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Molecular and epidemiological characterization of multidrug-resistant *Mycobacterium tuberculosis* isolates in Johannesburg, South Africa

Charmaine Khudzie Mlambo

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy

2011

Dedication

This work is dedicated to my friend
Edgar Semenya

Without your support, this would not have been possible.
Thank you.

Declaration

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

Signature: .....

Date: .....

Acknowledgments

Firstly, my thanks goes to my supervisor Dr Else Marais without whom this work would never have been possible. Thank you for your guidance, support and patience. I also wish to thank my co-supervisors Prof Robin Warren and Prof Tommie Victor, their guidance and advice was invaluable. I'd also like to sincerely thank our Head of Department and my co-supervisor Prof Adriano Duse for his support and guidance in the past years.

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To my Mom, my beautiful Masha (Vuyiswa), my brothers Lindelwa and Lizwe and my little sister Eyethu. Thank you for your unconditional love and your support even when you didn't fully understand my choices. I want to especially thank my Masha, you are the only person who can ALWAYS make me smile. To my friend Mashudu, thank you for all the years of support and friendship. Words cannot express how grateful I am for my friend Edgar Semenya, you have been a blessing to me and I will always be thankful for your support.

Summary

South Africa has a heavy burden of tuberculosis (TB) which is exacerbated by the concurrent epidemic of HIV. Molecular techniques have been used in most developed countries to investigate the dynamics of the TB epidemic, but despite the high prevalence of TB in sub-Saharan Africa, little data on strain types are available outside of the Western Cape. This study aims to provide information on the genotypic characteristics of multidrug-resistant (MDR) *Mycobacterium tuberculosis* strains in Johannesburg. Patient data obtained from the National Health Laboratory Service (NHLS) referral TB diagnostic laboratory and from Sizwe hospital, a MDR-TB referral hospital, were used to determine the risk factors for treatment outcomes in patients with MDR tuberculosis.

Multidrug-resistant *M. tuberculosis* isolates from over 100 clinics and hospitals in Johannesburg were stored for the study. Spoligotyping and MIRU-VNTR were used to genotype the strains. Drug susceptibility profiles showed that 238 (55%) of the 434 *M. tuberculosis* isolates tested were resistant to streptomycin and ethambutol, in addition to being resistant to rifampicin and isoniazid. A comparison of spoligotyping results with the international spoligotyping database (SITVIT2) showed a total of 50 shared international types (SITs). Forty-five shared types, containing 417 isolates (96%) matched a pre-existing shared type whereas 5 shared types (containing 11 isolates) were newly created. Diverse strain types were noted, with Beijing, LAM, EAI, T, S, H and X families being dominant. Spoligotype defined families were split into sub-clusters by MIRU typing, resulting in 76 MIRU international types (MITs), containing 389 isolates and 45 orphan isolates.

Spoligotyping showed lower discrimination (Hunter-Gaston discriminative index (HGDI) of 0.917) compared with MIRU typing (HGDI = 0.957) but there was no remarkable difference in

the discriminatory power of combined spoligotyping and MIRU (HGDI = 0.962) compared with MIRU typing used alone. Twenty-four loci MIRU-VNTR typing was performed on strains from Beijing and CAS, EAI and H families to identify loci with high discriminatory power in our region. The proposed 15 MIRU-VNTR locus combination, together with MIRU 39, was found to be sufficient as a secondary typing method for the routine epidemiological investigation of the Beijing family isolates. Non-Beijing families could be sufficiently differentiated by the 15 MIRU locus combination.

This study also describes the treatment outcomes of 351 MDR-TB patients at Sizwe hospital, who started treatment between 2004 and 2007, and investigates possible risk factors associated with poor outcomes. Final treatment outcome was available for 324 (92%) of the patients. Treatment success (completion and cure) was recorded in 158 (48.8%) of patients, while 73 (22.5%) had poor outcomes and 93 (28.7%) defaulted. Eleven (3.1%) patients were transferred out to another health facility and 16 (4.6%) had no recorded final outcome.

The proportion of successful treatment increased significantly over time. Univariable and multivariable analysis (P = 0.05) identified the year of MDR-TB diagnosis and spoligotype-defined families as factors associated with treatment outcome. No associations were found between treatment outcome and HIV status, previous TB and additional MDR resistance to either streptomycin or ethambutol. The patient isolates were also characterised molecularly, complementing the study of isolates from Johannesburg alone, and providing information for the Gauteng Province.

A sub-study illustrating genotypic diversity of the families constituting extensively drug-resistant TB (XDR-TB) strains in South Africa was conducted subsequent to the nosocomial outbreak in KwaZulu Natal (KZN). The results show that multiple, parallel development of resistance, rather than transmission alone, also plays an important role in the incidence of this extended form of resistance.

Publications and Presentations

C.K. Mlambo, R.M. Warren, X. Poswa, T.C. Victor, A.G. Duse, E. Marais. Genotypic diversity of extensively drug-resistant tuberculosis (XDR-TB) in South Africa. *International Journal of Tuberculosis and Lung Disease*, 12 (2008) 99-104.

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- Poster presentation at the Keystone Symposia on Molecular and Cellular Biology: Pathogenesis and Control of Emerging Infections and Drug-Resistant Organisms. Royal Orchid Sheraton Hotel, Bangkok, Thailand. October 22 – 27, 2008.

- Poster presentation at Faculty of Health Research day, University of the Witwatersrand, Johannesburg, South Africa. 20 August, 2008. Awarded Best Poster Prize in the category of Infectious Diseases.

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- Oral presentation at the Molecular and Cell Biology Group Symposium, University of the Witwatersrand, October, 2006.

- Poster presentation at Faculty of Health Research day, University of the Witwatersrand, August, 2006.

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List of Abbreviations

AIDS	Acquired immune deficiency syndrome
AMK	Amikacin
anti-TB	antituberculous
ART	Antiretroviral therapy
BCG	Bacille Calmette-Guérin
bp	Base pairs
CAS	Central Asian
CDC	Centers for Disease Prevention and Control
CM	Capreomycin
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DOTS	Directly Observed Treatment Short-course
DR	Direct Repeat
DST	Drug Susceptibility Testing
DVR	Direct Variable Repeat
EAI	East-African-Indian
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EMB	Ethambutol
ETH	Ethionamide
ETR	Exact Tandem Repeats
FQN	Fluoroquinolone
H	Haarlem
HGDI	Hunter Gaston Discriminatory Index
HIV	Human Immunodeficiency Virus
hr	Hour
INH	Isoniazid
IPT	Isoniazid Preventative Therapy
IS	Insertion Sequence

IUATLD	International Union Against Tuberculosis and Lung Disease
KM	Kanamycin
KZN	KwaZulu Natal
LAM	Latin-American-Mediterranean
M. tuberculosis	*Mycobacterium tuberculosis*
MDR-TB	Multidrug-resistant Tuberculosis
MGIT	Mycobacteria Growth Indicator Tube
min	Minute
MIRU	Mycobacterial Interspersed Repetitive Units
MIRU-VNTR	Mycobacterial interspersed repetitive units variable number tandem repeat
MIT	MIRU International Type
ml	Millilitre
MODS	Microscopic observation drug susceptibility
MPTR	major polymorphic tandem repeats
MRC	Medical Research Council
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NHLS	National Health Laboratory Services
NTP	National TB Programme
OFX	Ofloxacin
PAS	P-aminosalicylic acid
PCR	Polymerase Chain Reaction
PZA	Pyrazinamide
QUB	Queen's University Belfast
RFLP	Restriction fragment length polymorphism
RIF	Rifampicin
SCC	Short Course Chemotherapy
SDS	Sodium dodecylsulphate
sec	Second
SIT	Shared International Type

Spoligotyping	Spacer oligonucleotide typing
STR	Streptomycin
TB	Tuberculosis
TBE	Tris-Borate EDTA
TE	Tris-EDTA
Tris	Tris (hydroxymethyl)aminomethane
TZ	Terizidone
VNTR	Variable number tandem repeat
WHO	World Health Organization
XDR-TB	Extensively drug-resistant tuberculosis

CHAPTER 1: General Introduction

1.1 Tuberculosis

1.1.1 History

The burden of disease and death caused by tuberculosis (TB) makes it one of the most important and dangerous diseases in the history of human-kind. It was responsible for epidemics in Europe and North America during the 18th and 19th century, and has once again reached global epidemic proportions (Daniel 2006, Herzog 1998, WHO 2009b). A third of the world's population are currently thought to be infected with *Mycobacterium tuberculosis (M. tuberculosis)*, the causative agent for TB. In 2008, there were 9.4 million new cases estimated worldwide, with mortality estimated at 1.8 million. This equates to 4500 deaths a day (WHO 2009b).

M. tuberculosis has been present in the human population since the beginning of recorded history (Gutierrez *et al.* 2005). The genus *Mycobacterium* is presumed to have originated more than 150 million years ago (Daniel 2006) and an early progenitor of *M. tuberculosis* is thought to have been present in East Africa three million years ago, and to have co-evolved with early hominids at that time (Gutierrez *et al.* 2005). Modern members of the *M. tuberculosis* complex are thought to have originated from a common ancestor approximately 35,000 – 15,000 years ago (Gutierrez *et al.* 2005) and to have evolved clonally (Warren *et al.* 2001, Sola *et al.* 2001, Supply *et al.* 2003).

The earliest evidence of TB disease in Egypt was documented around 5000 years ago, and comes from skeletal deformities, including Pott's deformities observed in Egyptian mummies and also depicted in Egyptian art (Daniel 2006). The study of genetic material in such ancient tissues using modern molecular techniques has proven beyond any doubt that TB is an ancient disease and that it was widespread in ancient Egypt and Rome (Zink *et al.* 2003, Donoghue *et al.* 2004).

Written descriptions of tuberculosis (phthisis; consumption) are well documented in Greek literature around the time of Hippocrates (460 -370BC) (Daniel 2006). The disease is said to have been widespread, mostly affecting young populations, and that treatment at that time included fresh air, milk and sea voyages (Daniel 2006). Treatment of TB (King's Evil or scrofula) in the middle ages proposed cures such as the touch of the sovereigns of England and France (Daniel 2006, Herzog 1998) but since the etiology of the disease was poorly understood at that time, these efforts to cure the disease were ineffective. New theories on the etiology and transmission of TB emerged during the European Renaissance (Daniel 2006) but even with the new knowledge, there was still much debate about the etiology, with some researchers believing TB to tumorous and hereditary and others believing it to be infectious (Herzog 1998). More accurate pathological and anatomical descriptions of the disease began to appear in the 17th century (Daniel 2006). Evidence of the infectious nature of TB was first suggested by Benjamin Marten in 1790, describing the infectious agents as minute living creatures which, once they entered the body, produced lesions and symptoms of phthisis (Daniel 2006).

Subsequent experiments performed on rabbits by French military surgeon Jean-Antoine Villemin convincingly demonstrated the infectious nature of TB (Herzog 1998, Cardoso Leao, Portaels 2007). In 1882, Robert Koch presented definitive evidence on the causative agent, the tubercle bacillus, both grown on culture and visualized as rods under the microscope (Kaufmann, Schaible 2005), and finally settled the speculation regarding the causative agent.

1.1.2 *M. tuberculosis*: Bacteriological Overview

M. tuberculosis, the causative agent of TB in humans is a Gram-positive rod-shaped bacterium that is non-motile, has no capsule and does not form spores nor produce toxins (Ducati *et al.* 2006). The microbe's width and length vary from 0.3 to 0.6 µm and 1 to 5 µm, respectively. It is characterized by a thick and complex lipid rich cell wall which is commonly associated with pathogenesis (Zuber *et al.* 2008, Reed *et al.* 2004, Rocha-Ramirez *et al.* 2008) and considerably slow growth (Ducati *et al.* 2006). Currently, there are 130 known species among the *Mycobacterium* genus (Gutierrez *et al.* 2009) most of them being saprophytic soil bacteria and a minority of these species is pathogenic to humans, causing mycobacterial disease (*M. tuberculosis*, *M. bovis*, and *M. africanum*) and leprosy (*M. leprae*) (Smith *et al.* 2009, Monot *et al.* 2005). Members of the *M. tuberculosis* complex include *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. pinnipedii*, *M. caprae*, *M. microti* and *M. canetti* (Cousins *et al.* 2003, Aranaz *et al.* 2003, Pfyffer *et al.* 1998, van Soolingen *et al.* 1998, van Soolingen *et al.* 1997, Mostowy *et al.* 2004).

It is believed that mycobacterial ancestors came from the soil, and that the human bacillus may have been derived from the bovine form (*M. bovis*), probably through cattle domestication by pre-dynastic Egyptians (Gutierrez *et al.* 2005, Smith *et al.* 2009, Wirth *et al.* 2008).

The current modern strains of *M. tuberculosis* fall into six major lineages (Brudey *et al.* 2006, Filliol *et al.* 2002), which vary in their global distribution (Brudey *et al.* 2006, Filliol *et al.* 2002, Filliol *et al.* 2003).

TB is spread from person-to-person through the air by droplet nuclei which are produced when persons with pulmonary or laryngeal tuberculosis cough, sneeze, speak or sing (Frieden *et al.* 2003). Droplet nuclei can also be generated during aerosol treatments, sputum induction, aerosolization during bronchoscopy, and through manipulation of lesions or processing of tissue or secretions (Tang *et al.* 2006). Due to their small size, droplet nuclei can remain airborne for minutes to hours after expectoration (Nardell 2004). When inhaled, droplet nuclei are carried down the bronchial tree and implant in respiratory bronchioles or alveoli in the lungs. Whether or not an inhaled tubercle bacillus establishes an infection in the lung depends on both bacterial virulence and the inherent microbicidal ability of the alveolar macrophage that ingests it (Frieden *et al.* 2003, Dannenberg 1989). Individuals with dormant TB infection are not infectious and thus cannot transmit *M. tuberculosis*. Approximately 10% of individuals who acquire TB infection and are not given appropriate therapy will develop active TB disease, with the highest risk occurring in the first two years after infection (Comstock 1982).

TB accounts for extraordinarily high rates of morbidity and mortality worldwide primarily because of its ability to persist for long periods of time in the face of an active immune response, and to adapt rapidly to the changing conditions inside and outside the host (Ulrichs, Kaufmann 2002, Honer zu Bentrup, Russell 2001). *M. tuberculosis* remains dormant until the balance between bacillary persistence and the immune response gets disturbed (Honer zu Bentrup,

Russell 2001). Causes of an impaired host response include; age, malnutrition, excessive use of steroids, uncontrolled diabetes mellitus, renal failure, underlying malignant disease, chemotherapy, Human Immunodeficiency Virus **(**HIV). All the above mentioned factors can result in reactivation of bacilli resulting in clinical manifestations of tuberculosis (Honer zu Bentrup, Russell 2001, Wilkinson *et al.* 2000).

1.1.3 Global Burden of TB

After more than 30 years of decline, TB recaptured the attention of the global community when outbreaks of strains resistant to multiple anti-tuberculosis (anti-TB) drugs were reported in the early 90s in the United States, mainly associated with HIV-infected persons and causing high fatality rates (Centers for Disease Control (CDC) 1991). The World Health Organization (WHO) formally recognized TB as a major public health problem in 1993, resulting in an increase in global health efforts to control the disease (Weyer *et al.* 2004). The global trend in incidence however still seems to be increasing despite efforts to control the disease. In 2004 the TB incidence was an estimated 8.9 million cases, with approximately 1.7 million people dying from disease, including those co-infected with HIV (WHO 2004). There was a slight decrease in global TB incident cases in 2005 (8.8. million cases) and 7.4 million of these were from Asia and sub-Saharan Africa (WHO 2005). A total of 1.6 million people died of TB in that year, including 195 000 people infected with HIV (WHO 2005). Currently, TB incidence is estimated at 9.4 million cases worldwide (Figure 1.1), including 1.4 million cases among people with HIV (WHO 2009a). Twenty-two countries worldwide account for approximately 80% of the estimated number of new TB cases arising each year, and South Africa currently ranks third on that list (WHO 2009a).

The main reasons contributing to the exceptionally high TB burden in these countries include poverty, poor programme management, population increase and most importantly, the impact of HIV/AIDS (Corbett *et al.* 2003).

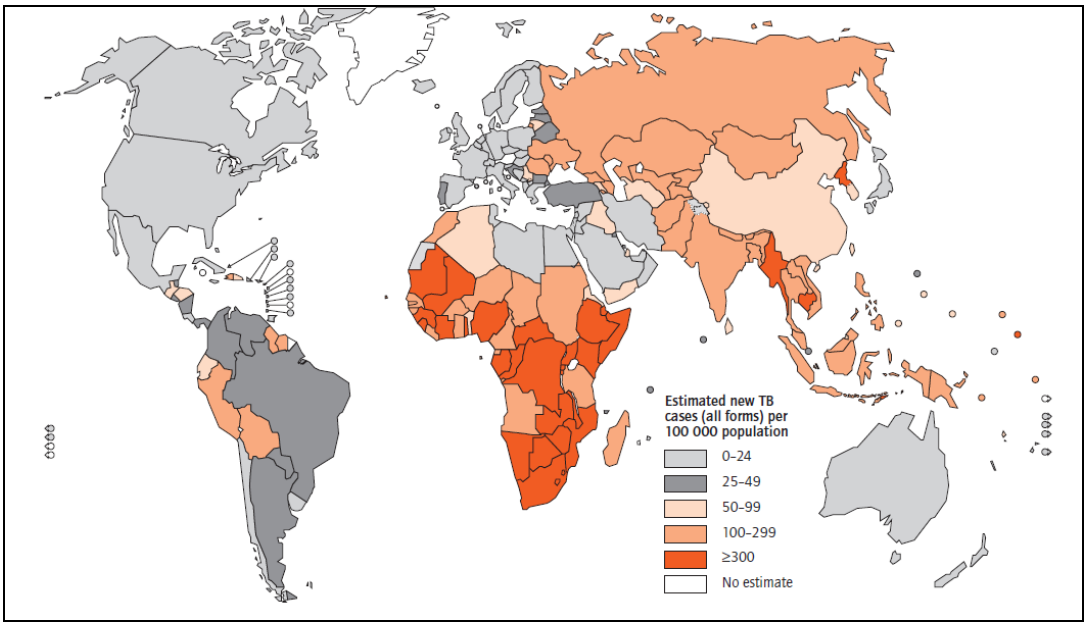


Figure 1.1: Estimated TB incidence rates, 2008. Source: WHO report, 2009

1.2 TB and HIV

TB and HIV form a lethal combination because they fuel each other's progress (Daley *et al.* 1992, Morozova *et al.* 2003). HIV not only increases the risk of reactivating latent *M. tuberculosis* infection, it also increases the risk of rapid progression to disease after infection or re-infection (Mariani *et al.* 2001, Goletti *et al.* 1996, Havlir, Barnes 1999, Small *et al.* 1993). In people not co-infected with TB and HIV, the lifetime risk of developing TB ranges from 5 to 10%. Co-infection, however, can increase the risk to above 10% per year (Corbett *et al.* 2003, Havlir, Barnes 1999).

TB is also the leading cause of death in the developing world among HIV-infected people; in 2008 alone there were an estimated 500 000 deaths among incident cases who were HIV-positive (WHO 2009a). The diagnosis of TB in HIV-positive persons is difficult because HIV infection complicates the clinical presentation of active TB through its effect on the immune system (De Cock *et al.* 1992). TB-HIV co-infected patients, and in particular patients with AIDS, together with active pulmonary TB often have sputum negativity, atypical chest radiographic results as well as increased incidence of extra-pulmonary TB (Geng *et al.* 2005).

These findings lead to misdiagnosis or delays in TB diagnosis when using conventional diagnostic methods and, as a result, higher morbidity and mortality (Geng *et al.* 2005, Yu *et al.* 2008, Saraceni *et al.* 2008). Treatment is also often challenging in HIV co-infected patients mostly due to interactions between drugs used in the treatment of both diseases, the severe side effects associated with these therapies (Dean *et al.* 2002), and the development of TB-associated immune reconstitution inflammatory syndrome (IRIS) (Murdoch *et al.* 2008). Furthermore, poor compliance with treatment can result in the development of drug-resistant strains (Wells *et al.* 2007).

Patients with TB-HIV co-infection have a high mortality risk (Ciglenecki *et al.* 2007, Haar *et al.* 2007) and this risk is elevated when patients are infected with drug-resistant TB (Wells *et al.* 2007, Cox *et al.* 2008, Gandhi *et al.* 2006, Gandhi *et al.* 2010). The effective management of TB in HIV-infected persons requires solutions to the above mentioned challenges.

Timing of the initiation of treatment must be optimized; TB-HIV co-infected patients need to be provided with safe and effective treatment combinations, and rapid and accurate diagnostic tools need to be developed for the detection of active and latent TB.

1.3 TB Chemotherapy and the Development of Drug Resistance

Anti-tuberculosis drug treatment started in 1944 with the discovery of streptomycin (STR) and P-aminosalicylic acid (PAS) (Daniel 2006, Herzog 1998). A study done in 1950 comparing the efficacy of SM and PAS both when used alone and in combination showed that the combined therapy was more effective (Wada 2007, Jawahar 2004). This resulted in the first multidrug anti-TB treatment approach. The addition of isoniazid (INH) in 1952 to the treatment regimen further improved treatment efficacy, increasing cure rates to up to 100% in some cases (Jawahar 2004). The duration of treatment was still lengthy, however, and lasted for 18 to 24 months; but this changed in 1960 when ethambutol (EMB) substituted PAS, and treatment duration was reduced to 18 months. The introduction of rifampicin (RIF) in the early 1970s further decreased treatment duration to 9 months and finally the addition of pyrazinamide (PZA) in 1980 resulted in the current recommended TB regimen which has treatment duration of 6 months (Wada 2007).

The internationally recommended TB chemotherapy strategy known as short-course chemotherapy (SCC) is aimed at completely eliminating active and dormant bacilli, and involves two phases of treatment using first-line anti-TB drugs (Narita *et al.* 2002). The initial (intensive) phase comprises the use of INH, RIF, ETH and PZA for 2 months, followed by 4 months' continuous phase treatment using INH and RIF, although in some cases, STR is substituted with EMB (Mitchison 2005).

Despite the effectiveness of anti-TB drugs in the management of the disease, the TB burden remains a global problem compounded by the development of drug-resistant strains which are difficult to treat. One of the major contributing factors to TB drug resistance is the lengthy chemotherapy required to treat and cure infection.

While drug-sensitive TB can be effectively treated with first-line drugs, treating multidrug-resistant (MDR) TB strains, defined as TB strains with *in vitro* resistance to INH and RIF, with or without resistance to the other first-line drugs, can exceed 2 years. The long treatment duration can significantly increase the risk of patient non-compliance as well as cost of treatment (WHO 2008b). The current recommended standardized regimen for MDR-TB treatment comprises 4 months' intensive phase using kanamycin (KM), ofloxacin (OFX), ethionamide (ETH), PZA, EMB or terizidone (TZ), followed by a continuous phase for 12 to 18 months comprising ETH, OFX, EMB or TZ. Treatment for MDR-TB is guided by culture conversion, whereby treatment is recommended for 18 months past the date of culture conversion and an extension to 24 months in chronic MDR-TB cases (WHO 2008b).

The development of TB strains with extensively-resistant (XDR) strains of *M. tuberculosis*, defined as MDR-TB strains with additional resistance to any fluoroquinolone and to at least one of the three injectable second-line drugs (kanamycin, amikacin and capreomycin) (Gandhi *et al.* 2006, Centers for Disease Control and Prevention (CDC) 2006), has further complicated the treatment strategies for TB. Treatment of XDR-TB cases is associated with even poorer outcomes than MDR-TB, especially in patients co-infected with HIV, and results in high early mortality (Gandhi *et al.* 2006, Gandhi *et al.* 2010, Kim *et al.* 2008, Migliori *et al.* 2008b, Dheda *et al.* 2010).

1.4 Drug Resistance and TB

1.4.1 Intrinsic and Non-intrinsic Resistance

Drug resistance in mycobacteria can either be intrinsic or non-intrinsic. Intrinsic resistance is mainly attributed to the highly hydrophobic multi-layer cell envelope which provides an effective permeability barrier (Brennan, Nikaido 1995, Jarlier, Nikaido 1990), and multidrug efflux pumps, especially associated with resistance to fluoroquinolones, that minimize drug uptake (Li, Nikaido 2004, Pasca *et al.* 2004, Cole *et al.* 1998, Louw *et al.* 2009). Non-intrinsic drug resistance is classified as either primary (transmitted) drug resistance or acquired (secondary) drug resistance. Acquired resistance occurs when susceptible bacteria become resistant to drugs as a result of mutations in chromosomal genes (Heym *et al.* 1994). Accumulation of mutations in different genes involved in resistance to individual drugs results in the development of multidrug-resistant strains, and in TB this has been attributed to human failure through irregular drug supply, inappropriate therapy and poor patient adherence to treatment (Zhang *et al.* 2000, Vareldzis *et al.* 1994). Primary drug resistance results from the spread or transmission of drug-resistant organisms by an infected source to other individuals.

1.4.2 Mechanisms of Drug Resistance

There has been progress in recent years in understanding the mechanisms of drug resistance in *M. tuberculosis*. Considerable knowledge has also been gained recently regarding the mode of action of the various drugs used in anti-TB therapy. The mechanisms of drug resistance are outlined in Table 1.1.

1.4.3 Diagnosis of TB and Detection of Resistance to Anti-TB drugs

The ability to diagnose TB and determine drug susceptibility remains one of the greatest obstacles to successful TB control. Sputum smear microscopy is the most commonly used method to diagnose active TB in regions with limited resources. The method is rapid, inexpensive and technically simple. The technique however lacks specificity compared to TB culture and requires 5,000 to 10,000 bacteria/ml to obtain a positive result. Culture of *M. tuberculosis* has both high sensitivity and specificity and is often used as the reference method for TB diagnosis (Kallenius *et al.* 1994). Culture is also essential for most methods used for species identification and drug susceptibility testing.

Traditionally, egg-based or agar-based solid media have been used in isolation of mycobacteria. Cultivation periods on egg-based media such as Lowenstein-Jensen slants can range between 3-6 weeks before growth of *M. tuberculosis* can be observed. More rapid growth is achieved by using liquid media such as Middlebrook broth. The introduction of the manual and automated Mycobacteria Growth Indicator Tube (MGIT) systems, and BACTEC™MGIT 960 (BD Diagnostics, Sparks, MD, USA), have reduced detection times to approximately 10 days (Tortoli *et al.* 1997). The BACTEC system, initially introduced for the rapid detection of mycobacterial growth, is currently the only rapid phenotypic method recommended by the WHO for drug resistance surveillance. Diagnosis of drug resistance in TB requires testing isolates for drug susceptibility to anti-TB drugs. Methods employed are classified as either phenotypic or genotypic diagnostic methods. Phenotypic methods, such as the BACTEC™MGIT 960 (BD Diagnostics, Sparks, MD, USA) system, detect the effects of anti-TB drugs on bacterial multiplication or metabolism compared to controls not exposed to the drugs.

Other phenotypic methods with similar specificity and sensitivity include the Microscopic Observation and Drug Susceptibility (MODS) assay (Caviedes *et al.* 2000, Moore *et al.* 2006, Arias *et al.* 2007), FastPlaque assay (Wilson *et al.* 1997) and colorimetric methods (Martin *et al.* 2007, Palomino *et al.* 2007, Perkins, Cunningham 2007). Genotypic drug susceptibility tests identify resistance-conferring mutations in the bacterial genome. Genotypic tests that have shown specificity and sensitivity similar to the recommended BACTEC™MGIT 960 (BD Diagnostics, Sparks,MD, USA), include Hain MTBDRplus assay (Hain Lifesciences, Nehren, Germany) (Barnard *et al.* 2008), and INNO LIPA RifTB line probe assay (Innogenetics, Ghent, Belgium)(Tortoli,Marcelli2007).

