1 Aim of study

Based on studies on genetic clonal heterogeneity mediated by mutations in genes involved in the evolution of drug resistance in MTB, and mixed infections determined by genotype analysis of colonies isolated from the same specimen/patient, as well as the reporting of occasional discordant DST results in the same MDR-TB patient at Sizwe Hospital, the first aim of the present study was to:

1. Investigate the occurrence of clonal heterogeneneity (including hetero-resistance) and mixed infections in five patients with drug-resistant pulmonary tuberculosis at Sizwe Hospital.

As it was the intention of the Centre of Tuberculosis (CTB) of the National Institute of Communicable Diseases (NICD) in collaboration with the Public Health Research Institute (PHRI [Newark, New Jersey]) and Sizwe Hospital to determine the presence of mixed infections, which may impact on patient management. The second aim of the study was to:

2. Gain experience in the techniques and safety requirements for research involving MDR- and XDR-TB. A pilot study was planned to address this important safety-related component of the research, before the main study for the MSc degree was embarked upon.

3.3.2 Specific objectives of the study

1. To determine appropriate laboratory techniques required for the detection of clonal heterogeneity and mixed infections in sputum samples by suitable dispersal of MTB organisms in sputum samples for the isolation of single colonies likely to be representative of MTB organisms in the lungs of patients with MDR-TB (Pilot study).

2. To perform under appropriate safety conditions, culture and DST on MTB colonies originating from a single sputum sample from selected MDR-TB patients for comparison with corresponding resistance genotypes.

3. To perform appropriate molecular techniques (i.e. IS*6110*-based genotyping and sequencing of drug resistance-linked genes) on MTB colonies from sputum samples obtained from five patients with drug-resistant pulmonary tuberculosis (i.e. MDR and pre-XDR patients) to determine the extent and nature of genetic heterogeneity including hetero-resistance relating to mixed clones and single strains, if present.

4. To characterise the MTB strain types present in the five patients with pulmonary drug-resistant tuberculosis using IS*6110*-based RFLP.

5. To identify the mutations responsible for drug-resistance in the five patients analysed.

6. To correlate genotypic and phenotypic findings from colonies from sputum of individual patients and investigate basis of any discordant findings that may be found.

CHAPTER 4

4 MATERIALS AND METHODS

The technical work involving the laboratory approach and methods used in the present study was conducted in two phases: a pilot study to determine optimal techniques for the isolation of single colonies to be used in the main study, and the main study itself. Ethics clearance was obtained from the Human Research Ethics Committee (medical) of the University of the Witwatersrand, Johannesburg (Clearance certificate number: M10128 [Appendix A]).

4.1 PILOT STUDY

A pilot study was conducted before the commencement of the major MSc project in order to determine optimal laboratory techniques required to produce single colonies from sputum samples obtained from patients with drug-resistant TB, as well as to gain experience in working in a Biosafety Level 3 (BL 3) laboratory with drug-resistant MTB strains. Extensive handling of MTB-infected sputum and MTB cultures was required for the main study to obtain single colonies for the detection of possible mixed infections and hetero-resistant populations of MTB in sputum samples collected from patients with MDR/XDR-TB. Safety concerns were of paramount importance. Since the main purpose of the study was to detect the presence of possible heterogeneity related to single strains and mixed infections, suitable methodology had to be investigated to obtain single colonies which ideally would be representative of the MTB populations in sputum samples.

4.1.1 Technical aspects explored in pilot study

The following aspects focussing on the optimal demonstration of heterogeneous MTB populations in sputum needed to be addressed in the pilot study:

- The type of procedure and processing of sputum specimens for producing single colonies. The following options were considered:

    a) Sediment after centrifugation in the N-acetyl-L-cysteine - Sodium Hydroxide (NALC-NaOH) concentration method on sputum samples to be used for plating on solid media to obtain single colonies

    b) Sputum digested by NALC, concentrated by centrifugation and plated for single colonies on solid media containing the antimicrobial selective mixture comprising polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA)

    c) MGIT culture of sputum samples onto solid media when the instrument (BACTEC MGIT 960 System, Becton Dickinson Diagnostic Systems, Sparks, MD) signals presence of growth

After careful consideration to maximize the likelihood of obtaining heterogeneity, a decision was made to use option a. For option b there were concerns regarding the inhibition of growth that may occur to the presence of antibiotics. For option c, expansion of the bacterial population in MGIT culture may result in loss of any transient heterogeneity due to competition effects.

- The type of solid medium/media to be used to isolate single colonies such as transparent agar plates (Middlebrook 7H11 agar or Dubos agar medium) or an opaque medium [Lowenstein Jensen (LJ) medium in plates] for colony morphology

- The optimal dispersion method for obtaining representative single colonies on solid media. This could either be obtained by:

    a) Sequential spreading from primary inoculum on surface of solid media in plates

    b) Spreading of standard volume from serial dilutions on solid media and incubation followed by colony counting

- Dispersion and breaking up of clumps/cords. Should cords of MTB bacilli be broken up by vortexing with glass beads in the presence of Tween 80 to provide single colonies or should less vigorous dispersion be used, based on the assumption that many bacilli in a single cord originate from a single or few MTB cells and that attempts to maximally break up cords would not be necessary?

- Number of colonies to be selected for characterisation and basis for selection (e.g. random or based on colony morphology)

4.1.2 Patients and specimens used in pilot study

Following informed consent, seven sputum samples from newly diagnosed MDR-TB patients at Sizwe Hospital were collected for routine processing at the Braamfontein TB Referral Laboratory (BTBRL) where conventional NALC-NaOH decontamination was performed

according to their standard operational procedure (SOP). After the final centrifugation step of the NALC-NaOH decontamination procedure, the pellet was re-suspended in ~ 2 ml phosphate buffer solution (PBS, pH 6.8) according to standard practice and 0.5 ml was inoculated into the MGIT growth tube (for diagnostic purposes). The remaining suspension was reserved for the pilot study. The sample pellets were collected from the BTBRL and taken to the CTB (previously, National Tuberculosis Reference Laboratory [NTBRL]) for processing in the pilot study.

4.1.3 Comparison of dispersion and spreading techniques on sediments.

4.1.3.1 Dispersion by vortexing with beads and Tween 80 versus vortexing alone

PBS was added to the sediment following treatment with the NALC-NaOH method at BTBRL to reach a final volume of 7.5 ml sediment suspension. 0.5 ml of the sample was inoculated into a MGIT tube and placed into the MGIT 960 system for culture and further study while the rest was used for dispersion studies. The need for vigorous dispersion in an attempt to break up MTB cords as opposed to gentle mixing was addressed comparing two dispersion methods: 1) by mixing the suspension in the presence of beads (3 mm) and a surface active agent (Tween 80 solution) and 2) by mixing the suspension in the absence of beads and Tween 80. 2 ml of the sediment (Sediment A) was used to disperse organisms vigorously by vortexing for 30 seconds following the addition of 0.05% Tween 80 in 7H9 broth solution (approximately 3 ml) and two sterile glass beads into a centrifuge tube (the total volume in the centrifuge tube was 5 ml). 2 ml of the sediment (Sediment B) was used to disperse the organisms gently. This

was achieved by adding 2 ml of the sediment to 3 ml of 7H9 broth solution and vortexing the solution for a total of 30 seconds.

4.1.3.2 Assessment of range of dilutions required for single colonies based on smear microscopy of sediments

A 20 µl drop of Suspension A was transferred to a microscope slide (smear was approximately 1.0 cm by 2.0 cm in size), fixed for an hour at 65°C and stained with auramine fluorescent staining according to the laboratory SOP. A 20 µl drop of Suspension B was also transferred to a glass slide for fluorescent staining. The smears from tube A and B were examined under high-dry objective (450X magnification). The number of fluorescing bacilli was counted per high field power for 20 fields and the mean number of acid fast bacilli (AFB) per field was determined. In order to obtain isolated colonies that were likely to be representative of a possible heterogeneous MTB population in the sputum sample, approximately 50 to 150 colony forming units (CFUs) on a culture plate was considered to be a realistic sample for the study. Dilutions based on the AFB concentrations on the smears that were likely to give this concentration were taken from the following table:

Table 4.1 Criteria for the selection of dilution sets based on smear microscopy

Dilution options for plate inoculation	Number of fluorescing bacilli observed/field
Undiluted, 10^{-1}, 10^{-2}, 10^{-3}	<25
10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}	25-250
10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}	>250

Dilution sets are based on the number of fluorescing bacilli per high power field using high-dry objective (450X magnification). This table is modified from Table 4, page 1231 from Woods et al., 2008 in the Manual of Clinical Microbiology. 9th Edition.

4.1.3.3 Dispersal procedures and plating out for single colonies

4.1.3.3.1 Plating out for single colonies by sequential streaking of inoculum

Because of its simplicity, the standard plating out by serial streaking of undiluted suspension following NALC-NaOH procedure was used to obtain isolated colonies and the findings of this procedure were compared with those of the conventional spreading of appropriate dilutions as set out in the table of specimen concentrates onto the surface of solid media in plates. 0.2 ml from each of the two dispersion tubes (Sediment A and Sediment B) was spread out in four consecutive plating out steps onto each of the three solid media. The aim was for each spreading step to sequentially dilute the organism load in the sediment to obtain evenly dispersed colonies.

4.1.3.3.2 Processing of dilutions from sediments dispersed by different vortexing procedures

For the vigorous dispersion method, ten-fold serial dilutions were made in a diluting fluid containing 7H9 liquid medium containing 0.05% Tween 80. The dilutions were made as follows: two sterile glass beads were added to 2 ml of the original concentrate suspension and vortexed for ten seconds. Dilutions were then made by serial transfer of 0.2 ml to 1.8 ml volumes of diluting fluids in tubes, each containing two glass beads. These tubes were then vortexed for ten seconds. For the gentle dispersion method, the same method was employed as for Suspension A, except 7H9 broth without the addition of Tween 80 was used as the diluting fluid after initial vortexing and no glass beads were added for the mixing steps. Appropriate dilution sets were obtained for Suspensions A and B based on the findings of the smear microscopy results (Table 4.1). 0.5 ml of sample was taken from each tube of the appropriate serial dilution set from Suspensions A and B and these were spread onto three different types of media using a sterile spreader. The three different types of media comprised Dubos agar, 7H11 Middlebrook agar and LJ agar poured into plates. Therefore there were a total of 24 plates for each patient sample using the NALC-NaOH pellet. Fluorescent microscopy indicated considerable dispersal to single cells in Suspension A compared to suspension B and this was reflected in the colony counts.

4.1.3.3.3 Incubation and plate reading

After inoculation, all plates were sealed and placed in a gas permeable bag to prevent dehydration during incubation. The plates were incubated at 37°C for a maximum period of

eight weeks. The plates were examined daily for five days then weekly thereafter for growth and contamination.

4.1.3.4 Conclusions and recommendations based on pilot study

The pilot study was useful in that it provided good experience for handling specimens and cultures containing drug-resistant MTB and afforded first-hand experience in the techniques required for research attaining to the main study. Caution was exercised in interpreting the findings of the pilot study because of the small numbers of specimens processed and the numbers of repetitive procedures required for valid conclusions. Despite this caveat, the findings of the pilot study were carefully interpreted in the light of experience of other researchers and publications in the literature and are summarised in the results section.

4.2 MIXED INFECTION AND CLONAL HETEROGENEITY STUDY

The following protocol is based on decisions and observations obtained from the pilot study and from the literature describing experience of other researchers in the field.

4.2.1 Sputum collection, processing and culture at the CTB, RSA



Figure 4.1 Photograph taken outside the Centre for Tuberculosis, Sandringham, South Africa

4.2.1.1 Patients

Sputum samples were collected from five patients diagnosed with drug-resistant TB at Sizwe hospital. Three newly admitted MDR-TB patients (Patients 3, 4 and 5) were selected for this study (one of which was diagnosed with primary MDR-TB) as well as two other patients with a history of drug resistant disease, treatment failure and/or relapse (Patient 1 and 2). One of the two patients with a history of TB had received treatment regimens based on first- and second-line anti-TB drugs. Patients were selected on the basis of their DST profiles and history of

disease in order to identify whether certain patients were more likely to contain heterogeneous/mixed populations of bacilli in their sputum (i.e. the frequency of multiple infections in re-treatment cases is expected to be much higher than that in new treatment cases) (Warren et al., 2004). Exclusion criteria for this study included: patients that were less than 18 years or greater than 65 years of age, pregnant patients, patients with a diagnosis of any other lung disease and patients with overt extra-pulmonary disease. A brief description of each patient is set out below:

- Patient 1 was placed on regimen 1 in April 2008. This was the patient's first episode of pulmonary TB and the patient was considered cured after completion of treatment. In 2009 the patient was admitted to Sizwe Hospital after being diagnosed with MDR-TB and was categorised at Sizwe Hospital as 'relapse after cure'. The patient started regimen 2 in June 2009 and the treatment outcome was recorded as 'treatment failure'. The patient was re-admitted in July 2010 after the patients sputum was culture positive for AFB after 23 days incubation. In addition, mycobacterial PCR (using the Hain line probe MTBDR*plus*® assay) on the culture showed that the sample was positive for MTB complex and the isolate was resistant to RIF but susceptible to INH. Patient 1 was on treatment when the sputum sample was collected for the present study. On the last admission, DST results indicated that patient was resistant to INH, RIF and ETH. Sputum was collected for the study on the 22nd of July 2010 and processed on the same day. At the time of sputum collection the patient was receiving OFX, EMB and terizidone.

- Patient 2 was placed on regimen 1 in January 2010. This was the patient's first episode of TB. The patient was admitted to Sizwe in May 2010 after being diagnosed with drug-resistant TB and was recorded in the category 'treatment failure'. Mycobacterial PCR results revealed that the patient was positive for MTBC and that the isolate was resistant to both RIF and INH. Prior to admission the routine DST indicated that the isolate was resistant to all seven drugs (INH, RIF, STREP, EMB, ETH, KAN and OFX). After admission, conflicting DST results were observed for KAN. Patient 2 was registered as an XDR patient in April 2010. Sputum was collected for the study on the 21 July 2010 and processed on the same day. At the time of sputum collection, the patient was receiving PAS, moxifloxacin, PZA, and INH

- Patient 3 was placed on regimen 1 in July 2010 and admitted to Sizwe in September 2010 after being diagnosed with MDR-TB. Regimen 1 was recorded at Sizwe as 'after failure of first treatment'. Patient was positive for AFB after 18 days incubation and mycobacterial PCR indicated resistance to both INH and RIF whilst DST indicated that the isolate was resistant to INH, RIF, STREP and EMB. The sputum sample was collected before the commencement of MDR treatment for this study.

- Patient 4 was placed on regimen 1 in January 2010 and patient category was recorded at Sizwe as 'after default'. Patient was admitted to Sizwe as an MDR-TB patient in September 2010. Patient was positive for AFB after nine days incubation and mycobacterial PCR results indicated that the isolate was now resistant to both RIF and INH. Routine DST results indicated that the isolate was resistant to INH, RIF, ETH, STREP and OFX. The sputum sample was collected for the study on the 9th of

September 2010 and processed on the same day. The sputum sample was collected before the commencement of MDR treatment.

- Patient 5 was enrolled in the study as a newly diagnosed MDR patient. This patient had received no previous treatment for TB. Patient category was recorded at Sizwe hospital as 'new patient'. Patient 5 was positive for AFB after 13 days incubation and mycobacterial PCR indicated that the isolate was resistant to both INH and RIF. DST indicated that the isolate was resistant to INH, RIF, STREP, EMB and ETH. The sputum sample was collected for investigation before the commencement of treatment.

  Further patient description is given in Table 6.1, Chapter 6 page 119.



Figure 4.2: Photograph taken at entrance to Sizwe Hospital, Sandringham, South Africa



Figure 4.3 Photograph taken of ward at Sizwe Hospital, Sandringham, South Africa

4.2.1.2 Sputum quality

Sputum samples were collected from Sizwe Hospital and taken immediately to the CTB for decontamination and culture onto Middlebrook 7H11 plates to obtain single colonies of MTB. Prior to processing, the volume and macroscopic appearance of the sputum samples were recorded and gram stains were performed according to the laboratory's SOP on each of the sputum samples in order to assess their quality. This was accomplished by using the Bartlett score which looks at the ratio of epithelial cells relative to the number of neutrophils present in the sample. The Bartlett score is determined by performing a gram stain in order to distinguish between organisms infecting the lower respiratory tract from potentially pathogenic bacteria colonising, but not infecting, the upper respiratory tract. The presence of polymorphonuclear (PMN) cells is used to indicate specimen collection from an area of active inflammation presumably from the infected lung or bronchus. Therefore, the best expectorated sputum samples will have many PMNs and few to no squamous epithelial cells. Such specimens are likely to include the etiologic agent (in this case MTB) of the infection in relatively high numbers and comparatively few contaminating organisms from the upper respiratory tract. The Bartlett scoring system is regarded as a not entirely reliable predictor of a good quality specimen, also because the inflammatory exudate of sputum from TB patients are likely to harbour increased numbers of lymphocytes and macrophages which are not readily identified in sputum. The criterion of regarding an excess of squamous epithelial cells in sputum as an indication of the presence of saliva may be a superior measure of sputum quality (Murray and Washington, 1975).

4.2.1.3 Sputum decontamination

Sputum samples were processed in the BL 3 laboratory and samples were decontaminated inside a class II A/B3 BSC using the NALC-NaOH digestion-decontamination method as per SOP. Briefly, an equal volume of 2% NaOH in NALC-NaOH solution was added to the sputum in a 50 ml screw-cap plastic centrifuge tube (final concentration of NaOH is 1%). The 50 ml conical tube was then mixed on a vortexer for 20 seconds and left to stand for 15 minutes with occasional shaking to promote digestion. More NALC was added to the sample if it was still mucoid. PBS was then added to the 50 ml mark on the centrifuge tube. The solution was then centrifuged in a refrigerated centrifuge for 15 minutes at 3000 x g. The supernatant was decanted into a container containing tuberculocidal disinfectant, leaving only the pellet behind.

4.2.1.4 Culture onto Middlebrook 7H11 and isolation of single MTB colonies

Following decontamination of the sputum samples, 2 ml of PBS was then added to each sample to re-suspend the pellet. In addition, two sterile glass beads were added to each sample using sterile forceps to aid in breaking up MTB cords. Each sample was then vortexed for a minimum time of 30 seconds to break up the MTB cords present in the sample as well as to homogenise the solution. In order to obtain isolated colonies that are likely to be representative of a possible heterogeneous MTB population in the sputum sample, approximately 50 – 150 CFUs on a culture plate was processed to approximate an appropriate colony sample for this study. Five Middlebrook 7H11 plates per patient sample were then placed in the 37°C incubator for 30 to 60 minutes before plating to get rid of any excess moisture that may have formed during storage of the plates in the fridge. After removing the

plates from the incubator, each plate was labelled with the patient ID number, date and dilution factor (undiluted – 10^4). A range of serial dilutions (10^1 to 10^4) were performed on each sample. Serial dilutions were made by adding 200 µl of sample to bijoux tubes containing 1.8 ml of 7H9 broth and two sterile glass beads. Each dilution tube was then vortexed for an additional ten seconds to further break up the MTB cords present in each sample. 100 µl from the neat sample as well as serial dilution was then transferred to Middlebrook 7H11 plates and a disposable loop was used to streak out the solution on the 7H11 plate in order to obtain single colonies. The five Middlebrook 7H11 plates were then sealed in a clear, gas permeable bag to prevent them from drying out, and incubated at 37°C for a maximum period of eight weeks. The cultures were examined weekly for growth as well as possible contamination. Once good growth was present, 20 colonies from each patient were selected from the 7H11 plate with the best dilution factor (i.e. dilution plate with 50 to 150 colonies present). Twenty colonies from the selected plates were picked off at random (colonies of different sizes and morphologies were selected) and inoculated into 1.5 ml tube containing 500 µl of 7H9 broth. 100 µl of this broth was subsequently inoculated onto an LJ slant, while the remaining 7H9 broth was placed in a -70°C freezer and stored for back up. Colonies picked from two of the five patients were inoculated onto segmented Middlebrook 7H11 plates as opposed to LJ slants as the 20 colonies picked from the original plate did not grow well on the LJ slants. The slants/ plates were examined weekly for growth. Once growth was sufficient, cells were scraped off the LJ slants using a disposable loop and inoculated into two Nalgene screw cap cryo-vials containing 0.5 ml of 7H9 broth. One set of isolates was kept at -70°C at the CTB and the other set of isolates was shipped to the PHRI in Newark, New Jersey, USA for genetic analysis.

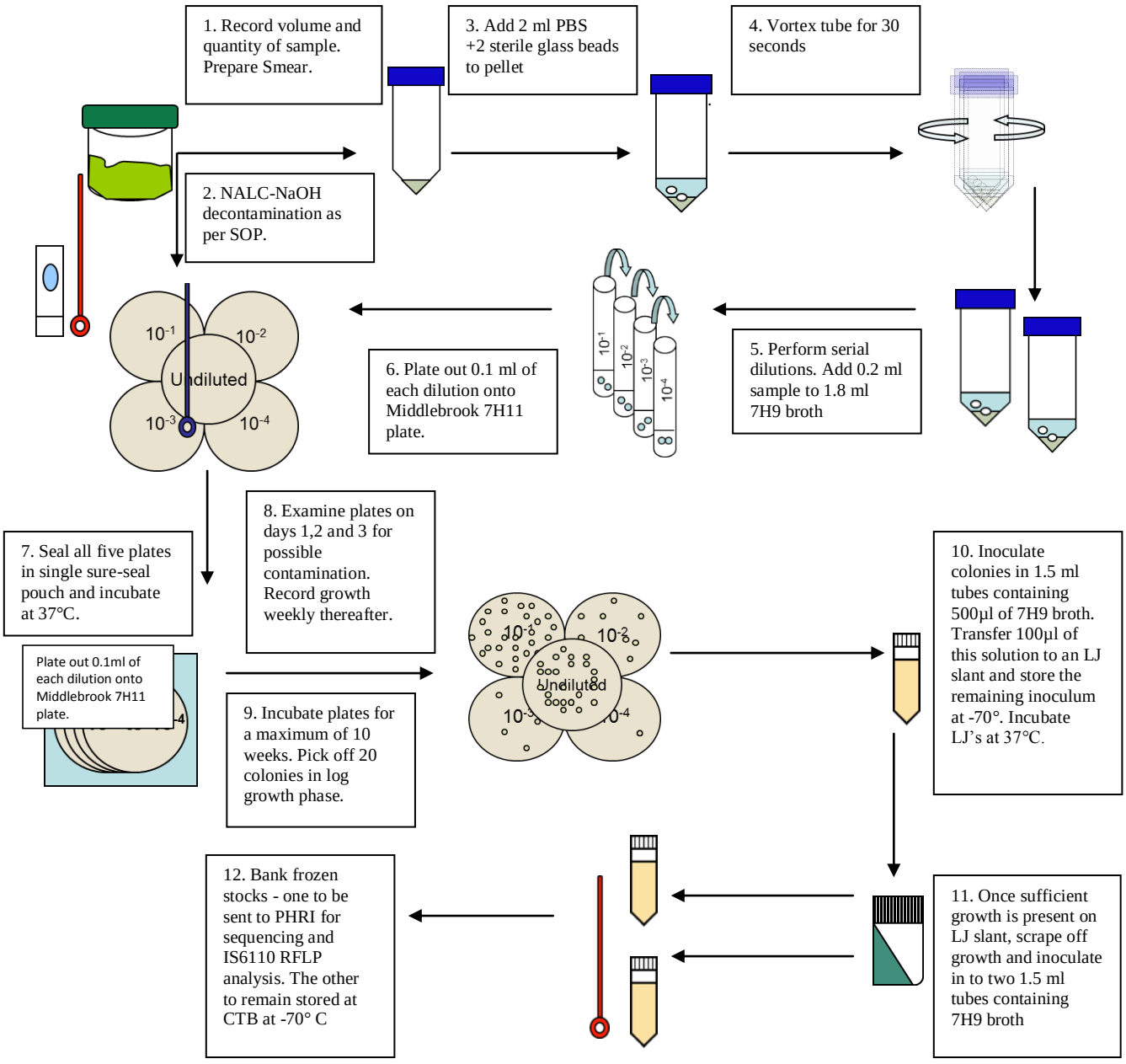

Figure 4.4 Simple illustration of steps taken to isolate single MTB colonies

4.2.1.5 Shipment of isolates to PHRI, Newark, New Jersey, USA

A total of 100 MTB colonies (20 colonies per patient) were shipped to PHRI for subsequent molecular analysis. Colonies were packaged according to World Courier standards.

4.2.2 Laboratory procedures and molecular analysis performed at PHRI, Newark, New Jersey, USA



Figure 4.5 Photograph taken of ICPH, PHRI in Newark, New Jersey, USA

4.2.2.1 Processing of MTB isolates in BL 3 laboratory

Before the commencement of laboratory work in the BL 3 laboratory at PHRI, various safety requirements had to be met. These requirements included BL 3 laboratory safety training, hands on respirator training using a Powered Air Purifying Respirator (PAPR), BL 3 medical clearance as well as other training procedures required by the facility.

4.2.2.1.1 MTB sub-culturing onto LJ slants

MTB cultures were received at the PHRI in Nalgene screw cap cryo-vials sealed with parafilm in an absorbent spill proof pouch that was placed inside a screw cap shipping container from RSA. After arrival, the shipping canister was transferred to the BL 3 laboratory and opened inside a class II A/B3 BSC. Appropriate PPE was worn in the BL 3 (this included 2 pairs of Nitrile gloves, Tyvek suit with hood and booties, a disposable blue apron and PAPR). Using a sterile Q-tip, an aliquot from the culture tube was transferred to an LJ slant and incubated at 37°C until there was good visible growth and colonies were ready for DNA isolation (approximately three to four weeks). The Nalgene screw cap cryo-vials containing the remaining MTB cultures were then placed in a 9x9 box and placed in the -80°C freezer for storage.

4.2.2.1.2 Extraction and isolation of DNA from MTB cells growing on LJ slants

After sufficient growth was observed on the LJ slants, DNA was now ready to be isolated and extracted from the MTB cells for molecular analysis. This procedure was performed in the BL 3. Colonies were transferred from LJ slants using a sterile Q-tip into 1.5 ml Eppendorf tubes

filled with 500 µl of sterile nuclease free water, as well as into 2 ml Nalgene cryo-vials filled with 750 µl 7H9 media with glycerol (these cryo-vials were placed in a -80°C freezer for long term storage of stock.) The Eppendorf tubes containing the water and MTB colonies was then transferred to a circular rack and placed in an 80°C water bath for an hour to heat kill the bacteria. The Eppendorf tubes were subsequently placed in an Eppendorf thermomixer set at 60°C. 70 µl of 10% Sodium dodecyl sulphate (SDS) solution followed by 50 µl of Proteinase K (10 mg/ml) was added to each sample and allowed to shake for one hour. 100 µl of pre-heated 5M NaCL and hexadecyltrimethyl ammonium bromide (CTAB) was then added to the tubes and allowed to shake for 15 minutes. Samples were then transferred to a rack and placed in a freezer at -80°C for 20 minutes. Samples were then transferred back into the Eppendorf thermomixer set at 60°C to defrost and 650 µl of 24:1 chloroform/isoamyl solution was added to each sample. Samples were mixed thoroughly and then centrifuged at 12,000 RPM for ten minutes. The upper aqueous layer (approximately 650 µl) was then transferred to 700 µl Isopropanol tubes and mixed. The Isopropanol Eppendorf tubes were then transported out of the BL 3 and placed at -20°C for 30 minutes or at 4°C overnight.

Samples were then centrifuged for ten minutes at room temperature at 12,000 RPM. The supernatant was discarded and the pellet was washed with 80% ethanol (this was accomplished using a squirt bottle containing 80% ethanol). The samples were re-centrifuged at room temperature for an additional ten minutes at 12,000 RPM. The supernatant was discarded and the tubes were left upside down on a paper towel for approximately one minute to remove any excess ethanol. The open Eppendorf tubes were subsequently transferred to a DNA Speed Vac (Savant Instruments, Inc. Farmingdale, New York, USA) and centrifuged for five minutes to completely evaporate excess alcohol from the pellet. 55 µl of 1X TE buffer

(Tris, EDTA) was added to each sample. The samples were left on a shaking platform for approximately 30 minutes to re-suspend the pellet. Samples were stored in a fridge at 4°C overnight.

4.2.2.1.3 Agarose gel to gauge DNA concentration

A 1% agarose gel was made by weighing 2 g of agarose powder and mixed into 200 ml of fresh 1X TBE (Tris, EDTA, Boric Acid) buffer in a 500 ml Erlenmyer flask. The agarose was placed in a microwave for about two minutes until it came to a boil. The agarose was then placed on a magnetic plate stirrer and left to cool until it reached approximately 55°C. When the agar was sufficiently cooled, 4 µl of Gelstar nucleic acid gel stain (Lonza) was added to the agarose and it was placed back onto the stir plate for an additional minute. Two thin 16 slot combs (for 60 samples and DNA ladder) were placed on the gel container accordingly to form wells. The cooled agarose gel was then poured into the gel casting container and left to set for 30 to 60 minutes. After the gel had set it was transferred to a gel box (Sub Cell GT System, Biorad) and covered with 1X TBE buffer. 2 µl of Ladder (DNA marker) was added to the first well of each row (2 rows for 60 samples). 5 µl of extracted genomic DNA was combined with 1 µl of loading dye and added to the appropriate well on the gel. The power supply was set to 80V and the samples were left to run for approximately one hour. The DNA samples were run on the 1% agarose gel to visually gauge the concentration of DNA present in each sample following extraction for IS*6110*-based RFLP analysis. DNA samples were diluted 1:100 or 1:50 (depending on the amount of visible growth present on the LJ slants) in nuclease-free water for PCR sequencing. 1.5 µl of diluted DNA was placed on the Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA) to

determine the concentration of DNA present for PCR sequencing (section 4.2.2.2.2). Diluted DNA samples for PCR were stored at -20°C until ready to process.

4.2.2.2 Molecular analysis in BL 2 laboratory

4.2.2.2.1 IS*6110*-based RFLP

IS*6110* RFLPs were performed on the five sets of DNA isolates obtained from each patient.

4.2.2.2.1.1 Digestion of MTB genomic DNA with restriction endonuclease *Pvu*II

For the restriction digest, genomic DNA and nuclease free water were added to a 1.5 ml sterile Eppendorf tube (the amount of DNA added was determined visually by the amount of DNA present on the 1% agarose gel – see section 4.2.2.1.3). In addition, 2.5 µl of buffer and 1.5 µl of *Pvu*II restriction endonuclease was added to each tube to a total volume of 22 µl. The tubes were labelled and placed in a water bath at 37°C for four hours. 5 µl of loading dye was added to each sample and the digested DNA was then subjected to electrophoresis on a 1% agarose gel (Sub cell GT system, Biorad) in order to perform southern transfer to a high strength, positively charged nylon membrane (zeta probe blotting membrane, Biorad).

4.2.2.2.1.2 Southern transfer

A positively charged zeta probe membrane was cut to a size of 20 cm x 15 cm and labelled in the upper right hand corner. The membrane was then briefly submerged into distilled water (dH_2O), followed by soaking for five minutes in 10 X SSC (20 X SSC: 3M NaCl, 0.3

Na_3citrate, pH 7.0 – diluted 1:2). The membrane was then placed on a support-gel supporting screen (Vacugene XL vacuum blotting unit, GT Healthcare) and covered with a plastic mask. The agarose gel was then carefully slid on top of the membrane. A vacuum was attached to the instrument and the power supply was set to 60 cm mbar. The gel was flooded with 1/100 diluted hydrochloric acid (HCL) and left for 20 minutes. The fluid was removed from the gel with a vacuum tip apparatus and then flooded with "Soak I" (0.5 M NaOH, 1.5 M NaCl; pH 7.2) and left for an additional 20 minutes. The fluid was removed and soaked for 20 minutes with "Soak 2" (0.5 M Tris, 1.5 M NaCL; pH 7.2) followed by 10 X SSC for 90 minutes. The vacuum was increased to 65 cm mbar during the final soak with 10 X SSC. After the southern transfer, the membrane was left to dry for a few minutes and then placed in a cross linker (UV Crosslinker FB-UVXL-1000, Fisher Scientific) to irradiate and permanently bind the DNA to the membrane.

4.2.2.2.1.3 Southern hybridisation

The membrane was placed in a glass cylinder and pre-hybridised with 30 ml of hybridisation buffer (Amersham ECL Direct nucleic acid labelling system, GE Healthcare) for 30 minutes at 5 RPM at 42°C in the hybridisation oven (Problot hybridisation oven, Labnet). For each membrane, 10 µl of 1:10 diluted probe and 5 µl of H_20 was aliquotted into an Eppendorf tube and placed in boiling water for five minutes. The probe used was made from a plasmid with the incorporated right side of IS6110 (450 kb) and universal primers. The Eppendorf tube was placed directly into ice for ten minutes. 15 µl of labelling agent and 15 µl of gluteraldehyde was then added to the probe and water. The tube was then placed in a water bath at 37°C for 15 minutes. The hybridisation buffer was then removed from the glass cylinder and poured

into a clean 50 ml conical tube. The labelled probe was then added to the hybridisation buffer in the conical tube and mixed. The hybridisation buffer was then poured back into the glass cylinder, attached to the clamps and left to hybridise overnight at 42°C at 6 RPM.

Hybridisation buffer was discarded the following morning followed by a quick rinse with 20 ml of primary wash buffer (for 1L: 360 g Urea, 25 ml 20 X SSC, 20 ml 20% SDS – dH_2O was added to a final volume of 1000 ml). 30 ml of primary wash buffer was then added to the cylinder and left to rotate in the hybridisation oven for 30 minutes. Primary wash buffer was then removed and 30 ml of secondary wash buffer (2 X SSC: 100ml 20 X SSC; 900ml dH_2O) was added to the glass cylinder to rinse. The membrane was removed from the glass cylinder and placed in glass pyrex dish containing secondary wash buffer on a shaking platform for ten minutes. The liquid was discarded and the last step was repeated for another ten minutes.

4.2.2.2.1.4 Detection of fluorescent label

The membranes were transferred from the glass pyrex dish to plastic containers. 9 ml of detection reagent (Luminata Forte Western Horse Radish Peroxidase Substrate) was poured onto the membranes and the plastic containers were rocked gently back and forth by hand to ensure the membranes were completely covered with reagent. The membranes were taken to a dark room for development. After four minutes the membranes were placed on a plastic film (cling wrap) and covered. A paper towel was used to remove any creases in the plastic film covering the membrane as well as any excess substrate. The membranes were placed in a cassette with the labelled side facing upwards. With the main lights switched off and just the red light left on in the developing room an X-ray film (Bluelight 8'' x 10'' Autorad film,

Genemate) was placed in the cassette on top of the membrane. After three minutes of exposure, the cassette was opened and the film was loaded into a Kodak X-OMAT 1000A processor (Eastman Kodak Company, Rochester, New York, USA) developing machine. Based on the intensity of the bands from the first film, a decision was made with regards to the exposure time for the second film if necessary. The X-ray film was scanned into the computer for analysis using BioImage Software version 4.3 (USA) to interpret the DNA fingerprints of each isolate. Refer to Appendix B for detailed description of solutions for IS*6110* RFLP.

4.2.2.2.2 Sequencing of genes associated with first- and second-line drug resistance

Sequencing of various genes associated with drug resistance was performed to demonstrate genetic heterogeneity in sputum samples based on mutations in genes that determine drug resistance to both the first- and second-line anti-TB agents. The first-line anti-TB drugs investigated included RIF, INH and STREP. The genes involved are the *rpoB* gene for RIF resistance, the *katG* and *inhA* gene for INH resistance and the *rrs* and *rpsL* gene for STREP resistance. The aminoglycosides (KAN and AMK) together with CAP and the FQs are the major groups of second-line drugs. The genes involved with second-line resistance are most commonly the *rrs* gene for KAN, AMK and CAP resistance, the *tlyA* gene for CAP resistance and the *gyrA* and *gyrB* gene for FQ resistance. The *pncA* gene was also sequenced with regards to PZA resistance.

4.2.2.2.2.1 PCR assay and preparation

The diluted DNA was removed from the -20°C freezer, placed in a rack and left to thaw at room temperature. Forward (Primer-F) and reverse (Primer-R) primers were ordered for the

different genes that needed to be sequenced. The primers were received in a powder form and were reconstituted using nuclease free water (this amount varied depending on the nmole amount indicated on the primer tube) to a final concentration of 100 µmole. The primers were further diluted 1:10 for a working stock solution for the PCR assay. The primer sequences for the various genes are given in the table below.

Table 4.2 Primers used for the sequencing of genes associated with MTB drug resistance

Primer Name	**Primer Sequence**
rpoB-F	5'-GGGAGCGGATGACCACCCA-3'
rpoB-R	5'-GCGGTACGGCGTTTCGATGAAC-3'
katG-F	5'-CCCATGGCCGCGGCGGTCGACATT-3'
katG-R	5'-CGCCGTCCTTGGCGGTGTATTGCC-3'
inhA (fabG1)-F	5’-CCTCGCTGCCCAGAAAGGGA-3'
inhA (fabG1)-R	5’-ATCCCCCGGTTTCCTCCGG-3'
rpsL-F	5'-GGCCGACAAACAGAACGT-3'
rpsL-R	5'-GTTCACCAACTGGGTGAC-3'
rrs-F	5'-CCATCGACGAAGGTCCGGGTTCT-3'
rrs-2R	5’-CGCGTCCTGTGCATGTCAAACC-3'
rrs-2F	5’-GTAGCTAACGCATTAAGTACC-3'
rrs-3R	5’-CACTACAGACAAGAACCCCTCACGG-3'
gyrA-F	5'-CCGGATCGAACCGGTTGACATC-3'
gyrA-R	5'-GGGCTTCGGTGTACCTCAT-3'
gyrB-F	5’-AACACCGAGGTCAAATCGTT-3'
gyrB-R	5’-CTGAATGCCGTCTTCCTTGT-3'
pncA-F	5'-TGGCCGCCGCTCAGCTGGTCATG-3'
pncA-R	5'-CCACCGCCGCCAACAGTTCAT-3'
tlyA-F	5'-GCATCGCACGTCGTCTTT-3'
tlyA-R	5'-GGTCTCGGTGGCTTCGTC-3'

The amount of primers, Taq (Denville Scientific Inc) and sterile water needed were calculated for each run. The calculations were as follows:

Primer-F (1:10): 0.4 µl/reaction x 104 = for 96 well plate with excess

Primer-R (1:10) 0.4 µl/reaction x 104 = for 96 well plate with excess

Taq (2X): 10 µl/reaction x 104 = for 96 well plate with excess

Sterile water: 9 µl/reaction x 104 = for 96 well plate with excess

DNA: 1 µl/reaction

Final volume = 20 µl in each well.