Table 1.1 Mechanisms of resistance in *M. tuberculosis* to first and second-line anti-TB drugs

Drug	Mechanism of action	Common genes involved in resistance	Gene product
Isoniazid	Inhibits mycolic acid biosynthesis	*kat G*	Catalase-peroxidase
		inhA	Enoyl ACP reductase
Rifampicin	Inhibits RNA synthesis	*rpoB*	β subunit of RNA polymerase
Ethambutol	Inhibits arabinogalactan synthesis	*embB*	Arabinosyl transferase
Streptomycin	Inhibits protein synthesis	*rpsL*	S12 ribosomal protein
		rrs	16S rRNA
Pyrazinamide	Interferes with pH balance	*pncA*	Nicotinamidase/Pyrazinamidase
Fluoroquinolones	Inhibit DNA gyrase	*gyrA*	DNA gyrase subunit A
		gyrB	DNA gyrase subunit B
Kanamycin/Amikacin	Inhibit protein synthesis	*rrs*	16S rRNA
Capreomycin	Inhibit protein synthesis	*tlyA*	rRNAse methyltransferase
Ethionamide	Inhibits mycolic acid biosynthesis	*etaA/ethA*	Flavin monooxygenase
PAS	Possibly inhibits folic acid and iron metabolism	*thyA*	Thymidylate synthase

ACP = acyl carrier protein; PAS = para-aminosalicylic acid

References:

Isoniazid: (Zhang *et al.* 1992, Timmins *et al.* 2004, Shoeb *et al.* 1985, van Soolingen *et al.* 2000, Marttila *et al.* 1998). **Rifampicin**: ((Mitchison 1985). **Ethambutol**: (Sreevatsan *et al.* 1997, Telenti *et al.* 1997). **Streptomycin**: (Honore, Cole 1994, Nair *et al.* 1993). **Pyrazinamide**: (Scorpio, Zhang 1996) **Fluoroquinolones**: (Riska *et al.* 2000). **Kanamycin/Amikacin**: (Alangaden *et al.* 1998). **Capreomycin**: (Maus *et al.* 2005).

1.5 Multidrug-resistant and Extensively Drug-resistant Tuberculosis

1.5.1 Epidemiology

Multidrug-resistant TB emerged as a threat to global TB control in the 1990s and has since been reported in all countries worldwide. A report by the WHO/IUATLD Global Project on Anti-tuberculosis Drug Resistance Surveillance (2008), representing data from 116 countries, revealed that new cases of MDR-TB had nearly tripled since 2006. In 2009, global TB data from 198 countries reported an estimated 0.5 million cases of MDR-TB worldwide, with 27 countries (15 of them in the European Region) accounting for 85% of all the cases (WHO 2009b). The number of XDR-TB cases worldwide is also increasing. In February 2008 XDR-TB had been detected in 45 countries, and by November 2009, 57 countries and territories had reported at least one case of XDR-TB (WHO 2009a). In the 2009 report, the reported prevalence of XDR-TB as a percentage of MDR-TB was highly variable, ranging from 0% in Rwanda and Tanzania to 12.8% in Baku, Azerbaijan, 15% in Donetsk, Ukraine and 23% in Estonia. Overall, of the MDR-TB cases reported to the WHO in 2009, 7% were XDR-TB (WHO 2009a). The global burden of MDR and XDR-TB is thought to be much higher than the current estimates because of limitations in the methods currently used in surveillance of drug-resistant cases (WHO 2004). The lack of routine drug susceptibility testing (DST) in many resource-limited countries, for example, contributes to the underestimation of MDR and XDR cases in these areas (WHO 2004). A study by Ben Amor and colleagues, confirms the underreporting of MDR-TB in Africa. Using estimates from 39 of 46 African countries, the authors reported high rates of MDR-TB, a contrast to WHO reports on the rates of MDR-TB in the African region (Ben Amor *et al.* 2008).

1.5.2 Treatment and Outcomes of MDR and XDR-TB

MDR and XDR-TB treatment involves using second-line anti-TB drugs which are less potent, more toxic and more expensive compared with the first-line TB drugs used to treat drug-susceptible TB. These factors contribute to the low cure and treatment completion rates as well as increased mortality seen when comparing outcomes from MDR and XDR-TB patients with patients with drug-susceptible TB.

With regards to specific treatment regimens, the choice largely depends on the DST profile, although many countries, especially in areas with limited resources have adopted a standardized regimen recommended by the WHO, which is based on the DST profile of the *M. tuberculosis* strains in a specific geographical area. Although the treatment outcomes for MDR and XDR patients are poor compared with patients with drug-susceptible TB, positive outcomes have been reported in predominantly HIV negative populations with both MDR and XDR-TB. In a study in Latvia, treatment of MDR-TB patients using information from regional drug resistance patterns or individualized drug susceptibility patterns for patients with DST data resulted in cure or treatment completion in 66% of HIV negative patients (Leimane *et al.* 2005). Other reports on XDR-TB patients among largely HIV negative populations in South Korea and Peru show survival rates of 55% and 60% respectively (Kim *et al.* 2007, Mitnick *et al.* 2008).

When comparing MDR-TB and XDR-TB treatment outcomes, XDR-TB patients have been reported to have poorer outcomes, especially with HIV-infected patients, where XDR-TB infection is usually associated with rapid death (Gandhi *et al.* 2006, Kim *et al.* 2008, Dheda *et al.* 2010, Migliori *et al.* 2007, Singh *et al.* 2007b).

The use of fluoroquinolones in the treatment of MDR and XDR-TB as well the integration of TB and HIV therapy seems to improve successful outcomes in MDR and XDR-TB patients (Leimane *et al.* 2005, Yew *et al.* 2000, Chan *et al.* 2004, Torun *et al.* 2007, Padayatchi *et al.* 2009, O'Donnell *et al.* 2009, Friedland *et al.* 2007, Dheda *et al.* 2010). One study in a population of predominantly HIV co-infected patients on antiretroviral therapy (ART) from South Africa reported a survival rate of 52% from XDR-TB patients after 6 months of therapy (O'Donnell *et al.* 2009). Several strategies have been proposed for the control of both MDR and XDR-TB and these include using standardized treatment regimens instead of individualized treatment based on population DST patterns in settings with limited resources and high burden TB (WHO 2008b, Van Deun *et al.* 2004): using empiric therapy, which included 4 or 5 oral second-line drugs for suspected MDR and XDR-TB patients awaiting DST results (Leimane *et al.* 2005, Van Deun *et al.* 2004, Mukherjee *et al.* 2004); scaling up infection control measures including administrative, environmental and personal measures (Bock *et al.* 2007, Escombe *et al.* 2007); and establishing community based treatment strategies in order to provide services to an increased number of patients as well as minimize nosocomial transmission (WHO 2008b, Heller *et al.* 2010, Padayatchi, Friedland 2008, Mitnick *et al.* 2003). Surgical resection has also been reported to be beneficial in the treatment of MDR and XDR-TB in patients with pulmonary TB but this procedure is often of unclear benefit in HIV-infected persons who often have disseminated disease (Chan *et al.* 2004, Shiraishi *et al.* 2008).

1.6 TB and South Africa

South Africa ranks third on the list of high burden TB countries in the world and fourth among the 27 countries with the highest rates of MDR-TB (WHO 2009a). The country had approximately 461,000 new cases of TB in 2007 with an incidence rate of an estimated 948 cases

per 100,000 - a major increase from 338 cases per 100,000 population in 1998 (WHO 2009a). The TB epidemic in the country is largely attributed to historical neglect, health service fragmentation and poor management of treatment strategies, compounded by the HIV epidemic (Abdool Karim *et al.* 2009, Metcalf 1991). Drug-resistant TB, especially MDR and XDR-TB has also emerged as a serious challenge in the management of TB in the country. Over 14,000 cases of MDR-TB were estimated to occur annually in South Africa in 2006 according to the latest WHO drug surveillance report (WHO 2008a). However, up-to-date incidence and prevalence estimates of drug-resistant TB are lacking because the last national drug resistance survey was done in 2001-2002 (Weyer *et al.* 2004).

The discovery of *M. tuberculosis* strains with extensive drug resistance in the KwaZulu Natal (KZN) Province of South Africa in 2005 focused global attention not only to the drug-resistant TB problem in the country but also to worldwide resistance (Gandhi *et al.* 2006). The initial causal strain in the KZN outbreak was later identified as the F15/LAM4/KZN genotype using molecular techniques (Pillay, Sturm 2010) and retrospective studies on the evolution of this strain in the province showed that the *M. tuberculosis* strain type has been responsible for cases of MDR-TB since 1994 and XDR-TB from 2001 (Pillay, Sturm 2010). XDR-TB strains have since been reported in all the country's provinces (WHO 2008a) and are composed of diverse strain families (Mlambo *et al.* 2008) as described by spoligotyping (Brudey *et al.* 2006, Kamerbeek *et al.* 1997).

Since the outbreak of XDR-TB in KZN, there has been an upgrade in the standard policies regarding the diagnosis and management of MDR/XDR-TB worldwide (WHO 2008b) and this

has resulted in a better understanding of the contribution of drug-resistant strains to the TB epidemic.

The most advocated method to address the challenges associated with the management of drug-susceptible and drug-resistant TB in South Africa has been the WHO recommended Directly Observed Treatment Short-course (DOTS) strategy. South Africa adopted the DOTS strategy in 1996 and it has since been implemented in all regions, although coverage varies within and among provinces. According to the latest WHO report, the DOTS strategy seems to be effective because the country surpassed the DOTS target of 70% TB case detection rate in 2006 and this further increased to 78% in 2007 (WHO 2009b). Treatment success rates for TB have also increased from 61% in 2001 to 74% in 2007, although the rate still falls short of the WHO recommended goal of 85%.

Diagnosis and treatment of drug-resistant TB however still pose major challenges in South Africa and unfavourable treatment outcomes such as default, failure and death have been reported in the few studies available from South Africa, especially associated with HIV co-infected persons (Brust *et al.* 2010, Holtz *et al.* 2006, Davies *et al.* 1999, Shean *et al.* 2008). Abdool Karim and colleagues proposed strategies which, when used together, could improve the management of TB in the country (Abdool Karim *et al.* 2009). The steps include:

- Determining cure rates in each health facility and developing locally appropriate plans to achieve the WHO recommended TB cure rate of 85% within a specified time.

- Improving TB case detection by intensified case finding using active screening for TB in every symptomatic patient presenting at a health facility and screening household contacts as well as HIV-infected persons attending health clinics.
- Integrating TB and HIV care so that 90% of TB patients are offered HIV testing and 90% of HIV patients are screened for active TB.
- Emphasizing the use of the WHOs 3 I's strategy of Intensified TB case finding, Isoniazid Preventative Therapy and TB Infection control (IPT) in TB-HIV care (WHO 2008c).
- Lastly, identifying and treating drug-resistant TB so that 85% of retreatment TB cases have culture and drug susceptibility testing.

Some of the strategies proposed above are already being evaluated for use in the country in an effort to control TB and the growing burden of drug-resistant TB. Rapid diagnostic assays for MDR-TB for example, have been evaluated in a public laboratory in Cape Town, with encouraging results (Barnard *et al.* 2008). The need to manage TB and HIV in the same patient is also facilitating the integration of TB and HIV services, with best examples found in the KZN Province (O'Donnell *et al.* 2009, Friedland *et al.* 2007).

The use of genotyping methods in the study of TB epidemiology has proven to be a valuable tool in understanding TB dynamics worldwide (van Soolingen 2001). An integration of such techniques into the National TB Programme (NTP) in South Africa would provide insight to questions about the current TB epidemic such as the population structure of *M. tuberculosis* in the different province and risk factors for TB transmission and modes of transmission (nosocomial, community, household, exogenous re-infection). Genotyping could also be used to confirm suspected mixed infections in TB patients.

In the Western Cape Province, where extensive molecular epidemiological studies have been undertaken, the population structure and TB transmission patterns of drug-susceptible and drug-resistant TB are well understood (van der Spuy *et al.* 2009) and this knowledge possibly contributes to the good functioning TB programme in that province.

1.7 Molecular Epidemiology of TB

1.7.1 Overview

The resurgence of TB worldwide has renewed interest in understanding the epidemiology and pathogenesis of the disease. Genotyping or DNA fingerprinting, made possible by the discovery of various genetic markers in the early 90s, has replaced phenotypic markers such as mycobacterial bacteriophage typing (Jones, Greenberg 1978) as the principal method for differentiating *M. tuberculosis* strains. The first reports of using insertion sequences (IS*986* or IS*6110*) to examine DNA polymorphisms in TB were published in 1990 (Hermans *et al.* 1990, McAdam *et al.* 1990), and their application in the study of epidemiology in TB resulted in the emergence of the field of molecular epidemiology of TB.

Many of the initial studies focused on evaluating genetic markers in order to determine their stability as well as effectiveness in differentiating *M. tuberculosis* (de Boer *et al.* 1999, Cohn, O'Brien 1998, Yeh *et al.* 1998). Information from strain genotyping has since been used in numerous studies to supplement traditional epidemiological methods and has provided invaluable insight on the transmission dynamics of TB disease, both globally and in specific regions. Also, molecular tools have given insight into *M. tuberculosis* population diversity and the various risk factors associated with drug-resistant TB. Some of the contributions of genotyping in the study of tuberculosis are detailed below.

Subsequent to the evaluation and standardization of genotyping methods in the 1990s, strain typing was mainly used to study outbreaks and transmission patterns of TB disease in institutional settings (Bifani *et al.* 1999, Frieden *et al.* 1996, Edlin *et al.* 1992). The approach was based on the principle that strains from a common ancestor have identical or similar DNA patterns. Genotype clusters therefore comprised of isolates with identical or highly similar genotype patterns (van Soolingen 2001) and generally indicated epidemiologically linked cases of recently transmitted diseases (van Soolingen 2001). The development and spread of TB disease in the institutional settings were often found to be associated with prior history of TB, overcrowding, drug and alcohol abuse as well as HIV co-infection.

Genotyping techniques have since been used extensively worldwide in an effort to understand the population structure of *M. tuberculosis* strains driving the global TB epidemic. Spoligotyping in particular, has greatly enhanced the understanding of the global dissemination of *M. tuberculosis* by using international databases to compare isolates from widespread geographic areas (Sola *et al.* 2001, Brudey *et al.* 2006, Filliol *et al.* 2002, Filliol *et al.* 2003). The use of spoligotyping, sometimes supplemented with other secondary genetic markers, has identified several *M. tuberculosis* genotype lineages and sub-lineages such as Beijing (van Soolingen *et al.* 1995), Haarlem (H) (Kremer *et al.* 1999), Central Asian (CAS), East African Indian (EAI) and Latin America Mediterranean (LAM) (Sola *et al.* 2001, Brudey *et al.* 2006, Filliol *et al.* 2003), LAM7_TUR (Zozio *et al.* 2005), F11/LAM3 (Victor *et al.* 2004), Cameroon (Niobe-Eyangoh *et al.* 2004), Manila (Douglas *et al.* 2003) and LAM11_ZWE (Easterbrook *et al.* 2004).

Genotyping methods have allowed for accurate distinction between relapse and re-infection of TB disease, and provided evidence of the contribution of exogenous re-infection to the burden of active TB in communities (Small *et al.* 1993, Kruuner *et al.* 2002, van Rie *et al.* 1999). DNA fingerprinting methods have also been used to demonstrate co-infections with multiple strains of TB (mixed infections) (Braden *et al.* 2001, Richardson *et al.* 2002a, Warren *et al.* 2004, Stavrum *et al.* 2009, Yeh *et al.* 1999, Mendez *et al.* 2009), a phenomenon that may result in conflicting drug susceptibility results. Some studies have also used strain genotyping to investigate cases of laboratory cross-contamination in the diagnosis of *M. tuberculosis*. This has proven useful not only in confirming false positive results due to laboratory cross-contamination, but also in confirming the source of contamination (e.g. contaminated reagents) (Martinez *et al.* 2006, Yan *et al.* 2005).

Using molecular genotyping techniques to identify strain families that are associated with drug-resistant outbreaks worldwide has allowed for the study of possible microbial factors associated with transmission and/or disease. The Beijing family, in particular, is widespread geographically and has been associated with drug resistance and outbreaks of drug-resistant TB (Glynn *et al.* 2002, Kruuner *et al.* 2001, Toungoussova *et al.* 2002, Bifani *et al.* 1996). This characteristic has prompted debates on the 'fitness' of such strains, and whether they are either more transmissible or more likely to cause disease once transmitted compared with other strains.

Some studies investigating mutations in strains of the Beijing family (Kruuner *et al.* 2001, Ebrahimi-Rad *et al.* 2003) have reported mutations resulting in better adaptability of Beijing strains to stress conditions and the possibility of an associated increased ability to spread in such

strains. Beijing strains have also shown high virulence in experimental animal models (Manca *et al.* 2005, Tsenova *et al.* 2005) compared with immunogenic reference strains. A recent review of the mechanisms underlying the emergence of Beijing strains (Parwati *et al.* 2010) however acknowledges that other ubiquitous genotypes, which have been associated with outbreaks of drug-resistant TB, such as Haarlem and African genotypes (Mardassi *et al.* 2005) may have fitness characteristics similar to Beijing strains. Further study is needed to understand the mechanisms underlying the transmissibility of such families (Parwati *et al.* 2010).

To date, a large number of genotyping tools for *M. tuberculosis* genotyping exist, of which IS*6110*-Restriction Fragment Length Polymorphism analysis (IS*6110*-RFLP), spacer oligonucleotide typing (spoligotyping) and Mycobacterial Interspersed Repetitive Unit Variable Number Tandem Repeats (MIRU-VNTR) typing have been internationally standardized and are the most commonly used.

1.7.2 IS*6110*-RFLP Analysis

IS*6110*-Restriction Fragment Length Polymorphism analysis (IS*6110*-RFLP) was the first method extensively used for strain differentiation of *M. tuberculosis*. The method is based on differences in the copy numbers and distribution of the insertion sequence (IS*6110*) in the *M. tuberculosis* genome. IS*6110*, first described in 1990, is 1,355 base pairs in size and a member of the IS3 family (McAdam *et al.* 1990, Thierry *et al.* 1990). The insertion sequence is distributed throughout the genome with copy numbers ranging from 0 to more than 20 (Hermans *et al.* 1990, Kurepina *et al.* 1998, van Soolingen *et al.* 1991, Sampson *et al*. 2001).

A standardized method for performing IS*6110* analysis was proposed by van Embden in 1993 and has since gained recognition as a gold standard for TB molecular typing (van Embden *et al.* 1993). The method uses the restriction enzyme, *Pvu*II, which cleaves IS*6110* at a single asymmetric site. The resulting fragments are hybridized to a probe specific to the right side of the IS and each hybridizing band corresponds to a *Pvu*II-*Pvu*II chromosomal fragment with a single IS*6110* restriction. Standardized internal and external weight markers are used to facilitate accurate computer-assisted analysis of the resultant RFLP fragments (Heersma *et al.* 1998).

IS*6110*-RFLP analysis has several limitations compared with the PCR-based genotyping methods of typing. The method is relatively slow, technically demanding and requires a significant quantity of good quality DNA from viable cultures. Inter-laboratory comparative analysis of RFLP patterns can be tedious and requires the use of sophisticated computer software. In addition, the method cannot be used reliably to type isolates with < 6 IS*6110* insertions because many of the sites of IS*6110* insertion are highly conserved in such strains (Soini *et al.* 2001). This problem is usually overcome by using additional typing techniques such as spoligotyping and MIRU-VNTR typing (Kamerbeek *et al.* 1997, Kremer *et al.* 1999, Bauer *et al.* 1999, Goyal *et al.* 1997).

1.7.3 Spoligotyping

Spacer oligonucleotide typing (spoligotyping) simultaneously identifies and differentiates members of the *M. tuberculosis* complex. The method is based on a DNA polymorphism present at the Direct Repeat (DR) region, which is uniquely present in *M. tuberculosis* complex strains (Kamerbeek *et al.* 1997). The DR region contains a variable number of 36 base pair direct repeats (DRs), interspersed by non-repetitive spacers, each 35 to 41 base pairs in length (Hermans *et al.* 1991). The DR and adjacent spacer are known as direct variable repeat (DVR) (Groenen *et al.* 1993). The DR was first described by Hermans and colleagues who sequenced the region in *M. bovis* BCG (Hermans *et al.* 1992). Additional spacers have since been discovered in the DR region, and the order of the spacers is well conserved (van Embden *et al.* 2000).

Spoligotyping relies on the *in vitro* amplification of the spacers between the direct repeats in the DR locus using primers DRa and DRb (biotinylated). The PCR products are then hybridized to a membrane which contains immobilized synthetic oligonucleotide spacer sequences derived from the DR of *M. tuberculosis* H37Rv and *M. bovis* BCG. Spoligotyping can be applied directly to a variety of specimen including cultured cells, non-viable samples and clinical samples (Kamerbeek *et al.* 1997). Results can be readily digitized and compared to an international database (Brudey *et al.* 2006, Dale *et al.* 2001). Spoligotyping has a turnaround time of 1-2 days and has been used extensively as a first-line screening technique supplemented by either IS*6110*-RFLP or MIRU-VNTR analysis to improve strain differentiation.

The main criticism of spoligotyping is that the method characterizes polymorphisms at a single genetic locus whereas IS*6110*-RFLP and MIRU-VNTR typing measure polymorphisms in the entire genome of *M. tuberculosis*. Other disadvantages include lower discriminatory power compared with IS*6110*-RFLP and MIRU-VNTR typing as well as convergent evolution, (Warren *et al*. 2002), which is a documented but rare phenomenon.

1.7.4 MIRU-VNTR Typing

Mycobacterial Interspersed Repetitive Unit Variable Number Tandem Repeat (MIRU-VNTR) typing is a PCR-based technique that provides better resolution than spoligotyping and resolution comparable to IS*6110*-RFLP, especially when using a combination of 24 MIRU-VNTR loci. Eleven initial VNTR loci comprising five major polymorphic tandem repeats (MPTR) (A-E) and 6 exact tandem repeats (ETR) (A to F) were first reported in 1998 (Frothingham, Meeker-O'Connell 1998). Additional VNTR loci have since been reported (Skuce *et al.* 2002, Roring *et al.* 2002, Supply *et al.* 2000, Magdalena *et al.* 1998). The MIRU-VNTR typing method currently employed in various laboratories worldwide has been standardized (Supply *et al.* 2006) and is based on variable numbers of tandem repeats of different classes of interspersed genetic elements known as mycobacterial interspersed repetitive units (MIRUs). The method evaluates the number of MIRUs distributed throughout the genome. It is based (firstly) on PCR amplification of multiple repeat loci using primers specific for the flanking regions of each repeat locus, and on the determination of sizes of the PCR products. Size determination can either be performed using a capillary system (Kwara *et al.* 2003, Supply *et al.* 2001) or gel electrophoresis (Mazars *et al.* 2001).

The discriminatory power of MIRU-VNTR typing is proportional to the number of loci evaluated and the choice of loci is guided by the study question. Generally though, the use of 12 and 15 loci is sufficient for first-line epidemiological investigations and an extension to 24 loci is useful for phylogenetic analysis, and has a discriminatory capacity comparable to that of IS*6110*-RFLP (Supply *et al.* 2006). Recent studies evaluating the use of 24 loci MIRU-VNTR typing in population-based investigations have reported varying results, with some studies advocating its feasibility (Alonso-Rodriguez *et al.* 2009, Allix-Beguec *et al.* 2008a, Oelemann *et al.* 2007) and others reporting insufficient discriminatory capacity, especially when compared to IS*6110*-RFLP (Hanekom *et al.* 2008, Iwamoto *et al.* 2007, Jiao *et al.* 2008).

MIRU-VNTR typing, like spoligotyping, can be used to facilitate global epidemiologic surveillance of TB. The development of high-throughput automated MIRU-VNTR analysis (Supply *et al.* 2001) and a MIRU genotyping website (Allix-Beguec *et al.* 2008b) provides a mechanism to compare global TB data.

1.7.5 Molecular Epidemiology of TB and South Africa

In South Africa, extensive molecular fingerprinting of mycobacterial strains, mostly using IS*6110*-RFLP and spoligotyping, is performed by few units, with the majority of studies emanating from the Western Cape. Studies done from this region have been instrumental in understanding the frequency, distribution and population structure of *M. tuberculosis* genotypes in the province and also in providing new insights into TB transmission.

The overall population of circulating drug-susceptible and drug-resistant TB strains is highly diverse in this province (Warren *et al.* 1996) and the TB epidemic is predominantly driven by

Beijing, LAM3/F11 and S/F28 families (Streicher *et al.* 2004, Hanekom *et al.* 2007b, Victor *et al.* 2007, Johnson *et al.* 2010, Nicol *et al.* 2005, Marais *et al.* 2006, Brudey *et al*, 2006, Lari *et al*, 2007).

Ongoing and recent transmission of both drug-susceptible and drug-resistant TB strains has been reported in the province, together with the risk factors associated with recent TB transmission (Middelkoop *et al.* 2009, Verver *et al.* 2004, van der Spuy *et al.* 2003, Victor *et al.* 1997). A recent study illustrated that transmission of TB infection in a high incidence area such as Cape Town, occurs at sites other than the household (Verver *et al.* 2004, Marais *et al.* 2009). Some of the work has also been pioneering in showing the existence and significance of exogenous re-infection in TB (van Rie *et al.* 1999) and also the presence of mixed infections and their association with retreatment cases (Richardson *et al.* 2002a, Warren *et al.* 2004). Spoligotyping has enabled reconstruction of the evolutionary history of low copy clades which could not be sufficiently differentiated using IS*6110*-RFLP (Streicher *et al.* 2007).

In other parts of the country, most published molecular epidemiological studies have been done in the North West Province (mostly in gold-mining communities) and in the (KwaZulu Natal) KZN Province.

The gold mines in the North West Province are associated with community health care centres which have well-functioning TB programmes (Calver *et al.* 2010). Several studies have been performed in these communities using traditional epidemiological methods in an effort to investigate the effect of factors such as HIV infection and silicosis on the TB epidemic in gold mines (Park *et al.* 2009, Glynn *et al.* 2008, Cowie 1995, Cowie 1994).

Limited work has also been done using molecular typing techniques in combination with traditional epidemiological methods to investigate the transmission patterns and risk factors associated with drug-susceptible and drug-resistant TB strains (Calver *et al.* 2010, Godfrey-Faussett *et al.* 2000) and to investigate the contribution of re-infection to the TB epidemic in the mining communities (Charalambous *et al.* 2008).

The KZN Province has recently been placed on the global stage with the description of XDR-TB associated with high mortality in HIV-infected persons (Gandhi *et al.* 2006). Molecular typing was instrumental in identifying the F15/LAM4/KZN strain responsible for the epidemic, and has also subsequently been used to illustrate the evolution of this strain from MDR to XDR (Pillay, Sturm 2007). Recent molecular studies from KZN have mainly focused on understanding the mutations conferring resistance to the highly transmissible F15/LAM4/KZN strain (Ioerger *et al.* 2009), as well as the transmission patterns of this strain both in nosocomial settings and in the community (Pillay, Sturm 2010). A recent study from KZN highlights the significance and contribution of exogenous re-infection to TB disease in high prevalence settings (Andrews *et al.* 2008). By using spoligotyping, the authors typed initial and follow-up isolates from 17/23 (74%) patients who developed MDR or XDR-TB after being treated for less drug-resistant forms of TB. In all the cases the spoligotypes from the follow-up isolates differed from the initial samples, indicating exogenous re-infection.

Very few studies have been performed on a national scale using molecular techniques to understand the TB epidemic in South Africa. A recent report by the Medical Research Council of South Africa (MRC) investigating drug-susceptible and drug-resistant TB isolates obtained during a national survey in 2001-2002, indicated a high diversity in the *M. tuberculosis*

genotypes in the country, with a majority of strains from the T family (ST53) exhibiting mixed infections (Stavrum *et al.* 2009).

This study was however limited by small sample sizes in some provinces. The Gauteng Province, for example, represented 13.8% (35/252) isolates despite having 10.5 million people, 22.8% of the country's population.

Although the investigation provides important baseline information on the circulating genotypes of both drug-susceptible and drug-resistant TB, the limited samples may not reflect the complete picture on the TB dynamics in the different provinces. More information is therefore still required in order to better understand the structure and distribution of strains circulating within specific regions in the country.

The majority of TB molecular epidemiological studies from South Africa have used IS*6110*-RFLP either alone or together with spoligotyping as the principal typing techniques. MIRU-VNTR typing is emerging as an efficient tool for differentiating *M. tuberculosis* and has been used in numerous studies worldwide (Zozio *et al.* 2005, Valcheva *et al.* 2008b, Millet *et al.* 2007, Dou *et al.* 2008a).

In South Africa, and indeed Africa, there is very limited information regarding the effectiveness of MIRU-VNTR typing, especially as a secondary typing method to differentiate the predominant *M. tuberculosis* families in this region. The few studies that have performed MIRU-VNTR typing to differentiate TB strains have mainly focused analysis on the Beijing family (Stavrum *et al.* 2009, Hanekom *et al.* 2008, Hanekom *et al.* 2007a). More information regarding

the most discriminatory loci for Beijing and the other prevalent non-Beijing *M. tuberculosis* strains in our region is required in order to allow for the effective use of fewer MIRU loci in epidemiological investigations when differentiating clusters identified by less discriminatory methods such as spoligotyping.

South Africa has a high TB prevalence and rapid and cost effective epidemiological methods are required to cope with the burden of disease. MIRU-VNTR typing together with spoligotyping is a fast and reliable means for epidemiological investigations and allows for the simultaneous identification and differentiation of *M. tuberculosis* strains. The digital format of results and the international databases (Brudey *et al.* 2006, Allix-Beguec *et al.* 2008b) also allow for the comparison of results with global data. More studies are required to investigate whether the combination of spoligotyping and MIRU-VNTR typing would be effective in differentiating the predominant strain types in this region.

1.8 Rationale for the Study

1.8.1 Choice of Study Area and Aim of the Study

Johannesburg is the financial hub of South Africa, the provincial capital of the Gauteng Province and the biggest city in South Africa. Johannesburg is comprised of seven administrative regions (Figure 1.2). The city has a population of approximately of 3.2 million people, with 49% under the age of 34 years. The latest available consensus (2001) reports that Black South Africans represent approximately 73% of the population, followed by Whites at 16%, Coloureds at 6% and Asians at 4%. The city's potential in providing job opportunities and its profile as a world-class city attracts people from all over the country and the world.

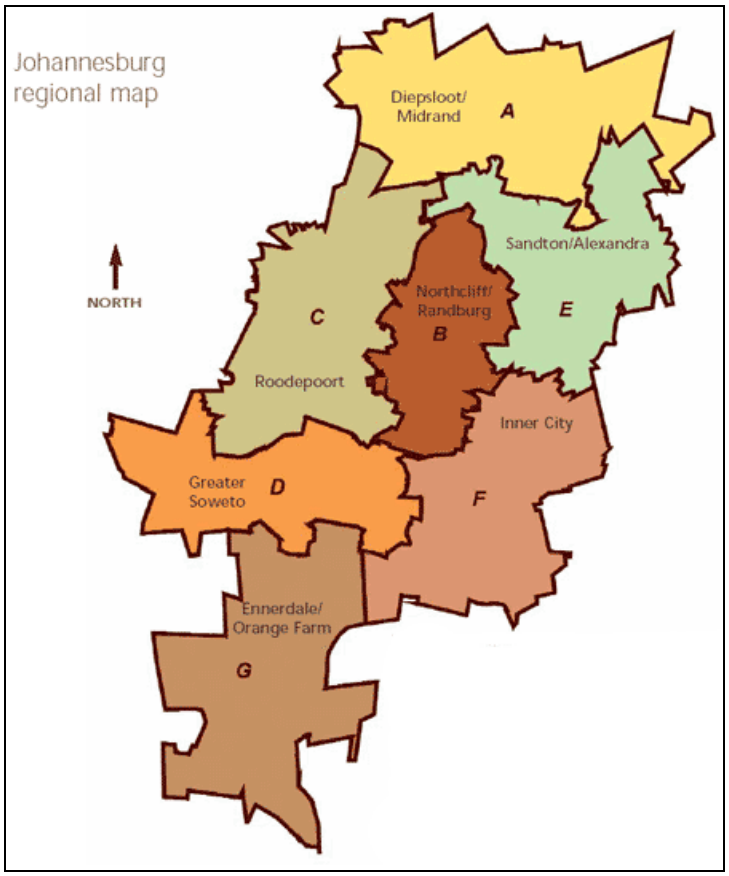


Figure 1.2: Map illustrating the seven administrative regions of Johannesburg (A to F). Source: the city of Johannesburg website, www.joburg.org.

The population is growing at a rate of 4.1% each year (Statistics SA 2009) which is partly facilitated by illegal immigrants from all over the African continent. People migrate to Johannesburg in search of jobs and better living conditions, but instead, most reside in low-cost informal settlements that are plagued by overcrowding, poverty and violence. With Johannesburg being the most populous, densely inhabited and industrialized of the country's cities, it is likely that the introduction and spread of mycobacterial strains is not the same as that seen in the other cities and provinces in the country.

1.8.2 General Aim

This study aimed to provide information on the genotypic characteristics, population structure and drug resistance profiles of MDR *M. tuberculosis* strains in the Johannesburg area, using molecular techniques in association with drug susceptibility results. Patient demographic and social characteristics were also used to determine factors associated with different treatment outcomes of MDR-TB in the region.

1.8.3 Study Objectives

1. To establish molecular typing of *M. tuberculosis* in our laboratory at the Department of Clinical Microbiology and Infectious Diseases.

2. To determine the population structure of clinical MDR-TB isolates from Johannesburg using molecular techniques and create a database of epidemiological (clinical, social and demographic) information.

3. To compare genotyping data from Johannesburg with national and global data.

4. To investigate drug susceptibility profiles associated with MDR-TB strains in Johannesburg.

5. To correlate MDR-TB genotypes, patient demographic and social characteristics with clinical outcomes in order to identify factors associated with various outcomes in the treatment of MDR-TB.

1.8.4 Thesis Layout

This thesis is divided into 7 chapters: Chapter 1 provides a background and introduction to the Study; Chapter 2 describes the materials and methods used in the Study. The results chapters (Chapter 3 to Chapter 6) are structured such that, Chapter 3 describes the molecular characterization and drug susceptibility patterns of multidrug-resistant (MDR) tuberculosis strains from Johannesburg; Chapter 4 focuses on the utility of 24 MIRU-VNTR typing for discriminating Beijing and non-Beijing MDR-TB isolates in Johannesburg; Chapter 5 reports on treatment outcomes of patients infected with MDR-TB at Sizwe Hospital, the MDR-TB referral hospital in Gauteng and Chapter 6 describes the genotypic diversity of "Pre-XDR" and XDR-TB isolates in South Africa, June 2005 – December 2006.

Chapter 7 summarises all the major findings for the Study and discusses possible future works. Appendices and References are also included at the end of the thesis. The thesis layout is illustrated in the flow chart below (Figure 1.3).

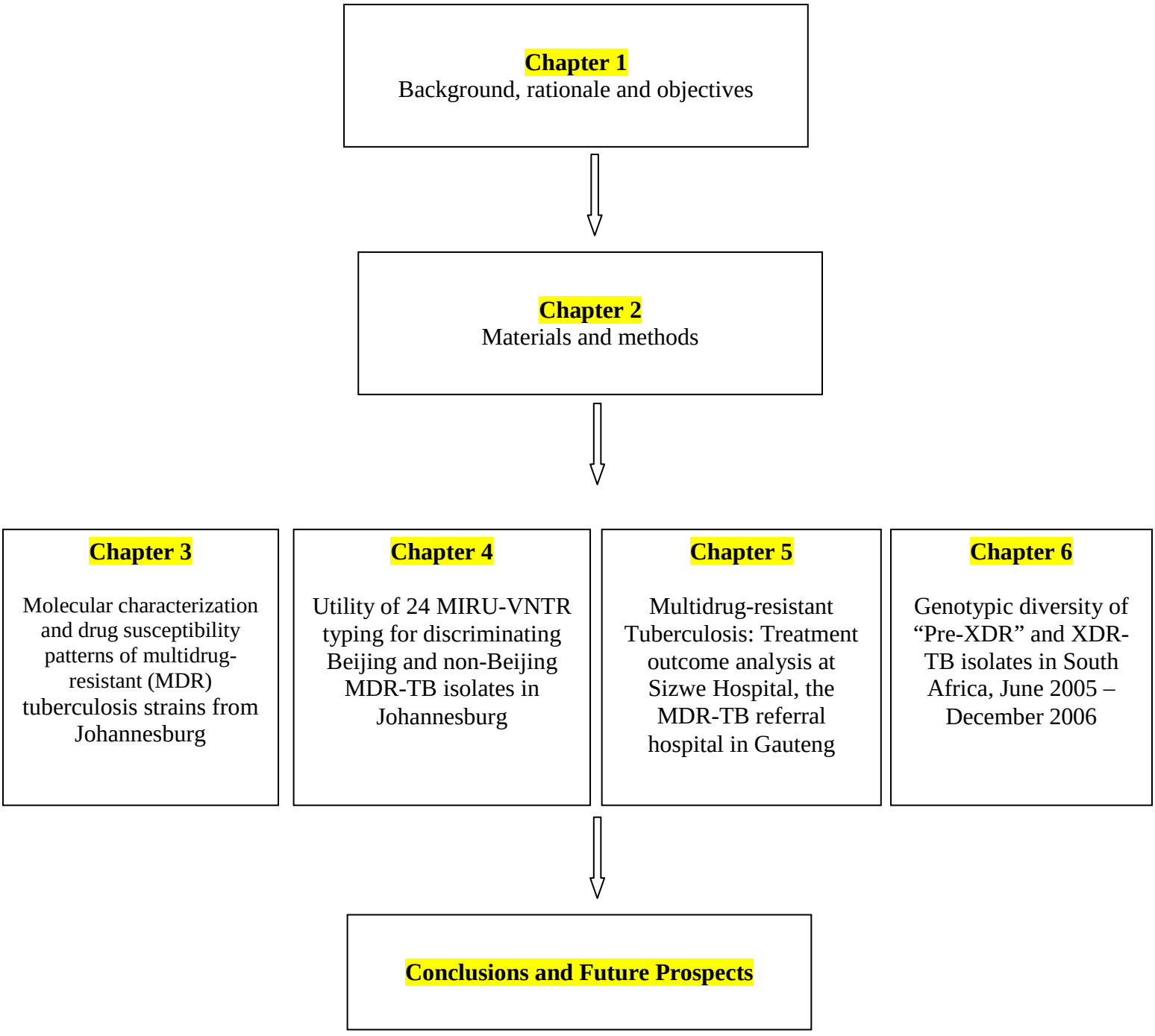


Figure 1.3: Thesis layout

CHAPTER 2: Materials and Methods

2.1 Sample Collection and Patient Data

2.1.1 The NHLS TB Referral Laboratory

The MDR-TB isolates used in the study were obtained from the National Health Laboratory Service Tuberculosis Referral Laboratory (NHLS TB Referral Lab) in Braamfontein, Johannesburg. This laboratory provides diagnostic services to the Department of Health, medical practitioners and to more than 100 clinics and hospitals, including Sizwe Hospital, the MDR-TB referral hospital in the Gauteng Province. The laboratory is the primary facility that performs drug susceptibility testing (DST) for first-line and second-line anti-TB drugs in the province and, for this reason, the samples submitted for testing to the lab form a good representation of MDR-TB strains circulating in the region.

2.1.2 Sample Collection

The isolates used were identified by searching the NHLS TB referral lab database for specimens submitted for drug susceptibility testing from the Johannesburg area and from Sizwe Hospital. The resultant list contained samples with various drug susceptibility patterns and also multiple samples per patient. These results were screened to remove duplicates and only the first known MDR-TB sample for each patient was selected for use in the study. The isolates included in the study exhibited one of four drug resistance profiles:

- resistance to isoniazid and rifampicin only
- resistance to isoniazid, rifampicin and streptomycin
- resistance to isoniazid, rifampicin and ethambutol
- resistance to all four anti-TB drugs

The current study sample reflects resistance patterns to first-line anti-TB drugs, since most of the samples do not have DST results for the second-line anti-TB drugs (kanamycin, ofloxacin and ethionamide). This is because second-line testing was not routinely performed in the NHLS TB referral lab and was only requested based on clinical suspicion prior to 2006. The policy has since been modified due to the development and spread of XDR-TB.

2.1.3 Patient Data Collection

MDR-TB samples from patients were divided into two groups based on the submitting health care facilities. The first group were patients with MDR-TB from any health care facility in the seven regions of Johannesburg. Information on these patients was obtained only from the NHLS TB referral database and included: age, gender, date of specimen collection, type of specimen, DST profile, clinic location and the region in Johannesburg from which the specimen was submitted. The second group were patients from Sizwe Hospital, the MDR-TB referral hospital (excluding any patients who were on the list from Johannesburg). This information included all the previously mentioned fields, together with additional information obtained from the patient files at the hospital. The additional information included; marital status, ethnicity, country of origin, employment status, housing status, association with the mines, association with prison, TB history, MDR treatment information, HIV serology, MDR-TB outcome and the date of the outcome. Permission to access patient files at Sizwe hospital was obtained from the Gauteng Provincial Department of Health (Appendix 2).

MDR-TB outcomes were defined using the standard definitions recommended by Laserson and colleagues as follows (Laserson *et al*. 2005):

- Cure: patient who has completed treatment according to the country's protocol and has been considered culture-negative (with at least five results) for the final twelve months of treatment. If only one positive culture is reported during that time, and there is no clinical evidence of deterioration, a patient may still be considered cured, provided that this positive culture is followed by a minimum of three consecutive negative cultures, taken at least thirty days apart.
- Treatment completed: patient who has completed treatment according to country's protocol but does not meet the definition for cure or treatment failure due to lack of bacteriological results (i.e. fewer than five cultures were performed in the final twelve months).
- Death: patient who dies for any reason during the course of MDR-TB treatment.
- Treatment default: patient whose MDR-TB treatment was interrupted for two or more consecutive months for any reason.
- Transfer out: patient who has been transferred to another reporting unit and for whom the treatment outcome is unknown.

2.1.3 Sample Processing

Samples were obtained from the NHLS TB referral lab as Mycobacteria Growth Indicator Tube (MGIT) culture (BD BioSciences, Sparks, MD, USA). Three aliquots were taken from each tube. The first was used for subculture in new MGITs (if growth in the original MGIT was very low).

The second was used for subculture onto Löwenstein–Jensen (LJ) slants, in preparation for RFLP analysis. And the third was heat-killed in preparation for spoligotyping and MIRU-VNTR typing. The original MGIT tubes were stored at 4°C.

2.1.3.1 Culture in MGITs

The materials provided by the manufacturer for culture in MGIT tubes included BBL Mycobacteria Growth Indicator Tube (MGIT), BACTEC™MGIT 960 supplement kit, containing BACTEC MGIT Growth Supplement and BBL MGIT™PANTA (Polymyxin, Amphotericin, Nalidixic acid, Trimethoprim, Azlocillin) antibiotic mixture (BD BioSciences, Sparks, MD, USA). Fifteen millilitres of the growth supplement was added to a lyophilized vial of the antibiotic mixture. Eight hundred microlitres of the growth supplement and PANTA mixture was then added to each MGIT tube, followed by 300µl of the specimen. The tubes were tightly closed and incubated at 37°C. Growth was detected by the presence of small 'grains' or flakes in the culture medium, usually after 3-6 weeks in culture. An unopened, un-inoculated MGIT tube was used as a control. Spoligotyping, described below in section 2.2, was used as a 'positive control' method to investigate possible cross contamination. Random samples were chosen and aliquots from the original MGIT and the subcultures were used to test for laboratory cross contamination of results.

2.1.3.2 Culture on LJ Slants

LJ slants (BD BioSciences, Sparks, MD, USA) were inoculated with 300µl of culture from the MGIT. The slants were incubated (positioned sideways) at 37°C overnight, then turned upright. The excess culture media from original inoculum was poured out after 7 days in culture.

The slants were aerated after every 3 days for 2 weeks, thereafter every week for 6 to 8 weeks depending on the growth observed. Growth was indicated by formation of granular white/cream colonies on the slants.

2.2 Spoligotyping

Spoligotyping was performed according to the manufacturer's instructions and using membranes and equipment provided with the spoligotyping kit (Isogen, Bioscience BV, Utrecht, The Netherlands). The protocol consisted of DNA extraction, PCR amplification, hybridization of PCR products, detection and interpretation results (Kamerbeek *et al*. 1997) as described below.

2.2.1 Sample Processing

Sample processing was performed in a Biosafety level III **(**BSL 3) laboratory due to the infectious nature of *M. tuberculosis*, using aerosol-resistant tips to avoid contamination. The sample preparation procedure was performed as described in the spoligotyping manual provided by the manufacturer (Isogen, Bioscience BV, Utrecht, The Netherlands). Two hundred microlitres of culture from MGIT was added in a screw-cap tube containing 500µl of 1 X TE buffer (refer to appendix for components) and incubated at 80°C for an hour in order to kill/lyse the bacterial cells. The screw-cap tubes were used to prevent aerosol formation during the heat killing process. The tubes were then centrifuged at 1800xg for 2mins, the supernatant discarded and pellet re-suspended in 500µl of 150mM NaCl. After a further 2mins centrifugation at 1800xg, the pellet was re-suspended in 300µl of 1 X TE buffer and stored at -20°C until further use.

2.2.2 Preparation of PCR Mixture

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

2.2.3 Hybridization

After the PCR reaction was completed, 20µl of the PCR product was added to 150µl 2 X SSPE/0.1%SDS in a microcentrifuge tube. The mixture was placed at 99°C for 10mins in order to denature the diluted PCR product. Immediately after denaturing, the tube was placed on ice for a further 5mins. The spoligomembrane (Isogen, Bioscience BV, Utrecht, The Netherlands) was washed in 2 X SSPE/0.1% SDS for 5mins, with constant shaking in a hybridization oven (MWG-Biotech AG, Ebersberg, Germany) at 57°C. The membrane was then placed on a support cushion in a miniblotter (Isogen, Bioscience BV, Utrecht, The Netherlands).

After preparation of the PCR product and assembly of the miniblotter/membrane, the equipment was moved to a biohazard safety cabinet located in a laboratory separate from the PCR lab. Any excess buffer remaining after pre-treating the membrane was aspirated. The slots were then slowly filled with the diluted denatured PCR product, with careful attention to avoid forming air bubbles in the slots. The first and last slots on the miniblotter we loaded with 2 X SSPE/0.1% SDS buffer, leaving the remaining lanes for samples and controls. After loading, the miniblotter/membrane was placed in a hybridization oven (MWG-Biotech AG, Ebersberg, Germany) for 60mins at 57°C, without shaking.

2.2.4 Detection

PCR products were removed by aspiration from the miniblotter slots after hybridization was complete. The membrane was washed twice in 2 X SSPE/0.5 SDS for 7mins at 57°C. Thirty millilitres of 2 X SSPE/0.5 SDS (42°C) was added with 7.5µl streptavidin peroxidase conjugate (500U/ml) in a 50 ml tube and gently mixed. The mixture was poured onto the membrane and incubated for 60mins at 42°C in a rolling bottle. After incubation, the membrane was washed twice with 2 X SSPE/0.5% SDS for 10mins at 42°C, followed by rinsing with 2 X SSPE for 5mins at room temperature. The detection step was achieved with the use of ECL detection liquid (Amersham Biosciences, Buckinghamshire, UK). The membrane was incubated in 10ml (5ml detection solution 1 and 5ml detection solution 2) for 90sec then transferred to a clear plastic bag. After heat–sealing, the bag was transferred into a 25 x 30 cm film cassette (Amersham Biosciences, Buckinghamshire, UK). The film was exposed to the membrane for periods ranging 5 to 20mins in a dark room at the Radiology Department at the Charlotte Maxeke Johannesburg academic hospital, and developed using a Kodak RPX-OXMAT Processor (Eastman Kodak Company, New York, USA).

2.2.5 Stripping the Membrane

Removal of the PCR products from the membrane (stripping) was done by washing the membrane twice in 1% SDS at 80°C for 30mins. The membrane was further washed in 20mM EDTA, pH 8, for 15mins at room temperature, then sealed in a plastic bag and stored at 4°C until further use.

2.2.6 Interpretation of Results

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

2.3 MIRU-VNTR typing

2.3.1 Sample Preparation

Samples for use in MIRU-VNTR typing were prepared as described previously in section 2.1.

2.3.2 PCR Amplification

PCR amplification of MIRU loci was performed using primers (described in Appendix 3) specific for the flanking regions of each locus. The PCR reaction mixture in all reactions contained 1X Master Mix: 0.05U/µl Taq DNA polymerase in reaction buffer, 2mM $MgCl_2$, 0.2Mm each for the deoxynucleotide triphosphates (Fermentas Life Sciences, Glen Burnie,

MD,USA), 2µM of the respective primer pair, DNA (1µg) and made up to a final volume of 25µl with sterile water.

The PCR amplification reaction was performed using an iCycler Thermocycler (Bio-rad Hercules, CA,USA) starting with a denaturing step for 10mins at 95°C, followed by 40 cycles of 1min at 94°C, 1min at 59°C and 30sec at 72°C. This was followed by a final extension step of 72°C for 10mins. DNA from *M. tuberculosis* strain H37Rv was included as positive control and sterile water was used a negative control. The primers used in the investigation included 12 MIRU primers (Supply *et al.* 2001, Mazars *et al.* 2001) , ETR (A, B, C) (Frothingham, Meeker-O'Connell 1998), QUB 11b and QUB 26 (Skuce *et al.* 2002) and QUB 4156 (Roring *et al.* 2002), Mtub (04, 21, 29, 30, 34 and 39) (Supply *et al.* 2006). Primer sequences are listed in Appendix 5.

2.3.3 Gel Preparation and Electrophoresis

PCR products were analyzed on 1.5% Seakem LE agarose gels (Lonza, Rockland, ME, USA) in 1X Tris-borate-EDTA (TBE) using the Sub-cell Model 96 apparatus (Bio-rad, Hercules, CA, USA). Eight microlitres of a 100bp marker (Fermentas Life Sciences, Glen Burnie, MD, USA) was loaded in both outside wells of the gel and also at 6 well intervals. This was done to facilitate easy determination of the sizes of the PCR products. Six microlitres of each PCR product was loaded. A control sample (H37Rv) was included with every gel. Standard sizes for H37Rv amplicons for the different loci are presented in Appendix 6 and Appendix 7. The gels were run at 100V for approximately 3hrs then stained with 400ml of 1X TBE buffer containing 1µg ethidium bromide for 15 to 30mins. Gels were photographed were using Gel Doc XR Documentation system (Bio-rad Laboratories, Hercules, CA, USA).

2.3.4 Sizing the Alleles

Amplification usually resulted in PCR products producing single sharp bands after gel electrophoresis. In a few cases, the sharp bands were accompanied by a ladder of much lower intensity bands (stutter peaks). In such cases, the PCR reaction was repeated as recommended in the protocol and the resultant band size was assigned. The sizes of the PCR products were either assessed manually or by using the Gel Doc XR Documentation system (Bio-rad Laboratories, Hercules, CA, USA). Sizing reference tables, shown in Appendix 6 and Appendix 7, were used to determine number of copies for each MIRU locus**.** H37Rv was used to verify the results for a particular locus by comparing with the allele number assigned for the H37Rv control for the locus used (Appendix 6 and Appendix 7).

2.4 Statistical Analysis

Univariable analysis was performed to examine associations between predominant MDR-TB strain clusters and age, gender, as well as drug susceptibility profiles using the Pearson's Chi-square test. Variables with *P* value of < 0.05 were regarded as statistically significant.

2.5 Ethics Approval

Ethics approval for this study was granted by the University of the Witwatersrand Human Research (Medical) Ethics Committee. The Ethics Clearance Certificate Number was M050628 (Appendix 1). Permission to access patient information from Sizwe Hospital was granted by the Gauteng Provincial Department of Health (Appendix 2).

CHAPTER 3: Molecular Characterization and Drug Susceptibility Patterns of Multidrug-resistant (MDR) Tuberculosis Strains from Johannesburg

3.0 Summary

Although TB is a major health problem in South Africa, knowledge regarding the circulating strains of *M. tuberculosis* and especially drug-resistant *M. tuberculosis* is limited. This chapter describes genotyping data and drug resistance profiles on a total of 434 multidrug-resistant (MDR) *M. tuberculosis* strains isolated from patients resident in Johannesburg between 2004 and 2007. Drug susceptibility testing for four major anti-TB drugs (isoniazid, rifampicin, streptomycin and ethambutol) showed that 238 (55%) of the *M. tuberculosis* isolates were resistant to all the four drugs. A comparison of spoligotyping results with the international spoligotyping database (SITVIT2) showed a total of 50 shared types. Forty-five shared types, containing 417 isolates, matched a pre-existing shared type whereas 5 shared types (containing 11 isolates) were newly created. Six isolates (orphans) were not identified in the database. High diversity was observed among the predominant families driving the MDR-TB epidemic (Beijing, Latin American Mediterranean (LAM), East-African-Indian (EAI), T, S, Haarlem (H) and X families). Differences were noted in the predominant spoligotypes in Johannesburg compared to the Western Cape Province (of particular interest was the under-representation of the SIT60/LAM4/KZN spoligotype and EAI family strains in the Western Cape).

Spoligotype-defined families were further split into sub-clusters by using 12 loci MIRU typing, resulting in 76 MIRU international types (MITs), containing 389 isolates and 45 orphan isolates and a clustering rate of 72.1%. The combined use of spoligotyping and MIRU typing decreased

the clustering rate to 66.8%. Spoligotyping showed the lowest discriminatory power (Hunter-Gaston discriminative index (HGDI) = 0.917) compared with MIRU typing (HGDI = 0.957) while combined spoligotyping and MIRU typing resulted in a HGDI of 0.962.

This chapter highlights the diversity of MDR-TB strains in Johannesburg. The high clustering rate suggests ongoing transmission and indicates that the other predominant MDR-TB strains are as transmissible as strains of the Beijing family. The findings also indicate the effectiveness of MIRU tying in differentiating spoligotype-defined clusters and illustrate the potential of these methods for the study of the molecular epidemiology of TB in the region.

3.1 Introduction

Genotyping is essential in the molecular epidemiology of *M. tuberculosis* since it identifies strains circulating in a population, making it possible to monitor prevalent strains or strain families that are overrepresented (Gori *et al.* 2005). Spoligotyping in particular has resulted in the construction of a well-used international database that not only assigns an identity to a given clinical isolate but also details the global phylogeography of *M. tuberculosis* complex strains (Brudey *et al.* 2006, Filliol *et al.* 2003).

Several studies using molecular typing techniques have identified seemingly stable associations between TB bacilli populations with their human hosts in various geographical areas (van Soolingen *et al.* 1995, Niobe-Eyangoh *et al.* 2004, Hirsh *et al.* 2004, Hermans *et al.* 1995, Niobe-Eyangoh *et al.* 2003). In East Asia for example, strains of the Beijing family predominate and represent more than 50% of the *M. tuberculosis* strain population (van Soolingen *et al.* 1995, Chuang *et al.* 2008, Dou *et al.* 2008b).

The Beijing family is the most well studied family in the world thus far, due mostly to its association with drug resistance and seemingly increased transmissibility (Glynn *et al.* 2002, Bifani *et al.* 1996). This genotype is thought to have been endemic in China for a long time (Qian *et al.* 1999) and has now spread worldwide (Brudey *et al.* 2006, Glynn *et al.* 2002). Other families, as defined by spoligotyping, have been found to predominate in other regions of the world, and these findings are summarized below.

The East-African-Indian (EAI) family is also prevalent in East, Middle and Central Asia, constituting 30% of circulating strains (Sola *et al*. 2001, Brudey *et al.* 2006, Douglas *et al.* 2003, Phyu *et al.* 2009). CAS1 (Central Asian)-Delhi family is predominant in the Middle East and in South and Central Asia (Phyu *et al.* 2009, Bhanu *et al.* 2002, Tanveer *et al.* 2008, Singh *et al.* 2004, Mathuria *et al.* 2008). In Europe the Haarlem family predominates while in South America approximately 50% of *M. tuberculosis* strains belong to the Latin American Mediterranean (LAM) family. The LAM family is also prevalent in the Mediterranean basin making up 36% of strains in Algeria and 55% in Morocco (Brudey *et al.* 2006). The X family, a well characterized IS*6110* low-banding family, is highly prevalent in North and Central America and is thought to be linked with Anglo-Saxon ancestry because it is mostly encountered in the UK and former British colonies (Sebban *et al.* 2002). In Africa, three major strain families are most frequently identified H, LAM and T family (Brudey *et al.* 2006, Victor *et al.* 2004, Mardassi *et al.* 2005, Streicher *et al.* 2004, Niobe-Eyangoh *et al.* 2003, Namouchi *et al.* 2008, Asiimwe *et al.* 2008, Tazi *et al.* 2007, Godreuil *et al.* 2007).