To prepare the master mix, the Primer-F, Primer-R, Taq and sterile water were mixed together in a 1.5 ml sterile Eppendorf tube. 19.5 µl of master mix was then transferred to each well in a 96 well Thermogrid PCR plate. 1 µl of DNA from each sample was then added to each corresponding well. Caps were then placed over each well to prevent evaporation during PCR. The plates were placed in the PCR machine (Eppendorf Mastercycler) and run on the following program:

95°C	5 min	Initial denaturing
95°C	30 sec	Denaturing
62°C	30 sec	Annealing
72°C	50 sec	Extension
35 Cycles		
72°C	2 min	Final elongation
1 Cycle		

Figure 4.6 PCR program for DNA amplification

After the PCR was run the plate was stored inside a zip lock bag at 4°C until a gel was run to gauge the PCR products.

4.2.2.2.2.1.1 Agarose gel to detect PCR products

A 1% agarose gel was prepared as set out in section 4.2.2.1.3. 4 µl of Gelstar nucleic acid gel stain was added to the agar when it had cooled to approximately 55°C. The gel was placed back onto the stir plate for an additional minute. Four 30 slot combs (for 96 samples) were placed in the gel container to form wells. The cooled agarose was poured into the gel container and left to set for approximately one hour. After the gel had set it was transferred to the Sub cell GT system for electrophoresis and submerged with 1X TBE buffer. 2 µl of 1 kb DNA Ladder (DNA marker) was added to the first well of each row (4 rows for 96 samples). 3 µl of PCR amplicon was then taken from each sample on the 96 well plate and combined with 1 µl of loading dye. The samples were then loaded into their corresponding wells on the agarose gel. The amplified DNA samples were then subjected to electrophoresis at 100V (0.04A) and run for approximately one hour. A picture of the gel was then taken on the Gel Doc 2000 (Biorad, Richmond, California) and using Quantity One Imaging Software 4.4.0 to view the PCR products.

4.2.2.2.2.2 Sending out PCR plates sequencing

For sequencing, 10 µl of PCR product was taken from the 96 well PCR plate and transferred to a Template V PCR plate. Caps were placed over each well and 2 pieces of plate sealer was used to seal the plates for transport. The plate was placed in a zip lock bag along with the Primer-F. Plates were then sent to Maryland, USA for sequencing.

4.2.2.2.2.3 Reading of sequences and data collection

The results for the sequencing data were downloaded from the Macrogen website (www.macrogenusa.com). AB1 files were placed in a separate file for analysis. A "sequence to align" template was designed which contained both the particular gene sequence for the H37Rv strain as well as the tracking numbers corresponding to the various well positions for each laboratory sample (for example A1 – Patient 1, Colony 1). Finch TV was the program used to view the chromatograms as well as to obtain the various sequences from the genes of interest. The sequences obtained for each gene from each set of patient isolates were then copied and pasted into the "sequence to align template" document alongside their appropriate tracking numbers. The website http://align.genome.jp/ was then used to align the genes of interest with the H37Rv gene sequence. The alignment results were copied and pasted into a "clustal alignment" document. Misalignments and mutations were identified by breaks in "stars" below each line of sequence. The codon number involving the single nucleotide was then identified. It was then determined whether a protein change resulted from this event.

4.2.3 Phenotypic susceptibility testing of colonies performed at the CTB

After returning to RSA, DST was performed on each set of colonies obtained for the five patients.

4.2.3.1 Sub-culturing of colonies for DST

In order to perform DST, a fresh MGIT culture was required for each isolate. The colonies stored at -70°C at the CTB, were thawed at room temperature after which 50 µl of each isolate

was inoculated into a MGIT tube containing 800 µl of OADC (Oleic acid, Albumin, Dextrose, Catalase) enrichment medium. Each MGIT tube was then scanned into the MGIT 960 system for incubation at 37°C. Once the tubes had flagged positive, they were removed and DST was performed within three days.

4.2.3.2 Drug Susceptibility Testing

DST was performed on each set of isolates obtained from each patient (i.e. a total of 99 tests were performed out of the 100 colonies isolated due to one colony from Patient 5 being contaminated). Drugs that were tested for susceptibility included two first line drugs, namely, RIF and INH and two second-line anti-TB drugs, namely OFX and KAN. DST was also performed on all isolates for high-level INH resistance (critical concentration 0.4 µg/ml). A lyophilised drug vial of INH and RIF [AST IR kit – Becton Dickenson (BD)] was reconstituted with 4 ml of sterile dH_2O. OFX and KAN [Diagnostic Media Products (DMP)] were removed from the -70°C freezer and left to thaw at room temperature. Tubes were set up for each isolate which included one tube for the growth control (a tube containing a 1:100 dilution of organism suspension and no drug) and another five tubes were designated for each drug tested [low level INH, high level INH, RIF, OFX and KAN (Critical concentrations: 0.1µg/ml, 0.4 µg/ml, 1.0 µg/ml, 2.0 µg/ml and 5.0 µg/ml respectively)]. Six bar-coded labels were printed for each specimen including the growth control tube and each drug tube. 800 µl of MGIT IR supplement or OADC supplement was aseptically added to all MGIT tubes (see Figure 4.7, step 1). 100 µl of the respective drug stock solution was then added to the appropriate MGIT tube (Figure 4.7, step 2). In order to prepare the growth control inoculums, 100 µl of the organism suspension was added to 9.9 ml of saline to make a 1:100 dilution

(Figure 4.7, step 3). After mixing, 500 µl of the 1 in 100 dilution was then inoculated into the growth control tube (Figure 4.7, step 4). Each drug containing tube was then inoculated with 500 µl of undiluted organism suspension (Figure 4.7, step 5). The tubes were all inverted three to four times to ensure thorough mixing. The tubes were then scanned and placed into the BACTEC MGIT 960 instrument.

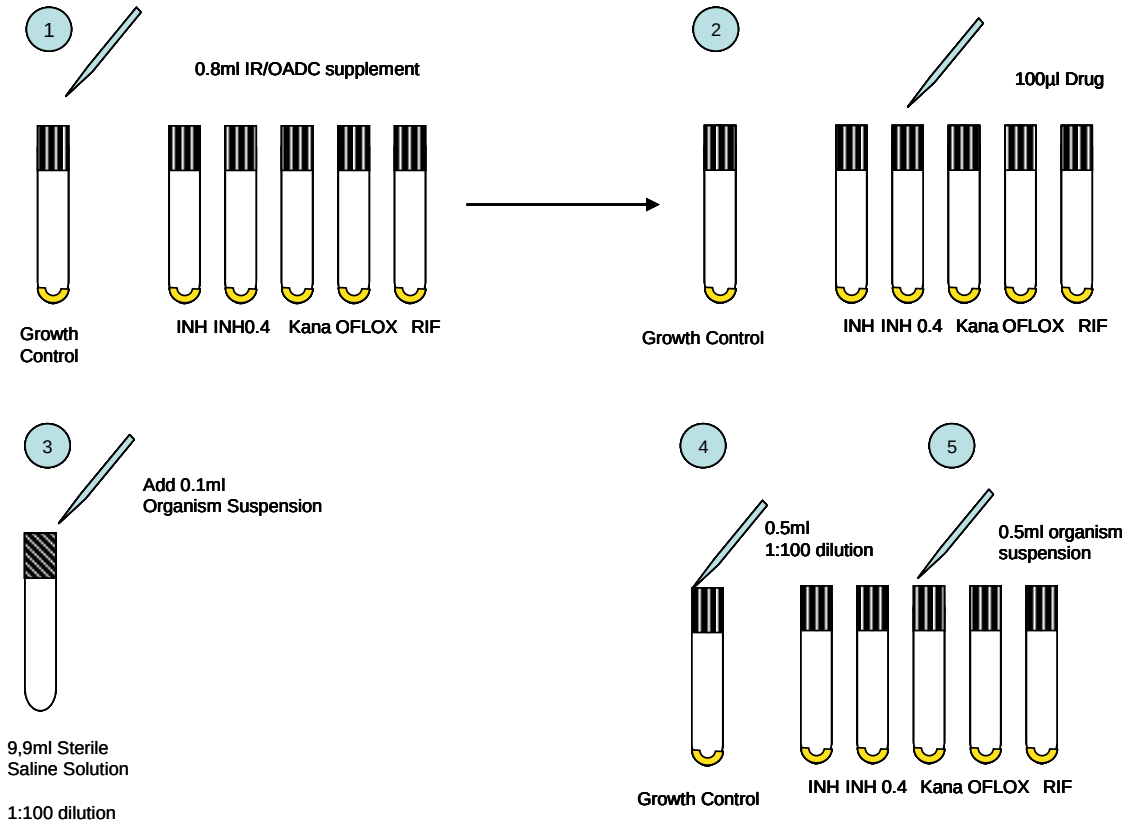

Figure 4.7 BD BACTEC™ MGIT™ 960 Preparation and Inoculation

4.2.4 Minimal Inhibitory Concentrations

After analysing the results obtained from the DST analysis, a decision was made to perform MICs on colonies from Patient 1 that yielded unexpected DST results to RIF.

4.2.4.1 Liquid culturing of MTB strains for MICs (DST/NRF Centre for TB Excellence)

Fresh cultures of the frozen stock colonies were cultured using disposable cellstar flasks. 6 ml of 7H9 broth solution was added to each culture bottle using a 10 ml pipette. It was ensured that the amount of media added was correct for the size of bottle used so that the culture would not enter the neck of the culture bottle when being incubated. The 6 ml of 7H9 media was inoculated with 50 µl of culture obtained from the frozen colonies that were stored at -70°C at the CTB. After inoculation, the flasks were closed immediately to prevent spillage. Before removal from the BSC, the flasks were wiped down with 70% ethanol and laid down flat in a secondary container. The container containing the culture flasks was then placed in an incubator at 37°C and was inspected every few days for MTB growth and/or contamination.

4.2.4.2 Preparation of MIC plates in BL 2 laboratory

MIC plates were set up for RIF for a sample of colonies that were selected from Patient 1 that yielded unexpected DST results when compared to the sequencing data obtained for these colonies. MIC plates were also set up for selected colonies from Patient 3 that yielded expected DST results according to sequencing data obtained for genes associated with resistance to RIF. The stock solution of RIF was 10 mg/ml. It was decided that a starting concentration 3.2 µg/ml was required to determine the MICs for the selected colonies. The

following calculations were performed in order to make up the appropriate concentrations of drugs required for the MICs.

For RIF:

The original 10 mg/ml stock solution of RIF was diluted 1:10 in DMSO to yield a working stock solution of 1 mg/ml of RIF. For 10 MIC plates, it was calculated that approximately 2.5 ml of RIF at a final concentration of 3.2 μg/ml was required to perform the experiment. The following calculation was performed:

$C1V1 = C2V2$

$1{,}0 \times 10^{-3}\text{g} \times x = 3.2 \times 10^{-6}\text{g} \times 2.5 \times 10^{-3}\ell$

$x = \dfrac{3.2 \times 10^{-6}\text{g} \times 2.5 \times 10^{-3}\ell}{1.0 \times 10^{-3}\text{g}}$

$x = 8 \times 10^{-6}\ell$

$x = 8\ \mu\text{l}$

Therefore, it was calculated that 8 μl of the 1:10 RIF dilution was added to 2.5 ml of DMSO to yield a 3.2 μg/ml concentration of RIF (this is diluted to 1.6 μg/ml after addition of MTB culture). From preliminary work, a RIF MICs ranging from 0.1 to 0.4μg/ml was obtained using broth microdilution with culture grown to an optical density of 0.5 (diluted further). A starting concentration of 1.6μg/ml was used to ensure that the MIC (defined by no growth), fell roughly within the dilution range on the microtitre plate. The 1.6μg/ml starting concentration was used to obtain an MIC level determining extent of susceptibility and not to determine the susceptibility status for which critical concentrations are used.

Half a 96 well plate (Nunc, Denmark) was used to perform MICs for a single colony. 50 µl of 7H9 broth was added to all wells using a multi-channel pipette, i.e. 50 µl of broth was added to every well excluding the first column of the plate. 100 µl of 7H9 media was then added to column one, row one to act as a media control. 100 µl of DMSO was then added to column one, row two to act as a control for DMSO. 100 µl of 3.2 µg/ml of RIF was then added to column one, rows three and four. After each respective reagent/drug had been added to the respective wells, two fold serial dilutions were performed. 50 µl of sample was taken from each well in column one and transferred using a multi-channel pipette to column two. Once transferred, the solutions were mixed by pipetting the fluid up and down approximately ten times. Again, 50 µl from each well in column two was transferred to each corresponding well in column three and so forth. This was repeated for all twelve columns. The remaining 50µl in the last column was discarded so that each well contained a final volume of 50 µl.

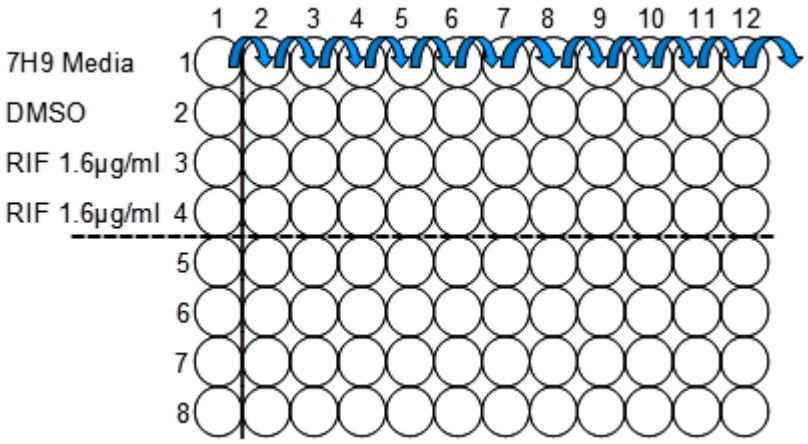

Figure 4.8 Illustration of MIC plate set-up indicating final concentrations

4.2.4.3 Addition of MTB culture to MIC plates in BL 3 laboratory

Once the MIC plates had been set up, they were taken into the BL 3 laboratory in order to add the freshly grown culture from the selected colony to each well. Before inoculating each well, the cultures were diluted in 7H9 broth to obtain a turbidity matching approximately that of a McFarland Standard of 1. Once the cultures were diluted, 50 µl of diluted culture was then added to all wells using a multi-channel pipette to give a final volume of 100 µl per well (making the starting concentration of RIF 1.6 µg/ml). The plates were then placed in a plastic sleeve and incubated at 37°C for approximately two weeks before reading the MICs.

4.2.4.4 Reading of MIC plates

MICs were read two weeks following incubation. The MIC was recorded as the concentration of the test drug in the first well of the serial dilutions where no MTB growth was visible.

CHAPTER 5

5 RESULTS AND DISCUSSION OF FINDINGS

5. 1 FINDINGS OF PILOT STUDY: OPTIMIZATION OF METHODS AND MEDIA

In order to obtain MTB bacterial cells representative of organism heterogeneity that may have developed during patient treatment/infection, a pilot study was carried out to evaluate different methods of organism dispersion in sputum diluted out to optimise laboratory conditions and procedures to obtain single MTB colonies. This approach was based on the premise that CFUs on solid media inoculated with patients' sputum represent clones developing from single MTB cells in sputum. It was also determined which of three solid TB media, namely Dubos, Middlebrook 7H11 and LJ (media poured in plates), would best demonstrate differences in colony morphology which could possibly be linked to heterogeneous subpopulations of MTB or to mixed infections, as well as yield the greatest number of single MTB colonies .

Seven sputum samples collected from newly recruited MDR-TB patients at Sizwe Hospital were included in the pilot study. Of the seven samples, the first five were only processed after delays of four to six days, while specimen 6 and specimen 7 were processed within 48 hours. The findings of the pilot study are given below:

5.1.1 Smear microscopy for acid-fast bacilli

Only specimen 4 and specimen 5 were positive for AFB and both specimens were graded with a positivity rating of +++, i.e. equivalent to more than 10 AFB per immersion field (100 X oil immersion and 10 X eye piece).

5.1.2 Time to flag positive for MTB in MGIT liquid culture

Specimen 4: 6 days

Specimen 5: 6 days

Specimens 1, 3, 6, and 7 were culture negative on the MGIT system while specimen 2 flagged positive at 5 days due to bacterial contamination; not MTB.

5.1.3 Time to visible growth of MTB on agar and LJ plates

Specimen 4: 2 weeks

Specimen 5: 2 weeks

The low culture positivity rate was of concern, although many of these patients were on treatment at the time of admission which may have contributed to the low culture positivity rate. The delays in the transport of specimens from Sizwe Hospital to the BTBRL were traced back to delays in entering specimen/patient details into an IT database at the hospital. This was

due to the fact that the clerk responsible for this task was only available twice a week. This problem affected specimens 1 -5. Specimen 6 and specimen 7 were transported directly to the BTBRL used for routine testing, allowing the specimens to be processed within 48 hours. However, both specimens contained very little sputum (< 2ml) which may have contributed to the negative culture results. Sputum specimen quality and quantity were identified as important aspects to be addressed before the commencement of the main heterogeneity study.

5.1.4 Growth support on solid media

After 3-5 weeks' incubation of decontaminated Specimen 4 and Specimen 5, three colony sizes were observed on all three solid media. Colony sizes at 5 weeks were: large (~2mm) in diameter (~60% of total); small (~1mm) in diameter (~40% of total) and intermediate size (`~1.5mm) in diameter (<5% of total).

Colony size following incubation on Dubos agar and 7H11 agar was similar but colonies were marginally larger on Dubos agar plates, while on LJ plates, colony variation was also observed and all colony variants were slightly smaller on this medium, compared with the two agar-based media. To illustrate these findings, the relative colony sizes on the three solid media were estimated based on a scale from a 4+ allocation for a fully sized large colony variant, present as single entities in large numbers on Dubos agar medium, to a 1+ for a pinpoint size colony on this medium (see Table 5.1).

Table 5.1 Relative colony size on solid media five weeks' incubation

Specimen	Dubos	7H11	LJ
4	++++	+++	++
5	++++	+++	++

Colony size according to scale + to ++++ as follows:

+ = Pinpoint size colonies (~ < 1 mm in diameter)

++ = Small intermediate size colonies (~ 3mm in diameter)

+++ = Large intermediate size colonies (~ 5mm in diameter)

++++ = Colony size after 5 weeks' incubation on Dubos medium (>5mm in diameter)

5.1.5 Organism Dispersal by vortexing in the presence and absence of Tween 80 plus beads

The performance of two approaches to disperse TB organisms in sputum following decontamination and concentration of specimen 4 and specimen 5 is summarized in Table 5.2. The vigorous approach involved dispersion of TB bacilli by vortexing the concentration sediments previously diluted and suspended in PBS containing 0.05% Tween 80 (total volume 5 ml) in tubes with two glass beads each for ten seconds. For the gentle approach, the same volume (5 ml) of similarly diluted sediment suspensions in PBS was used but without Tween 80 or glass beads and was also vortexed for ten seconds (see Materials and Methods section).

Table 5.2 Numbers of CFUs on solid media following vigorous and gentle dispersion of sediments from specimen 4 and specimen 5

Specimen	Dispersion Method	Dilution Factor	Dubos	7H11	LJ
4	Vigorous*	10^1	Contam§	Contam§	Contam§
		10^2	178	0	0
		10^3	16	Contam§	Contam§
		10^4	0	0	0
		10^5	1	0	0
4	Gentle**	10^1	>1000	>1000	>1000
		10^2	502	172	Contam§
		10^3	Contam§	Contam§	Contam§
		10^4	0	0	0
5	Vigorous*	10^1	406	280	310
		10^2	29	28	7
		10^3	Contam§	Contam§	Contam§
		10^4	0	0	1
5	Gentle**	10^1	278	215	120
		10^2	17	26	19
		10^3	Contam§	Contam§	Contam§
		10^4	0	0	0

* NALC-NaOH sediments vortexed in presence of Tween 80 and glass beads

** NALC-NaOH sediments vortexed in absence of Tween 80 and glass beads

Contam§ = Contaminated

It is clear that limitations inherent to the plate-dilution method for colony counting do not allow for reliable quantitative comparisons but our findings suggest that dispersal in Tween 80 is unlikely to yield substantially larger numbers of colonies and may inhibit subpopulations of MTB which could have happened in the case of the dispersal of specimen 4.

It was not possible to assess the correlation between smear microscopy and dilutions of NALC-NaOH sediment producing single colonies. The three-plus (+++)-positive sputum specimens produced single colonies on plates inoculated with specimens diluted 1 in 10 and 1 in 100 respectively. However, the delays in processing the two specimens may have resulted in some loss of viability. Also, for the main study, a 10^3 dilution would most likely also be required for a three-plus-positive smear microscopy specimen. The extent of contamination in the pilot study was a major problem and is addressed in section 5.1.8.

5.1.6 Comparison of colony variation on three solid media

Colony morphology on the three solid media was studied based on size, transparency, colour/pigment, surface structure and type of edge of single colonies at approximately three and five weeks of incubation. Typical yellow to buff colonies with wrinkled surfaces were observed on LJ plates while the colonial morphology on Dubos and 7H11 agar plates was similar and typical for MTB colonies but the colonies were more translucent and less pigmented than those on the LJ plates (See photographs in Figure 5.1 and Figure 5.2). Changes in the surface structure of colonies associated with duration of incubation resulted in cumbersome and imprecise characterisation of morphotypes and colony size at different durations of incubation lent itself better for this purpose.

In both specimen 4 and specimen 5, two predominant colony types were found:

Large colonies: These colonies appeared after two to three weeks' incubation. The colonies were a semi-opaque, milky colour, with wrinkled surface and serrated edges. Large colonies constituted approximately 60% of all colonies on all three solid media.

Small colonies: The small colonies possessed similar characteristics compared to the large colonies but were smaller and appeared marginally later than the large colonies. These constituted approximately 40% of all colonies.

Intermediate-sized colonies: The morphology of these colonies was similar to that of the large and small colonies but they were fewer in number (<5% of total).

The appearance of the MTB colonies on the three solid media was similar with regard to the surface structure; however, the colonies were smaller and more pigmented on the LJ plates. In addition, all three colony morphotypes demonstrated similar degrees of cord formation on microscopy of the cultures on the solid media compared with cords seen on Ziehl-Neelsen staining of liquid (MGIT) cultures.

For differentiation based on colony morphology, the use of any of the three solid media would be suitable. Colonies on Dubos and 7H11 agar media were similar in morphology and additional variation on LJ medium was not of sufficient value to warrant its use for this purpose alone. Based on these findings it was concluded that the use of either Dubos or 7H11 agar would be suitable choices for colony differentiation. It was finally decided that 7H11 agar which is widely used in research settings, would be appropriate for the main study.

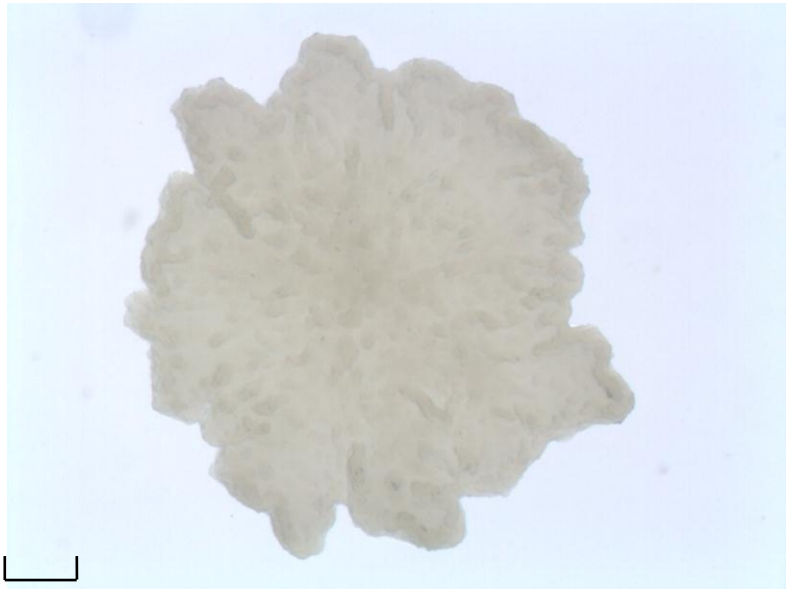

Figure 5.1 Image taken of fully developed large (~5mm) MTB colony on 7H11 agar medium using plate microscope (magnification X15)



Figure 5.2 Image taken of fully developed large (~3 mm) MTB colony on LJ medium using plate microscope (magnification X25)

5.1.7 Comparison of methods of dispersal of MTB organisms on solid media

The procedure of conventional spreading of organisms on plates by streaking of inoculum was compared with the dilution method to obtain single colonies. The results from these two different methods are summarised below:

5.1.7.1 Inoculum streaking method

The spreading out procedure entailed four sequential steps comprising primary inoculation streaking followed by three further streaking steps, each aimed at decreasing the number of micro-organisms in the specimen across the surface of the solid medium. This method resulted in growth of isolated colonies but at best produced semi-quantitative assessment of organism load in a specimen and contamination was more common than encountered with the dilution method.

Table 5.3 CFUs developing on solid media following sequential streaking of sediments subjected to two dispersal methods

Specimen	Solid Medium	Spreading Steps	Vigorous: CFU	Gentle: CFU
4	Dubos	1	Confluent streaks	Confluent streaks
		2	12	20
		3	5	2
		4	0	0
4	7H11	1	50	0
		2	0	0
		3	0	0
		4	0	0
4	LJ plate	1	>100	Contam§
		2	4	Contam§
		3	0	Contam§
		4	0	Contam§
5	Dubos	1	>50 (merging)	Contam§
		2	4	Contam§
		3	1	Contam§
		4	0	Contam§
5	7H11	1	12	0
		2	4	0
		3	0	1
		4	0	0
5	LJ plate	1	Contam§	Contam§
		2	Contam§	Contam§
		3	Contam§	0
		4	Contam§	0

Contam§ = Contamination

Contamination was a major problem using the streaking method and more than 15 single colonies per specimen could only be obtained on six out of the 12 plates. This was mainly

attributed to contamination. Dubos plates performed best by supporting the growth of more and larger colonies when compared to Middlebrook 7H11 and the LJ plates. Small and large colony morphotypes could be distinguished on all three types of media, as was in the case with colonies on the dilution plates. Compared to the dilution method, the plate spreading method is simple to perform; however, the results indicate that if contamination is picked up in the primary inoculum area and then transferred to subsequent areas for inoculation, no separable MTB colonies may be obtained from a culture positive specimen. This occurred in three out of 12 (25%) plating out procedures, two on LJ plates (Specimen 4, gentle dispersion and Specimen 5, vigorous dispersion) and one on a Dubos medium plate (Specimen 5, gentle dispersion). In addition, colony yields were on occasion lower than expected, even in the absence of contamination. No colony formation was seen on the 7H11 plate from specimen 4 using the gentle dispersion method compared with excellent results on the Dubos medium. It is possible that faulty technique may have been responsible, e.g. using an inadequately cooled loop for spreading of neat fraction.

The results in Table 5.3 indicate that in the absence of contamination, and with good technique a sufficient number of single colonies are likely to be obtained using the spreading method; however, it was decided that these findings were not consistent enough to replace the dilution method.

5.1.7.2 Inoculum dilution method

(See Table 5.2)

5.1.8 Contamination

Contamination was a major problem in the pilot study and will be described at various stages of sediment processing.

5.1.8.1 Contamination and media preparation stage

Batches of Dubos agar, 7H11 agar and LJ plates were controlled for contamination in the media preparation facility, DMP before release. At the time of inoculation of plates with different dilutions, no contamination was observed on the plates. All the dilutions were therefore spread on plates that showed no evidence of contamination. All the plates were dried in the incubator for approximately one hour prior to inoculation. This was accomplished by placing the medium-containing compartment facing down, resting the inverted lid at an angle of ± 45° to allow space for air drying. Drying was performed on the metal surface of the incubator treated ("sterilized") by a peracetic acid formulation (Ultraseptin), followed by 70% alcohol.

5.1.8.2 Contamination encountered on plates inoculated with 10-fold dilutions of first five specimens

The table below indicates the number of plates found to be contaminated during the processing of the first five specimens in the pilot study.

Table 5.4 Contamination associated with the processing of five sputum specimens

		Number of plates inoculated/contaminated					
		After 1 week incubation			After 4 weeks incubation		
Solid Medium	Dispersal Method	Contaminated	Inoculated	%	Contaminated	Inoculated	%
Dubos	Vigorous	11	24	46	11	24	46
	Gentle	12	24	50	12	24	50
7H11	Vigorous	1	24	3	3	24	13
	Gentle	1	24	5	5	24	21
LJ	Vigorous	4	24	17	7	24	29
	Gentle	2	24	8.3	7	24	29
Total	Vigorous	16	72	22	21	72	29
	Gentle	15	72	21	24	72	33.3
	Both	31	144	21	45	144	31
	Dubos	23	48	48	23	48	48
	7H11	2	48	4.2	8	48	17
	LJ	6	48	13	14	48	29

5.1.8.3 Contamination on plates related to dilutions of specimens yielding MTB

Considerable contamination was encountered during preparation of the dilutions of sediments of specimen 4 and specimen 5 after four weeks' incubation (see Table 5.2).

A total of 72 dilution plates for vigorous dispersion were inoculated; of these, 11 Dubos plates, 3 7H11 plates and 7 LJ plates were contaminated. Therefore approximately 29% of the total vigorous dispersion plates were contaminated. A total of 72 dilution plates for gentle dispersion were inoculated; of these, 12 Dubos, 5 7H11 and 7 LJ plates were contaminated. Therefore approximately 33% of the total gentle dispersion plates were contaminated,

indicating that there was no difference in the contamination rates between the two different dispersal methods.

5.1.9 Summary of findings and conclusions drawn from pilot study

The pilot study was conducted for two purposes:

1) To gain information on and expertise in the techniques required for the processing of sputum samples to produce MTB clones in the form of CFUs that would possibly represent genetic heterogeneity in MTB loads in sputum samples and

2) To gain experience in the collection and processing of sputum samples from patients with MDR-TB under optimal safety conditions.

Based on the findings reported above, the following factors emerged as important and had to be addressed before the start of the main study.

5.1.9.1 Sputum collection

Sputum collection was performed according to standard practice at Sizwe Hospital. In the pilot study, some of the samples were very low in volume and there was no guarantee that the sample was collected satisfactorily. It was recommended that sputum samples be collected under staff supervision with the required safety features in place. It was decided that for the purpose of this study a microscopic examination of a Gram-stain to determine the ratio between squamous epithelial cells and inflammatory cells (Bartlett score) be performed on

each specimen before processing, in order to assess the quality of the sputum sample collected. The macroscopic appearance and volumes of each sputum sample were also recorded before processing in order to monitor the quality of the specimens collected for the main study. A sputum sample with a Bartlett score of 0, -1 and -2 suggests a poor specimen and in such cases consideration should be given to collect another specimen.

5.1.9.2 Specimen transport and labelling

Specimen transport, labelling and processing needed to be revised to circumvent problems identified in the pilot study. It was clear that specimens be transported directly to the laboratory for the procedures and processing required for the main study. A separate sputum sample would therefore be required for the research study, in addition to the sample collected for routine testing. This would require specific ethical approval from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand. The research sample should be transported immediately to the CTB for processing in the main study and a labelling and numbering system put into place before the commencement of the study. In addition, informed consent should be obtained and documented before samples are collected for the study.

5.1.9.3 Selection of Solid media

The use of different solid media to provide a greater variety of colony morphotypes which may be associated with heterogeneous subpopulations due to clonal heterogeneity or mixed infection with different clones was investigated. In the very limited experience of two

specimens producing colonies on three solid media there was evidence of subpopulations of colonies of different sizes (mainly two different sizes: small and large colonies) in both specimens and this was visible on all three solid media. It was concluded that only one solid medium was required and Middlebrook 7H11 agar plates was chosen for the major study.

5.1.9.4 Dispersal of MTB organisms

Comparison between the two dispersal methods used did not provide conclusive information and the decision to discontinue the streaking technique in favour of the plate dilution method only was made after discussions between scientists at the PHRI and the CTB. It was recommended only one method to disperse NALC sediment should be employed in the study and that 0.05% Tween 80 should not be used to enhance dispersal of MTB organisms. It was suggested that all sediments should be dispersed by the addition of two sterile beads to the sediment followed by 30 seconds of vortexing. Tween 80 was used to disperse MTB organisms in suspensions from decontaminated sediments (Mukamolova et al.); however, based on the results observed in the pilot study it was not certain as to whether a surface active agent such as Tween 80 may have a toxic effect thus affecting the growth of certain MTB subpopulations which may have affect the study. In an early study, it was shown that Tween 80 does have a bacteriostatic effect on MTB (Sattler and Youmans, 1948).

5.1.9.5 Selection of dilution factors based on smear microscopy findings

Only two specimens, both three plus positive for AFB could be used to correlate dilution factor with AFB's on smear microscopy, suggesting that 10^1 to 10^3 dilutions may be appropriate. Due to the delays in transport before the specimens were processed, it was suggested that a 10^4 fold dilution step be included in the main study.

5.1.9.6 Method of streaking out for single colonies

Contamination was identified as a major problem with this method. It was decided that the dilution strategy would be used instead of this method for the isolation of single colonies.

5.1.9.7 Contamination

Contamination was a problem in the performance of the pilot study. In the dispersion and dilution experiments a contamination rate of 45 out of 144 dilution steps (31%) was experienced. This was probably, at least in part due to relative inexperience in the processing of sputum samples, although contamination following NALC-NaOH decontamination for MTB culture is a universal problem. In a meta-analysis of ten studies dealing with the use of the MGIT system, a mean contamination rate of 8.6% with 95% confidence intervals of 8.2% to 9.9% was reported (Di Perri et al., 1989). Although the metal surfaces of the incubators were sterilized with peracetic acid and 70% alcohol, it was also suggested that the plate drying procedures may have contributed to the high level of contamination. Rigid precautions would have to be maintained in the main study to minimise the possibility of introducing

contamination during this procedure. The question of whether or not to use PANTA to reduce the risk of contamination was also addressed, however it was anticipated that with the use of a simplified protocol requiring fewer manipulations during specimen processing should help minimise the level of contamination. In addition, it was suggested that PANTA may have an inhibitory effect on certain MTB populations. Therefore the decision to not include PANTA in the solid media was made for the main study.

5.2 MAIN STUDY RESULTS

The main study was constructed in various phases and the various experiments were performed at three different laboratories, namely the CTB in Sandringham, RSA, the PHRI in Newark, New Jersey, USA and the DST/NRF Centre of Excellence for Biomedical TB Research (CBTRL) in Braamfontein, RSA.

5.2.1 Isolation of single colonies

Procedures involving sputum decontamination, culture and isolation of single colonies were conducted at the CTB. Twenty colonies were selected from sputum cultures of each patient and subsequently shipped to the US for molecular analysis. The details are given below:

5.2.1.1 Sputum quality as assessed by the Bartlett score

Based on the results from the pilot study, it was decided that the sputum quality for each specimen could conveniently be assessed according to the Bartlett score. A final score of 0 or less usually indicates a lack of active inflammation or contamination of the specimen with

saliva. Generally a repeat specimen should be requested if the sputum sample has a Bartlett score of 0 or less. Four of the five sputum samples had a good score of ≥1 indicating a good sputum sample. Specimen 3 had a Bartlett score of 0. A repeat specimen was not requested because smear microscopy indicated a P++ grading of AFB (see smear microscopy section 5.2.1.2) and growth on the Middlebrook 7H11 plates yielded sufficient MTB colonies for the study (see table 5.5).

5.2.1.2 Smear Microscopy for AFB

Fluorescence microscopy was performed on the sediment from each decontaminated specimen and the results are given below. All five specimens were positive for AFB. Smear microscopy was performed in order to provide information on the quantity of AFB present in the sputum sample as a rough guide to the bacterial load of MTB in a patient's sputum. The quantity of AFB in sputum, using x250 fluorescent microscopy, was recorded, based on guidelines for public health laboratories issued by the Centres for Disease Control (CDC) and Prevention, Atlanta, USA (Kent and Kubica, 1985), as follows:

Scanty: 1 to 9 AFB seen per 300 immersion fields

P+: 1 to 10 AFB seen per 10 immersion fields

P++: 1 to 10 AFB seen per immersion field

P+++: 10 to 90 AFB seen per immersion field

P++++: More than 90 AFB seen per immersion field

The microscopy findings on the study patients were:

Patient 1: P+++

Patient 2: Scanty 6 (per 300 fields)

Patient 3: P++

Patient 4: P++

Patient 5: P+++

5.2.1.3 Growth on Middlebrook 7H11 plates

Ten-fold dilutions, comprising neat, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were performed on each decontaminated patient sample before the plating out procedure commenced. Each dilution was plated out using disposable loops in order to obtain single colonies that could possibly be representative of the different MTB populations occurring in the sputum sample. The plating out procedures were performed in triplicate for increased accuracy of colony counting, but also as a back-up in case one set of plates may have become contaminated at some point during the eight week incubation time (based on the high contamination rates seen in pilot study). The plating out procedure was done in duplicate for Patient 5 once it was noted that contamination was not a major issue in the main study. The plates were labelled with the patient number, dilution factor date and the three sets of plate were labelled A, B and C.

Table 5.5 Mean number of CFUs from three sets of Middlebrook 7H11 plates at eight weeks' incubation

Patient	Dilution Factor				
	Undiluted	1 in 10	1 in 100	1 in 1000	1 in 10 000
1	>1000	>50	7	1	1
2	47	10	4	1	0
3	Semi-confluent	>1000	78	6	0
4	Confluent	>1000	>100	15	3
5	Confluent	Semi-confluent	>1000	>100	90

Twenty colonies were picked off the 7H11 plates and transferred onto LJ slants and incubated further. Once sufficient growth was present, scrapings of these slants were suspended in 0.5 ml of 7H9 broth and shipped to UMDNJ-PHRI for subsequent molecular analysis and strain typing.

5.2.2 Results from Molecular Characterization performed at UMDNJ-PHRI

Strain typing was performed at the UMDNJ-PHRI using IS*6110*-based RFLPs. In addition, sequencing of genes associated with first- and second-line drug resistance was performed on each set of colonies. The results obtained from the work conducted in the US are indicated below:

5.2.2.1 IS*6110*-based RFLP results

IS*6110*-based RFLP analysis was performed on 99 colonies out of the 100 colonies that were shipped to the US for molecular analysis. Once the isolates arrived at the PHRI laboratory,

they were immediately subbed onto LJ slants in order to obtain sufficient growth for the DNA extraction procedure. IS*6110*-based RFLP was not performed on colony 16 that was obtained from Patient 5 due to this sample being contaminated on arrival.