Even though most of the work from the African region has identified the above mentioned families, the sub-lineages and proportions of T, H and LAM families is not homogenous throughout the region. In some countries, a few predominant strain families fuel the TB epidemic (Niobe-Eyangoh *et al.* 2004, Easterbrook *et al.* 2004, Mardassi *et al.* 2005, Niobe-Eyangoh *et al.* 2003, Namouchi *et al.* 2008, Chihota *et al.* 2007) while in others, a high diversity of circulating TB strain families is observed (Stavrum *et al.* 2009, Kibiki *et al.* 2007, Homolka *et al.* 2008). In North Africa, the prevalent LAM strain type is SIT 42, together with H (SIT50) and T (ST53) (Namouchi *et al.* 2008). In the West Africa, LAM_10 CAM (SIT 61, the Cameroon family) predominates together with H and T family (Niobe-Eyangoh *et al.* 2003, Godreuil *et al.* 2007, Homolka *et al.* 2008). In East Africa, CAS_KILI, LAM, EAI, Beijing are prevalent, together with the T2 family (Kibiki *et al.* 2007, Eldholm *et al.* 2006, Githui *et al.* 2004) and in Southern Africa, a LAM strain, LAM11_ZWE (SIT 59) predominates in Zimbabwe and Zambia, together with LAM and T family (Easterbrook *et al.* 2004, Chihota *et al.* 2007).

In South Africa, extensive molecular fingerprinting of mycobacterial strains is performed by few units, with the majority of studies describing the local distribution of circulating strains from the Western Cape Province. Several studies from other regions have shown that even within a country, strains driving TB disease can differ and that it is important to focus TB control efforts at a local level (Abadia *et al.* 2009, Al-Hajoj *et al.* 2007, Singh *et al.* 2007c). Johannesburg therefore, being the most populous of the country's cities, could have a different strain composition of *M. tuberculosis* compared with Cape Town and the rest of the country's cities.
For TB control, it is important to understand whether specific genotype families are overrepresented and if these strains are successfully transmitted within the community.

The discriminatory ability of spoligotyping can be improved by applying a secondary typing method to clustered strains (Goguet de la Salmoniere *et al.* 1997). Spoligotyping in combination with MIRU-VNTR tying has a discriminatory power comparable to that of IS*6110*-RFLP, and much higher in isolates with low copy numbers of IS*6110* (Supply *et al.* 2001, Mazars *et al.* 2001, Lee *et al.* 2002, Cowan *et al.* 2002, Cowan *et al.* 2005, Kremer *et al.* 2005). Moreover, because of its high resolution, simplicity, reproducibility and easy laboratory comparison, MIRU-VNTR typing has been found to be highly suitable for global epidemiological surveillance of TB (Supply *et al.* 2003, Mazars *et al.* 2001, Sola *et al.* 2003, Kanduma *et al.* 2003, Mokrousov *et al.* 2004, Ferdinand *et al.* 2004). Numerous studies worldwide have used MIRU typing to improve discrimination and assess clonal diversity in the families identified by other typing methods (Kwara *et al.* 2003, Supply *et al.* 2001, Valcheva *et al.* 2008b, Sola *et al.* 2003, van Deutekom *et al.* 2005, Dou *et al.* 2009). MIRU typing has however only been used in limited studies in South Africa, mostly in association with Beijing family strains (Stavrum *et al.* 2009, Hanekom *et al.* 2008, Hanekom *et al.* 2007a).

This chapter details the MDR-TB strain families circulating in Johannesburg as identified using spoligotyping and MIRU analysis and also investigates associations between strain diversity, drug susceptibility profiles, and patient demographic information (age and gender). The chapter also describes the use of 12 loci MIRU typing in a diverse strain population and investigates the utility of 12 MIRU typing for differentiating MDR-TB strain families identified by spoligotyping in our setting.

3.2 Study Design

3.2.1 Sample Selection

434 MDR-TB isolates, collected from the National Health Laboratory Services (NHLS) Central TB lab in Braamfontein, Johannesburg, from March 2004 to December 2007 were analyzed in this study. One strain per patient was included (first MDR isolate). The samples were primarily sputum and were submitted to the NHLS TB referral laboratory from over 100 hospitals and clinics in Johannesburg. The NHLS TB referral lab records contain limited patient information and no clinical information e.g. history of treatment. The available demographic data (age, gender and geographic region) were obtained from NHLS TB referral lab records and analyzed in this study.

3.2.2 Molecular Genotyping

All MDR-TB isolates from the study population were typed using spoligotyping and MIRU analysis (Kamerbeek *et al.* 1997, Mazars *et al.* 2001). Spoligotyping was performed as described in Chapter 2. Individual spoligotyping patterns were entered in an Excel spreadsheet and compared to those of the international database SITVIT2, of the Pasteur Institute in Guadeloupe. MIRU typing (12 loci) was performed using primers and conditions as described in Chapter 2. MIRU data were sent to the Institut Pasteur de Guadeloupe for comparison and entered into an 'in-house' database containing 12 loci MIRU patterns referred to as MITs (MIRU international types).

3.2.3 Definition of Spoligotype-MIRU Clusters

Cluster analysis was performed for spoligotyping, MIRU typing and a combination of both spoligotyping and MIRU typing. A cluster was defined as two or more strains with identical genetic patterns, and strains with unmatched genetic patterns were considered non-clustered. Clusters were assumed to have arisen from recent transmission, and the clustering rate was used to estimate the amount of recent transmission in the population (Small *et al.* 1994). The minimum estimate of the proportion of TB cases related to recent transmission (clustering rate) was calculated as (number of clustered patients - number of clusters)/total number of patients.

3.2.4 Statistical Analysis

Univariable analysis was performed to examine associations between predominant MDR-TB strain clusters and age, gender, as well as drug susceptibility profiles using the Pearson's chi-square test. Variables with P value of < 0.05 were regarded as statistically significant.

3.3 Results

3.3.1 Study Population and Drug Resistance Profiles

The study population constituted 61% males, 38.5% females and gender information was not available for 0.5% of the patients. Patients in the group aged 16 to 35 years had the highest number (46.3%) of isolates, followed by those in age group 36 to 75 years (36.4%) and age group <1 to 15 years (7.8%). Data on age were not available for the remaining 9.5% of the patients. Drug susceptibility testing for four major anti-TB drugs (INH, RIF, STR and EMB) showed that 238 (55%) of the 434 *M. tuberculosis* isolates were resistant to all the four drugs. Ninety-four (22%) were resistant to INH and RIF only and sensitive to STR and EMB.

Fifty-seven isolates (13%) were resistant to INH, RIF and STR and 40 isolates (9%) were resistant to INH, RIF and EMB. Few isolates (1%) only had results for INH and RIF without additional DST for the other first-line drugs.

3.3.2 Spoligotyping

A total of 50 shared types were identified. Forty-five shared types, containing 417 isolates, (96%) of our sample matched a pre-existing shared type whereas 5 shared types (containing 11 isolates) were newly created shared types. The newly created shared types, were designated 2996 (n=2), 2997 (n=6), 2998 (n =1), 2999 (n=1) and 2196 (n=1) (Table 3.1). Six isolates were not identified in the database ("orphans"): one corresponded to the LAM family, one to H1 (Haarlem family), one to S family while three were of unknown origin (Table 3.2). Twenty eight clusters (2-71 isolates per cluster) were identified from the 50 shared types (Table 3.1). Among these, ten SITs were grouped as predominant/major shared types and included more than 10 isolates each (Table 3.3). These predominant SITs represented 337/434 (78%) of all the isolates. A description of the predominant spoligotypes and their worldwide distribution in the SITVIT 2 database is shown in Table 3.3.

Table 3.1: Description of 50 shared types (SIT) containing 434 MDR-TB isolates from Johannesburg, South Africa

SIT	Clade	Spoligotype Description	Total strains in this study	% in this study
1	Beijing	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	71	16.36
1092	CAS1-Delhi	■■■□□□□■□■■■■■■■■■■■■■□□□□□□□□□□□■■■■■■■■■	1	0.23
21	CAS1-Kili	■■■□□□□■■□■■■■■■■■■□□□□□□□□□□□□□□□■■■■■■■■	7	1.61
48	EAI1-SOM	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■□■■■	17	3.92
806	EAI1-SOM	■■■■□■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■□■■■	20	4.61
2997*	EAI1-SOM	■■■■■□■□■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■□■■■	6	1.38
2998*	EAI1-SOM	■■■■■■■■■■■■■□□■■■■■■■■■■■■□□□□■□■■■■■□■■■	1	0.23
47	H1	■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■■■■■■	2	0.46
62	H1	■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■□■■■	3	0.69
2375	H1	■■■■■□■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■■■■■■	5	1.15
36	H3	■■■■■■■■■■■■□■■■■■■■■■■■■■■■□■□□□□■■■■■■■■	1	0.23
49	H3	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■□■■■	1	0.23
50	H3	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■■	37	8.53
168	H3	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■□■■■■	1	0.23
2999*	LAM	■■■■■■■■■■■■■■□□□■□□□□□□■■■■■■■■□□□□■■■□■■■	1	0.23
20	LAM1	■■□■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■■	3	0.69
815	LAM11-ZWE	■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■■□□□□■■■□■■■	1	0.23
2196*	LAM11-ZWE	■■■■■■■■■■■■■■■■□■■■□□□□■■□□□□■■□□□□■■■□■■■	1	0.23
17	LAM2	■■□■■■■■■■■■□■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■■	2	0.46
4	LAM3	□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■□□□□■■■■■■■■	1	0.23
33	LAM3	■■■■■■■■□□□■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■■	16	3.69
719	LAM3	■■■■■■■■□□□■■■■■■■■□□□□□■■■■■■■■□□□□■■■■■■■■	3	0.69
60	LAM4	■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■□■■■	69	15.9
811	LAM4	■■■■■■■■■■■■■■■■■■■■□□□□■□□□□□■■□□□□■■■□■■■	4	0.92
42	LAM9	■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■■	6	1.38
81	LAM9	■■■■■■■■■□■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■■	1	0.23
766	LAM9	■■■■■■■■■■■■■■□□□■□□□□□□■■■■■■■■□□□□■■■■■■■■	2	0.46
803	LAM9	■■■■■■■■■■■■■■□□□□□□□□□□□■■■■■■■■□□□□■■■■■■■■	1	0.23
2165	LAM9	■■■■■■■■■■■■■■□□■■■■■■□□□□■■■■■■■■□□□□■■■■■■■■	1	0.23
1634	MANU2	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□■■■■■■■■■	1	0.23
2992	MANU2	■■■■■■■■■■■■■■■■■■■■■■■■□□■■■■■■■■□□■■■■■■■■■	1	0.23
34	S	■■■■■■■■□□■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■	32	7.37
789	S	■■□■■■■■■□□■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■	1	0.23

Continued on next page

***Table 3.1**: Continued from previous page*

SIT	Clade	Spoligotype Description	Total strains in this study	% in this study
73	T	■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	2	0.46
53	T1	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	45	10.37
244	T1	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■	10	2.3
344	T1	■■■□□□□□□□□□■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	1	0.23
519	T1	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■■■■■■	1	0.23
649	T1	□■■■■■■■■■■■■■■■■■■□□□■□□■■■□■■■□□□□■■■■■■■	3	0.69
926	T1	■■■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	5	1.15
2531	T1	■■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■	6	1.38
52	T2	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	1	0.23
37	T3	■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	5	1.15
46	Unknown	■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□□□□□□□□	3	0.69
563	Unknown	■■■■■■■■■■■■□■■■■■■■■■■■□□□□□□□□□□□□□□□■■■■	1	0.23
1516	Unknown	■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■■	1	0.23
2996*	Unknown	■■■□■□□□□□□□□■□□□■■□□□□□□□□□□□□□□□□□□□■■■■■	2	0.46
2016	X2	■■■□■■■■■■■■■■■■□■■■■■■■■■■■■■■■□□□□■■□□□□■	1	0.23
92	X3	■■■□□□□□□□□□■■■■■■□■■■■■■■■■■■■■■■□□□□■■■■■■■	20	4.61
200	X3	■■■□□□□□□□□□■■■■■■□■■■■■■■■■■■■■■■□□□□■■■□□□□	1	0.23

Note that an additional 6 orphan patterns are shown in Table 3.2.
SIT* followed by an asterisk indicates "newly created shared-type" after matching with another orphan in the SITVIT2, or due to 2 or more strains belonging to a new pattern within this study: SIT2997 matched an orphan from Australia, SIT2998 matched an orphan from South Africa, SIT 2999 matched an orphan from Poland, SIT2996 was created by 2 isolates in the present study. Clade designations are according to SITVIT2 using revised SpolDB4 rules. Unk: Unknown patterns within any of the major clades described in SITVIT2.

Table 3.2: Description of the orphan strains from the study

Isolation Number	Year of isolation	Spoligotype pattern	Octal number	Clade	Drug resistance			
					INH	RIF	STR	EMB
J328	2004	■■■■■■■■□□■■■■■■■■■■■□■■□■■■■■■■□□□□■■■■■■■	776377733760771	S	R	R	R	R
J289	2004	■■■□□□□■■□■■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■■	703377777761771	Unknown	R	R	R	R
J271	2005	■■■■■■■■■■■■■□□□□■□□□□□□■■■■■■■■□□□□■■□□□□■	777741007760601	LAM	R	R	R	S
J279	2005	■■■■□■■■□□□■■■■■■■■■□□□□■■■■■■■■□□■■■■■■■■■	756177607763771	Unknown	R	R	R	S
J290	2005	■■■□□□□□□□□□■□■■■□■■■■■■■■■■■■■■□□□□■■■■■■■	700056777760771	Unknown	R	R	R	S
J273	2006	■■■■■■■■■□■■■■■■■■■■■■■■■□□□□□□■□□□□■■□□■□■	777377774020621	H1	R	R	R	S

Clade designations according to SITVIT2 using revised SpolDB4 rules; Unk: Unknown patterns within any of the major clades described in SITVIT2. LAM = Latin American Mediterranean family, H = Haarlem family, S = S family. INH = isoniazid, RIF = rifampicin, EMB = ethambutol. R = resistant, S = susceptible.

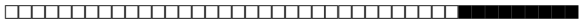
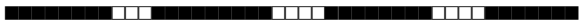
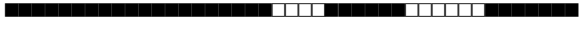
Table 3.3: Description of predominant MDR *M. tuberculosis* shared-types representing ≥2% strains, (9 or more strains) from this study, and their distribution in Africa and worldwide in the SITVIT2 database.

SIT*	Clade	Total strains in this study	% in this study	% in South Africa (excluding this study) according to SITVIT2**	Distribution in regions from AFRICA with ≥5% of a given SIT***	World-wide distribution in countries with ≥5% of a given SIT****
SIT 1	Beijing	71	16.36	19.93	AFRI-S (18.41), AFRI-E (1.65), AFRI-N (0.85), AFRI-W (0.31)	ZAF (18.41), USA (13.69), JPN (11.81), RUS (9.92)
SIT 60	LAM 4	69	15.90	23.67	AFRI-S (39.49), AFRI-N (7.25), AFRI-W (6.88), AFRI-E (1.09)	ZAF (39.49), BRA (10.87), VEN (5.43)
SIT 53	T1	45	10.37	8.72	AFRI-S (7.85), AFRI-N (4.51), AFRI-E (3.11), AFRI-W (2.10), AFRI-M (0.11)	ZAF (7.80), ITA (6.70), BRA (6.03), TUR (5.89)
SIT 50	H3	37	8.53	3.54	AFRI-N (5.12), AFRI-S (4.68), AFRI-E (1.36), AFRI-W (1.25), AFRI-M (0.16)	ESP (9.96), ITA (7.46), BRA (6.97), AUT (6.80), USA (5.61)
SIT 34	S	32	7.37	20.04	AFRI-S (22.88), AFRI-N (3.64), AFRI-E (1.56)	ZAF (22.88), CAN (12.82), ITA (11.09), BRA (7.45), USA (7.11)
SIT 806	EAI1-SOM	20	4.61	16.67	ST <5%	ST <5%
SIT 92	X3	20	4.61	65.25	AFRI-S (61.76), AFRI-E (0.98), AFRI-W (0.33), AFRI-N (0.33)	ZAF (61.76), USA (8.82), BRA (7.19)
SIT 48	EAI1-SOM	17	3.92	8.37	AFRI-S (13.64), AFRI-E (2.27), AFRI-N (1.52)	NLD (17.80), ZAF (13.64), BGD (11.74), DNK (10.98), IND (7.58), SAU (5.68)
SIT 33	LAM 3	16	3.69	45.70	AFRI-S (37.97), AFRI-N (2.01), AFRI-E (0.63), AFRI-W (0.50)	ZAF (37.97), ESP (11.03), BRA (10.65), ARG (7.14), PER (6.77), USA (5.64)
SIT 244	T1	10	2.30	13.33	AFRI-S (24.32), AFRI-E (5.41), AFRI-W (2.70)	ZAF (24.32), PRT (21.62), BRA (16.22), BGD (8.11), FRA (5.41)

* Predominant shared types are defined as SITs representing 2% or more strains in a given dataset (9 or more strains in this study);
Clades are designated according to SITVIT2 using revised SpolDB4 rules.
** % of each SIT compared to what is available in SITVIT2 from South Africa (excluding this study). E.g. if according to SITVIT2 and SIT in the world (937) and in South Africa (ZAF) (294), excluding this study : (294/937)*100 = 31.4%
*** Worldwide distribution is reported for regions with ≥5% of a given SIT as compared to their total number in the SITVIT2 database. The definition of geographical regions and sub-regions is according to the United Nations (http://unstats.un.org/unsd/methods/m49/m49region.htm).
**** The 3 letter country codes are according to http://en.wikipedia.org/wiki/ISO_3166-1_alpha-3; Countrywide distribution is only shown for SITs with≥5% of a given SITs as compared to their total number in the SITVIT2 database.

Table 3.4 shows a comparison of the predominant spoligotypes from Johannesburg compared with the major spoligotypes of drug-resistant *M. tuberculosis* strains circulating in the Western Cape Province during the study period (2004 -2007). The Western Cape data were obtained from an 'in house' database of the Department of Science and Technology/National Research Foundation Centre of Excellence in Biomedical Tuberculosis Research, Medical Research Centre (MRC) Centre for Molecular and Cellular Biology, Stellenbosch University. A total of 984 isolates collected in the Boland/Overberg and Karoo/Southern Cape regions during the study period were represented in the database. Only the predominant spoligotypes (≥ 10 isolates) from this sample are shown in Table 3.4.

Table 3.4: Description of predominant MDR-TB shared-types representing ≥2% strains (i.e. 10 or more strains) from the Western Cape Province, 2004 – 2007, and comparison with this study.

Clade*	SIT	Spoligotype pattern	Octal code	Western Cape	Johannesburg
				No. (total %) of strains	No. (total %) of strains
Beijing	1*		000000000003771	368 (39.4)	71 (16.36)
X1	119		777776777760771	92 (9.85)	
LAM3	33*		776177607760771	72 (7.71)	16 (3.69)
T1	53*		777777777760771	47 (5.03)	45 (10.37)
X1	1329		777776777560771	36 (3.85)	
X3	92*		700076777760771	30 (3.21)	20 (4.61)
T4_CEU 1	39		777777347760471	19 (2.03)	
U	1241		777777607700771	19 (2.03)	
T1	1067		774037707760700	15 (1.61)	
X1	336		777776777760731	14 (1.5)	
X2	137		777776777760601	14 (1.5)	
H3	50*		777777777720771	13 (1.39)	37 (8.53)
T5	44		777777757760771	13 (1.39)	
S	34*		776377777760771	10 (1.07)	32 (7.37)

SIT and Clade designations are according to SITVIT2 using revised SpolDB4 rules. SIT*indicates shared types also predominant in Johannesburg region. Source for Western Cape data: Department of Science and Technology/National Research Foundation Centre of Excellence in Biomedical Tuberculosis Research, Medical Research Centre (MRC) Centre for Molecular and Cellular Biology, Stellenbosch University.

3.3.3 MIRU Typing

All the 434 MDR clinical isolates were typed by MIRU typing and compared with an 'in-house' database of the Institut Pasteur de Guadeloupe which in addition to spoligotyping patterns also contains 12-loci MIRU patterns (referred to as MIRU international types, MITs). The 50 shared types previously identified by spoligotyping were readily distinguished from each other by MIRU typing and further split into 76 MITs containing 389 isolates (Table 3.5) together with 45 orphan isolates (Table 3.6). MIRU analysis of the newly created types confirmed the grouping obtained by spoligotyping grouping; SIT 2996 (MIT 313), SIT 2997 (MIT 65), SIT 2998 (MIT 64) and SIT 2999 (MIT 132) (Table 3.7). The MITs obtained when predominant spoligotypes were sub-clustered by MIRU typing are shown in Table 3.8.

Table 3.5: Description of MIRU shared types from this study. A total of 76 MITs containing 389 isolates matched a pre-existing shared type in the SITVIT2 database.

12 loci MIRU pattern	MIT* for 12 MIRU	Total (%) in this study
223125153324	8	23 (5.3)
223226143321	10	1 (0.23)
223325173533	17	17 (3.92)
224225153324	23	1 (0.23)
224325153224	31	1 (0.23)
224325153322	32	2 (0.46)
224325153323	33	6 (1.38)
224325153324	34	4 (0.92)
224326143324	39	1 (0.23)
225323153323	43	1 (0.23)
254326223513	64	3 (0.69)
254326223613	65	6 (1.38)
223325183533	84	3 (0.69)
223325171531	96	5 (1.15)
223325173543	99	8 (1.84)
223325173523	101	4 (0.92)
222325173543	104	1 (0.23)
223325143324	112	1 (0.23)
224325143324	117	4 (0.92)
223226153321	128	3 (0.69)
123326153224	132	1 (0.23)
124326153224	140	3 (0.69)
223125153322	156	1 (0.23)
223125153224	158	4 (0.92)
224325153321	161	1 (0.23)
224126152321	163	2 (0.46)
233325153324	212	8 (1.84)
224326153323	213	10 (2.3)
224326153324	215	1 (0.23)
223326153321	224	6 (1.38)
224325173533	229	1 (0.23)
223125143324	231	1 (0.23)
333225143324	232	5 (1.15)
223326153323	236	2 (0.46)
223126152321	237	4 (0.92)
223325174533	245	4 (0.92)

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Table 3.5*: continued from previous page*

12 loci MIRU pattern	MIT* for 12 MIRU	Total (%) in this study
124326153324	246	67 (15.44)
224326173323	248	1 (0.23)
223325163531	254	10 (2.3)
233325153325	256	3 (0.69)
227425113434	261	1 (0.23)
223326153322	289	1 (0.23)
224326133324	313	2 (0.46)
224325163323	343	1 (0.23)
223326143321	402	1 (0.23)
124326153325	593	1 (0.23)
225325143323	611	1 (0.23)
223326153311	699	1 (0.23)
233325143325	804	1 (0.23)
123325153323	897	5 (1.15)
224126153321	953	1 (0.23)
124326153322	991	1 (0.23)
234326153321	1061	1 (0.23)
223325174523	1202	3 (0.69)
223325123521	1203	2 (0.46)
223325173542	1204	2 (0.46)
223225173523	1205	4 (0.92)
223325163521	1206	2 (0.46)
227425112434	1207	4 (0.92)
254126223413	1208	8 (1.84)
254226223413	1209	6 (1.36)
254326223414	1210	19 (4.38)
225425183324	1211	39 (8.99)
223327163321	1212	2 (0.46)
224316153323	1213	1 (0.23)
224327153323	1214	2 (0.46)
223226143221	1215	4 (0.92)
333325153224	1216	8 (1.84)
333225153324	1217	2 (0.46)
233325154225	1218	3 (0.69)
124326153223	1219	1 (0.23)
124316153225	1220	2 (0.46)

Continued on next page

Table 3.5*: continued from previous page*

12 loci MIRU pattern	MIT* for 12 MIRU	Total (%) in this study
224315173221	1221	2 (0.46)
223415153323	1222	4 (0.92)
224436155322	1223	3 (0.69)
225436155324	1224	18 (4.15)
	orphan	45 (10.37)
Total		434

*12 loci MIRU sequence: MIRU 02, 04, 10, 16, 20, 23, 24, 26, 27, 31, 39, 40
All strains matching a pre-existing pattern in the SITVIT2 database are classified as MITs (in case of no match, they are designated as "orphan" as shown in Table 3.6).

Table 3.6: Description of the orphan MIRU patterns (n=45) from this study and corresponding spoligotyping-based information (octal code, SIT, and clade).

Strain number	12 loci MIRU (orphan pattern)	Octal code	SIT	Clade
J7	223325152523	000000000003771	1	Beijing
J29	223325176523	000000000003771	1	Beijing
J36	223225174533	000000000003771	1	Beijing
J51	223325121521	000000000003771	1	Beijing
J118	227223153323	777777774020731	62	H1
J74	227425123434	703377400001771	21	CAS1-Kili
J78	227325112434	703377400001771	21	CAS1-Kili
J106	254426283414	757777777413731	806	EAI1-SOM
J110	22432612341_	757777777413731	806	EAI1-SOM
J128	22_326153323	777777777720771	50	H3
J20	2233251735_3	000000000003771	1	Beijing
J273	2253231_3323	777377774020621	Orphan	H1
J69	223325123531	000000000003771	1	Beijing
J94	254_26223513	777777777413731	48	EAI1-SOM
J120	226223153323	777777774020731	62	H1
J160	225225153_24	777377607760771	81	LAM9
J173	124316153323	776177607760771	33	LAM3
J177	224326173324	776177607760771	33	LAM3
J192	123326153321	777777607760731	60	LAM4
J264	22332517___3	777777777723771	1634	MANU2
J266	22532_153433	777777747763771	2992	MANU2
J272	22__25113_3_	777377777761771	1516	Unknown
J279	22_32_1_3__3	756177607763771	Orphan	Unknown
J289	22__25113__4	703377777761771	Orphan	Unknown
J295	2243251_3323	677777777760601	2531	T1
J297	233325144223	776377777760771	34	S
J305	333325123323	776377777760771	34	S
J317	2_3325143325	776377777760771	34	S
J327	233325154224	776377777760771	34	S
J354	224437145221	777777777740371	519	T1
J355	224536155322	777737770000071	563	Unknown
J359	225436155323	677777777760601	2531	T1
J360	225437165324	700076777760771	92	X3
J372	224436165531	700076777760771	92	X3
J377	226536195324	700076777760771	92	X3
J385	213326143324	777777777760771	53	T1

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***Table3.6**: Continued from previous page*

Strain number	12 loci MIRU (orphan pattern)	Octal code	SIT	Clade
J389	_2232615332_	777777777760771	53	T1
J390	22_125153326	777777777760771	53	T1
J391	222425152221	777777777760731	52	T2
J402	223125143222	777777777760771	53	T1
J412	223124153325	777777777760771	53	T1
J415	223325143332	777777777760771	53	T1
J423	2243251_332_	777777777760771	53	T1
J427	22_32515332_	777777777760771	53	T1
J432	22_125153324	777777777760771	53	T1

12 loci MIRU sequence: MIRU 02, 04, 10, 16, 20, 23, 24, 26, 27, 31, 39, 40
SIT and Clade designations are according to SITVIT2 using revised SpolDB4 rules
_denotes alleles that could not be amplified at particular MIRU loci (missing alleles)

Table 3.7: MIRU profiles of the five newly created shared types (11 isolates)

Strain number	Spoligotype (SIT)	MIRU 2	MIRU 4	MIRU 10	MIRU 16	MIRU 20	MIRU 23	MIRU 24	MIRU 26	MIRU 27	MIRU 31	MIRU 39	MIRU 40	**MIT profiles**
J267	2196	2	2	4	1	2	6	1	5	2	3	2	1	163
J287	2996	2	2	4	3	2	6	1	3	3	3	2	4	313
J288	2996	2	2	4	3	2	6	1	3	3	3	2	4	313
J280	2997	2	5	4	3	2	6	2	2	3	6	1	3	65
J281	2997	2	5	4	3	2	6	2	2	3	6	1	3	65
J282	2997	2	5	4	3	2	6	2	2	3	6	1	3	65
J283	2997	2	5	4	3	2	6	2	2	3	6	1	3	65
J284	2997	2	5	4	3	2	6	2	2	3	6	1	3	65
J285	2997	2	5	4	3	2	6	2	2	3	6	1	3	65
J268	2998	2	5	4	3	2	6	2	2	3	5	1	3	64
J269	2999	1	2	3	3	2	6	1	5	3	2	2	4	132

Note: Concordance between newly described spoligotypes (SITs) and MIRU profiles (MITs).