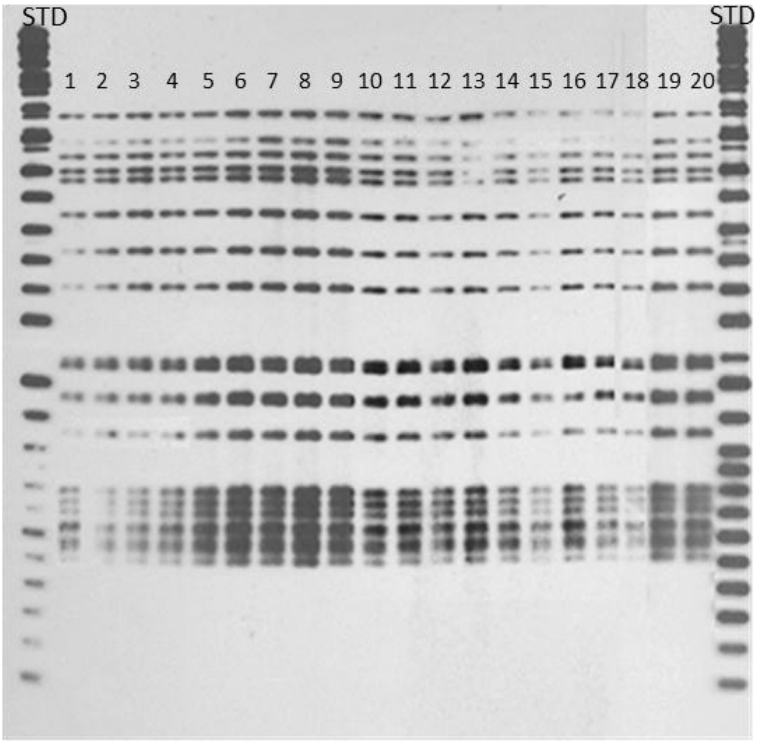

Figure 5.3 IS*6110* RFLP fingerprints from 20 MTB colonies isolated from Patient 1

(STD = molecular weight standard)

Figure 5.3 shows the RFLP fingerprint patterns obtained for the 20 colonies isolated from Patient 1. The strains were analysed and matched against the PHRI IS*6110* RFLP database. The PHRI TB Centre has a collection of more than 17 000 clinical MTB isolates acquired from countries throughout the world, including a large number of strains from different regions in RSA. All of these strains have been genetically characterised by IS*6110* RFLP typing, and the epidemiologic information together with the DNA fingerprints are catalogued

in an accessible database. The 20 colonies analysed for Patient 1 were all identified as the W799 strain which falls into PGG1, cluster II (see Figure 5.8). No evidence of heterogeneity or mixed infections were observed in the RFLP fingerprint patterns of Patient 1 in that all 20 colonies possessed the same number of bands present (roughly 20 bands) and identical RFLP banding patterns were observed. Mixed infection can sometimes present as a faint banding pattern behind a dark banding pattern (LIBs), however with single colonies this should not occur.

It is important to note that the nomenclature of IS*6110* fingerprints is not standard, and that different methods are used in different laboratories. The review article by Gagneux and Small compares the terminology and molecular markers for six main lineages of MTB and *M.africanum* (Gagneux and Small, 2007). The nomenclature used in this project is the same as that used by the PHRI TB laboratory.

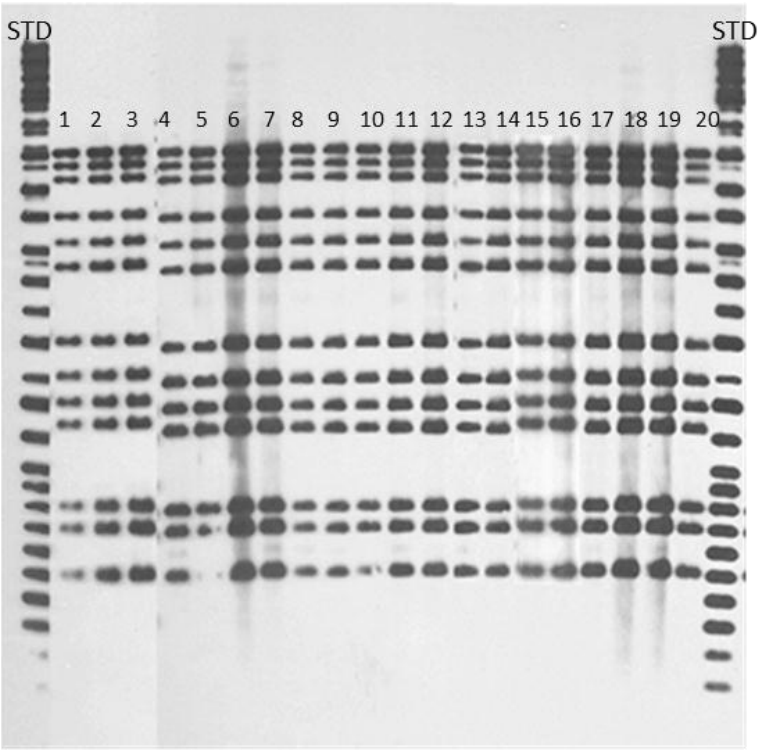

Figure 5.4 IS*6110* RFLP fingerprints from 20 MTB colonies isolated from Patient 2

(STD = molecular weight standard)

Figure 5.4 shows the IS*6110*-based RFLP fingerprint patterns obtained for colonies one to 20 isolated from Patient 2. The 20 colonies were analysed at the PHRI TB Centre and were identified as MH strains using pattern recognising software. The MH strain falls into PGG1, cluster II on the phylogenetic tree (see Figure 5.8). All 20 colonies possessed 13 IS*6110* bands and had identical RFLP fingerprints, thus no evidence of mixed infection or heterogeneity was observed in Patient 2.

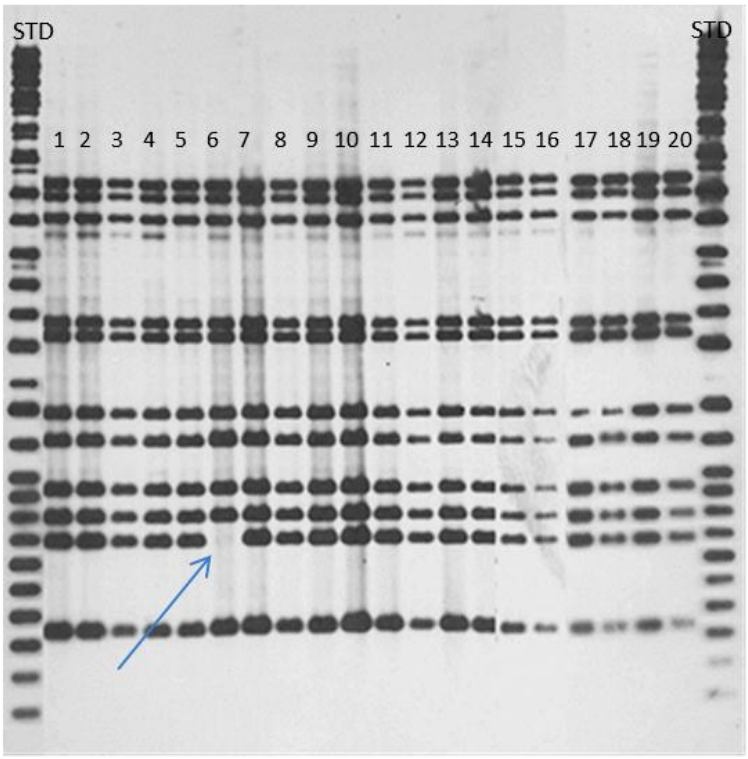

Figure 5.5 IS*6110* RFLP fingerprints from 20 MTB colonies isolated from Patient 3

(STD = molecular weight standard**)**

Figure 5.5 shows the IS*6110*-based RFLP profiles that were obtained for colonies one through 20 for Patient 3. All 20 colonies were identified as an AI variant, i.e. these profiles closely matched the AI strains that had been previously captured and saved into the PHRI database but were not identical. Nineteen of the 20 strains were completely identical in their RFLP banding patterns and contained the same number of bands (11 bands present). Colony six had a band missing compared to the other 19 colonies isolated from Patient 3 (only 10 bands present). The absence of the band (indicated by the arrow) could be attributed to one of two possibilities: 1) the missing band may have occurred due to a true deletion of an IS*6110* transposable element or 2) a SNP may have occurred in the *Pvu*II site in the flanking region of the DNA, thus resulting in a shift in the size of the band so that it is no longer apparent (i.e. band may have run off the gel if it was too small, or it may have been shifted to the same size

as another band in the pattern so the DNA fragments run together as a single band). In order to investigate this, the nylon membrane with hybridised DNA was subsequently hybridised with a probe specific to the left side of IS*6110*. The final blot obtained from the left-sided probe indicated 20 identical IS*6110* RFLP banding patterns, each containing the same number of bands (data not shown), suggesting that the missing band in colony six was a result of a change in the *Pvu*II site and not a deletion of an IS*6110* element. The AI strain falls into PGG2, cluster VI as seen in Figure 5.8.

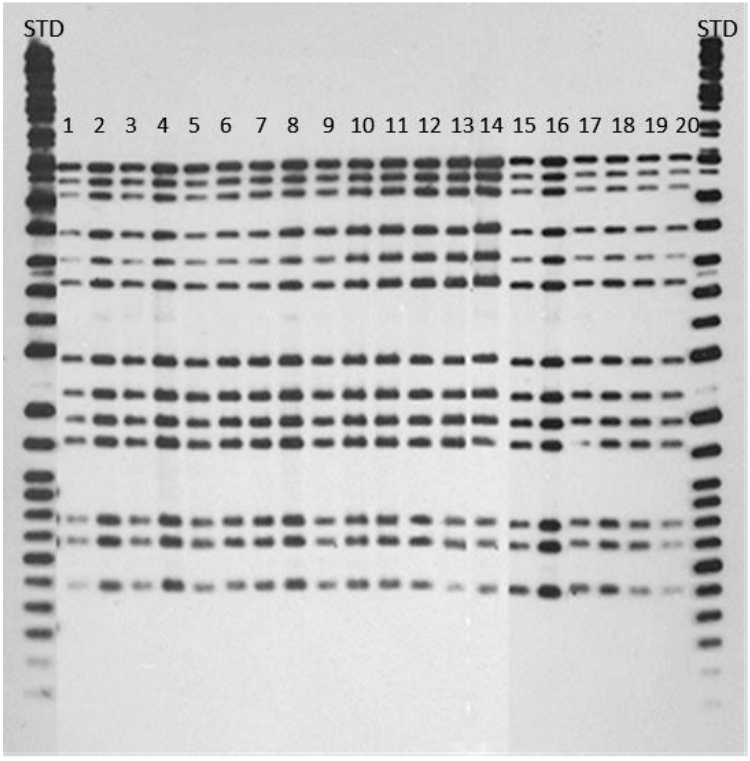

Figure 5.6 IS*6110* RFLP fingerprints from 20 MTB colonies isolated from Patient 4.

STD = molecular weight standard

Figure 5.6 displays the IS*6110* RFLP banding patterns obtained for the 20 colonies isolated from Patient 4. The 20 colonies all displayed 13 bands and identical IS*6110*-based RFLP

banding patterns. Using pattern recognising software available at the PHRI TB Centre, the colonies were identified as a MH strain. The MH strain falls into PGG1, cluster II as illustrated in Figure 5.8. It is interesting to note that the exact same strain was identified in Patient 2 suggesting the possibility of an epidemiological link between these two patients.

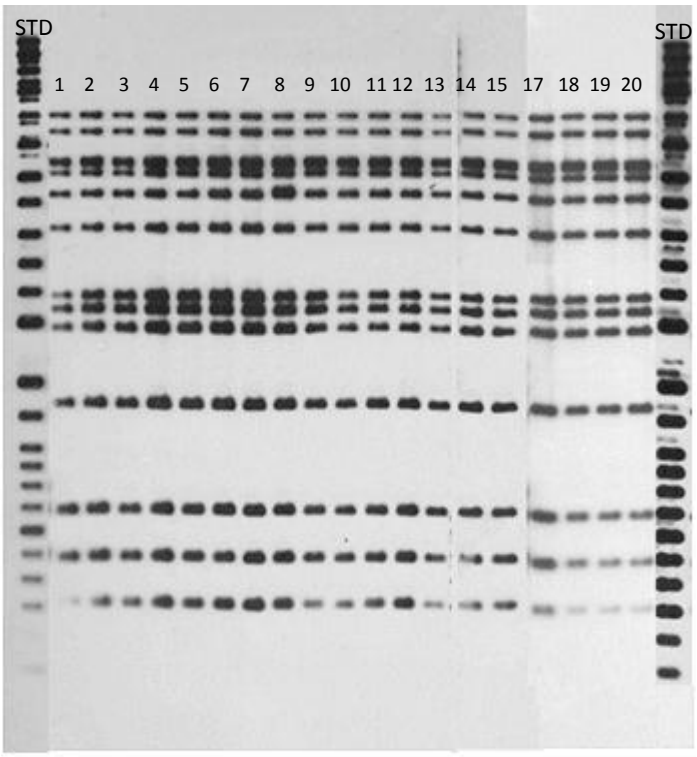

Figure 5.7 IS*6110* RFLP fingerprints obtained from 19 MTB colonies isolated from Patient 5

STD = molecular weight standard

Figure 5.7 shows the IS*6110* RFLP fingerprint patterns that were obtained from the 19 colonies that were isolated from Patient 5. Although 20 colonies were picked from this patient, colony 16 was contaminated on arrival in the USA. This single colony was therefore immediately disposed of to prevent any further spread of fungal contamination in the

laboratory. All 19 colonies demonstrated identical fingerprint patterns, each containing a total of 13 bands. Using pattern recognising software available at the PHRI TB centre, the colonies were all identified as the HP81 strain. The HP81 is also known as the KZN XDR strain which is notorious for the outbreak that it caused in Tugela Ferry, a rural settlement in KZN in 2006 (Pillay and Sturm, 2007). HP81 falls into PGG 2 cluster VI (as seen on the phylogenetic tree, Figure 5.8) No evidence of mixed infections or heterogeneity was observed in this patients as the fingerprint patterns from all 20 colonies were identical.

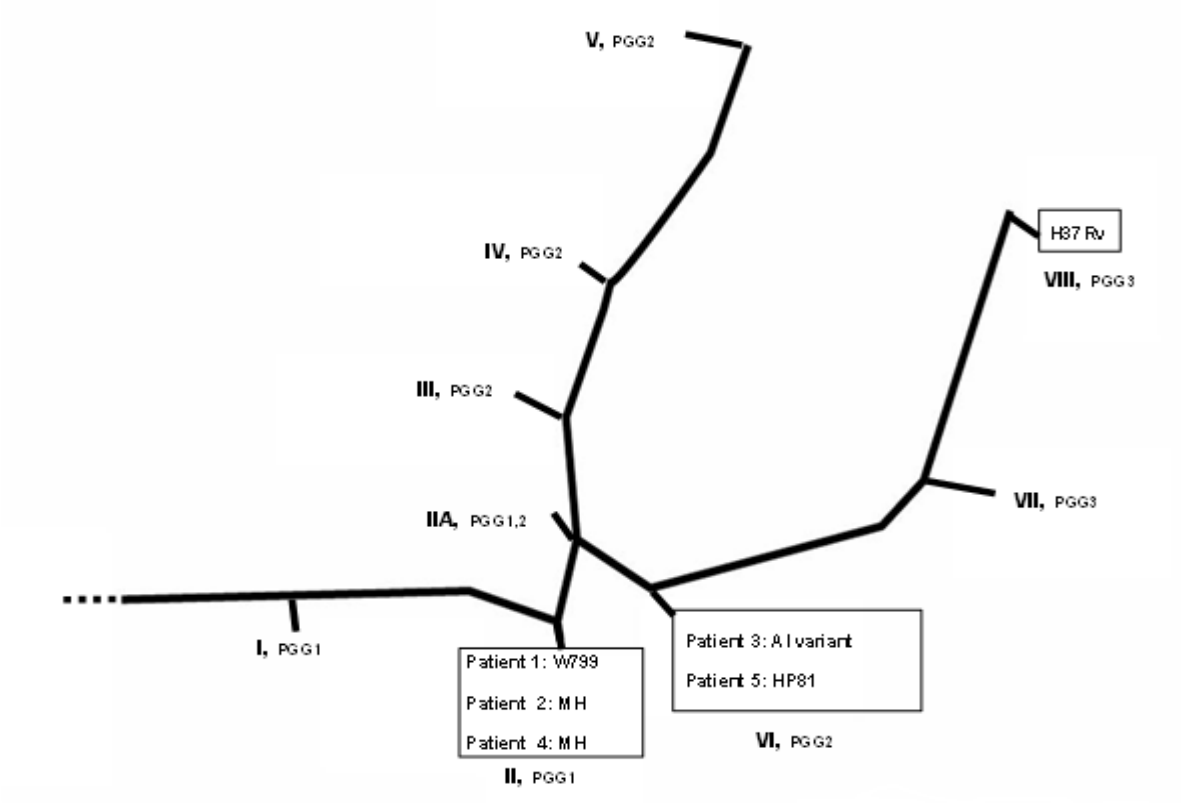

Figure 5.8 Patients 1 to 5 superimposed on the SNP-derived phylogenetic framework of MTB. Modified from Mathema et al., 2006.

The RFLP data obtained from Patient 1 identified all twenty colonies as the W799 strain. W799 falls within PGG 1 and cluster II on the phylogenetic tree. Colonies from Patients 2 and 4 were identified as MH strains and also fall within PGG1, cluster II. The colonies isolated from Patient 3 were identified as an AI variant using IS*6110* analysis and pattern recognizing software. It was classified as an AI variant in that it closely resembled the AI strains that were captured within the PHRI data collection, but were not completely identical to any of the strains in this collection. The AI strains fall within PGG2 and cluster V1 on the phylogenetic tree. Lastly, all 19 colonies examined from Patient 5 were identified as the HP81 strain.

5.2.2.2 Sequencing of genes associated with first- and second-line drug resistance

Sequencing of genes associated with drug resistance was performed on all 20 colonies for each patient to demonstrate the presence of possible genetic hetero-resistance within each set of patient isolates. The main genes associated with first-line anti-TB drug resistance include *rpoB* gene for RIF resistance and *katG* and *inhA* genes for INH resistance. STREP, EMB and PZA are also classed as first-line drugs. The genes sequenced that are associated with resistance to these drugs include the *rrs* and *rpsL* genes for STREP resistance, the *rrs* gene for KAN, AMK and CAP resistance and the *tlyA* gene for CAP resistance. The *embB* gene which is associated with EMB resistance was not sequenced as phenotypic and genotypic susceptibility to *embB* has been shown to correlate poorly (Mokrousov et al., 2002). The *pncA* gene was also sequenced and is associated with PZA resistance. The *gyrA* and *gyrB* gene were sequenced for OFX resistance. The sequencing data obtained from the five sets of patient isolates were all clonal and are summarised in Table 5.6.

Table 5.6 Sequencing data from 20 colonies isolated from five patients

Patient	*rpoB*	*katG*	*inhA-fabG*	*pncA*	*rpsL*	*rrs*	*tlyA*	*gyrA*	*gyrB*
1	H526L	wt	wt	wt	wt	wt	wt	wt	wt
2	S531L	S315T	wt	C14R	wt	A514C	wt	D94G	wt
3	S531L	S315T	wt	C14W	wt	A514C	wt	wt	wt
4	S531L	S315T	wt	C14R	wt	A514C	wt	D94G	wt
5	S531L	S315T	T-8A	del T pos 389 (codon 130)	wt	wt	wt	wt	wt

H = histidine; L = leucine; S = serine; T = threonine; A = alanine; D = aspartate and G = glycine; wt = wild type

Patient 1 harboured the H526L mutation in the *rpoB* region. Interestingly, DST results obtained for Patient 1 indicated that the patient was an MDR-TB patient (i.e. isolate resistant to both INH and RIF); however, no mutations were observed in any of the 20 colonies sequenced for *katG* or *inhA*. This patient may have had mutations in *kasA* or *ahpC* or the INH target gene *ndh* which codes for NADH dehydrogenase. None of these genes were investigated in this study. All the other resistance determining genes sequenced for Patient 1, (namely *pncA*, *rpsL*, *rrs*, *tlyA*, *gyrA* and *gyrB*) showed wild type sequences which were in agreement with the DST results that were performed on routine testing. Patient 2, 3, 4 and 5 displayed the common S531L mutations in *rpoB* region and the S315T mutation in *katG* gene. Patient 5 had a mutation present in both the *katG* and *inhA* regions which are both associated with INH resistance.