Table 3.8: 12 loci MIRU-based sub-clustering of spoligotype-defined clusters of predominant *M. tuberculosis* shared-types from Johannesburg

SIT* (Octal code) Clade	Total strains (%) in this study	MIRU-based sub-clustering (MIT** designation and number of strains per cluster)
SIT1 (000000000003771) Beijing	71 (16.36)	MIT17 (n=17), MIT84 (n=3), MIT96 (n=5), MIT99 (n=8), MIT101 (n=4), MIT104 (n=1) MIT254 (n=10), MIT1202 (n=3), MIT1203 (n=2), MIT1204 (n=2), MIT1205 (n=4), MIT1206 (n=2), Orphan (n=6)
SIT60 (777777607760731) LAM4	69 (15.90)	MIT34 (n=1), MIT215 (n=1), MIT246 (n=64), MIT593 (n=1),MIT99 (n=1), orphan (n=1)
SIT53 (777777777760771) T1	45 (10.37)	MIT8 (n=19), MIT31 (n=1), MIT33 (n=1), MIT34 (n=1), MIT156 (n=1), MIT158 (n=3) MIT224 (n=5), MIT231 (n=1), MIT246 (n=2), MIT953 (n=1), MIT1061 (n=1), Orphan (n=9)
SIT50 (777777777720771) H3	37 (8.53)	MIT23 (n=1), MIT1211(n=35), Orphan (n=1)
SIT34 (776377777760771) S	32 (7.37)	MIT212 (n=6), MIT232 (n=5), MIT256 (n=3), MIT804 (n=1), MIT1216 (n=8) MIT1217 (n=2), MIT1218 (n=3), Orphan (n=4)
SIT806 (757777777413731) EAI1_SOM	20 (4.61)	MIT1210 (n=18), Orphan (n=2)
SIT92 (700076777760771) X3	20 (4.61)	MIT1224 (n=17), Orphan (n=3)
SIT 48 (777777777413731) EAI1-SOM	17 (3.92)	MIT64 (n=2), MIT1208 (n=8), MIT1209 (n=6), Orphan (n=1)
SIT33 (776177607760771) LAM3	16 (3.69)	MIT213 (n=8), MIT236 (n=2), MIT246 (n=1), MIT1210 (n=1) MIT1213 (n=1), MIT1214 (n=1), Orphan (n=2)
SIT 244 (777777777760601) T1	10 (2.30)	MIT32 (n=2), MIT34 (n=1), MIT39 (n=1), MIT117 (n=4), MIT161 (n=1), MIT289 (n=1)

* Predominant shared types are defined as SITs representing 2% or more strains in a given dataset (i.e., 7 or more strains in this study); clades are designated according to SITVIT2 using revised SpolDB4 rules.

3.4 Discussion

The current study is the first study in Johannesburg to report on the strain diversity of a substantial sample of MDR-TB strains. MDR-TB is a major healthcare burden in resource poor countries that may lack adequate or uninterrupted supplies of second-line drugs and where the methods to detect drug resistance are limited. In such countries the lack of resources hinders regular drug resistance surveys and therefore the magnitude of the problem remains largely unknown. A national survey published by the Medical Research Council in 2004 detailed information on TB drug resistance in South Africa. In the report, drug resistance to all first-line drugs tested in new cases was 0.5% and in re-treatment cases, 1.4%. In our study of MDR-TB isolates, we observed that 55% (238/434) of the isolates showed resistance to all the first-line drugs.

In countries like South Africa, where TB is endemic, it is important to identify predominant strains in order to monitor changes in strain composition within communities and to understand the epidemiology of the disease in the country as a whole. In Johannesburg the spoligotype patterns showed diversity in the predominant strain families and even in shared types within certain families. A comparison of the predominant strain families and their worldwide distribution in the SITVIT 2 database showed that the families were represented in all regions but in varying proportions. The three strain families (H, LAM and T) reported to be prevalent in the African continent (Brudey *et al.* 2006) were indeed present in all the regions of the continent as shown on the SITVIT 2 database.

There were however some differences in the circulating predominant shared types when the Johannesburg strains were further compared with published studies from specific African regions.

The LAM spoligotype SIT 42, which is prevalent in North Africa (Namouchi *et al.* 2008) as well as LAM 11_ZWE (SIT 59), which predominates in neighbouring Zambia and Zimbabwe (Easterbrook *et al.* 2004, Chihota *et al.* 2007) were not well represented in our sample. The study sample also did not identify any spoligotypes of the LAM_10 CAM (SIT 61, the Cameroon) family which predominates in West Africa (Niobe-Eyangoh *et al.* 2003, Godreuil *et al.* 2007, Homolka *et al.* 2008). These observations were unexpected because Johannesburg attracts and receives a diverse population of legal and illegal immigrants from all over the African continent.

The lack and/or under representation of these spoligotypes in our study sample could be explained by a number of reasons. Firstly, even though the NHLS TB Reference laboratory receives specimens from public hospitals in all the regions of Johannesburg, services at these hospitals are not free for foreign nationals. This situation is even worse for illegal immigrants seeking such services because documentation displaying a valid visa as well as payment is required prior to treatment. It could therefore be that the majority of samples sent to the TB laboratory exclude such patients. Other explanations could include; insufficient time for the strains to establish in the population; inherent microbial characteristics or even the characteristics of the host population in Johannesburg. Further analysis of MDR-TB spoligotypes from other regions of the continent would be required to investigate difference between those strains and the local predominant MDR-TB strains.

The diversity in the predominant strains observed in the study is consistent with other studies from South Africa (Mlambo *et al.* 2008, Stavrum *et al.* 2009, Streicher *et al.* 2004). A detailed comparison with strains from this study with drug-resistant strains in the Western Cape during the study period revealed some differences. Interestingly, SIT 60 was not predominant in the Western Cape whereas it was the second predominant spoligotype in Johannesburg. SIT 60 (LAM4/F15/KZN) is often referred to as the KZN strain because this strain type was responsible for an outbreak of XDR-TB in the KZN Province, where it was identified in 85% of the patients (Gandhi *et al.* 2006). A more extensive comparison with the global spoligotyping database indicated this strain type was in fact poorly represented in all African regions except for Southern Africa, and in particular, South Africa. Since the strain family was not represented in the Western Cape, it may be that the high representation in SITVIT 2, excluding this study, is due to information submitted from KZN.

This study also showed that the EAI1-SOM family (SIT 48, 806) is predominant in Johannesburg. According to the SITVIT 2 database, SIT 48 has been reported in East, North and Southern Africa as well as Europe, Asia and Middle East. SIT 806 however showed low distribution both in Africa and globally. Data from the Western Cape showed that the EAI family did not form part of the predominant families and was generally not well represented (4/984) in the population of circulating strains in 2004-2007. EAI ancestral strains are thought to have spread back to Africa from Asia through India along with human migrations (Filliol *et al*. 2003) and currently predominate in East Africa and India. The representation of this strain in Johannesburg compared to the Western Cape could be explained by differences in the ethnic populations in the provinces.

The X family, especially SIT 92, was predominant both in Johannesburg and in the Western Cape. This family is however not well represented in the rest of Africa according to the SITVIT 2 database and, in fact, most of the X family strains in the Western Cape (SITs 336, 137, 1329, 119) were not identified in the Johannesburg sample. The X family is thought to be linked with Anglo-Saxon ancestry (Sebban *et al.* 2002) and the overrepresentation of this family in the Western Cape could reflect previous colonization history and the use of Cape Town as the main port of entry.

The most prevalent strain family in the study, Beijing family, has a well documented association with drug resistance in the literature (Marais *et al.* 2006, Almeida *et al.* 2005, Cox *et al.* 2005, Park *et al.* 2005, Toungoussova *et al.* 2004, Bifani *et al.* 2002). The worldwide distribution of this strain family has led to speculation that it has selective advantage over other clinical isolates to cause disease (Bifani *et al.* 2002). Hypotheses explaining the successful transmission of Beijing include: reduced susceptibility to anti-TB drugs, resulting in a large expansion in Asia and worldwide; resistance to *Mycobacterium bovis* BCG-induced immunity; introduction to naive populations and alteration in gene function due to IS*6110* insertions (Bifani *et al.* 2002, Beggs *et al.* 2000, Abebe, Bjune 2006, Gagneux *et al.* 2006).

The presence of the Beijing family was expected in the current study sample because the strain family had been identified in various regions in the African continent (Kibiki *et al.* 2007, Homolka *et al.* 2008, Githui *et al.* 2004, Glynn *et al.* 2005), sometimes associated with drug resistance (Stavrum *et al.* 2009, Streicher *et al.* 2004).

Other families that have been associated with drug resistance and outbreaks in the region include the Haarlem family (SIT 50), which was associated with outbreaks in North Africa (Mardassi *et al.* 2005); LAM (SIT 60) was associated with an outbreak of XDR-TB among primarily HIV-positive patients in KZN (Gandhi *et al.* 2006) and LAM3/F11,which is one of the strains driving the drug-resistant TB epidemic in the Western Cape (Victor *et al.* 2004, Streicher *et al.* 2004). These drug-resistant *M. tuberculosis* strain families pose a potential threat for outbreaks of MDR and XDR-TB in the region, especially among immunocompromised persons.

Interpreting data on the significance of prevalent strain populations for the clinical benefit of patients is challenging because the factors that contribute to the success of any particular *M. tuberculosis* genotype are not well understood. Abundance alone is not necessarily an indicator of virulence in bacterial populations and therefore a better understanding of the effects of host genetic and environmental variability on disease presentation is required (Malik, Godfrey-Faussett 2005). The prevalence of TB in large urban areas such as Johannesburg is further complicated by close human-to-human contacts and potential multiple sources of *M. tuberculosis* from the diverse ethnic groups migrating to the city from different regions in South Africa and the rest of the continent in search of better work opportunities

In the current study, the spoligotype-defined families were further typed by MIRU typing in order to further differentiate and assess clonal diversity within the spoligotype families. As with studies of *M. tuberculosis* strain populations from other countries (Valcheva *et al.* 2008b, Sola *et al.* 2003), this study showed that the combination of the two techniques improved the discriminatory power (HGDI = 0.962) compared to spoligotyping alone (HGDI=0.917).

Using the combined methods also decreased the clustering rate to 66.8%, whereas spoligotyping alone identified 87.1% clustering and MIRU used alone identified 72.1% clustering. In epidemiological studies patients whose isolates have identical patterns (i.e. a cluster) are considered likely to have been infected recently and can therefore be targeted for epidemiological investigation to identify the chain of transmission (van Soolingen 2001). Interpreting the proportion of disease due to recent transmission directly derived from a clustered proportion is however complicated (Glynn *et al.* 1999). In urban areas, clustering observed in epidemiological data can suggest recent on going transmission of TB, while in geographically stable rural populations, clustering may result from simultaneous reactivation of infection acquired from the same source in the distant past (Braden *et al.* 1997).

Some studies have also suggested that the relationship between clustering and the proportion of disease attributable to recent transmission depends on different factors such as the patients' age, the geographical study area and the duration of the study (van Soolingen 2001) as well as the number of patients studied and the virulence and transmissibility of the strains (Glynn *et al.* 1999).

In the current study however, no association was found between clustering and age ($P = 0.73$) when using univariable analysis. Gender and distribution in Johannesburg were however associated with clustering ($P = 0.033$ and $P = 0.006$ respectively) as was additional drug resistance in MDR-TB isolates with either streptomycin or ethambutol ($P = 0.001$).

The clustering rates from this study were higher than estimates of recent transmission and rates of clustering reported in other studies performed in other African countries (Easterbrook *et al.* 2004, Verver *et al.* 2004, Godfrey-Faussett *et al.* 2000, Tudo *et al.* 2004, Lockman *et al.* 2001). This could either reflect differences in transmission dynamics or the result could be influenced by the molecular typing techniques used in this study. Also, the current study investigated transmission in MDR TB strains only, the other above mentioned studies from high TB incidence countries investigated transmission patterns in samples of drug susceptible and MDR-TB strains and therefore, the noted differences in transmission patterns could be influenced by these differences. A more comparable analysis would require the further use of a more discriminatory typing technique such as 24 MIRU-VNTR typing or IS*6110*-RFLP typing. Nevertheless, as with the other studies, the high clustering rate observed could imply ongoing transmission in the region, and since the strains transmitted strains are MDR-TB strains, this observation has dire consequences for the control of TB disease.

A common pattern in the drug-susceptible, MDR and XDR-TB strains was observed in the region. The predominant MDR-TB strain families are the same families driving drug-susceptible TB and XDR-TB (Mlambo *et al.* 2008). In countries like South Africa, with many MDR-TB cases (WHO 2008a), it is likely that strains with greater fitness could establish themselves and be transmitted and generate disease at the same pace as susceptible strains (Caminero 2010).

3.5 Conclusions

The results from the current chapter illustrate the diversity of MDR-TB strains in Johannesburg and confirm other studies from South Africa regarding the abundance of *M. tuberculosis* strain types in the country. We observed the similarity in the predominant MDR strains, XDR and

drug-susceptible TB strains in the country and propose that the drug-resistant strains are sustaining transmission and generating disease with similar effectiveness as drug-susceptible strains.

The study has several limitations: Firstly, only MDR-TB isolates were selected for study. However, a comparison with a recent molecular epidemiological study of drug-susceptible and drug-resistant isolates, including 109 MDR-TB isolates from 8 of the 9 provinces in South Africa, provided a basis of discussion regarding the *M. tuberculosis* genotypes circulating in South Africa. Extensive molecular typing of drug-susceptible and mono-resistant *M. tuberculosis* isolates in Johannesburg would however provide a more accurate analysis. Secondly, the use of 12 MIRU loci as a secondary typing technique limits the interpretation of epidemiological links. IS*6110*-RFLP typing has been shown to improve the discrimination of clusters identified by less discriminatory methods, therefore the use of this technique in this study could possibly lower the clustering rate and provide more accurate interpretation of transmission patterns. The use of IS*6110*-RFLP typing however proved challenging in this study mainly due to loss of viability of cultures as well as technical problems with the procedure. We however believe that the combination of spoligotyping and MIRU analysis in this study provides insight into the population structure of MDR-TB strains in Johannesburg and identifies communities that can be targeted for further study using the more discriminatory typing methods.

This observation has important implications for TB control in the region. We assume that disease transmission, reflected by the high clustering rate, occurs either because the MDR-TB contact is immunocompromised and progresses rapidly to active TB disease after exposure, or because the

source patient delays seeking medical attention, resulting in the potential for many secondary cases. If this is the case, increased emphasis should be placed on the early identification of MDR-TB cases through an upscale in drug susceptibility testing and also, improved referrals to MDR-TB hospitals and treatment follow-up until cure.

Still, these recommendations are challenging in a high TB/HIV incidence region like South Africa and would require substantial resources.

CHAPTER 4: Utility of 24 MIRU-VNTR Typing For Discriminating Beijing and Non-Beijing MDR-TB Isolates in Johannesburg

4.0 Summary

The previous chapter described the spoligotype and 12 MIRU loci-defined population structure of MDR-TB strains in Johannesburg, which was dominated by several globally distributed strain types. In this chapter, 24 loci MIRU-VNTR typing was performed on strains from Beijing, CAS, EAI and H families. The objective was to assess the ability of the method to differentiate strains as compared with the 12 loci MIRU combination and also to identify specific MIRU-VNTR loci with high discriminatory power among the predominant strain families in our region. The proposed 15 MIRU-VNTR locus combination together with MIRU 39 was found to be sufficient as a secondary typing method for the routine epidemiological investigation of the Beijing family MDR-TB *M. tuberculosis* isolates. The other families could also be sufficiently differentiated by the 15 MIRU locus combination. However, additional typing either by IS*6110*-RFLP or by use of 24 MIRU loci plus hypervariable VNTR loci (3232, 3820 and 4120) should be considered for population-based molecular epidemiological studies investigating transmission patterns in order to obtain maximal resolution.

4.1 Introduction

The Beijing, Haarlem and EAI families are some of the predominant families driving the drug-resistant TB epidemic in South Africa (Mlambo *et al.* 2008, Stavrum *et al.* 2009, Streicher *et al.* 2004, Johnson *et al.* 2010).

In an effort to understand the molecular epidemiological dynamics of TB disease in South Africa, the 'gold standard' for typing *M. tuberculosis* strains, IS*6110*-RFLP, has been used in the Western Cape Province (van der Spuy *et al.* 2009). However, this method is cumbersome, time-consuming and requires large quantities of DNA from viable samples, therefore other effective methods need to be evaluated for the characterization and differentiation of predominant *M. tuberculosis* strain families in this region. MIRU-VNTR typing has been shown to have discriminatory capacity close to that of IS*6110*-RFLP, especially when using 24 MIRU-VNTR loci (Supply *et al.* 2006). MIRU-VNTR loci however do not exhibit uniform discriminatory power across *M. tuberculosis* families in different areas and therefore may need to be selected for each particular region, especially where resources are limited, in order to minimize cost (Mokrousov *et al.* 2004, Han *et al.* 2007).

The Beijing genotype strains constitute a significant proportion of circulating strains worldwide (Brudey *et al.* 2006, Glynn *et al.* 2002, Bifani *et al.* 2002), and often demonstrate some important pathogenic features such as increased BCG virulence (Lopez *et al.* 2003), association with drug resistance (Cox *et al.* 2005) and the ability to multiply more rapidly in human macrophages (Zhang *et al.* 1999). Generally, strains of the Beijing family are closely related and therefore difficult to differentiate using most of the currently used typing techniques. The standard method to identify Beijing is spoligotyping, which results in highly similar patterns showing an absence of hybridization signals in spacers 1 to 34 (Glynn *et al.* 2002, Bifani *et al.* 2002, Kremer *et al.* 2004). In regions such as East Asia where the genotype constitutes more than 50% of circulating strains, the method cannot be used in epidemiological investigations to trace transmission patterns.

The same concerns arise in countries like South Africa, where Beijing strains are associated with mono- and multidrug resistance (Streicher *et al.* 2004, Johnson *et al.* 2010) and also extensively drug-resistant (XDR) TB (Mlambo *et al.* 2008).

As previously mentioned, IS*6110*-RFLP has been found to be the most discriminatory method for more detailed typing of Beijing strains (van Soolingen *et al.* 1995, Kremer *et al.* 2004). Unfortunately, the limitations of this method necessitate the evaluation of more rapid, reliable, reproducible and user-friendly methods. The 12 MIRU typing method has been used in numerous studies as a secondary typing method to either spoligotyping or IS*6110*-RFLP to confirm clonality (Zozio *et al.* 2005, Valcheva *et al.* 2008b, Millet *et al.* 2007, Dou *et al.* 2008a, Sola *et al.* 2003, Mokrousov *et al.* 2004, Baboolal *et al.* 2009, Candia *et al.* 2007, Aktas *et al.* 2008, Durmaz *et al.* 2007, Sharma *et al.* 2008, Allix *et al.* 2004, Nikolayevskyy *et al.* 2006, Ali *et al.* 2007).

Even though the 12 loci MIRU method has shown limited ability to discriminate Beijing strains, the data obtained from these studies have been invaluable for characterizing global diversity and phylogenetic reconstruction (Mokrousov *et al.* 2004). A proposed 24 loci MIRU-VNTR system (Supply *et al.* 2006), which includes a sub-set of 15 MIRU-VNTR loci recommended for use in molecular epidemiological investigations, as well as the full set of 24 MIRU-VNTR loci which is recommended for more extensive phylogenetic analysis, has shown marked improvement in differentiating *M. tuberculosis* strains compared with the 12 loci typing method (Oelemann *et al.* 2007, Jiao *et al.* 2008, Abadia *et al.* 2009, Valcheva *et al.* 2008a, Maes *et al.* 2008, Alonso-Rodriguez *et al.* 2008).

Population based studies evaluating the use of the proposed 24 loci MIRU-VNTR studies in comparison with IS*6110*-RFLP have however reported varying results. Studies from Spain, Germany and Belgium (Alonso-Rodriguez *et al.* 2009, Allix-Beguec *et al*. 2008a, Oelemann *et al.* 2007) reported good concordance between MIRU-VNTR and IS*6110*-RFLP. Other studies from Beijing endemic regions (Iwamoto *et al.* 2007, Jiao *et al.* 2008) and from South Africa (Hanekom *et al.* 2008) report a slightly better strain discriminating ability when typing Beijing strains using IS*6110*-RFLP and the South African study (Hanekom *et al.* 2008) identified discordant results when comparing MIRU-VNTR and IS*6110*-RFLP, especially when identifying transmission patterns. The studies advocate the use of either the IS*6110*-RFLP or 24 loci MIRU-VNTR with additional hypervariable loci VNTR 3820, VNTR 3232 and VNTR 4120 when investigating epidemiological links in stains of the Beijing family.

When using a MIRU-VNTR typing system, a preliminary evaluation to assess the diversity of particular loci in local strains for each geographical region prior to implementing MIRU typing for routine use is recommended since not all the standard 12, 15 or 24 loci are necessarily required to define all the unique isolates in a given situation (Supply *et al.* 2006, Mokrousov *et al.* 2004). Selecting loci with high discriminatory ability depending on the strain families (Brudey *et al.* 2006) known to be prevalent in a particular region could minimize time and expense. In South Africa, where the MDR and XDR-TB epidemic is driven by Beijing strains together with other families (LAM, S, X, T, EAI) (Mlambo *et al.* 2008, Stavrum *et al.* 2009), investigations of effective MIRU-VNTR loci need to be expanded to all these families.

Limited studies have used MIRU-VNTR analysis to characterize *M. tuberculosis* strains in South Africa thus far (Stavrum *et al.* 2009, Hanekom *et al.* 2008, Hanekom *et al.* 2007a, Savine *et al.* 2002). A study by Hanekom and colleagues compared Beijing MIRU types from Cape Town with those from the Beijing endemic region of East Asia using 12 MIRU profiles and identified nine Beijing MIRU types that were shared between the two regions (Hanekom *et al.* 2007a). While it provides essential baseline information regarding the MIRU types in the region, this study was limited to using the 12 MIRU typing method.

A more recent study by Hanekom and colleagues investigated concordance between IS*6110*-RFLP and 24 loci MIRU-VNTR (Hanekom *et al.* 2008) using Beijing strains from the Western Cape. Even though discordance was reported between the methods, especially regarding investigations on transmission patterns, the study showed the improvement in discriminatory power between 12, 15 and 24 MIRU-VNTR typing and also advocated the use of 24 MIRU-VNTR loci together with other hypervariable loci in population studies involving the Beijing family. Another recent study by Stavrum and colleagues, using 15 loci MIRU-VNTR to type Beijing strains from different Provinces in the country identified QUB-11b as highly discriminatory for Beijing strains and MIRU 40, Mtub 39, QUB-11b and QUB-26 as highly discriminatory for T family (SIT53). The identification of such effective MIRU loci and their associated families could eventually result in optimized first-line MIRU-VNTR typing geared to rapidly differentiate the specific, predominant strain families in the region.

This chapter describes the most discriminatory loci for typing strains from the Beijing (SIT1) and non-Beijing strain families (Haarlem (SIT50), EAI (SIT48, ST806) and CAS (SIT21, ST1092)

using 24 MIRU-VNTR combination (Supply *et al.* 2006) in the region. We also suggest the optimal MIRU-VNTR loci combinations for differentiating the predominant *M. tuberculosis* strain families in Johannesburg, in an effort to minimize both time and expense.

4.2 Study Design

4.2.1 Sample Selection and Molecular Typing

To further differentiate the patterns identified by 12 loci MIRU typing, 24 MIRU-VNTR typing was used on a set of isolates consisting of Beijing (n=71), EAI (n=44), CAS (n=8) and H (n=41) family strains. The discriminatory index for the 12 MIRU versus 24 MIRU locus system was calculated using the Hunter Gaston Discriminatory Index (HGDI)(Hunter, Gaston 1988). The HGDI was also calculated for each of the MIRU loci. MIRU-VNTR analysis was performed as described in Chapter 2.

4.3 Results

4.3.1. Beijing Family

The proposed 24 MIRU-VNTR locus combination was used to differentiate 71 MDR-TB isolates previously shown to belong to the Beijing family on the basis of spoligotyping (Chapter 3). Interpretable results on the 12 MIRU loci were obtained for all 71 Beijing strains, which resulted in 19 MIRU profiles corresponding to a total of 64 clustered and 7 unique isolates. When the patterns were compared to a MIRU database at the Pasteur Institute in Guadeloupe, our sample identified 13 MITs and 6 orphans (Table 4.1).

Application of the 24 MIRU-VNTR typing method decreased the sizes of the clusters and sub-divided the 12 MIRU-defined clusters into several new clusters (Table 4.1). The use of the 12 locus combination was the least discriminatory (HGDI 0.90), while using the 15 MIRU loci typing method provided better resolution (HGDI 0.95). The full set of 24 provided the highest discriminatory power (HGDI 0.97). The discriminatory power also differed significantly among the 24 MIRU-VNTR loci.

Table 4.2 shows a comparison of the discriminatory capacities of the different MIRU-VNTR loci for Beijing family, with the QUB-11b locus (HGDI: 0.73) found to be the most discriminatory locus among the Beijing strains. The use of the proposed 15 MIRU locus combination (Supply *et al.* 2006) showed only eight loci with high to moderate discriminatory power in our study (Table 4.2). The supplemental nine loci showed moderate discrimination in only three loci (MIRU 27, MIRU29 and MIRU39). The lowest discrimination was found for the monomorphic loci (MIRU2, MIRU4, MIRU20, MIRU23, MIRU24, MIRU 31 and ETRB) (Table 4.2).

Table 4.1: Beijing family (SIT1) sub-clustered using 12 loci MIRU and 24 loci MIRU-VNTR typing (n=71)

12 loci MIRU	24 loci MIRU-VNTR	
MIT 17 223325173533 n=17	22332517353332458244433 22332517353332458244435 22332517353342468248433 22332517353342428245233 22332517353342448235443 22332517353342428245233 22332517353342428245243 22332517353342448235443 223325173533332452444433	n=3 n=2 n=1 n=1 n=1 n=2 n=4 n=2 n=1
MIT 84 223325183533 n=3	22332518353342468245433	n=3
MIT 96 223325171531 n=5	22332517153142448245433 22332517153142347245233 22332517153142448245433 22332517153142458245433	n=1 n=1 n=1 n=2
MIT 99 223325173543 n=8	22332517354342428245233 22332517354342428243233	n=7 n=1
MIT 101 223325173523 n=4	22332517352332468245433 22332517352332458244435 22332517352342465345433 22332517352342467245433	n=1 n=1 n=1 n=1
MIT 104 222325173543 n=1	222325173543424662454434	n=1
MIT 245 223325174533 n=4	22332517453342458245633 22332517453342458244433 22332517453342458245433	n=1 n=1 n=2
MIT 254 223325163531 n=10	22332516353142569334433 22332516353142468334433	n=9 n=1
MIT 1202 223325174523 n=3	22332517452342458245433	n=3
MIT 1203 223325123521 n=2	22332512352142569334433	n=2

Continued on next page

Table 4.1*: continued from previous page*

12 loci MIRU	24 loci MIRU-VNTR	
MIT 1204		
223325173542	22332517354242428245 2433	n=2
n=2		
MIT 1205	22322517352342558245 4434	n=3
223225173523	22322517352342458243 3433	n=1
n=4		
MIT 1206		
223325163521	22332516352142568334 4433	n=2
n=2		
Orphan		
223325152523	22332515252342457245 4431	
223325176523	22332517652342458245 4433	
223225174533	22322517453342458245 4433	n=6
223325121521	22332512152142448245 4433	
2233251735_3	2233251735_3424_82454433	
223325123531	22332512353142569334 4433	

_denotes alleles that could not be amplified at particular MIRU loci (missing alleles)

Table 4.2: HGDI of each MIRU-VNTR locus (24 loci) in MDR *M. tuberculosis* Beijing genotype strains from Johannesburg

MIRU-VNTR	MIRU-VNTR locus[a]	MIRU-VNTR alias	No. of alleles	No. of repeats	HGDI	Discrimination
15 MIRU	2163b	QUB-11b	4	2 - 6	0.73	high
	1955	Mtub 21	4	3 - 8	0.5	moderate
	2996	MIRU 26	5	2 - 8	0.46	moderate
	802	MIRU 40	3	1 - 3	0.46	moderate
	4052	QUB-26	7	2 - 11	0.46	moderate
	577	ETR-C	3	3 - 5	0.41	moderate
	424	Mtub 04	2	2 - 3	0.41	moderate
	4156	QUB-4156	2	2 - 3	0.36	moderate
	3690	Mtub 39	4	2 - 5	0.24	poor
	2165	ETR-A	2	3 - 4	0.2	poor
	2401	Mtub 30	3	2 - 6	0.19	poor
	1644	MIRU 16	2	2 - 3	0.13	poor
	960	MIRU 10	2	2 - 3	0.03	poor
	580	MIRU 4	1	2	0	poor
	3192	MIRU 31	1	5	0	poor
Supplemental 9	4348	MIRU 39	3	2 - 4	0.6	moderate
	3007	MIRU 27	5	1 - 6	0.39	moderate
	2347	Mtub 29	2	2 - 3	0.37	moderate
	3171	Mtub 34	2	3 - 4	0.08	poor
	2461	ETR-B	1	2	0	poor
	2531	MIRU 23	1	5	0	poor
	2059	MIRU 20	1	2	0	poor
	154	MIRU 2	1	2	0	poor
	2687	MIRU 24	1	1	0	poor

a The loci within different MIRU-VNTR locus combinations are listed in descending order of allele diversity. Abbreviations: MIRU-VNTR: mycobacterial interspersed repetitive units-variable number of tandem repeats; HGDI, Hunter -Gaston diversity index; ETR, exact tandem repeats. Highly discriminatory: >0.6, moderate: 0.3-0.6, poor:<0.3 (Sola *et al*., 2003).