5.2.3 Phenotypic susceptibility testing and sequencing results

The DST by MGIT and corresponding sequencing findings on drug resistance-linked genes are summarised in Table 5.7.

Table 5.7 Drug susceptibility status and corresponding mutations relating to RIF, INH (low- and high-level) OFX and KAN resistance for the 20 colonies isolated from five patients

Patient	RIF	*rpoB*	INH 0.1	INH 0.4	*katG*	*inhA*	OFX	*gyrA*	*gyrB*	KAN	*rrs*
1	**R/S**	H526L	**R/S**	S	wt	wt	S	wt	Wt	S	wt
2	R	S531L	R	R	S315T	wt	R	D94G	Wt	S	wt
3	R	S531L	R	R	S315T	wt	S	wt	Wt	S	wt
4	R	S531L	R	R	S315T	wt	R	D94G	Wt	S	wt
5	R	S531L	R	R	S315T	T-8A	S	wt	Wt	S	wt

R = resistant; S = susceptible; wt = wild type

H = histidine; L = leucine; S = serine; T = threonine; A = alanine; D = aspartate and G = glycine

Bold type denotes a mixture of both susceptible and resistant DST results amongst patient colonies

DST was performed on each set of patient isolates for RIF, INH (low and high level resistance), OFX and KAN. The DST results are summarised in Table 5.7, along with the genotypic profiles that correspond to the selected drugs. All 20 colonies for Patient 1 harboured the H526L mutation in the *rpoB* region; however a mixture of both susceptible and resistant DST results were observed amongst the 20 colonies. In addition, mixtures of susceptible and resistant phenotypes were observed in Patient 1 for low level INH resistance, yet no mutations were observed in the 20 colonies isolated for both *katG* and *inhA*. The

discrepant results seen for Patient 1 are depicted more clearly in Table 5.8 where the DST results for each individual colony are illustrated. The DSTs were repeated twice to confirm the discordant results that were initially observed in the first set of DSTs. The only other discordant result observed between genotypic and phenotypic data was seen in patient 5, where routine DST showed resistance to STREP, yet both *rpsL* and *rrs* were wild type.

Table 5.8 DST results for INH (0.1 µg/ml) and RIF as well as the sequencing data for *rpoB, katG* and *inhA* obtained for 20 colonies isolated from Patient 1

	Rifampicin DST results			Rifampicin muation	INH DST results			INH muation	
Colony No	**1**	**2**	**3**	**rpoB**	**1**	**2**	**3**	**katG**	**inhA**
1	R	C	S	H526L	R	C	R	wt	wt
2	S	S	R	H526L	R	R	R	wt	wt
3	S	R	R	H526L	R	R	R	wt	wt
4	S	NG	R	H526L	R	NG	R	wt	wt
5	R	S	S	H526L	R	R	R	wt	wt
6	S	S	S	H526L	R	R	R	wt	wt
7	S	S	S	H526L	R	S	R	wt	wt
8	S	R	S	H526L	S	R	R	wt	wt
9	S	S	R	H526L	R	R	R	wt	wt
10	S	S	S	H526L	S	S	R	wt	wt
11	S	S	R	H526L	R	R	R	wt	wt
12	S	S	NG	H526L	R	R	NG	wt	wt
13	S	S	R	H526L	R	R	R	wt	wt
14	S	S	R	H526L	R	R	R	wt	wt
15	S	S	R	H526L	S	R	R	wt	wt
16	R	S	S	H526L	R	R	R	wt	wt
17	R	S	R	H526L	R	R	R	wt	wt
18	R	S	R	H526L	R	R	C	wt	wt
19	R	S	S	H526L	R	R	R	wt	wt
20	S	S	R	H526L	R	S	R	wt	wt

The DST results for RIF and INH are shown in addition to the corresponding mutations obtained from sequencing data. The DSTs were performed on three occasions

R = resistant; S = sensitive; C = contaminated MGIT; NG = no growth (growth control failed to grow).

MICS were performed for RIF on colonies highlighted in grey

As seen in the table above, a significant proportion of the colonies showed phenotypic susceptibility towards RIF despite harbouring the common H526L mutation. In fact in the first

DST experiment, 70% of the colonies indicated susceptibility towards RIF. In the second DST experiment the growth control tube from colony four failed to grow and colony one was contaminated. It was calculated that approximately 89% of the colonies in the second experiment showed susceptibility towards RIF. In the third round of DST experiments, the growth control from colony 12 failed to grow. In the third experiment approximately 42% of colonies showed susceptibility towards RIF. Poor genotypic and phenotypic correlation was observed in Patient 1 with regards to RIF resistance. A decision was made to determine MICs for a sample of the 20 colonies isolated from Patient 1 to determine whether or not the MICs for these colonies would also differ?

DST with regards to INH was also performed. In addition, a mixture of both susceptible and resistant phenotypes was observed with regards to low level INH resistance, but to a lesser degree compared to RIF. In the initial DST experiments, approximately 85% of colonies indicated resistance towards low level INH, whereas sequencing results showed that all 20 colonies were wild type for both *inhA* and *katG*. In the second set of DST experiments, 83% of colonies indicated resistance towards low level INH. In the third set of DST experiments, the growth control tube for colony 12 failed to grow. In addition the INH (0.1 µg/ml) test MGIT tube for colony 18 was contaminated. The remaining 18 colonies all indicated resistance to INH in the third round of DSTs.

Poor genotypic and phenotypic correlation was observed between the colonies selected for Patient 1. As mentioned earlier, these findings may suggest that the colonies isolated from this patient may have contained mutations present in other genes associated with INH resistance such as *kasA*, *ahpC* (Ramaswamy and Musser, 1998) or in the more recently identified INH

target gene *ndh*, which codes for NADH dehydrogenase (Lee et al., 2001). None of these genes were sequenced in this study.

5.2.4 MICs performed on selected colonies from Patient 1 for RIF

MICs for RIF were determined for certain colonies taken from Patient 1. Colony three, seven, nine, ten, 16, 18 and 19 were selected as these contained a selection of isolates that either remained consistently sensitive or switched from resistant to sensitive or the other way around. In addition, MICs were performed on three colonies taken from Patient 3 to act as a control as this patient demonstrated good correlation between genotypic and phenotypic data. An H37Rv strain was also included in the experiment. The results for the MICs were read after approximately two weeks of incubation. The MICs for all seven colonies taken from Patient 1 had identical MICs of 0.4μg/ml. In addition, the MICs from the three colonies isolated from Patient 3 also demonstrated identical MICs of 0.4 μg/ml. An H37Rv plate indicated an MIC of 0.1 – 0.2 μg/ml. MICs were not conducted on Patient 1 for INH since DST using the MGIT revealed that all 20 colonies were susceptible at high level INH (see Table 5.7).

CHAPTER 6

6 DISCUSSION OF MAIN STUDY FINDINGS

The present study was designed as an observational study aimed at examining in-depth for the possible presence of mixed MTB infections and/or clonal heterogeneity (including clonal hetero-resistance) in five patients with drug-resistant TB. Mixed infections are defined as the presence of two or more MTB strains with distinct fingerprints occurring during a single infectious episode, whereas clonal heterogeneity denotes slight variations in DNA fingerprint patterns or mutations in resistance-linked genes in single MTB strains, resulting in the presence of subpopulations of such strains in sputum specimens. These variations are due to mutations which act as evolutionary events such as sSNPs or nsSNPs that occur within an isolate originating from a single strain infecting a patient (Braden et al., 2001, Cave et al., 1994, Shamputa et al., 2004). Four out of the five patients selected for the study were "high-risk" for genetic heterogeneity in that they had received prolonged exposure to anti-TB drugs. In addition, discordant routine DST results had been documented in patients' hospital records in four out of the five patients (in two of these patients discordance involved ETH and EMB which could have been due to DST problems associated with these two anti-TB agents (Lin et al., 2009, Madison et al., 2002)), and being hospitalised, they were exposed to the risk of nosocomial transmission and mixed infections. Patient characteristics as well as treatment information for the five patients is given in Table 6.1.

Table 6.1 Patient characteristics, MTB strain types, treatment information and discordant routine DST results

Patient	Age (yrs)	Sex [a]	Diagnosis [b]	Strain [c]	Treatment History [d]	Previous TB treatment, months	On treatment at time of sputum collection [e]	Discordant DSTs? [f]
1	51	M	MDR	W799	R1,F2	> 18	Yes K,O,Z,Te	Yes R*
2	31	M	Pre-XDR	MH	F1	~ 8	Yes P,M.Z,H	Yes K, S*,O*Eth*
3	33	F	MDR	AI variant	F1	~ 6	No	Yes Eth
4	36	M	Pre-XDR	MH	D1	<6	No	Yes E*
5	29	F	Primary MDR	HP81	N	0	No	No

[a] M: Male, F: Female

[b] MDR: multidrug-resistant TB; XDR: extremely drug-resistant TB

[C] Strain identified by IS*6110*-based RFLP

[d] F1: failure after WHO category1 treatment, F2: failure after WHO category 2 treatment, D1: default after category 1 treatment, R1: relapse after WHO category 1 treatment N: new case

[e] K: kanamycin, O: ofloxacin, Z: pyrazinamide, E: ethambutol, Te: terizidone, P: para-aminosalicyclic acid. M: moxifloxacin, H: isoniazid

[f] R: rifampicin, Eth: ethionamide, E: ethambutol; S: streptomycin, K: kanamycin O: ofloxacin

* Discordant results seen after collection of sputum sample for study

Treatment outcome definitions used in Table 6.1:

- Relapse in a patient was defined as a situation where a patient who was "cured" or labelled as "treatment completed", is subsequently diagnosed with bacteriologically positive TB on sputum smear microscopy or culture.

- Treatment failures were patients who had begun treatment for smear-positive pulmonary TB but remained smear positive (or became smear positive again) after five months or during the course of treatment.
- Defaulters were defined as patients who interrupted their TB treatment for at least two months after having been on treatment.
- A new patient was defined as a TB patient who had received no prior anti-TB treatment (WHO, 2003)

IS*6110*-based RFLP genotyping was performed on the 20 colonies isolated from a single sputum sample from each of the five patients to investigate the possible occurrence of mixed infections, and/or clonal heterogeneity of MTB bacilli in the sputum samples. Sequencing of genes associated with drug-resistant TB was performed on the 20 colonies isolated from each patient to investigate possible genotypic hetero-resistance in a single strain. In addition, DST was performed on each set of 20 colonies to investigate the presence of phenotypic hetero-resistance within strains. MICs were performed on colonies from the same sputum sample, shown to belong to the same IS*6110* genotype and exhibiting phenotypic hetero-resistance on DST using the BACTEC MGIT 960.

6.1 DETECTION OF MIXED INFECTIONS USING IS*6110*-BASED RFLP

IS*6110*-based RFLP results from the 99 colonies analysed indicated that mixed infections were not present in any of the five patients that were recruited for this study. This was seen by the identical banding patterns observed among the 20 colonies (19 colonies for Patient 5) picked for each patient. In the past, it was generally accepted that TB was the result of an

infection with a single MTB strain and that infection with this strain was able to provide protection against infection with a secondary MTB strain (Stead, 1967); However, the occurrence of mixed populations of MTB in a single patient is no longer disputed but relatively few studies on their importance, epidemiology and frequency have been recorded in the literature.

Table 6.2 highlights six key studies that investigated the occurrence of mixed infections in patients infected with pulmonary MTB in different regions and settings. Of the six studies, two were conducted in Cape Town, South Africa, one in Bangladesh, one in a prison TB-hospital setting in Georgia, and the remaining two were carried out in Zambia and Malawi.

The frequency of mixed infections in the six studies varied from 1.2% in cultured isolates from the Karongo district in northern Malawi (Mallard et al., 2010) to 19% in sputum isolates from Cape Town, South Africa (Warren et al., 2004). Mixed strains were not observed in a comprehensive study conducted in the Netherlands (low-incidence setting) where 1,277 IS*6110* RFLP isolates were examined for the presence of mixed infections (de Boer et al., 2000; study not shown in table).

Table 6.2 Key studies on mixed infections in patients with pulmonary MTB

Year	2002	2004	2004	2006	2010	2010
Author	Richardson et al.	Warren et al.	Shamputa et al.	Shamputa et al.	Mulenga et al.	Mallard et al.
Location	Cape Town, South Africa	Cape Town, South Africa	Bangladesh (Mymensingh District)	Georgia (Prison TB Hospital)	Zambia (4 Diagnostic TB Centres)	Malawi (Karongo District)
DNA fingerpring technique employed	IS6110 RFLP Direct repeat (DRr)	PCR amplification (Beijing and non-Beijing)	IS6110 RFLP Spoligotyping MIRU-VNTR	IS6110 RFLP MIRU-VNTR	Spoligotyping MIRU-VNTR	PCR amplification (LAM and non-LAM) IS6110 RFLP Spoligotyping
Patients						
No of patients enrolled	210	407	132	385	361	72 and 347
No of patients analyzed	131	186	97	199	273 (156 for MIRU)	72 (Sputum) 347 (Culture)
First-time	210	200	1	134/199	Yes	Yes (62)
Retreatment records	No	207	131	65/199	Yes	Yes (10)
HIV positive status	All HIV Negative	NA	NA	NA	NA	60% of culture positive patients
Specimens						
Single	Yes (74)	Yes	Yes	No	Yes	Yes
Multiple	Yes (136)	No	No	Yes	Yes	Yes
AFB +ve	Yes	Yes	Yes	Yes	Yes	Not Given
Sputum	No	Yes*	No	No	No	Yes/No
Culture	Yes	Yes/No	Yes	Yes	Yes	Yes
Sputum collected before commencement of treatment?	Yes	Yes (200)	Yes	Yes	Yes/No	Yes/No
DST status	NA	NA	Yes	Yes	NA	NA
Colonies from primary culture	NA	NA	10	NA	NA	NA
No of patients with mixed infections (%)	3/131 (2.3%)	35/186 (19%;4.8% by spoligotype)	2/97 (2.1%)	26/199 (13.1%; 9% by IS6110)	5/156 (3.2%)	2/160 (Sputum: 2.8%) 4/347 (Culture: 1.2%)

* Sputum specimens were cultured in BACTEC medium (BD, Frankilin Lakes, NJ) afterwhich crude DNA preparations were obtained
NA = Not available

Two of the studies presented in Table 6.2 stand out in that they both observed a relatively high rate of mixed infections in comparison to the other four studies (Shamputa et al., 2006, Warren et al., 2004). Warren et al., (2004) used a novel PCR technique and demonstrated that 19% of the patients analysed in their study were simultaneously infected with a Beijing and non-Beijing MTB strain. Shamputa and colleagues, (2006) analysed several pre-treatment isolates from adult inmates admitted to a prison TB hospital in Georgia using standard IS*6110* DNA fingerprinting followed by MIRU-VNTR and observed mixed infections in 13.1% of patients analyzed. The high mixed infection rates seen in these two studies occurred in settings of overcrowding coupled with a high incidence of TB. In Cape Town the incidence was 251 cases per 100 000 (Beyers et al., 1996), while in the prison population in Georgia it was an estimated 5,995 per 100 000 population (WHO, 2000). Although a high incidence of TB may have accounted for the high rate of mixed infections seen in the study by Warren et al., (2004), Richardson et al., (2002) also conducted their study in Cape Town but found the frequency of mixed infections to be markedly lower (2.3% vs. 19%). This result is similar to the frequency of mixed infections seen in Bangladesh (2.1%) (Shamputa et al., 2004). These latter two studies used IS*6110* RFLP analysis as their primary fingerprinting techniques. In the Bangladesh study, a single sputum sample per patient from 97 patients was obtained but 10 colonies per sample from these patients were analysed (Shamputa et al., 2004), whereas the Richardson et al., (2002) study in Cape Town analyzed serially collected sputum samples (210 in total) from 131 patients.

The variation in the frequency of mixed infections observed between the two Cape Town studies is most likely due to a) differences in the processing of sputum samples e.g. neutralized deposits of smear microscopy-positive sputum samples or MTB cultures, prior to

DNA extraction, or other innovative ways such as direct PCR on sputa cultured for seven days in MGIT broth (Warren et al., 2004), or b) DNA fingerprinting techniques used to detect mixed infections. Richardson et al., (2002) used IS*6110*-based RFLPs and additional fingerprinting using the direct repeat probe (DRr) on cultured samples, whereas Warren et al., (2004) used a novel PCR reaction with primers to detect Beijing and non-Beijing strains directly on sputum samples cultured for seven days in BACTEC medium (7H9 broth) before performing PCR. The sensitivity of the mycobacterial culture and fingerprinting techniques used for the identification of mixed strains by Richardson et al., (2002), is likely to be lower than the PCR-based method used by Warren et al., (2004) because of the ability of PCR to amplify minimal amounts of specific DNA. The authors however, point out that their assay detects only mixed infections between Beijing and non-Beijing strains but not mixed infections involving different Beijing strains or different non-Beijing strains and therefore represent a conservative estimate of the frequency of mixed infections present in this particular setting. However, when spoligotyping, which is less discriminatory than IS*6110*, was used on cultures by Warren et al., (2004) in the same study, the frequency of the mixed infections identified dropped from 19% to 4.8% which is still appreciably higher than the 2.3% frequency of mixed infections recorded by Richardson et al., (2002).

Shamputa and co-workers, (2006) also recorded a high frequency of mixed infections in a Georgian prison TB hospital. The investigators used IS*6110* RFLP as an initial screening method on multiple pre-treatment sputum samples and observed mixed infections in 9% of the patients analysed. When MIRU-VNTR was used as a secondary typing tool, mixed infections were observed in 13.1% of the patients. Based on the premise that LIBs in an IS*6110* RFLP profile of an MTB isolate may be present as a result of multiple infections, de Boer et al,

(2000) noted that IS*6110* RFLP as an initial screening method may lead to an underestimation of mixed infections within a single isolate since it is unclear as to what extent low ratios of one of the strains present in a mixture may have been reflected in LIBs. It is also possible that these strains may not have been detected at all. In this prison hospital mixed infections missed by IS*6110* were detected using a more discriminatory method in three patients out of 30 (10%) whose strains possessed identical IS*6110* banding patterns but displayed double alleles at multiple loci using MIRU-VNTR. In addition five patients out of 30 (17%) showed evidence of mixed infection by MIRU-VNTR typing but only displayed minor differences (≤ 3 bands) using IS*6110* based RFLPs reflecting clonal subpopulations as opposed to mixed infections (Shamputa et al., 2006). Using extrapolation, the authors noted that they may have missed up to 7% of mixed infections in the remaining 139 patients whose isolates were not subjected to MIRU-VNTR analysis.

In addition to the type of fingerprinting method used, Shamputa et al., (2006) noted that analysis of a single pre-treatment isolate (as opposed to multiple pre-treatment isolates) will have led to missed mixed infections in 14 of the 26 mixed infection cases that were observed in this study. Although mixed infections in a single and/or multiple sputum samples are likely to originate from separate lesions in the lungs that contain different MTB strains, the above mentioned findings suggest that a single sputum sample may not be representative of the entire bacillary population in the lung and that the occurrence of mixed infections (and heterogeneous subpopulations) in the lung may be frequently underestimated when analysing a single sputum sample.