4.3.2 Non-Beijing Families (EAI, CAS and H)

Application of the 12 MIRU loci typing method to the Haarlem family (SIT 36, 47, 50, 62, 168, 2375) confirmed the subtypes originally identified by spoligotyping (Table 4.3). The majority of strains (96.4%) of the most predominant spoligotype SIT 50 were grouped into MIT 1211 (225425183324) and further typing using 24 MIRU-VNTR loci did not appreciably differentiate the groups identified by MIRU 12 (Table 4.3).

Table 4.3: H family sub-clustered using 12 loci MIRU and 24 loci MIRU-VNTR typing (n=41)

Spoligotype	**12 Loci MIRU**	**24 loci MIRU-VNTR**	
SIT 36 n=1	**MIT 611** 225325143323 n=1	22532514332323633234343	(n=1)
SIT47 n=2	**MIT 1211** 225425183324 n=2	22542518332432327323 4323	(n=2)
SIT 50 n=28	**MIT 1211** 225425183324 n=27 22_326153323 n=1	22542518332432327323 4343 22_326153323224473444143	(n=27) (n=1)
SIT 62 n=3	**MIT 43** 225323153323 n=1 227223153323 n=1 226223153323 n=1	22532315332332358322 2333 22722315332322357323 2343 22622315332322357323 2333	(n=1) (n=1) (n=1)
ST168 n=1	**MIT 1211** 225425183324 n=1	2254251833243232_3234343	(n=1)
ST 2375 n=5	**MIT 897** 123325153323 n=5	12332515332332366323 4343	(n=5)
Orphan n=1	2253231_3323 n=1	2253231_3323323583222343	(n=1)

A similar result was observed for strains of the CAS family, where MIRU typing successfully identified and differentiated strains from CAS1_KILI (SIT 21) and CAS1_DELHI (SIT 1092) families by both 12 and 24 loci combinations (Table 4.4). The predominant spoligotype, ST21 was further split into two MIT types (MIT 1207 and 261) but typing using 24 MIRU-VNTR loci did not further sub-cluster the other 12 loci-defined groups (Table 4.4).

Table 4.4: CAS family (SIT21, SIT 1092) sub-clustered using 12 loci MIRU and 24 loci MIRU-VNTR typing (n=8)

Spoligotype	**12 loci MIRU**	**24 loci MIRU-VNTR**	
SIT 21	**MIT 1207**		
	227425112434	22742511343442225243432 2	n=4
	n=4		
	MIT 261		
	227425113434	22742511343442225243432 2	n=1
	n=1		
	Orphan		
	227425123434	22742512343442223243432 2	n=1
	227325112434	22732511243442225242432 2	n=1
SIT 1092	**MIT 229**		
	224325173533	22432517353342227244432 3	n=1
	n=1		

MIRU typing of the EAI family (ST 48, 806, 2997, 2998) using the 12 loci combination resulted in 6 MITs and 3 orphan patterns compared to the four SITs identified by spoligotyping (Table 4.5). 24 MIRU-VNTR typing further sub-divided MIT 1210 (n=20) into 6 MIRU patterns, the largest consisting of 75% of the strains (Table 4.5). The discriminatory power of the 24 MIRU-VNTR combination increased (HGDI: 0.85) compared with 12 MIRU combination (HGDI: 0.77).

Table 4.5: EAI1_SOM family sub-clustered using 12 loci MIRU and 24 loci MIRU-VNTR typing (n=44)

Spoligotype	**12 Loci MIRU**	**24 loci MIRU-VNTR**	
SIT48	**MIT 64**	254326223513913942311211323 6	n=1
n=17	254326223513	254326223513942311211323 7	n=1
	n=2		
	MIT 1208	254126223413942321211323 7	n=8
	254126223413		
	n=8		
	MIT 1209	254226223413942321211323 7	n=5
	254226223413	2_422622341394232112113237	n=1
	n=6		
	254_26223513	254_26223513942321211323 7	n=1
	n=1		
SIT806	**MIT 1210**	254326223414644145121332 12	n=15
n=20	254326223414	__4326223_146442512133212	n=1
	n=18	2_43262234142223372442 11	n=1
		2_432622341464414512133212	n=1
	254426283414	254426283414644145121332 12	n=1
	n=1		
	254426283414	2243261_3___64414512133212	n=1
	n=1		
SIT2997	**MIT 65**		
n=6	254326223613	254326223613624843210321 2	n=6
	n=6		
	MIT 64		
SIT 2998	254326223513	254326223513942332211323 7	n=1
n=1	n=1		

_denotes alleles that could not be amplified at particular MIRU loci (missing alleles)

Table 4.6: HGDI of each MIRU-VNTR locus (24 loci) in MDR *M. tuberculosis* non-Beijing genotype strains from Johannesburg

MIRU-VNTR locus[a]	HGDI	Discrimination
QUB-26	0.76	high
QUB-11b	0.74	high
Mtub 21	0.73	high
ETR-A	0.72	high
ETR-C	0.66	high
Mtub 39	0.66	high
MIRU 16	0.65	high
MIRU 26	0.65	high
MIRU 10	0.63	high
MIRU 31	0.61	high
QUB-4156	0.59	moderate
MIRU 39	0.58	moderate
MIRU 23	0.55	moderate
Mtub 29	0.55	moderate
Mtub 30	0.53	moderate
MIRU 4	0.5	moderate
MIRU 24	0.5	moderate
ETR-B	0.48	moderate
MIRU 40	0.47	moderate
Mtub 34	0.42	moderate
Mtub 04	0.2	poor
MIRU 2	0.12	poor
MIRU 27	0.1	poor
MIRU 20	0	poor

[a] The loci within different MIRU-VNTR locus combinations are listed in descending order of allele diversity. Abbreviations: MIRU-VNTR: mycobacterial interspersed repetitive units-variable number of tandem repeats; HGDI, Hunter -Gaston diversity index; ETR, exact tandem repeats. Highly discriminatory: >0.6, moderate: 0.3-0.6, poor:<0.3 (Sola *et al*., 2003).

4.3.3 Simultaneous Co-infection and Clonal Variants in EAI Family

Table 4.7 illustrates the use of MIRU-VNTR typing in detecting possible mixed infection and clonal variants in *M. tuberculosis* specimens. The sample shown is from the EAI family. All the EAI family isolates were fully typed using 24 MIRU-VNTR typing and of the 44 isolates, 6 (J85, J94, J99, J111, J115 and J110) showed either a double allele in a single MIRU-VNTR locus, thus suggesting the presence of 2 closely related clonal variants or 2 or more alleles in more that two loci, suggesting co-infection with multiple strains of *M. tuberculosis*.

Table 4.7: Six isolates from EAI1_SOM family (n=44) showing either clonal variation (2 alleles in 1 locus) or mixed infection (2 or more alleles > 2 loci).

Strain number	Spoligotype Family	STI	MIRU-2	MIRU-4	MIRU-10	MIRU-16	MIRU-20	MIRU-23	MIRU-24	MIRU-26	MIRU-27	MIRU-31	MIRU-39	MIRU-40	ETR-A	ETR-B	ETR-C	QUB-11b	QUB-26	QUB-4156	Mtub04	Mtub21	Mtub29	Mtub30	Mtub34	Mtub39	12 MIRU loci	MIT profile	24 MIRU loci
J80	EAI1-SOM	48	2	5	4	1	2	6	2	2	3	4	1	3	9	4	2	3	2	1	2	11	3	2	3	7	254126223413	1208	254126223413913423212113237
J86	EAI1-SOM	48	2	5	4	1	2	6	2	2	3	4	1	3	9	4	2	3	2	1	2	11	3	2	3	7	254126223413	1208	254126223413913423212113237
J87	EAI1-SOM	48	2	5	4	1	2	6	2	2	3	4	1	3	9	4	2	3	2	1	2	11	3	2	3	7	254126223413	1208	254126223413913423212113237
J89	EAI1-SOM	48	2	5	4	1	2	6	2	2	3	4	1	3	9	4	2	3	2	1	2	11	3	2	3	7	254126223413	1208	254126223413913423212113237
J90	EAI1-SOM	48	2	5	4	1	2	6	2	2	3	4	1	3	9	4	2	3	2	1	2	11	3	2	3	7	254126223413	1208	254126223413913423212113237
J91	EAI1-SOM	48	2	5	4	1	2	6	2	2	3	4	1	3	9	4	2	3	2	1	2	11	3	2	3	7	254126223413	1208	254126223413913423212113237
J92	EAI1-SOM	48	2	5	4	1	2	6	2	2	3	4	1	3	9	4	2	3	2	1	2	11	3	2	3	7	254126223413	1208	254126223413913423212113237
J93	EAI1-SOM	48	2	5	4	1	2	6	2	2	3	4	1	3	9	4	2	3	2	1	2	11	3	2	3	7	254126223413	1208	254126223413913423212113237
J81	EAI1-SOM	48	2	5	4	2	2	6	2	2	3	4	1	3	9	4	2	3	2	1	2	11	3	2	3	7	254226223413	1209	254226223413942312113237
J82	EAI1-SOM	48	2	5	4	2	2	6	2	2	3	4	1	3	9	4	2	3	2	1	2	11	3	2	3	7	254226223413	1209	254226223413942312113237
J83	EAI1-SOM	48	2	5	4	2	2	6	2	2	3	4	1	3	9	4	2	3	2	1	2	11	3	2	3	7	254226223413	1209	254226223413942312113237
J84	EAI1-SOM	48	2	5	4	2	2	6	2	2	3	4	1	3	9	4	2	3	2	1	2	11	3	2	3	7	254226223413	1209	254226223413942312113237
J85	EAI1-SOM	48	2	2+5	4	2	2	6	2	2	3	4	1	3	9	4	2	3	2	1	2	11	3	2	3	7	254226223413	1209	2_42262234139423212113237
J96	EAI1-SOM	48	2	5	4	2	2	6	2	2	3	4	1	3	9	4	2	3	2	1	2	11	3	2	3	7	254226223413	1209	254226223413942312113237
J88	EAI1-SOM	48	2	5	4	3	2	6	2	2	3	5	1	3	9	4	2	3	3	1	2	11	3	2	3	6	254326223513	64	254326223513913423312113236
J95	EAI1-SOM	48	2	5	4	3	2	6	2	2	3	5	1	3	9	4	2	3	3	1	2	11	3	2	3	7	254326223513	64	254326223513913423312113237
J94	EAI1-SOM	48	2	5	4	2+3	2	6	**2**	2	3	5	1	3	9	4	2	3	2	1	2	11	3	2	3	7	254_26223513	orphan	254_26223513942312113237
J97	EAI1-SOM	806	2	5	4	3	2	6	2	2	3	4	1	4	6	4	4	14	5	1	2	13	3	2	1	2	254326223414	1210	254326223414646414512133212
J98	EAI1-SOM	806	2	5	4	3	2	6	2	2	3	4	1	4	6	4	4	14	5	1	2	13	3	2	1	2	254326223414	1210	254326223414646414512133212
J99	EAI1-SOM	806	1+2	2+5	4	3	2	6	2	2	3	3+4	1	4	6	4	4	2	5	1	2	13	3	2	1	2	254326223414	1210	__4326223_146442512133212
J100	EAI1-SOM	806	2	5	4	3	2	6	2	2	3	4	1	4	6	4	4	14	5	1	2	13	3	2	1	2	254326223414	1210	254326223414646414512133212
J101	EAI1-SOM	806	2	5	4	3	2	6	2	2	3	4	1	4	6	4	4	14	5	1	2	13	3	2	1	2	254326223414	1210	254326223414646414512133212
J102	EAI1-SOM	806	2	5	4	3	2	6	2	2	3	4	1	4	6	4	4	14	5	1	2	13	3	2	1	2	254326223414	1210	254326223414646414512133212
J103	EAI1-SOM	806	2	5	4	3	2	6	2	2	3	4	1	4	6	4	4	14	5	1	2	13	3	2	1	2	254326223414	1210	254326223414646414512133212
J104	EAI1-SOM	806	2	5	4	3	2	6	2	2	3	4	1	4	6	4	4	2	5	1	2	13	3	2	1	2	254326223414	1210	25432622341464412512133212
J105	EAI1-SOM	806	2	5	4	3	2	6	2	2	3	4	1	4	6	4	4	14	5	1	2	13	3	2	1	2	254326223414	1210	254326223414646414512133212
J107	EAI1-SOM	806	2	5	4	3	2	6	2	2	3	4	1	4	6	4	4	14	5	1	2	13	3	2	1	2	254326223414	1210	254326223414646414512133212
J108	EAI1-SOM	806	2	5	4	3	2	6	2	2	3	4	1	4	6	4	4	14	5	1	2	13	3	2	1	2	254326223414	1210	254326223414646414512133212
J109	EAI1-SOM	806	2	5	4	3	2	6	2	2	3	4	1	4	6	4	4	14	5	1	2	13	3	2	1	2	254326223414	1210	254326223414646414512133212
J111	EAI1-SOM	806	2	2+5	4	3	2	6	2	2	3	4	1	4	2	2	3	3	7	2	4	4	4	2	1	1	254326223414	1210	2_4326223414222337244211
J112	EAI1-SOM	806	2	5	4	3	2	6	2	2	3	4	1	4	6	4	4	14	5	1	2	13	3	2	1	2	254326223414	1210	254326223414646414512133212
J113	EAI1-SOM	806	2	5	4	3	2	6	2	2	3	4	1	4	6	4	4	14	5	1	2	13	3	2	1	2	254326223414	1210	254326223414646414512133212
J114	EAI1-SOM	806	2	5	4	3	2	6	2	2	3	4	1	4	6	4	4	14	5	1	2	13	3	2	1	2	254326223414	1210	254326223414646414512133212
J115	EAI1-SOM	806	2	2+5	4	3	2	6	2	2	3	4	1	4	6	4	4	14	5	1	2	13	3	2	1	2	254326223414	1210	2_43262234146441451213321
J116	EAI1-SOM	806	2	5	4	3	2	6	2	2	3	4	1	4	6	4	4	14	5	1	2	13	3	2	1	2	254326223414	1210	254326223414646414512133212
J106	EAI1-SOM	806	2	5	4	4	2	6	2	8	3	4	1	4	6	4	4	14	5	1	2	13	3	2	1	2	254426283414	orphan	254426283414646414512133212
J110	EAI1-SOM	806	2	2	4	3	2	6	1	2+5	3	3+4	1+2	3+4	6	4	4	14	5	1	2	13	3	2	1	2	22432612341_	orphan	2243261_3___64414512133212
J280	EAI1-SOM	2997	2	5	4	3	2	6	2	2	3	6	1	3	6	2	4	8	4	3	2	10	3	2	1	2	254326223613	65	254326223613624843210312
J281	EAI1-SOM	2997	2	5	4	3	2	6	2	2	3	6	1	3	6	2	4	8	4	3	2	10	3	2	1	2	254326223613	65	254326223613624843210312
J282	EAI1-SOM	2997	2	5	4	3	2	6	2	2	3	6	1	3	6	2	4	8	4	3	2	10	3	2	1	2	254326223613	65	254326223613624843210312
J283	EAI1-SOM	2997	2	5	4	3	2	6	2	2	3	6	1	3	6	2	4	8	4	3	2	10	3	2	1	2	254326223613	65	254326223613624843210312
J284	EAI1-SOM	2997	2	5	4	3	2	6	2	2	3	6	1	3	6	2	4	8	4	3	2	10	3	2	1	2	254326223613	65	254326223613624843210312
J285	EAI1-SOM	2997	2	5	4	3	2	6	2	2	3	6	1	3	6	2	4	8	4	3	2	10	3	2	1	2	254326223613	65	254326223613624843210312
J268	EAI1-SOM	2998	2	5	4	3	2	6	2	2	3	5	1	3	9	4	2	3	3	2	2	11	3	2	3	7	254326223513	64	254326223513913423221113237

4.4. Discussion

MIRU genotyping can be performed using automated high throughput equipment which facilitates rapid and highly reproducible results. Resource-limited countries usually cannot afford the cost and maintenance of such equipment, and therefore for such settings MIRU typing can be done using simple agarose gel electrophoresis. This method basically requires the same quality control precautions as required for all PCR-based methods and the use of affordable gel electrophoresis equipment. Moreover, knowledge of the predominant families circulating in a given setting together with the MIRU loci that show high discriminatory power in these families could result in saving costs as only those selected loci would be used when genotyping. In the current study, a detailed analysis of the MIRU loci (24 MIRU-VNTR) that show high discrimination for Beijing and non-Beijing strains in the region will be discussed.

An analysis of the Beijing family strains from the study sample supported a previous study by Mokrousov and colleagues, who identified MIRU 26 and MIRU 39 to have good discrimination for South African Beijing strains (Mokrousov *et al.* 2004). The overall results from our study do however differ slightly with the aforementioned study in that while MIRU 10 was also recommended for differentiation of South African Beijing strains (Mokrousov *et al.* 2004), we found that the MIRU 10 locus showed poor discrimination for our sample (HGDI 0.03).

Another report by Jiao and colleagues (2008) found that the supplemental nine loci are not polymorphic in Beijing genotype strains (Jiao *et al.* 2008). Our results however suggest that MIRU 27 (HGDI 0.37), MIRU (HGDI 0.39) and especially MIRU 39 (HGDI 0.6) could be useful in differentiating Beijing strains in the study region.

When using the 24 MIRU-VNTR loci typing method we identified QUB-11b to be highly discriminatory for Beijing family strains (HGDI: 0.73). This locus has also been found in various studies to sufficiently differentiate Beijing and non-Beijing strains (Supply *et al.* 2006, Jiao *et al.* 2008, Millet *et al.* 2007, Valcheva *et al.* 2008a, Wada *et al.* 2007, Kam *et al.* 2006).

The complete set of MIRU-VNTR loci that were useful in discriminating Beijing isolates in this study were: MIRU26, MIRU27, MIRU39, MIRU40, ETR-C, QUB-11b, QUB-26, QUB-4156, Mtub 04, Mtub 21 and Mtub 29. Since most of these discriminatory loci are included in the proposed 15 loci combination (Supply *et al.* 2006), with the exception of MIRU 27, MIRU 39 and Mtub 29, we suggest that the proposed 15 MIRU locus combination with additional MIRU 39 (HGDI: 0.6) can be used as a typing method in routine epidemiological investigation of the predominant MDR-TB *M. tuberculosis* isolates in the region. Additional typing either by IS*6110*-RFLP or by use of 24 MIRU loci plus hypervariable VNTR loci (3232, 3820 and 4120) would be required for maximal resolution in phylogenetic and transmission studies (Hanekom *et al.* 2008). The study also identified MIRU2, MIRU4, MIRU20, MIRU23, MIRU24, MIRU 31 and ETRB to be monomorphic in typing Beijing family strains. Several studies have also identified either one or all of these MIRU loci (2, 20, 23 and 24) to be monomorphic in Beijing strains (Mokrousov *et al.* 2004, Valcheva *et al.* 2008a, Kam *et al.* 2005) and even recommended their exclusion in epidemiological investigations involving this genotype (Mokrousov *et al.* 2004).

The discriminatory index of the combined sample of non-Beijing strains (n=93) was calculated for each of the MIRU-VNTR loci; ten loci were highly discriminatory (MIRU 10, MIRU 16, MIRU 20, MIRU 31,ETR-A, ETR-C, QUB-11b, QUB-26, Mtub21and Mtub39). Another ten loci were moderately discriminatory (MIRU 4, MIRU 23, MIRU24, MIRU 39, MIRU 40, ETR-B, QUB-4156, Mtub 29, Mtub30 and Mtub34) and four were poorly discriminatory (MIRU 2, MIRU20, MIRU27 and Mtub04). The poor discriminatory loci have been reported in other studies (Jiao *et al.* 2008, Sola *et al.* 2003, Han *et al.* 2007, Nikolayevskyy *et al.* 2006, Valcheva *et al.* 2008a, Alonso-Rodriguez *et al.* 2008, Kam *et al.* 2005). Since these loci are not included in the 15 MIRU-VNTR typing method recommended for epidemiological investigation, we suggest that the 15 loci combination is sufficient for typing of non-Beijing strains in our region, especially the predominant families identified in chapter 3. Population-based molecular epidemiological studies of TB would however require the additional use of IS*6110*-RFLP or 24 MIRU-VNTR typing, with additional hypervariable loci for maximal resolution (Hanekom *et al.* 2008).

Mixed/multiple infections are a major concern in the diagnosis and treatment of TB. Double alleles in a single MIRU-VNTR locus suggest the presence of 2 closely related clonal variants whereas 2 or more alleles in more that two loci suggest co-infection with multiple strains on *M. tuberculosis* (Supply *et al*. 2006, Allix *et al*. 2004, Garcia de Viedma *et al*. 2005). Simultaneous co-infection by more than one strain of *M. tuberculosis* has been demonstrated in several studies (Richardson *et al.* 2002a, Warren *et al.* 2004, Garcia de Viedma *et al.* 2005, Bandera *et al.* 2001). Confirming such multiple infections can however be a complicated and laborious process since it requires either the use of a highly discriminating genotyping method or the culture and

analysis of multiple single colonies from each specimen (Richardson *et al.* 2002a, Warren *et al.* 2004).

IS*6110*-RFLP has been the method of choice for exploring clonally complex cases of TB due to its high discriminatory power, and the existence of more than one clone is usually detected by the presence of low intensity bands, and the interpretation of such results requires expertise (Warren *et al.* 2004). MIRU-VNTR typing has proven useful for simplifying and optimizing the characterization of clonally complex cases (Garcia de Viedma *et al.* 2005), since it produces a single allele for each locus resulting in single band patterns after PCR amplification. If two different strains are present, two different alleles should be observed in a single locus thereby suggesting clonal variants.

MIRU loci have been reported to be more stable than IS*6110* (Mazars *et al.* 2001), therefore it is unlikely that the result seen in our sample could be due to random variations caused by instability of the loci. Laboratory contamination is a possible explanation for this result and therefore additional genotyping using IS*6110*-RFLP would be necessary in order to confirm the suspected mixed infections identified in this study. The importance of correctly identifying co-infections with multiple *M. tuberculosis* strains is of particular significance in a high burden TB area like South Africa. Such infections can have significant impact on TB chemotherapy, especially if the infecting *M. tuberculosis* strains have differing drug resistance profiles. The overall findings in this chapter are that the use of 15 loci MIRU-VNTR typing is feasible for a high TB incidence region like Johannesburg, South Africa. The high rate of TB in the region however increases the possibility of simultaneous co-infections with multiple *M. tuberculosis*

strains and therefore, if such cases are suspected, subsequent analysis using IS*6110*-RFLP would be required for confirmation.

4.5 Conclusions

The overall findings in this chapter are that the use of 15 loci MIRU-VNTR typing is feasible for a high TB incidence region like Johannesburg, South Africa. The high rate of TB in the region, however, increases the possibility of simultaneous co-infections with multiple *M. tuberculosis* strains and therefore, if such cases are suspected, subsequent analysis using IS*6110*-RFLP would be required for confirmation.

Chapter 5: Multidrug-resistant Tuberculosis: Treatment Outcome Analysis at Sizwe Hospital, the MDR-TB Referral Hospital in Gauteng

5.0 Summary

Multidrug-resistant tuberculosis disease is associated with lengthy treatment, expensive and potentially toxic regimens, with high rates of poor treatment outcomes often observed (treatment failure and death). This study describes the treatment outcomes of MDR-TB patients at Sizwe Hospital, Johannesburg, who started treatment between 2004 and 2007 and investigates possible risk factors associated with poor outcomes. Treatment success (completion and cure) was recorded in 158 (48.8%) of patients, while 73 (22.5%) had poor outcomes, 93 (28.7%) defaulted, 11(3.1%) were transferred out to other health facilities and 16 (4.6%) had no recorded final outcome. The proportion of successful treatment significantly increased over time. Univariable and multivariable analysis (level of significance $P = 0.05$) identified the year of MDR-TB diagnosis and spoligotype-defined families as factors associated with treatment outcome. No associations were found between treatment outcome and HIV status, previous TB, and additional MDR resistance to either streptomycin or ethambutol.

The study also describes the molecular characteristics of the MDR-TB isolates obtained from the patients at Sizwe Hospital and complements the characterization of isolates from Johannesburg (Chapter 3) by extending the study area to include the rest of the Gauteng Province. MDR-TB cases identified in this province are ideally referred to Sizwe Hospital for treatment and follow-up therefore, this sample is a good representation of the MDR-TB strains circulating at the MDR-TB referral hospital, and in Gauteng.

The strain diversity of the isolates from Sizwe Hospital was determined using spoligotyping and MIRU analysis and the results were similar to the Johannesburg-only sample (Chapter 3), with Beijing, LAM, EAI, T and H families forming the largest groups in the MDR-TB population in the region.

5.1 Introduction

Multidrug-resistant TB disease is characterized by difficulties in treatment compared with drug-susceptible TB, since the MDR-TB strains are resistant to the two most efficient anti-tuberculosis drugs, rifampicin and isoniazid (WHO 2008b, WHO 2008a). MDR-TB treatment is often associated with high rates of treatment failure of at least 50%, which is even higher in countries with less developed TB programmes (Espinal *et al.* 2000) and often results in high mortality rates (Migliori *et al.* 2009, Migliori *et al.* 2008a).

Knowledge of risk actors associated with poor outcomes of MDR-TB treatment is crucial for developing more effective treatment solutions (Caminero 2010). Several studies have been performed in developed countries to evaluate the treatment outcomes in MDR-TB patients and also to identify the predictors of poor outcomes (Chan *et al.* 2004, Flament-Saillour *et al.* 1999, Bang *et al.* 2010, Eker *et al.* 2008). The risk factors in these settings often include prior treatment with anti-TB drugs; resistance to fluoroquinolones (FQN); resistance to capreomycin; HIV infection; history of imprisonment; older age; male gender and extra-pulmonary TB (Yew *et al.* 2000, Chiang *et al.* 2006, Orenstein *et al.* 2009, Chan, Iseman 2008).

There is, however, limited information on both the treatment outcomes of MDR-TB patients and the factors associated with poor outcomes in high TB and HIV prevalence regions such as sub-Saharan Africa. Some of the available studies have either been limited by sample numbers or focused mostly on investigating factors associated with default of patients from MDR-TB treatment (Holtz *et al.* 2006, Davies *et al.* 1999, Shean *et al.* 2008, Seung *et al.* 2009). A recent study from the KZN Province does, however, highlight the magnitude of TB drug resistance as well as the negative effects of HIV infection, prior history of TB, year of diagnosis and male sex on poor treatment outcomes in the KZN Province (Brust *et al.* 2010). South Africa currently ranks fourth among all countries in terms of MDR-TB burden (WHO 2009b) and the recent discovery of XDR-TB in the KZN Province (Gandhi *et al.* 2006) as well as subsequent reports of XDR-TB in other provinces (WHO 2008a) emphasize the magnitude of the drug-resistant TB epidemic in the country.

The treatment regimen for MDR-TB is generally determined by considering drug susceptibility profiles of the infecting *M. tuberculosis* isolate and includes the use of second-line anti-TB drugs (WHO 2008b). The selected regimen can vary between a standardized and individualized structure depending on the country's treatment policy and resources (WHO 2008b, Yew *et al.* 2000, Chan *et al.* 2004, Van Deun *et al.* 2004, Flament-Saillour *et al.* 1999, Tahaoglu *et al.* 2001, Shin *et al.* 2006). In South Africa, the management of MDR-TB involves referral of MDR-TB patients to specialized MDR-TB hospitals situated in the various provinces. Sizwe Hospital is the referral hospital for the Gauteng Province and MDR-TB patients identified in medical centres in the province are ideally referred to Sizwe Hospital for treatment.

A standardised treatment recommended by the World Health Organization is used for treatment (Weyer 2004), which consists of a four month intensive phase with kanamycin, pyrazinamide, ofloxacin, ethionamide and either terizidone or ethambutol, followed by a 12-18 months continuous phase of ofloxacin, ethionamide and either terizidone or ethambutol (WHO 2008b, Weyer 2004).

The treatment strategy of providing MDR-TB patient management in specialized institutions, often accompanied with individualized treatment regimens, has been used extensively and with great success in developed countries with low TB/HIV prevalence (Chan *et al.* 2004, Eker *et al.* 2008, Ferrara *et al.* 2005, Talay *et al.* 2008). Regions with high burden of TB and limited resources usually opt for the WHO recommended treatment strategy (Weyer 2004) as well as community-based treatment (Mitnick *et al.* 2003) mainly because of the high load of TB cases, as well as lack of resources including medication, medical personnel and beds in treatment facilities.

The current study aimed to investigate treatment outcomes of MDR-TB patients who had been treated at Sizwe Hospital for≥ 2 years and to identify possible risk factors associated with p oor treatment outcomes (treatment failure and death). We also used molecular typing to characterize the MDR-TB genotypes from patients at Sizwe Hospital and investigated possible associations between genotypes and treatment outcomes.

5.2 Study Design

5.2.1 Study Population

A total of 351 patients with culture-confirmed MDR-TB who started treatment at Sizwe Hospital between 2004 and 2007 were included in this study. The study group included patients from all areas in Gauteng but excluded any patients from Johannesburg identified in Chapter 3 in order to avoid duplicate analysis in molecular typing. Patients who were either transferred out or had no record of final outcome were excluded from the final analysis, resulting in a final study population of 324 patients. Ethical approval for the study was granted by University of the Witwatersrand Human Research (Medical) Ethics Committee. Permission to access patient information from Sizwe Hospital was obtained from the Gauteng Provincial Department of Health.

5.2.2 Outcome Definitions

Standard definitions for cure, treatment completion, treatment failure, death, default and transfer out were used as described in Chapter 2 (Laserson *et al.* 2005). Successful treatment was further defined as either cure and/or completion and poor treatment was defined as either treatment failure and/or death (Veen *et al.* 1998). The duration of treatment for all patients was $\geq$ 2 years.

5.2.3 Molecular Characterization of MDR-TB Isolates

All the 351 MDR-TB isolates from the study population were typed using spoligotyping and MIRU analysis. Only the first MDR sample was used per patient for genotyping analysis. Spoligotyping was performed as described in Chapter 2.

The individual spoligotyping patterns were entered in an Excel spreadsheet and compared to those of the international database SITVIT2 (Institut Pasteur de Guadeloupe, available online at http://www.pasteurguadeloupe.fr:8081/SITVITDemo). MIRU typing (12 loci) was performed with primers and conditions as described in Chapter 2. MIRU data were sent to the Institut Pasteur de Guadeloupe for comparison and entered into an 'in-house' database containing 12 loci MIRU patterns referred to as MITs (MIRU international types).

5.2.4 Statistical Analysis

Univariable analysis was performed to examine associations between baseline characteristics and treatment outcomes using the Pearson's chi-square test. Characteristics examined included; age, gender, year of MDR-TB diagnosis, drug susceptibility profiles, prior history of TB, HIV status and spoligotype-defined clades. Variables with $P < 0.05$ on univariable analysis were then incorporated into a multivariable logistic regression model to estimate the predictors of poor outcome. Odds ratios were estimated at 95% confidence intervals, and a P value of < 0.05 was regarded as statistically significant.

5.3 Results

5.3.1 Analysis of Treatment Outcomes

Of the 351 patients whose strains were selected for analysis at the beginning of the study, 16 (4.6%) patients had no recorded final treatment outcome and 11 (3.1%) patients were transferred out to other regions for continued treatment. Final outcome was available for 324 (92%) of the patients and only these patients were included in the final analysis. The baseline characteristics (gender, age, HIV status, previous TB, and drug resistance profiles) for the 324 patients with known final outcome are shown in Table 5.1.

Among these patients, 93 (28.7%) defaulted, 73 (22.5%) had poor outcome and 158 (48.8%) had successful outcomes (Figure 5.1).

Table 5.1: Baseline characteristics

	n(%)
Total	**324**
Gender	
Female	154 (47.5)
Male	170 (52.5)
Age, years	
≤15	9 (2.8)
16 - 35	167(51.5)
≥36	145(44.8)
Missing	3 (0.9)
HIV status	
HIV-positive	203(62.7)
HIV-negative	72(22.2)
Missing	49 (15.1)
Prior history of TB	
Yes	182(56.2)
No	97(29.9)
Missing	45 (13.9)
Resistance to TB drugs	
INH + RIF only	30 (9.3)
INH + RIF + STR	30(9.3)
INH + RIF + EMB	26(8)
INH + RIF + STR + EMB	232(71.6)
INH + RIF, no additional DST	6(1.9)

HIV: human immunodeficiency virus; TB: tuberculosis

The proportion of cases with successful treatment increased from 2004-2007 with a commensurate decrease in the proportion of default and poor outcome (Figure 5.1). Univariable analysis revealed that later year of starting treatment, male gender and strain families other than the Haarlem family were associated with successful rather than poor treatment outcome (Table 5.2).

Although there was a trend towards more successful outcomes among those who were not resistant to both ethambutol and streptomycin, this was not significant (Table 5.2). There was no evidence of association between successful outcomes and either HIV or previous TB among those for whom data were available. In multivariable analysis, year of MDR-TB diagnosis and strain family remained significantly associated with treatment outcomes, while gender showed only weak evidence of an association (Table 5.2).

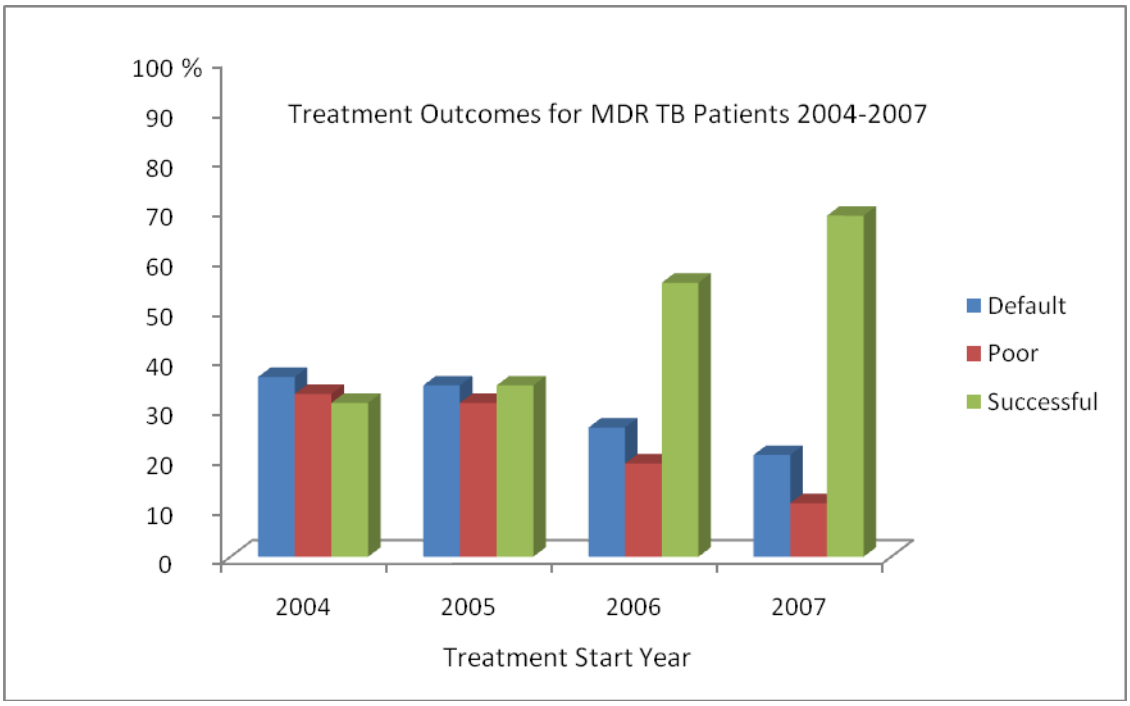

Figure 5.1: Treatment outcomes for MDR-TB patients starting treatment in 2004-2007

Table 5.2: Odds ratio (OR) for successful treatment versus poor treatment among MDR-TB patients at Sizwe Hospital

	Successful outcome		Univariable analysis			Multivariable analysis		
Variables	N	%	OR	95% CI	*P* value	OR	95% CI	*P* value
Start year								
2004	18/37	48.6%	1		< 0.001			< 0.001
2005	30/57	52.6%	1.17	0.51-2.69		0.83	0.35-2.03	
2006	53/71	74.6%	3.1	1.35-7.18		2.62	1.08-6.42	
2007	57/66	86.4%	6.68	2.58-17.35		5.32	1.94-14.60	
Gender								
Female	67/113	56.8%	1		0.004			0.065
Male	91/118	77.1%	2.31	1.31-4.10		1.81	0.96-3.42	
Strain family								
LAM	43/56	76.8%	1		0.005			0.041
T	29/41	70.3%	0.73	0.29-1.83		0.85	0.32-2.25	
Beijing	28/41	68.3%	0.65	0.27-1.61		0.64	0.24-1.70	
H	16/38	42.1%	0.21	0.09-0.54		0.24	0.09-0.64	
Other	42/55	76.4%	0.98	0.41-2.40		0.78	0.30-2.01	
Age (yrs)								
0-15	7-Jun	85.7%	1		0.208			
16-35	71/114	62.3%	0.27	0.03-2.36				
≥ 36	79/107	73.8%	0.47	0.05-4.08				
Missing	2/3	66.7%	0.33	0.01-8.18				
MDR additional resistance								
embRstrR	107/168	63.7%	1		0.122			
embRstrS	14/18	77.8%	2	0.63-6.33				

Continued on next page

***Table 5.2**: continued from previous page*

	Successful outcome		Univariable analysis			Multivariable analysis		
Variables	N	%	OR	95% CI	*P* value	OR	95% CI	*P* value
MDR additional resistance								
embSstrR	17/21	81%	2.42	0.78-7.53				
embSstrS	17/21	81%	2.42	0.78-7.53				
HIV status								
No	42/55	76.4%	1		0.131			
Yes	105/155	67.8%	0.65	0.32-1.32				
Missing	21-Nov	52.4%	0.34	0.12-0.98				
Previous TB								
No	54/72	75%	1		0.215			
Yes	91/136	70%	0.67	0.36-1.28				
Missing	13/23	56.5%	0.43	0.16-1.16				

CI, confidence interval; HIV, human immunodeficiency virus; TB, tuberculosis; embR= ethambutol resistant; strR = streptomycin resistant; embS = ethambutol susceptible = strS, streptomycin susceptible; LAM = Latin American Mediterranean; H = Haarlem; T = "ill-defined T family"

5.3.2 Molecular Characterization of MDR-TB Isolates

5.3.2.1 Spoligotyping

When spoligotyping results were compared to the updated international spoligotyping database (SITVIT 2) all 351 isolates matched existing shared spoligotype international types (STIs), resulting in 41 different shared types belonging to clades; Beijing, CAS, EAI, H, LAM, S, T, X and unknown clade (ST 46) (Table 5.3). Spoligotyping grouped 339 isolates into 29 clusters, resulting in a high clustering rate of 88.3% (339-29/351). A comparison of MDR-TB strains from Sizwe Hospital (Gauteng) with strains circulating in Johannesburg (Chapter 3) showed a similarity in the predominant strains (Table 5.3).

5.3.2.2 MIRU Analysis

Secondary typing using MIRU analysis identified 101 MITs (MIRU international types) and 78 orphan patterns when compared to an 'in-house' database (SITVIT 2) of the Institut Pasteur de Guadeloupe. A total of 61 clusters, containing 233 isolates were identified, with a resultant reduced clustering rate of 49% (233-61/351) compared with spoligotyping. The sub-clusters obtained when predominant spoligotypes were differentiated by MIRU typing are shown in Table 5.4. This table also shows the global regions in which the spoligotypes identified in this region predominate as identified by the international spoligotyping database, SITVIT2.

Table 5.3: Comparison of spoligotype shared types (SIT) identified from Sizwe Hospital (Sizwe) compared with Johannesburg (JHB).

SIT*	Clade**	Spoligotype description	Sizwe (No of isolates) n=351	JHB (No. of isolates) n=434
1	Beijing	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	59 (16.81)	71(16.36)
60	LAM4	■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■□■■■	54(15.38)	69(15.90)
50	H3	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	51(14.53)	37(8.53)
53	T1	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	26(7.41)	45(10.37)
34	S	■■■■■■■■□□■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	19(5.41)	32(7.37)
92	X3	■■■□□□□□□□□■■■■■□■■■■■■■■■■■■■□□□□■■■■■■■	16(4.56)	20(4.61)
48	EAI1_SOM	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■□■■■	15(4.27)	17(3.92)
2531	T1	■■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■	15(4.27)	6(1.38)
33	LAM3	■■■■■■■■□□□■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	12(3.42)	16(3.69)
37	T3	■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	7(1.99)	5(1.15)
42	LAM9	■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	7(1.99)	6(1.38)
926	T1	■■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	7(1.99)	5(1.15)
18	X2	■■□■■■■■■■■■■■■■□■■■■■■■■■■■■■■□□□□■■□□□□■	6(1.71)	
46	Unknown	■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□□□□□□□□	5(1.42)	3(0.69)
766	LAM9	■■■■■■■■■■■■■■□□□■□□□□□□■■■■■■■■□□□□■■■■■■■	4(1.14)	2(0.46)
815	LAM11_ZWE	■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■■□□□□■■■□■■■	4(1.14)	1(0.23)
4	LAM3	□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■□□□□■■■■■■■	3(0.85)	1(0.23)
71	S	■■■■■■■■□□■■□■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	3(0.85)	
811	LAM4	■■■■■■■■■■■■■■■■■■■■□□□□■□□□□□■■□□□□■■■□■■■	3(0.85)	4(0.92)
1536	LAM9	■■■■■■■■□□■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	3(0.85)	
2997	EAI1_SOM	■■■■■□■□■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■□■■■	3(0.85)	6(1.38)
47	H1	■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■■■■■	2(0.57)	2(0.46)
62	H1	■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■□■■■	2(0.57)	3(0.69)
73	T	■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	2(0.57)	2(0.46)
129	EAI6_BGD1	■■■□□□□□□■■■■■■■■■■■■■□□■■■■□□□□■□■■■■■■■■■	2(0.57)	
137	X2	■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■□□□□■■□□□□■	2(0.57)	
719	LAM3	■■■■■■■■□□□■■■■■■■■□□□□□■■■■■■■■□□□□■■■■■■■	2(0.57)	3(0.69)
806	EAI1_SOM	■■■■■□■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■□■■■	2(0.57)	20(4.61)
21	CAS1_Kili	■■■□□□□■■□■■■■■■■■■□□□□□□□□□□□□□□□□■■■■■■■■	1(0.28)	7(1.61)
26	CAS1_Delhi	■■■□□□□■■■■■■■■■■■■■■□□□□□□□□□□□□□■■■■■■■■■	1(0.28)	
51	T1	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□□□□	1(0.28)	
59	LAM11_ZWE	■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■■□□□□■■■■■■■	1(0.28)	
158	T3	■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■□□□□■■□□□□■	1(0.28)	
244	T1	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■	1(0.28)	10(2.30)
393	T1	■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	1(0.28)	
498	T1	■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	1(0.28)	

Continued on next page

***Table 5.3**: continued from previous page*

SIT*	Clade**	Spoligotype Description	Sizwe (No of isolates) n=351	JHB (No. of isolates) n=434
1355	LAM6	■■■■■■■■■■■■■■■■■■■■□□□□□■■■■□■■■□□□□■■■□■■■	1(0.28)	
1597	T1	■■■□□□■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	1(0.28)	
1607	LAM11_ZWE	■■□□□□□□■■■■■■■■■■■■□□□□■■□□□□■■□□□□■■■■■■■	1(0.28)	
1915	S	■■■■■■■■□□■■■■■■■■■■■□■■■■■■■■■■□□□□■■■■■■■	1(0.28)	
17	LAM2	■■□■■■■■■■■■□■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■		2(0.46)
20	LAM1	■■□■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■		3(0.69)
36	H3	■■■■■■■■■■■■□■■■■■■■■■■■■■■■■□■□□□□■■■■■■■		1(0.23)
49	H3	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■□■■■		1(0.23)
52	T2	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■		1(0.23)
81	LAM9	■■■■■■■■■■□■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■		1(0.23)
168	H3	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■□■■■■		1(0.23)
200	X3	■■■□□□□□□□□□■■■■■□■■■■■■■■■■■■■■□□□□■■■□□□□		1(0.23)
344	T1	■■■□□□□□□□□□■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■		1(0.23)
519	T1	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■■■■■■		1(0.23)
563	Unknown	■■■■■■■■■■■■□■■■■■■■■■■□□□□□□□□□□□□□□■■■■		1(0.23)
649	T1	□■■■■■■■■■■■■■■■■■■□□□□■□□■■■□■■■□□□□■■■■■■■		1(0.23)
789	S	■■□■■■■■■□□■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■		1(0.23)
1092	CAS1_Delhi	■■■□□□□■□■■■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■		1(0.23)
1516	Unknown	■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■■		1(0.23)
1634	MANU2	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□■■■■■■■■■		1(0.23)
2016	X2	■■■□■■■■■■■■■■■■□■■■■■■■■■■■■■■■■□□□□■■□□□□■		1(0.23)
2165	LAM9	■■■■■■■■■■■■■■□□■■■■■■□□□□■■■■■■■■□□□□■■■■■■■		1(0.23)
2375	H1	■■■■■■□■■■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■■■■■		5(1.15)
2992	MANU2	■■■■■■■■■■■■■■■■■■■■■■■■□□■■■■■■■■□□■■■■■■■■■		1(0.23)
2196	LAM11_ZWE	■■■■■■■■■■■■■■■■■□■■■□□□□■■□□□□■■□□□□■■■□■■■		1(0.23)
2996	Unknown	■■■□■□□□□□□□□■□□□■■□□□□□□□□□□□□□□□□□□□■■■■■		2(0.46)
2998	EAI1_SOM	■■■■■■■■■■■■■■■□□■■■■■■■■■■■■□□□□■□■■■■■□■■■		1(0.23)
2999	LAM	■■■■■■■■■■■■■■■□□□■□□□□□□■■■■■■■■□□□□■■■□■■■		1(0.23)

*SIT designations in SITVIT2 database. ** Clade designations according to SITVIT2 using revised SpolDB4 rules; Unk: Unknown pattern within any of the major clades described in SITVIT2

Table 5.4: Description of predominant *M. tuberculosis* shared-types from Sizwe Hospital and their worldwide distribution in the SITVIT2 database, followed by 12-loci MIRU-based subclustering of spoligotype-defined clusters

SIT*(Octal Code) Clade	Number (%) in this study	Distribution in Regions with ≥5% of a given SIT ** (%)	MIRU-based sub-clustering (MIT***designation and number of strains per sub cluster)
SIT1 (000000000003771) Beijing	59 (16 .81)	AMER-N (30.39), ASIA-SE (13.77), AFRI-S (12.54), ASIA-E (11.09), ASIA-N (8.27), ASIA-S (5.16)	MIT 17(n=5), MIT 83 (n=3), MIT 84 (n=2), MIT 96 (n=2), MIT 99 (n=4), MIT 229 (n=3), MIT 245 (n=1), MIT 254 (n=8), MIT 431 (n=2), MIT 469 (n=1), MIT 886 (n=2), MIT 1364 (n=2), MIT 1365 (n=2), MIT 1368 (n=2), MIT 1371 (n=1), MIT 1372 (n=1), MIT 1382 (n=1), MIT 1387 (n=2), Orphan (n=15)
SIT33 (776177607760771) LAM3	12 (3.42)	AFRI-S (32.02), AMER-S (22.07), AMER-N (15.86), EURO-S (13.60), EURO-W (5.42)	MIT 38 (n=2), MIT 169 (n=1), MIT 213 (n=2), MIT 236 (n=1), MIT 247 (n=2), MIT 807 (n=1), MIT 1214 (n=2), Orphan (n=1)
SIT34 (776377777760771) S	19 (5.41)	AMER-N (28.57), AFRI-S (20.95), EURO-S (15.24), AMER-S (11.16), EURO-W (7.62)	MIT 178 (n=2), MIT 212 (n=1), MIT 232 (n=1), MIT 247 (n=1), MIT 256 (n=3), MIT 487 (n=1), MIT 1216 (n=4), MIT 1218 (n=1), MIT 1379* (n=1), MIT 1388 (n=1), Orphan (n=3)
SIT37 (777737777760771) T3	7 (1.99)	EURO-W (15.18), AMER-N (14.85), ASIA-W (14.52), EURO-N (12.21), EURO-E (6.93), AMER-S (5.94), EURO-S (5.61), AFRI-S (5.61), ASIA-S (5.28)	MIT 27 (n=1), MIT 34 (n=1), MIT 157 (n=1), MIT 382 (n=1), MIT 1222 (n=1), MIT 1370 (n=1), Orphan (n=1)
SIT42 (777777607760771) LAM9	7 (1.99)	AMER-S (29.85), AMER-N (16.29), EURO-S (12.79), EURO-W (7.03), AFRI-N (5.07)	MIT 10 (n=1), MIT 246 (n=2), MIT 1215 (n=2), MIT 1375 (n=1), Orphan (n=1)
SIT48 (777777777413731) EAI1-SOM	15 (4.27)	EURO-N (25.32), ASIA-S (20.51), EURO-W (14.43), AFRI-S (12.91), AFRI-E (9.62)	MIT 1210 (n=1), MIT 1362 (n=2), MIT 1367 (n=2), MIT 1380 (n=2), Orphan (n=8)
SIT50 (777777777720771) H3	51 (14.53)	AMER-N (22.22), AMER-S (16.16), EURO-W (15.61), EURO-S (12.84), EURO-E (6.57), AFRI-S (5.00)	MIT 46 (n=1), MIT 140 (n=1), MIT 740 (n=1), MIT 1029 (n=2), MIT 1211 (n=20), MIT 1363 (n=2), MIT 1369 (n=6), MIT 1373 (n=2), MIT 1374 (n=3), MIT 1377 (n=3), MIT 1386 (n=2), Orphan (n=8)

Table 5.4: *Continued from previous page*

SIT*(Octal Code) Clade	Number (%) in this study	Distribution in Regions with ≥5% of a given SIT ** (%)	MIRU-based sub-clustering (MIT***designation and number of strains per sub cluster)
SIT53 (777777777760771) T1	26 (7.41)	AMER-N (19.67), AMER-S (14.46), EURO-W (12.82), EURO-S (10.02), ASIA-W (8.69), AFRI-S (6.50)	MIT 8 (n=1), MIT 23 (n=1), MIT 45 (n=1), MIT 46 (n=1), MIT 117 (n=1), MIT 128 (n=1), MIT 149 (n=2), MIT 208 (n=2), MIT 224 (n=2), MIT 699 (n=1), MIT 733 (n=2), MIT 771 (n=1), MIT 1029 (n=1), MIT 1062 (n=1), MIT 1385(n=1), Orphan (n=7)
SIT60 (777777607760731) LAM4	54 (15.38)	AFRI-S (45.53), AMER-S (16.48), AFRI-W (7.54), AMER-N (6.15), AFRI-N (5.59), EURO-S (5.03)	MIT 215 (n=1), MIT 246 (n=15), MIT 249 (n=2), MIT 593 (n=2), MIT 1361 (n=2), MIT 1366 (n=14), MIT 1374 (n=1), MIT 1375 (n=2), MIT 1378 (n=3), Orphan (n=12)
SIT92 (700076777760771) X3	16 (4.56)	AFRI-S (50.25), AMER-N (23.04), AMER-S (9.07)	MIT 34 (n=9), MIT 46 (n=1), MIT 215 (n=1), MIT 462 (n=1), MIT 676 (n=1), MIT 1383 (n=1), Orphan (n=2)
SIT926 (773777777760771) T1	7 (1.99)	AFRI-S (55.17), ASIA-W (17.24), AMER-N (13.79), AMER-S (6.90)	MIT 8 (n=2), MIT 10 (n=1), MIT 23 (n=1), MIT 208 (n=2), Orphan (n=1)
SIT2531 (677777777760601) T1	15 (4.27)	AFRI-S 91.30, AMER-S 8.70	MIT 22 (n=3), MIT 33 (n=5), MIT 116 (n=1), MIT 188 (n=1), MIT 1389 (n=1), Orphan (n=4)

* Predominant shared types are defined as SITs representing 2% or more strains in a given dataset (i.e., 7 or more strains in this study); clades are designated according to SITVIT2 using revised SpolDB4 rules.

** Worlwide distribution is reported for regions with ≥5% of a given SIT as compared to their total number in the SITVIT2 database. The definition of macro-geographical regions and sub-regions is according to the United Nations (http://unstats.un.org/unsd/methods/m49/m49regin.htm); Regions: AFRI (Africa), AMER (Americas), ASIA (Asia), EURO (Europe), and OCE (Oceania), subdivided in: E (Eastern), M (Middle), C (Central), N (Northern), S (Southern), SE (South-Eastern), and W (Western).

*** 12 loci MIRU pattern designations in SITVIT2 database, a database of the Pasteur Institute in Guadeloupe.

5.4 Discussion

Currently, the estimated proportion of MDR-TB cases among TB cases in South Africa ranges from 1.8% among all new cases and 6.7% among previously treated TB cases (WHO 2009b). The treatment of such cases is lengthy, expensive and is associated with high mortality rates and high rates of default from treatment (Gandhi *et al.* 2006, Shean *et al.* 2008). Most of the available studies on MDR-TB treatment outcomes, from countries with a low HIV prevalence and often limited samples sizes, indicate higher rates of successful treatment (51 – 77%) (Kim *et al.* 2007, Chan *et al.* 2004, Mitnick *et al.* 2003, Bang *et al.* 2010, Chiang *et al.* 2006, Orenstein *et al.* 2009, Tahaoglu *et al.* 2001, Migliori *et al.* 2007, Kliiman, Altraja 2009, Goble *et al.* 1993) when compared to the current study (48.8%). In South Africa, MDR-TB treatment involves hospital-based treatment for several months at the start of treatment, which ensures effectiveness, adherence and also removes infectious patients from the community (Singh *et al.* 2007a). This system however means that patients are separated from their families and from daily routines for long periods and therefore can result in treatment interruption (default) as patients attempt to leave the hospitals to attend to their lives (Holtz *et al.* 2006). The default rates observed in the current study decreased over the years 2004 to 2007 (36.3% – 20.5%), this is consistent with other studies in South Africa (Brust *et al.* 2010, Holtz *et al.* 2006, Shean *et al.* 2008) and from other high TB prevalence countries (Chiang *et al.* 2006, Palmero *et al.* 2004, Park *et al.* 2004). The decreased default observed in the study corresponds to a time when the country was implementing health care reform measures in response to the discovery of XDR-TB in the KwaZulu Natal Province.

The importance of improving programmatic factors such as for example, communication between personnel and patients and increased patient supervision, have been shown to improve treatment completion in other studies (Thiam *et al.* 2007). Infection control measures, data capture, admission and discharge guidelines as well as security measures were scaled up at Sizwe Hospital subsequent to the XDR-TB outbreak in KZN (M.P Grobusch, personal communication) and these improvements may have contributed to the decrease in default from treatment.