More recently, a study was conducted to detect the occurrence of mixed infections of MTB strains in sputum samples taken from patients in Malawi (Mallard et al., 2010). The investigators used a PCR-based approach and applied two lineage-specific PCR assays to sputum samples, targeting the Latin American Mediterranean (LAM) and non-LAM genotypes (the most common genotypes present in northern Malawi), as well as Beijing and non-Beijing genotypes. The authors found mixed infections in 2.8% of patient sputum samples, with the LAM - non-LAM assay, significantly lower than the 19% mixed infection rate observed in the Cape Town study that investigated the occurrence of Beijing and non-Beijing strains in a single sample. Mallard et al., (2010) proposed that the differences found between their study and the earlier Cape Town study could be explained by differences in the annual risk of infection (3% in Cape Town (Kritzinger et al., 2009) versus 1% in the rural Karongo district in Malawi (Crampin et al., 2009)), or the laboratory methods employed in the studies. In the Mallard et al. study, (2010) DNA was extracted directly from the sputum samples, whereas in the Cape Town study the sputum samples were first cultured in BACTEC medium, thus possibly enhancing the sensitivity of the assay. On the other hand, culturing the sputum sample may have had a negative influence on mixed populations in that this may have resulted in overgrowth of one MTB strain over another. Although both studies used a PCR based approach, there were also fundamental differences in the PCR method used. For example, the Beijing/non-Beijing assay used by Warren at al., (2004) was based on non-competitive primers. In addition the investigators used an extra five PCR cycles which may also have enhanced the sensitivity of the assay.

It is also possible that the DNA fingerprinting technique employed in our study (IS*6110*-RFLP) was not sufficiently sensitive to detect the presence of mixed infections. As seen in the

study conducted by Shamputa et al., (2004) three separate techniques were used and the frequency of mixed infections found was still low (2%). Although IS*6110* RFLP is the gold standard technique that is used for the genotyping of MTB isolates, certain studies have found other fingerprinting techniques or genotyping methods to be superior in identifying mixed infections (Shamputa et al., 2006). A novel PCR technique, such as that used by Warren et al., (2004) or MIRU-VNTR (also a PCR-based technique) and may have been more sensitive tools for identifying mixed infections.

Other factors that may have decreased the chances of finding mixed infections in the present study may include the manner in which sputum samples are routinely processed and decontaminated. Sputum decontamination may have decreased the possibility of finding mixed infections, i.e. the decontamination process and culture technique may have introduced an element of strain selection. The study protocol was designed to limit the amount of specimen manipulation and culturing to reduce the possibility of the selection of one strain over another. For example, the use of Tween 80 was excluded when vortexing the specimen to break up the cords as there was a possibility that this reagent may have had an inhibitory effect on the growth of certain MTB populations, although this has not been demonstrated. In addition, Shamputa et al., (2006) also proposed that a single sputum sample may lead to an underestimation of the frequency of mixed infections. In the present study design, single colonies were selected from a single sputum sample. This may possibly explain why no mixed infections were observed in this study. It is possible that a single sputum sample is not representative of the total bacillary population in the lung and bacilli occurring at different pulmonary infection sites are not necessarily released in the same sputum sample. In fact, Shamputa and co-workers, (2006) indicated that if only a single pre-treatment sputum sample

was analysed using IS*6110* based RFLP or MIRU-VNTR, none to only half of the mixed infection samples would have been identified. The collection of multiple sputum samples taken from the same patients may have increased the possibility of identifying mixed infections or hetero-resistant populations occurring in the lung. Organisms isolated from separate physical locales in the lung may also develop independent mutations resulting in the acquisition of drug resistance compared to organisms located in separate parts of the lung. In addition, we also looked at a small sample size in-depth and an increase in patient numbers may have revealed the presence of mixed infections.

6.2 DETECTION OF CLONAL HETEROGENEITY BY IS*6110*-BASED RFLP

Although mixed infections were not observed amongst the 20 colonies isolated in the present study, clonal heterogeneity was observed in terms of a variation in fingerprinting patterns amongst the 20 colonies isolated from one of the five patients. One colony (colony six) from Patient 3 had a band missing (only ten bands present on the blot) on the IS*6110* blot, compared to the other 19 colonies (11 bands present on the blot) that were genotyped. This finding could suggest that approximately 5% of the colonies taken from this sputum may display this particular mutation, although the absence of the missing band is most likely to be a result of a random event. The absent band would have been missed if individual colonies were not picked and if IS*6110* RFLP was performed on the culture alone in that the missing band would have been disguised by the presence of the "absent" band in the remaining 19 colonies.

6.2.1 IS*6110* band shift in colony six isolated from Patient 3

It was investigated whether the absence of the band in colony six was due to the transposition /loss of an IS*6110* element or whether it was due to a loss or gain of a *Pvu*II site that would have occurred due to a SNP occurring in the DNA flanking region of the *Pvu*II site. It is more likely that a SNP would have resulted in a loss of the *Pvu*II site as a six-base restriction site (CAGCTG) would not show up via random de novo mutations unless there may have already been a near perfect site in the DNA, for example, a site with one nucleotide difference that would have resulted in a new restriction site via a SNP. A change in a *Pvu*II cleavage site (either gain or loss of a *Pvu*II site) would result in a change in the size of the DNA fragment. If the cleaved DNA fragment was smaller than the original DNA fragment due to a gain in a *Pvu*II site, the DNA may have run off the gel, therefore not appearing as a visible band on the IS*6110* blot (therefore appearing absent compared to the other colonies). In addition, the new DNA fragment may have been larger (due to a loss in a *Pvu*II site) and would thus be located further up on the blot, however this was not seen in the instance of colony six. It is however possible that the new DNA fragment may have had a similar molecular weight (similar size) to another fragment of DNA that was already present. This would result in the two DNA fragments of similar size moving together on the agarose gel when subjected to electrophoresis and thus presenting as a single band on the IS*6110* RFLP blot (the band containing the two DNA fragments may be slightly thicker compared to other bands only containing one DNA fragment, for example in the remaining 19 colonies where the SNP did not occur). In order to further investigate the absent band present, the original nylon membrane with the hybridised DNA was re-hybridised with an IS*6110* probe specific to the left side of the insertion sequence, IS*6110*. Hybridisation with a left-side probe allowed for the

visualisation of the DNA this time looking at the "left side" of the MTB chromosomal DNA sequences flanking the site of the IS*6110* insertion. Results of the left-probe blot displayed 20 identical colonies with the same number of IS*6110* bands suggesting that the absence of the band was in fact due to a SNP in the *Pvu*II site. If colony six was still missing a band when compared to the other 19 colonies when using the left probe, the missing band would then have been attributed to a loss of an IS*6110* insertion element. Since the absence of the band was most likely due to a SNP in a *Pvu*II site, this was most likely a random event. Evolution involves a selection process whereby less fit organisms eventually die out, whilst advantageous mutations are selected for and thus grow and reproduce. There is probably no selective advantage for the variant *Pvu*II site in colony six; however, a small variant sub-population could still outgrow other MTB populations if it is in a separate location from the predominant population – for example, within a granuloma that enlarges to form a cavity where the mutant strain can reproduce. This kind of neutral genetic change could thus result in a genetic drift.

6. 3 DETECTION OF CLONAL HETERO-RESISTANCE BY SEQUENCING OF GENES ASSOCIATED WITH DRUG RESISTANCE

Hetero-resistance was investigated by analyzing mutations in genes that determine drug resistance to first- and second-line anti-TB agents. The genes that were sequenced included *rpoB*, *katG*, *inhA*, *gyrA*, *gyrB*, *rrs*, *rpsL*, *tlyA* and *pncA*. Hetero-resistance was not observed in any of the sputum samples of the patients enrolled in this study as identical genotypic profiles were observed amongst the 20 colonies isolated from each patient. None of the 20 colonies

(or 19 in the case of Patient 5) showed evidence of wild type and mutant sequencing profiles indicative of hetero-resistance.

Three key studies demonstrating clonal heterogeneity, including hetero-resistance, feature in Table 6.3. The phenomenon of hetero-resistance was demonstrated by the analysis of lesions taken from the lungs of patients subjected to therapeutic pneumonectomy (Kaplan et al., 2003). The investigators performed sequencing on genes associated with MTB drug resistance. A mixture of both drug-susceptible and drug-resistance MTB strains was observed in three out of three culture-positive patient samples. In another study by Post et al., (2004) in which serial sputum isolates from chronic MDR patients were examined, sequencing was also performed on selected genes associated with MTB drug resistance. In addition to some of the patients acquiring drug-resistance mutations during the study period, the investigators demonstrated the existence of clonal hetero-resistance in four out of 13 patients analysed. It was suggested that patients infected with hetero-resistant MTB populations may require anti-TB treatment targeting both drug-susceptible and drug-resistant phenotypes (Post et al., 2004). In a study where ten individual colonies, selected from each MTB sputum culture, were typed by spoligotyping, IS*6110* RFLP and MIRU-VNTR, clonal heterogeneity (not linked to resistance related genes) was observed in eight out of 97 patients based on variations in colony spoligotypes and IS*6110* RFLP fingerprints (Shamputa et al., 2004). It has been suggested that the presence of such bacterial subpopulations may be a result of gradual evolutionary changes in MTB that occur post-infection, following adaptation in a new host environment (de Boer et al., 1999).

Table 6.3 Comparison of three key studies with the present study on clonal heterogeneity, including hetero-resistance, in patients infected with pulmonary MTB

Year	2003	2004	2004	2012
Author	Kaplan et al	Post et al	Shamputa et al	Present Study
Location	Cape Town, South Africa	Cape Town, South Africa	Bangladesh (Mymensingh District)	Sizwe Hospital Gauteng, South Africa
Genes sequenced	*rpoB* , *katG* , *inhA* , *pncA* , *embB* , *rpsL* , *rrs* , *gyrA*	*rpoB, katG, mabA-inhA, pncA, embB, rpsL, rrs, gyrA*	Not Done	*rpoB, katG, inhA, pncA, rpsL, rrs, gyrA, gyrB, tlyA*
Drug Susceptibility Testing	ND	RIF, INH, EMB, STREP, KANA, OFLOX, Eth, Th	RIF,INH, EMB,STREP	RIF,INH (0.1µg/ml; 0.4µg/ml) KANA, OFLOX
Fingerprinting techniques employed	IS6110-based RFLP	Spoligotyping IS6110-based RFLP	Spoligotyping, IS6110-based RFLP, MIRU-VNTR	IS6110-based RFLP
Patients				
No of patients enrolled	6	13	132	5
No of patients analysed	3	13	97	5
Treatment records	Yes	Yes	131/132	Yes
Duration of treatment (mo)	7 to 24	3 to 16	NA	0-18
HIV positive status	All HIV negative	All HIV negative	NA	All Positive
Specimens				
Specimen Type	pneumonectomy	sputum	sputum	sputum
Drug resistant isolates	2 MDR-TB	All chronic MDR-TB	NA	Yes
Serial isolates examined?	No	Yes	No	No
AFB positive	Yes	Yes	Yes	Yes
Culture	Yes	Yes	Yes	Yes
DST status	NA	Yes	Yes	Yes
Sample collected before the commencement of treatment?	No	No	Yes	Yes and No
Colonies from primary culture	No	No	10	20
No of patients displaying clonal heterogeneity (%)	3/3 (100%)*	4/13 (31%)*	8/97 (8.2%)[§]	1/5 (20%)[§]

NA = Not available

mo = months

* Hetero-resistance associated with resistance determining genes

[§] Clonal heterogeneity based on DNA fingerprinting

6.4 ABSENCE OF SINGLE-STRAIN HETERO-RESISTANCE AND MIXED INFECTIONS IN FIVE PATIENTS OF PRESENT STUDY

Although the present study was designed with the expectation that, based mainly on the Cape Town studies of Kaplan et al., (2003) and Post et al., (2004), single strain hetero-resistance and/ or mixed strain are common in MDR-TB patients receiving treatment, and would be detected in a small sample size of high-risk patients, it was performed as an antecedent to a much larger prospective study which was specifically designed to detect and study the evolution of drug resistance in MDR-TB patients and the occurrence of mixed infections and hetero-resistance.

6.4.1 Factors in present study favouring genetic heterogeneity

In the design of the study, the following factors were considered to favour hetero-resistance:

- Information available in the literature suggesting that the presence of genetic heterogeneity was common in chronic MDR-TB patients (Kaplan et al., 2003, Post et al., 2004). The present study was designed with the assumption that similar rates of mixed infections, as well as hetero-resistance would be observed in patients from Sizwe Hospital. Kaplan et al., (2003) examined pneumonectomy specimens from six treatment refractory patients, of which three were culture positive. Histological examination of the three culture-specimens revealed hetero-resistance in all three patients in at least one out of the eight genes analysed by sequencing. In addition, Post et al. (2004) demonstrated the occurrence of clonal hetero-resistance in serially

recovered sputum in four out of 13 patients analysed (4/33 specimens from four patients [21.1%]). Both studies identified high rates suggesting that mixed infections and clonal heterogeneity in MTB are common. The present study was designed on the assumption that similar rates of mixed infections and clonal heterogeneity would be present.

- Prolonged exposure to both first- and second-line anti-TB drugs in four out of the five patients

Four of the five patients selected for the study had received previous treatment for TB and were thus considered to be "high-risk" for the presence of mixed infections and/or hetero-resistance. Patients 1 and 2 had received both first and second-line anti-TB drugs before the commencement of the study and had been treated for ± 18 and eight months respectively. Patient 3 and 4 had both received first-line anti-TB therapy. Patient 3 was admitted at Sizwe Hospital as a treatment failure patient, whereas Patient 4 was admitted as "after default" of first-line therapy. Patient 5 was admitted with primary MDR-TB and had therefore not received any previous treatment.

- Discordant routine DST was reported in four out of the five patients.

Discordant DST results were initially observed in Patient 2 for KAN and the patient was registered as an XDR patient at Sizwe Hospital. Subsequent DST indicated that the isolate was susceptible to KAN. After sputum collection, additional discordant routine DST results were

observed for OFX, STREP and ETH. In Patient 3, discordant DST results were observed for ETH. For ETH, *inhA* and *katG* were sequenced. The *ethA* gene was not sequenced in the present study. Approximately one month after sputum collection for the study, discordant DST results in Patient 1 were observed for RIF, and discordant DST results in Patient 4 were observed for EMB, although DST for EMB has been reported to be problematic (Madison et al., 2002).

- Increased chance of transmission and or super-infection in hospitals and TB clinics.

Two out of five patients had been admitted to the same hospital (Sizwe Hospital) for prolonged periods, (Patient 1, >12 months; Patient 2, > 2 months), increasing the chances of transmission and/or super-infection of MTB strains. In addition, the two newly admitted MDR patients (Patient 3 and Patient 4) would have also been exposed to other TB patients at hospitals and TB clinics thus increasing the chances of transmission and super-infection. Shamputa et al., (2006) found high rates of mixed infections in a prison TB-hospital setting where the likelihood of super-infection with additional MTB strains was high.

- Number of colonies tested.

Shamputa et al., (2004) selected ten individual colonies which were screened for detectable mixed infections (2.1%) and heterogeneity (8.2%). In the present study, 20 individual colonies were selected per patient/ specimen but only 5 patients were included in the study.

6.4.2 Evidence from literature indicating realistic sample sizes for demonstration of mixed infections and hetero-resistance in patients receiving anti-TB treatment

Assessment of the literature suggests that the occurrence of mixed infections and clonal hetero-resistance may not be as common as that observed in the two studies mentioned in section 6.4.1. In their earlier study, Shamputa et al., (2004) detected clonal heterogeneity in eight out of 97 patients (8.2%) and found the frequency of mixed infections to be 2.1%. Two years later, the investigators looked at multiple sputum isolates obtained from a prison TB hospital in Georgia and found mixed infections in 13.1% of the patients analysed. Furthermore, in a study conducted in KZN, spoligotyping and MIRU-VNTR performed on 56 necropsy cultures indicated that five (9%) were mixed and four (7%) displayed clonal heterogeneity (Cohen et al., 2011). The authors also stated that MDR-TB is not a significant factor for the presence of hetero-resistance (mixed infection or clonal hetero-resistance) (OR 0.47; 95% CI 0.05-4.33; p=0.51). These authors also indicated that prolonged treatment in time would tend to reduce the frequency of hetero-resistance and mixed infections. They stated: "While it did not reach the level of statistical significance, there is a suggestion that being on treatment for TB at the time of death reduced the possibility of complex infection (OR, 0.27; 95% CI 0.06-1.20; p=0.08). This may reflect the selective pressure of standard drug treatments, which we expect should rapidly eliminate drug-sensitive bacteria; this hypothesis is supported by the strong positive association between being on treatment at the time of death and the detection of MDR-TB, among these individuals (OR, 9.0; CI 1.06-76.48; p=0.04)."

6.5 STRAINS IDENTIFIED BY IS*6110* RFLP

Four different MTB strains were identified from the five patients enrolled in this study. The 20 colonies isolated from Patient 1 were all identified as W799 - a W-Beijing strain. This strain falls within PGG1 [katG codon 463 CTG (Leu) and gyrA codon 95 ACC (Thr)], cluster II on the phylogenetic tree (Gutacker et al., 2002). The strain identified in Patient 2 was an AI variant which falls into PGG2, cluster VI. Patient 2 and Patient 4 both had identical IS*6110* RFLP banding patterns which were both identified as MH strains. Furthermore, both Patient 3 and Patient 4 displayed identical mutations in the sequencing data (*rpoB*: S531L; *katG*: S315T; *gyrA*: D94G). It is distinctly possible that there is an epidemiological link between Patient 2 and Patient 4, although it is unlikely that this may be due to super-infection at Sizwe Hospital since Patient 4 was a newly admitted patient, whereas Patient 2 was a pre-XDR patient that had been on treatment at Sizwe for approximately two months before the sputum sample was collected for the study. It is also possible that the MH strain may be a highly prevalent strain in the Gauteng region. Further epidemiological research is required on the distribution of MTB strains occurring in South Africa to substantiate this possibility. The MH strains fall under PGG1, cluster II on the phylogenetic tree. The MTB strain identified in Patient 5 was the HP81 strain. This strain is also known as the KZN/LAM4/F15 strain which caused the outbreak of XDR-TB in Tugela Ferry, RSA in 2006 (Pillay and Sturm, 2007). HP81 falls into PGG II, cluster VI on the phylogenetic tree.

6.5.1. W-Beijing strains

MTB colonies isolated from Patient 1 were identified as belonging to W799, a member of the W-Beijing family. W-Beijing strains have been extensively reported in the literature. The W-Beijing family of MTB strains have been found to highly prevalent throughout Asia and countries of the former Soviet Union and have been reported in many other geographical settings as well. In fact, the epidemiological settings of W-Beijing are diverse and these strains have been known to cause outbreaks in institutional and nosocomial settings as well as resulting in ongoing transmission. Strain W (a W-Beijing strain) was identified as the cause of an MDR-TB outbreak in New York City in the early 1900's which was associated mainly with crowed homeless shelters and HIV seropositive individuals (Bifani et al., 1996, Frieden et al., 1996, Valway et al., 1994). W-Beijing isolates were also reported to comprise approximately 20% of all MTB isolates found in the Western Cape (van Helden et al., 2002). Key molecular characteristics that define the W-Beijing family of strains include:

1) They all belong to SNP-defined PGG 1 (Sreevatsan et al., 1997),
2) They have an IS*6110* insertion in the origin of replication (AI insertion) (Kurepina et al., 1998)
3) They have the same spoligotype pattern, S00034 (Bifani et al., 1999),
4) All W-Beijing strains have a single IS*6110* insertion in the NTF region (this genotype distinguishes these strains from the NYC W strain and its decedents) and
5) These isolates have a rare di-nucleotide change in codon 315 of katG (AGE →ACA) (Ramaswamy and Musser, 1998).

6.5.2. KZN XDR-outbreak strain, HP81

The 19 colonies genotyped from Patient 5 were identified as HP81 [also known as F15/LAM4/KZN (according to the South African classification of MTB strains)], which is the strain that is known to have caused an XDR-TB outbreak in Tugela Ferry in 2006. The spoligotype for this strain is listed in the spoligotyping database as ST 60. In addition, the organism belongs to the F15 family which forms part of the Latino American and Mediterranean (LAM) family and corresponds with the LAM4 subgroup (Streicher et al., 2007). The KZN strain is now referred to as the F15/LAM4/KZN strain (Pillay and Sturm, 2007). Although this strain was known to have caused an XDR outbreak, the strain isolated in this study was most likely a primary MDR infection (resistant to RIF and INH) as no record of previous TB treatment was found for this particular patient. In fact as early as 1994, F15/LAM4/KZN was responsible for a number of MDR-TB cases which indicated the strains ability to cause cases of primary drug-resistant TB. After 1994, MDR isolates with additional drug resistance had been reported and in 2001 the first XDR isolate was recorded. In 2006, a cluster of persons infected with the "KwaZulu-Natal strain" was reported (Ghandi et al., 2006) in which the infecting organism was resistant to RIF, INH, KAN and the FQs. The development of resistance over time was reported in the F15/LAM4/KZN strain which is responsible for a significant proportion of transmissible MTB (Pillay and Sturm, 2007). The authors concluded that the development of drug resistance in this strain coincided with the implementation of anti-TB control programs such as the DOTS program and that in the absence of DST may have resulted in the emergence of a highly transmissible XDR strain. It was also suggested that the high incidence of HIV infected individuals and individuals infected with susceptible strains of TB in the area may have contributed to the outbreak in

2006. As mentioned earlier, the F15/LAM4/KZN strain is responsible for a large number of primary MDR-TB cases. Patient 5 was admitted to Sizwe Hospital with primary MDR-TB. It has recently been shown that the KZN strain, along with other XDR-TB strains (variants of MTB strain families Haarlem, EAI, LAM and X), are now present in a number of other provinces in South Africa (Mlambo et al., 2008). The DNA sequences of several clinical isolates from KZN using whole genome sequencing was determined (Ioerger et al., 2009). Analysis of the direct repeat region indicated a spoligotype pattern missing spacers 21-24, 33-36 and 40, which was consistent with the F15/LAM4/KZN spoligotype reported by Pillay and Sturm (2007). The HP81 strain isolated in the present study contained mutations in *rpoB* and *pncA* that are different from those present in the MDR (KZN-V2475) and XDR (KZN-R506) strains isolated by Ioerger et al., (2009). These are depicted below:

Table 6.4 Mutation profiles in *rpoB* and *pncA* in members of the F15/LAM4/KZN strain

	*rpoB**	*pncA***
KZN-V2475	D435Y	G132A
KZN-R506	D435G, L452P, I1106T	+g in A152[a]
HP81	S531L	-t pos 389 (codon 30)[b]

Mutations in coding regions are numbered as amino acid substitutions (upper case). Numbering of base substitutions (lower case) is given relative to the H37Rv genome.

* D: aspartate; Y: tyrosine; G: glycine; L: leucine; P: proline; I: isoleucine; T: threonine; S: serine

** G: glycine; A; alanine

[a] Frame shift mutation in amino acid 152 caused by insertion of 1 bp

[b] Frame shift mutation at position 389 (codon 30) caused by deletion of 1 bp

The development of drug resistance in F15/LAM4/KZN from MDR to XDR is a clear indication for DST to be performed to guide treatment of Patient 5 in order to ensure appropriate treatment. Such treatment would decrease chances of further acquisition and transmission of drug resistance mutations. It is also extremely important that improved infection control be implemented in order to contain the spread of resistant disease. Patients with drug-resistant TB are often hospitalised for extended periods and come into contact with other patients and healthcare workers. This can lead to nosocomial infections as was seen in KZN (Ghandi et al., 2006).

6.6 PHENOTYPIC HETERORESISTANCE

DST using the BACTEC MGIT 960 instrument was conducted on the 20 colonies isolated from each patient for RIF, INH (low- and high-level INH; 0.1µg/ml and 0.4µg/ml respectively), OFX and KAN. For four out of the five patients, no discordant phenotypic results were observed between the 20 colonies that were isolated from each patient. Thus, good correlation (100%) was observed between the phenotypic and genotypic data obtained for the 20 colonies isolated from these four patients (Patients 2, 3, 4 and 5). This is in agreement with results found in other studies (Escalante et al., 1998, Laszlo et al., 2002, Torres et al., 2000). Patient 1 showed 100% correlation with regards to phenotypic and genotypic data obtained for both OFX and KAN; however; phenotypic hetero-resistance was observed in Patient 1 with regards to both RIF and INH (low-level) resistance.

6.6.1. DSTs conducted on colonies isolated from Patient 1 with discordant INH susceptibility results

Initial DSTs conducted on the 20 colonies isolated from Patient 1 yielded a mixture of susceptible and resistant results amongst the 20 colonies analysed for low-level INH resistance. In the initial DST experiments conducted for INH susceptibility, 17 out of 20 colonies demonstrated phenotypic resistance to INH, yet no mutations were observed in any of the 20 colonies for both INH resistance-related genes tested i.e. *inhA* and *katG*, as determined by sequencing. DST results were obtained on 18 out of the 20 colonies when the DST on the 20 colonies was conducted a second time. In this experiment, a growth control from one of the 20 colonies failed to grow (colony four) and one set of test MGIT tubes for another colony was contaminated (colony one). Fifteen out of the 18 colonies (approximately 83%) exhibited low-level INH resistance. The experiment was completed for a third time where results were obtained for 19 out of the 20 colonies (growth control for colony 12 failed to grow). All 19 colonies (100%) were resistant to low level INH. DST performed in the first set of INH experiments showed that all 20 colonies displayed susceptibility at the high-level INH breakpoint concentration. Poor correlation was observed between the genotypic and phenotypic data obtained for Patient 1 with regards to low-level INH resistance. Although no *katG* or *mabA-inhA* mutations were identified for Patient 1, it is likely that mutations may have been present in other INH resistance related genes such as *kasA*, *ahpC* or *ndh* which may account for the high percentage of phenotypic resistance observed when looking at low level INH resistance.

6.6.2 DSTs conducted on colonies isolated from Patient 1 with discordant RIF susceptibility results

DSTs for RIF resistance were conducted on the 20 colonies selected for Patient 1 on two additional occasions to confirm the mixed resistance results that were observed amongst the 20 colonies on the initial set DST experiments. With regards to the initial DST results, 14 out of 20 colonies displayed phenotypic susceptibility to RIF despite all 20 colonies harbouring the H526L mutation in the *rpoB* region. The test was repeated for a second time to confirm these results. In the second experiment, the growth control of one of the 20 colonies failed to grow (colony four) and one set of test MGIT tubes for another colony was contaminated (colony one). Again it was observed that 16 out of a total of 18 (approximately 89%) colonies for Patient 1 were susceptible to RIF. The test was done for a third time on the 20 colonies isolated from Patient 1. In this round of DSTs, results were obtained from 19 of the 20 colonies due to the failure of the growth control for colony 12. Eight out of 19 colonies were susceptible to RIF (42%). Poor correlation was seen between the phenotypic and genotypic data obtained for the 20 colonies isolated from Patient 1. The discordant results observed in patient one with regards to RIF warranted further investigation to determine whether or not these results could be attributed to 1) true hetero-resistance, which may pertain to compensatory mutations occurring outside the *rpoB* region, or that the H526L mutation may not be sufficient for high-level RIF resistance, or 2) technical shortcomings with regards to performing DST using the BACTEC MGIT 960 instrumentation and protocol for performing this test. Published data by van Deun et al., (2009) illustrated discordant RIF DST results seen during proficiency testing that was performed by nine Supra-National Tuberculosis Reference Laboratories (SRLs). These SRLs tested the RIF susceptibilities of 19 MTB strains using

standard culture-based methods. A MTB strain isolated from Bangladesh harbouring the H526L mutation was amongst the strains tested in the proficiency test which yielded a susceptible result by the radiometric BACTEC 460 and the BACTEC MGIT 960 methods. However, the majority of strains with *rpoB* mutations that were found to be susceptible using the BACTEC radiometric method or BACTEC 960 method were found to be resistant by the proportion method using either LJ or Middlebrook 7H10 agar. The authors concluded that the reason for these discordant results most likely relates to the existence of MTB strains with borderline susceptibility. It was also noted that laboratories using either the BACTEC radiometric or BACTEC 960 method were likely to report a borderline strain as susceptible and strains were only reported as resistant if the average ratio of the MIC and the critical concentration was at least 4. A study by Traore et al. also showed that genotypically resistant results have been reported as susceptible (approximately 10% of isolates) when using the BACTEC radiometric method (Traore et al., 2007).

6.7 Rifampicin MICs conducted on selected colonies from Patient 1

In order to further investigate the discrepant RIF DST results observed for Patient 1, MICs were performed on a few selected colonies from this patient to determine whether or not these discrepancies were a true reflection of phenotypic hetero-resistance or rather whether these results related to the particular MTB strain in question being borderline resistant.

The MICs obtained for all seven colonies selected from Patient 1 were 0.4µg/ml. The result obtained suggests that the H526L mutation may only encode low-level RIF resistance and that resistance associated with this particular mutation can easily be missed when using the

BACTEC 960 method (and perhaps other liquid-based methods like the BACTEC 460 radiometric method). Susceptible DST results with genotypically resistant RIF strains have been observed and reported using the BACTEC radiometric method (Lambert et al., 2003, Traore et al., 2007, van Deun et al., 2009) as well as the BACTEC 960 method (van Deun et al., 2009). In the study conducted by Traore et al, to test the performance of a multi-well plate assay using mycobacteriophage D29, RIF resistant isolates from Uganda with mutations in codon 511, 516 or 533 were missed when DSTs were performed using the BACTEC radiometric method but were found to be resistant when they performed resistance testing using the phage assay (Traore et al., 2007). The mutations in these isolates were identified by nucleotide sequencing of the *rpoB* gene when strains were found to be resistant by either the BACTEC or phage assay. In fact, four false negative results (reported as sensitive) were found using the BACTEC 460 assay and the authors noted that they found the phage assay to be more sensitive and specific assay when comparing the two. The results from our findings suggest that the H526L mutation, by encoding low-level resistance, may have clinical significance, in line with other RIF mutations that code for low-level RIF resistance. Important questions that need to be addressed are: a) Are there additional low-level RIF mutations that may be missed using the BACTEC MGIT 960 method that have not yet been identified? and b) How frequent are these mutations in a particular setting? If these mutations are common, this may have a negative impact on TB treatment in that many RIF resistance strains may be missed. Certain *rpoB* mutations may be associated with a fitness cost which may weaken or disappear during patient treatment due to compensatory mutations. The most prevalent *rpoB* mutation, which was observed in the four other patients in this study, is the Ser531Leu mutation. The S531L mutation has does not appear to be associated with a fitness cost; however, less common mutations occurring in *rpoB* are associated with a fitness burden

and can result in poor growth in certain assays (Mariam et al., 2004, O'Sullivan et al., 2005). Generally the S531L mutation along with some mutations in codons 513 and 526 have been associated with high-level RIF resistance and cover approximately 90%o of isolates displaying phenotypic RIF resistance (van Deun et al., 2009). Resistant strains that were frequently missed in the van Deun study that also resulted in the lowest MICs had the Leu511Pro and Leu533Pro mutations. In fact, some authors have considered these strains to be susceptible (Ohno et al., 1996). The results obtained in the present study, confirmed by the results obtained by other studies, may suggest that the standard approach used to conduct DSTs, particularly the BACTEC MGIT 960, may require modification. For instance, when solid media are used to perform DST, the incubation time of borderline strains can be extended to yield a resistant result, but this is not possible when using an automated methods (such as the BACTEC 960 or the BACTEC 460 systems). A possible reason for BACTEC failures therefore may relate to endpoint readings occurring too early. It is possible that an initially sensitive borderline strain may yield a resistant result if the MGIT incubation period is extended. In addition, increasing the amount of inoculum added may also alter a borderline strain result from susceptible to resistance, as these strains may grow poorly as a result of a fitness cost incurred by that particular *rpoB* mutation. Other authors have also made the recommendation of lowering the RIF breakpoint to 0.5μg/ml when using the BACTEC radiometric method (Suo et al., 1988).

The labeling of a clinically RIF resistant strain as susceptible can have serious consequences for anti-TB treatment which may ultimately result in default from treatment, treatment failure or death. Cases resulting in default and treatment failure will exacerbate the TB problem by ultimately resulting in the transmission of RIF resistant or MDR strains in the community.

Low-level, but clinically probable RIF resistance, linked to specific *rpoB* mutations requires further investigation. These strains are likely to be missed using rapid, automated, broth-based systems such as the BACTEC MGIT 960. It would be important to investigate the frequency of these strains in South Africa. A high frequency of these strains in a particular setting may ultimately require an adaptation of some of the current automated methods used for DST.

CHAPTER 7

7 SUMMARY OF MAIN FINDINGS AND CONCLUDING REMARKS

An in-depth analysis was conducted to investigate the presence of mixed infections and/or clonal heterogeneity in five patients infected with drug-resistant TB admitted to Sizwe Hospital. IS*6110*-based RFLP analysis was conducted to characterise the MTB strains present in these five patients as well as to identify mixed/heterogeneous MTB strains in a single patient. Although, mixed infections were not found in any of the five patients using IS*6110*-based RFLP, clonal heterogeneity was observed in one of the five patients (20%), involving 1 out of 99 colonies (1%) using IS*6110*-based RFLP typing.

A slight variation in the DNA fingerprint was observed in terms of an "absent" band in colony six when compared to the fingerprint patterns from the other 19 colonies isolated from Patient 3. Hybridisation of the original nylon membrane with a "left-side" probe indicated that the absent band was most likely due to a SNP that occurred in the flanking region of the DNA in the *Pvu*II site. This finding could suggest that approximately 5% of the MTB strains present in the sputum sample collected could contain this particular mutation, although the mutation identified is most likely due to a random event.

Four different MTB strains were identified from the five patients analysed: W799 (W-Beijing family) from Patient 1, MH from Patient 2 and Patient 4, AI (variant) from Patient 3 and HP81 from Patient 5 were the strains identified according to the nomenclature used by Centre for Tuberculosis at the PHRI, Newark New Jersey, USA. Two patients were infected with the

MH strain of which both had identical sequencing and DST results, suggesting the possibility of an epidemiological link between these two patients. One patient was infected with the HP81 strain which is related to the strain that was shown to have been responsible for the XDR-TB outbreak in Tugela Ferry, KZN, RSA in 2006 but harboured different mutations in the *rpoB* and *pncA* genes.

Sequencing of genes associated with drug resistance was performed on each set of 20 colonies isolated from the five patients. No evidence of clonal hetero-resistance was seen in the various genes sequenced amongst the 20 colonies isolated from each patient.

Phenotypic heterogeneity affecting both RIF and INH was demonstrated in the sputum sample from Patient 1. In the case of RIF, it was shown that inconsistent discrepancies on MGIT DST were obtained and on MIC determination these colonies had low RIF MICs (0.4µg/ml). The phenotypic heterogeneity encountered in Patient 1 is in accordance with the findings of van Deun et al., (2009) and probably relates to problems inherent to the MGIT DST system as indicated by these authors. The INH discrepant DST findings among the 20 colonies also relate to low-level INH resistance and need to be investigated further by MIC testing and DST performed on both MGIT and solid medium methods.

7.1 LIMITATIONS OF STUDY

Little heterogeneity was observed in the study aside from a band shift that was observed in one of the 20 colonies isolated from Patient 3. Section 6.4.2 outlines various aspects that explain why this was missed. The following factors are highlighted:

- The number of patients selected for the study was insufficient.
- Twenty colonies were selected based on decisions from the literature. Twenty colonies may not be sufficient to demonstrate within host heterogeneity

7.2 FUTURE WORK

- The phenotypic heterogeneity involving INH susceptibility encountered with the 20 colonies isolated from Patient 1 needs to be investigated further by MIC testing and determination of the genetic basis for this low-level resistance not linked to the common *katG* and *inhA* mutations.

- Additional colonies from the five patients should be investigated to increase chances of identifying the presence of hetero-resistance and/or mixed infections in these patients. Additional colonies are available for fingerprinting (approximately 80 additional colonies are available from each of the five patients). These are stored as frozen stocks at the CTB of the NICD.

- Additional fingerprinting techniques such as MIRU-VNTR and/or novel PCR-based on original 20 colonies plus additional colonies could be performed to increase likelihood of identifying mixed infections other forms of genetic heterogeneity.

- More patients need to be analysed to increase the chances of finding clonal heterogeneity and mixed infections in patients treated at Sizwe Hospital. This will be accomplished through the involvement of the candidate in the Gilla Kaplan longitudinal study where strains from 100 HIV positive and 100 HIV negative patients will be determined and sequenced for hetero-resistance.

- To augment the detection of mixed infections, IS*6110*-based RFLP can be used to identify mixed infections without isolating single colonies by the identification of multiple LIBs in the IS*6110* banding patterns of cultures from sputum samples. This detection of LIBs could be used as a ‘screening method’ to identify patients that are potentially infected with mixed strains.

APPENDECES

APPENDIX A: ETHICS CLEARANCE

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

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Miss Amanda Axcell

CLEARANCE CERTIFICATE	**M10128`**
PROJECT	Genotypic and Phenotypic Heterogeneity of Mycobacterium Tuberculosis Recovered from Patients with Pulmonary Disease Involving Multiple-Drug Resistant Tuberculosis
INVESTIGATORS	Miss Amanda Axcell.
DEPARTMENT	Virology & Communicable Diseases
DATE CONSIDERED	29/01/2010
DECISION OF THE COMMITTEE*	Approved unconditionally

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

DATE 29/01/2010 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Prof HJ Koornhof

DECLARATION OF INVESTIGATOR(S)

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

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

APPENDIX B: SOLUTIONS FOR IS6110-BASED RFLP

10x TBE
For 8L:
Tris 864g
Boric acid 440g
EDTA 74.4g

Soak I
0.5 M NaOH
1.5M NaCl

For 3L:
60g NaOH
262.98g NaCl

For 4L:
80g NaOH
350.64 NaCl

Soak II
0.5M Tris → pH 7.2 with HCl
1.5NaCl

For 2L:
125.2g Tris
175.33g NaCl
80mL HCl

For 4L:
242.3g Tris
350.65g NaCl
160mL HCl

ECL protocol buffer
For 1 probe 30mL:
27mL Hybridization buffer
3mL 5M NaCl
1.5g Blocking agent

For 300mL:
270mL Hybridization buffer
30mL 5M NaCl
15g Blocking agent

*Aliquot 30mL in
50mL Falcon tubes

Primary Washing Buffer
For 1L:
360g Urea
25mL 20xSSC
20mL 20%SDS or 4g SDS

For 2L:
720g Urea
50mL 20xSSC
40mL 20%SDS

Secondary Washing Buffer
2x SSC

*SSC: Sodium chloride and sodium citrate
*SDS: Sodium dodecyl sulfate
*CTAB: hexadecyltrimethylammonium bromide

20xSSC
3M NaCl
0.3M Na citrate ⟶ pH 7.0 with few drops HCl

For 1L:	For 4L:
175g NaCl	700g NaCl
88g Na citrate	352g Na citrate

Loading dye

50 ml of Loading Dye from 1% Double Dye (DD) stock:

10x TBE	5 ml
Glycerol	25 ml
H2O	15 ml
1% DD	5 ml

1% DD in H20 from Bromphenol Blue and Xylene cyanole.

IS6110 Probe preparation:

Primers:	F5' GTA-AAA-CGA-CGG-CCA-GTG;
	R5'GGA-AAC-AGC-TAT-GAC-CAT-G.
PCR:	30 cycles, 94-30'', 55-30'', 72-1'

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