Several studies have reported high mortality and poor treatment outcomes associated with HIV-infected patients (Brust *et al.* 2010, Flament-Saillour *et al.* 1999, Kliiman, Altraja 2009). The poor outcomes and especially rapid time to death have mostly been observed in the absence of effective antiretroviral treatment (Wells *et al.* 2007, Gandhi *et al.* 2006, Frieden *et al.* 1996, Seung *et al.* 2009). A recent study from the KZN Province, the first extensive study detailing the treatment outcomes of MDR-TB patients in a TB referral hospital in South Africa, reported an association between HIV co-infection and death. Since the study period was prior to the availability of ART therapy in the public sector (2000-2003), the authors attributed the HIV/death association to the lack of ART therapy and recommended an integration of ART with second-line anti-TB treatment in the management of MDR-TB patients (Brust *et al.* 2010). The current study showed no association between HIV status and poor treatment outcomes. This difference in association could be due to several factors.

Firstly, it could be a reflection of the effectiveness of integrating second-line anti-TB treatment together with antiretroviral therapy (ART), as is done at Sizwe Hospital. Administering ART together with MDR-TB treatment has been noted to improve treatment outcomes of MDR-TB patients in previous reports (O'Donnell *et al.* 2009, Dheda *et al.* 2004) and the rapid time of death that is frequently associated with HIV/MDR-TB co-infected patients is greatly decreased with effective early treatment of both HIV and MDR-TB.

Secondly, our observation may be due to the death of the most ill TB-HIV co-infected patients before they are admitted to Sizwe Hospital. Hence, those patients who do get to the MDR referral hospital may be healthier and, for example, have higher CD4 cell counts at the initiation of MDR-TB treatment, which combined with appropriate ART could account for good treatment outcomes. Further investigations would be necessary to evaluate the associations between CD4 cell counts and other parameters at diagnosis and treatment outcomes in this setting.

The proportion of isolates resistant to all four drugs was higher in this sample (72%) compared with 55% observed in the sample from Johannesburg (Chapter 3). This observation could reflect patients with higher levels of drug resistance (resistant to all four first-line drugs) are likely to be referred to Sizwe hospital from the hospitals and clinics in Gauteng. The observed high resistance to the majority of first-line anti-TB drugs strongly highlights the necessity for regular surveillance of drug resistance at a

national level in order to get a better picture of the actual resistance situation in South Africa.

Comparing the genotyping data from Johannesburg (Chapter 3) with genotyping data from Sizwe Hospital confirmed the diversity of strains circulating in our region as well as the predominance of the SITs responsible for MDR-TB in the province. The distribution of these strains, according to SITVIT2, revealed that most of the predominant strains, with the exception of LAM9/SIT42, which is prevalent in North Africa, (Namouchi *et al.* 2008) are well represented in the African region (≥5%).

An association between strain family and treatment outcome was observed in the study ($P = 0.041$). All the strain families with the exception of the Haarlem (H) family seemed to have similar percentages of successful treatment outcome whereas the H family was associated with less chance of a successful treatment outcome. Data on the associations between strain family and outcomes need to be interpreted with caution in regions with high MDR-TB prevalence. Some patients in such regions may be infected with either mixed infections of *M. tuberculosis* and/or exogenous re-infection by different *M. tuberculosis* strains. The infecting *M. tuberculosis* isolates can sometimes differ in strain families and even drug susceptibility profiles (Mendez *et al.* 2009, Garcia de Viedma *et al.* 2005). Further studies investigating serial isolates of *M. tuberculosis* strains during MDR-TB treatment would provide better information on the associations of strain families and treatment outcomes.

A history of previous TB infection and treatment is often associated with the development of acquired MDR-TB in areas with a high prevalence of TB (Leimane *et al.* 2005, Brust *et al.* 2010, Kliiman, Altraja 2009, Faustini *et al.* 2006). This is usually attributed to the failure of TB control efforts due to poor case management, interruptions of drug supply and the use of inadequate drug regimens or treatment defaults. In the current study, a high proportion (56%) of MDR-TB patients had a history of previous TB. New MDR-TB cases were noted in 30% patients, while 14% of patients had a missing record of prior TB history. The higher number of retreatment TB cases observed in this study agrees with the studies of other investigators mentioned above and suggests that previous exposure to anti-TB drugs may result in acquired drug-resistant TB.

Statistical analysis showed no association between treatment outcomes and previous TB. The lack of association between the treatment outcomes of new versus retreatment MDR-TB cases could be explained by the fact that at the initiation of MDR-TB treatment all the patients were already infected with an MDR-TB strain and were receiving standard treatment from a specialized referral centre; factors other than the method of infection (acquired or transmitted) with a drug-resistant strain determined the treatment outcome.

In studies investigating treatment outcomes of MDR-TB patients, additional risk factors such as resistance to fluoroquinolones (FQN), prior use of FQN, resistance to capreomycin, history of imprisonment, low body mass index, extra-pulmonary TB, underlying illness (excluding HIV infection), alcohol consumption and smoking were associated with poor outcomes (Chan, Iseman 2008).

One of the main limitations of the current study is that it relied on accurate capture of data by staff members at admission and on patient information from patient files at the hospital. The data collection sheet used in this study (Appendix 8) was originally designed to capture information on the above mentioned risk factors often associated with MDR-TB in the literature (Orenstein *et al.* 2009, Chan, Iseman 2008). These variables had to be excluded from analysis because of incomplete data capture and therefore other potential factors that may have affected the observed treatment outcomes could not be evaluated. Improved methods on recording patient data in the MDR-TB referral hospitals as well as complete clinical information would allow for further insight into factors associated with treatment outcomes for MDR-TB patients. This knowledge would also aid in developing appropriate interventions in order to improve treatment outcomes.

The process used for referring patients to Sizwe Hospital also needs to be re-evaluated. Of the 434 patients identified in Johannesburg (Chapter 3), only 10% were found to have patient files at Sizwe Hospital. This suggests that some of the patients may have died before they could have been admitted, that others did not show up for admission, or were treated elsewhere. Since resources for patient admission and patient follow-up are limited, this situation seems to have not been addressed. The situation results in incomplete data sets for epidemiological studies focused on MDR-TB in the region, since only a proportion of MDR-TB patients reach the referral hospitals. In order to increase the proportion of MDR-TB patients that receive proper medical treatment in high TB burden areas such as South Africa, community-based treatment or satellite in-patient

treatment centres should perhaps be considered (Padayatchi, Friedland 2008, Mitnick *et al.* 2003).

Another limitation of this study was that during the study period, drug susceptibility testing for second-line anti-TB drugs was not mandatory in the NHLS referral TB lab and thus treatment DST results for MDR-TB treatment were not available. XDR-TB has been associated with poor outcomes in several studies (Migliori *et al.* 2008b, Migliori *et al.* 2009, Cox *et al.* 2007) and therefore, the poor outcomes of some of the patients in this study could be linked to the development of XDR-TB during treatment. Policies regarding testing for second-line drug resistance have since been modified in the country subsequent to the discovery of XDR-TB in all the provinces, and further investigations that include a more comprehensive drug susceptibility profile of MDR-TB isolates should provide a better understanding of the contribution of XDR-TB to treatment outcomes.

5.5 Conclusions

The findings of the current study have clear implications for TB control efforts in our province, since the incidence of MDR-TB continues to increase and predominant MDR-TB strains may be transmitted with similar effectiveness to drug-susceptible TB strains. It has been reported that cure rates of >60% are required to interrupt the transmission of drug-resistant TB and that a cure rate of ≥ 80% is needed to achieve a 10 fold reduction in global MDR-TB incidence in the next 20 years (Dye, Williams 2000). The current study, together with other studies from South Africa (Brust *et al.* 2010, Shean *et al.* 2008) show successful treatment rates of less than 50% in MDR-TB cases and highlights the challenge that still exists in managing and treating multidrug-resistant TB in the country.

According to our results, Sizwe hospital, a specialized treatment centre which is equipped with trained personnel, a HIV clinic that provides ART, improved infection control strategies and efficient security measures, seemed to show steady yearly improvements in managing MDR-TB cases. This was reflected in the annual decrease in default and poor outcomes rates as well as the improved successful treatment outcomes. Improved treatment outcomes have been reported in other programs from resource-limited countries with high MDR-TB, low HIV prevalence such as Estonia, Latvia, Peru, the Philippines, and the Russian Federation (Nathanson *et al.* 2006) when treatment in administered in accordance with WHO recommended guidelines.

During the study period of this thesis, standardized methods to determine drug susceptibility testing to second-line drugs and rapid molecular testing for detection of MDR-TB were being evaluated in the country's laboratories (Barnard *et al.* 2008). This could eventually result in earlier MDR-TB case detection and in more cases being referred to Sizwe Hospital. Hence, the typical patient in the hospital could change in the next few years and continued reporting of MDR-TB treatment outcomes is necessary in the referral centres in order to detect changes in the risk factors associated with poor treatment outcomes.

Early diagnosis of MDR-TB patients has been shown to improve overall treatment outcomes in countries with more effective MDR-TB treatment programmes (Leimane *et al.* 2005). Clinicians in resource-limited countries unfortunately usually only diagnose MDR-TB after failed treatments using first-line anti-TB drugs, by which time patients may have a worse prognosis and have transmitted the disease in their communities. Attention to early diagnosis and early MDR-TB treatment, particularly focused on HIV/MDRTB co-infected patients would likely result in improved treatment outcomes.

The current study highlights the challenges associated with MDR-TB treatment in this high TB and HIV prevalence region. The diverse and highly transmissible MDR-TB strain population, as well as inadequate system used to refer patients to specialized MDR-TB centres contribute to the continuous rise in the incidence of MDR-TB in the region.

Routine use of rapid diagnostic methods, drug susceptibility testing of new and re-treatment TB cases, a better referral and monitoring system, an integrated treatment for HIV and TB are still all required in order to effectively manage the drug-resistant TB epidemic in the country.

CHAPTER 6: Genotypic Diversity of "Pre-XDR" and XDR-TB Isolates in South Africa, June 2005 –December 2006

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6.0 Summary

Extensively drug-resistant tuberculosis (XDR-TB), which is defined as multi-drug-resistant *Mycobacterium tuberculosis* resistant to any fluoroquinolone and kanamycin or capreomycin or amikacin, is emerging as a major threat to TB control worldwide. Treatment of such cases is extremely difficult and is complicated by the HIV/AIDS pandemic, especially in developing countries. Little is known about the genotypic population structure of XDR-TB and whether these strains have primarily been transmitted or have each individually acquired the mechanisms for resistance. The objective of the study was to gain insight into the genotypic population structure of "pre-XDR" and XDR *Mycobacterium tuberculosis* strains in South Africa using a molecular approach and thereby determine whether XDR-TB is mainly acquired or transmitted. Forty one XDR-TB strains resistant to ofloxacin and kanamycin were isolated from 699 patients at the NHLS TB referral laboratory in Johannesburg, South Africa, from June 2005 to December 2006. Sixty seven 'pre-XDR' isolates, resistant to either ofloxacin or kanamycin, together with ethionamide, were also identified. All of these isolates were genotyped by spoligotyping. A total of 33 different spoligotypes and 9 genotypic families were identified from 42 hospitals/clinics in 5 provinces in the country.

This study was the first description of the genotypic population structure of extensively-resistant *M. tuberculosis* strains in South Africa. The high level of genotypic diversity observed suggests that acquisition of resistance to second-line drugs is an important factor in the TB epidemic in South Africa.

6.0 Introduction

To date, extensively drug-resistant TB (XDR-TB) has been identified in more than 50 countries worldwide and poses a serious challenge to TB management. Extensive drug resistance was first noted in the late 1980s and 1990s, and in 2004 the WHO and USA Centers for Disease Control and Prevention (CDC) surveyed selected national reference laboratories to estimate the rates of XDR-TB (Centers for Disease Control and Prevention 2006). It was found that between 2000 and 2004, 20% of the strains tested were MDR-TB and that 10% of these were XDR-TB, using the previous definition of XDR-TB (resistance to 3 of the 6 classes of second-line drugs). The data collected showed that rates of XDR-TB varied across the globe, with Asian and East European countries showing the highest rates. In October 2006, the WHO re-defined XDR-TB as MDR-TB with additional resistance to any fluoroquinolone and to at least one of three injectable second-line anti-TB drugs used in treatment (capreomycin, kanamycin or amikacin), mainly because currently drug susceptibility testing is reliable only for capreomycin, kanamycin or amikacin and the fluoroquinolones, among the second-line anti-TB drugs. In 2006, an outbreak of XDR-TB in KwaZulu Natal (KZN) was associated high mortality rates and attracted a lot of international attention (Gandhi *et al.* 2006). Subsequent to the KZN outbreak, XDR-TB was identified in 60 locations in KZN and in all 9 provinces in South Africa (Madariaga *et al.* 2008).

XDR-TB has also been reported in 53 countries worldwide and is associated with poor outcomes, especially in HIV co-infected persons (Gandhi *et al.* 2006, Kim *et al.* 2008, Migliori *et al.* 2009, Migliori *et al.* 2008a, Migliori *et al.* 2007).

Our study was the first in South Africa to report on the diversity on XDR-TB strains, as identified by spoligotyping. Prior to that, the only published genotypic information of XDR-TB strains worldwide was from an Iranian study of 12 patients, where a chain of transmission of Haarlem and East African Indian-3 strains was identified (Masjedi *et al.* 2006). Few studies have since reported on the use of molecular genotyping techniques in the study of XDR-TB transmission (Calver *et al.* 2010, Lai *et al.* 2010, Sun *et al.* 2008). A study from Iran recently reported on potentially highly transmissible super extensively drug-resistant TB strains, which are resistant to most of the available second-line anti-TB drugs and belong to diverse *M. tuberculosis* strain families (Haarlem, Beijing, EAI and CAS families) (Velayati *et al.* 2009).

The aim of our previous/published study (Mlambo *et al.* 2008) was to use a molecular epidemiological approach to gain an insight into the genotypic diversity of XDR-TB *M. tuberculosis* strains in South Africa and thereby to determine whether XDR-TB is mainly acquired (secondary resistance) or transmitted (primary resistance). Since MDR-TB is a precursor for XDR-TB, is it also important to understand the drug susceptibility patterns and genetic characteristics of “pre-XDR-TB” strains especially in regions with high TB prevalence, in order for effective control of the development of XDR-TB.

The published data focused mainly on the molecular characteristics of the 41 XDR-TB strains. This chapter aims to further describe the molecular characteristics, distribution and transmission patterns of the "pre-XDR" TB strains.

6.2 Study Design

6.2.1 Sampling and Drug Susceptibility Testing

In this study a convenience sample was used, representing all MDR-TB isolates submitted to the NHLS TB referral laboratory, Johannesburg, from June 2005 to December 2006. This laboratory is the primary government facility able to test for second-line resistance in the country during this period. The indirect susceptibility procedure using BACTEC 460/BACTEC 12B medium (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) was used as per the manufacturer's instructions to test for resistance to second-line drugs. Second-line testing was performed using ofloxacin (OFX) (2.0 µg/ml), KM (5.0 µg/ml) and ethionamide (ETH) (2.5 µg/ml). DST was not performed for all second-line drugs at the NHLS TB referral lab for several reasons: CM has not been available in South Africa for treatment, AMK may exhibit cross-resistance with the other aminoglycoside agents (such as KM), and there were no guidelines for testing para-aminosalicylic acid (PAS). Due to the difficulties associated with retrospective data collection in South Africa, the only patient information available was sex, age and clinic/hospital of sample origin. Ethical clearance for the study was obtained from the University of the Witwatersrand Human Research (Medical) Ethics Committee.

6.2.2 Spoligotyping

Only the first heat-inactivated culture received from each patient was used for genotypic analysis; all follow-up heat-inactivated cultures were excluded from the analysis. Spoligotyping was performed according to the internationally standardized protocol as described in Chapter 2 (Kamerbeek *et al.* 1997). To assess whether laboratory contamination could have influenced the interpretation of the genotyping data, serial isolates from 13 patients were randomly sampled and spoligotyped. All of these results were concordant. The SpolDB4 database (http://www.pasteur-guadeloupe.fr/tb/spoldb4) was used to assign genotype families to the spoligotypes obtained (Brudey *et al.* 2006). Culture material was unavailable for restriction fragment length polymorphism analysis.

6.3 Results

MDR-TB isolates (n=845) from 699 patients were submitted from 7 of the 9 provinces of South Africa for testing for resistance to second-line drugs, from June 2005 to December 2006. Of these, 22 (3%) were resistant to ofloxacin only, 7 (1%) to kanamycin only and 117 (16.5%) were resistant to ethionamide only. Resistance to 2 or 3 second-line drugs was observed in isolates from 108 (15%) patients (Table 6.1) and molecular analyses were performed on these isolates. Sixty two percent of the samples resistant to 2 or more drugs were from male patients. The year of birth was available for 106 of the patients, with the average age being 37 years and the median age 36 (range 2-56). Of the isolates resistant to 2 or more second-line drugs, 41 isolates from 16 clinics in 4 provinces were found to be resistant to kanamycin and ofloxacin, defining them as XDR-TB. Thirty were also resistant to ethionamide (Figure 6.1).

Table 6.1: The percentage and distribution of "pre-XDR" and XDR-TB strain families in the provinces of South Africa

Spoligotype family	Province	XDR-TB		MDR+OFX +ETH		MDR+KM +ETH	
		Number	%	Number	%	Number	%
Beijing	North West	4	34	1	12	2	47
	Northern Cape	5		2		4	
	Limpopo	3		0		0	
	Gauteng	2		3		0	
	Free State	0		0		1	
LAM	North West	0	12	4	17	2	13
	Northern Cape	0		0		0	
	Limpopo	1		0		0	
	Gauteng	4		5		0	
EAI	North West	2	10	3	6	0	0
	Limpopo	1		0		0	
	Gauteng	1		0		0	
X	North West	1	2	9	19	0	7
	Gauteng	0		1		1	
T	North West	2	10	5	13	0	0
	Northern Cape	0		1		0	
	Gauteng	2		1		0	
H	North West	1	5	2	13	1	20
	Northern Cape	0		1		1	
	Limpopo	0		1		0	
	Gauteng	1		3		1	
S	North West	1	2	1	2	0	0
CAS	Gauteng	0	0	1	2	0	0
Manu1	Gauteng	0	0	0		1	7
Not in SpolDB4	North West	6	24	5	15	1	7
	Limpopo	2		2		0	
	Gauteng	2		1		0	
TOTAL		**40**		**52**		**15**	

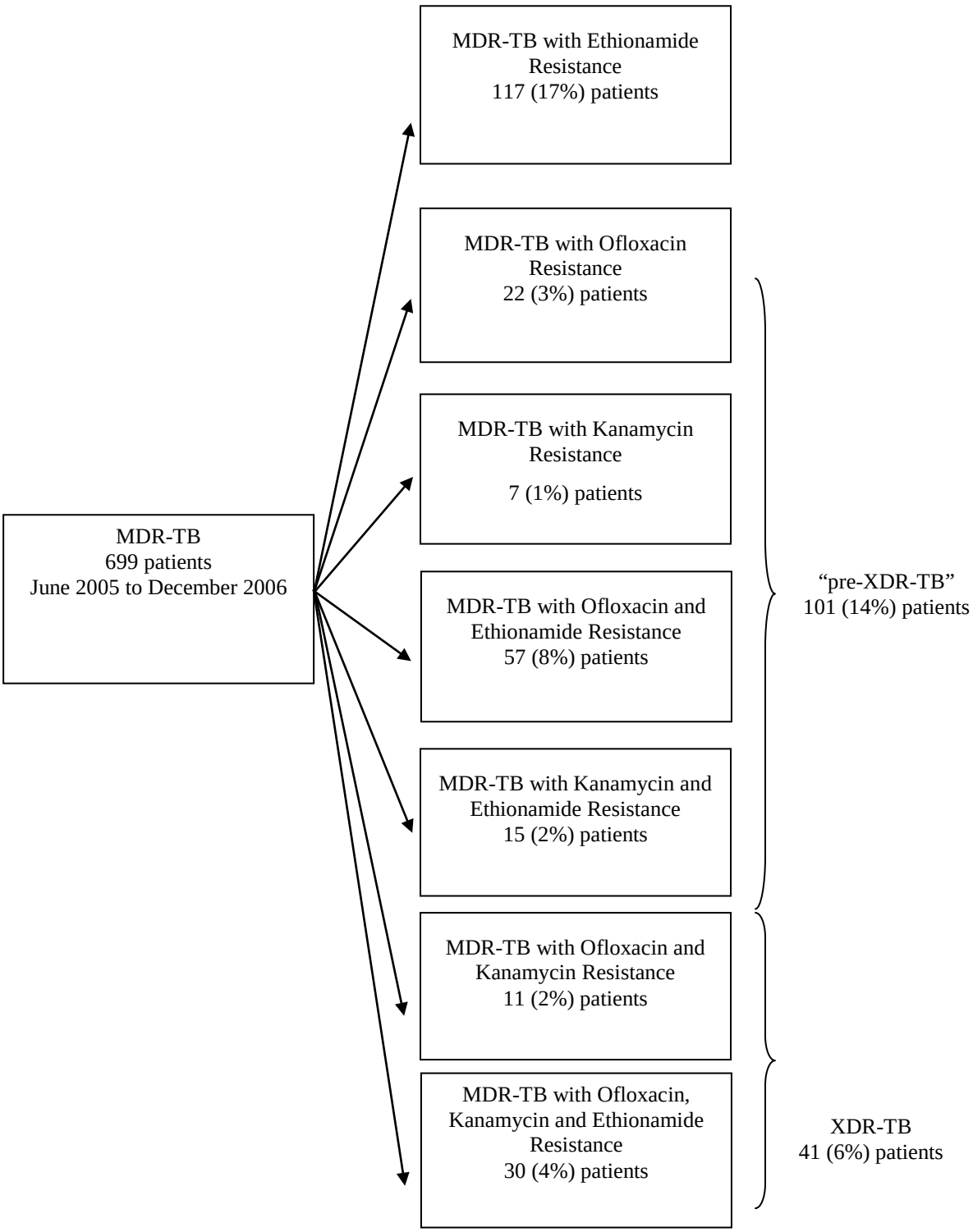
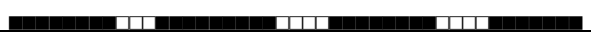
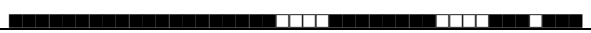
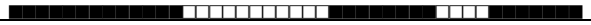


Figure 6.1 Second-line DST results of the 699 submitted MDRTB isolates. MDR = multidrug-resistant; TB = tuberculosis; ETH = ethionamide; OFX = ofloxacin; KM = kanamycin; XDR = extensively drug-resistant; DST = drug susceptibility testing.

Of these isolates, 8 (20%) originated from the Gauteng MDR-TB referral hospital (Sizwe), 7 (17%) from a TB hospital in Limpopo, another 7 (17%) from a hospital serving a gold mining community in North West Province and 6 (15%) from a nearby hospital. One to two strains were submitted from 12 other hospitals and clinics (Table 6.1). Seventeen spoligotypes and seven internationally recognised spoligotype families were observed amongst the XDR isolates, of which the Beijing genotype family formed the largest group with 14 (34%) (Table 6.1).

Sixty-seven patients were identified as having MDR-TB with resistance to either ofloxacin or kanamycin, together with ethionamide resistance, and were termed a 'pre-XDR' group. Isolates originated from 30 hospitals/clinics in 5 provinces (Table 6.1). Twenty one spoligotypes and 9 genotypic families were seen (Table 6.2). Resistance to ofloxacin and ethionamide was observed in 52 isolates (78%) and kanamycin and ethionamide resistance in 15 isolates (22%). The spoligotypes families found among the ofloxacin and ethionamide resistant group and kanamycin and ethionamide resistant group are shown in Figure 6.1. To assess whether molecular laboratory contamination contributed to the similarity of strain types seen, subsequent isolates from 13 patients were randomly sampled and typed. All spoligotype results for serial isolates were concordant.

Table 6.2: Description of "pre-XDR-TB" strains circulating in South Africa

Spoligotype family	SIT	Spoligotype pattern	MDR+OFX+ETH	MDR+KM+ETH
Beijing	1		6 (12%)	7 (46%)
LAM	LAM3/33		9 (17%)	2 (13%)
	LAM4/60			
	LAM11/ZWE/82			
EAI	EAI1_SOM/48		3 (6%)	0
	EAI1_SOM/1649			
X	X3/92		10 (18%)	1 (7%)
	X2/18			
T	T1/53		7 (14%)	0
	T1/244			
	T1/803			
	T1/1597			
Haarlem (H)	H3/50		7 (14%)	3 (20%)
	H3-T3/36			
S	S/71		1 (2%)	0
CAS	CAS1_DELHI/25		1 (2%)	0
Manu 1	100		0	1 (7%)
Not in database			8 (15%)	1 (7%)
TOTAL			**52**	**15**

6.4 Discussion

This is the first study to demonstrate that a significant proportion of MDR-TB cases in South Africa have XDR-TB. The calculated proportion of XDR-TB maybe an under-estimate, given that this study only analyzed a convenience sample of isolates which were largely collected before the description of XDR-TB in South Africa (Gandhi *et al.* 2006). In the absence of awareness, attending clinicians may not have submitted MDR-TB specimens for routine second-line DST. This study also raises concerns over the significant proportion of MDR-TB cases (14%) that were resistant to a single marker of XDR-TB (either OFX or KM). As drug resistance in *M. tuberculosis* is due to the accumulation of mutations in the genome, this pool of 'pre-XDR-TB' strains are at risk of developing extensive resistance if not managed appropriately. The higher proportion of OFX resistance as compared to KM resistance seen in this study could be due to the widespread use of FQs to treat other ailments (such as diarrhoea and urinary tract infections) (Grimaldo *et al.* 2001). In contrast, the clinical use of KM to treat other infections is limited.

Genotyping of the isolates by spoligotyping showed that a range of strain types had developed XDR-TB. These XDR-TB strains represented at least seven different genotype families, with the Beijing genotype making up 34% of the isolates. In previous studies, the Beijing genotype has been associated with drug resistance (European Concerted Action on New Generation Genetic Markers and Techniques for the Epidemiology and Control of Tuberculosis 2006) and has been found to be overrepresented in children with drug-resistant TB in the Western Cape, South Africa (Marais *et al.* 2006).

The Beijing family constitutes approximately 17% of MDR-TB in Gauteng and 39% of strains in the Western Cape (Chapter 3). The proportion of Beijing strains seems to have doubled compared to earlier studies (Streicher *et al.* 2004, Richardson *et al.* 2002b) in the Western Cape and this could be evidence for the transmissibility of this family in that region. Analysis of the spoligotyping data suggest that the majority of XDR-TB cases (63%) in South Africa have developed extensive drug resistance through acquisition, as defined by the presence of a unique spoligotype within a defined geographical setting. This estimate may be as high as 75% if it is assumed that each cluster (within a defined geographical setting) was initiated by a source case who acquired extensive drug resistance (Small *et al.* 1994).

One reason for this high level of acquisition could be the high number of MDR-TB patients who default from treatment in South Africa. A study by Holtz and colleagues showed that 30% of patients defaulted within the first 6 months of treatment, and even more at later stages (Holtz *et al.* 2006). Acquisition of resistance reflects negatively on the success of the National Tuberculosis Programme in the country and is an issue that has to be addressed to prevent the spread of this potentially incurable disease (Van Rie, Enarson 2006). This study also raises the concern that XDR-TB may be transmitted between patients in the community and/or hospitals and clinics. A likely instance of transmission was observed where six patients admitted to a hospital in the North West Province presented with XDR-TB with an identical *M. tuberculosis* strain (previously undescribed spoligotype). Another possible case of transmission in the North West Province was noted where two patients from the same health care facility had isolates with the Beijing spoligotype.

All of the XDR-TB isolates analyzed from this province originated from gold mining hospitals or health care facilities in mining areas. It therefore appears that transmission may play an important role in this setting, which is consistent with an increased risk of transmission expected in the crowded working and residential conditions typically associated with mines. Identical XDR-TB strains (with identical spoligotypes) were isolated from two or more patients attending the same hospital/clinic in three additional instances. However, we acknowledge that the discriminatory power of spoligotyping may not be able to accurately define transmission in all instances, particularly in the case of strains with the Beijing genotype. In future studies, it will be important to use high resolution genotyping methods (IS*6110*-RFLP or MIRU-VNTR typing) to accurately quantify the contribution of transmission to the XDR-TB epidemic.

6.5 Conclusions

The first MDR-TB case in South Africa was recorded in 1985 (Weyer *et al.* 1995). Since then the number of MDR-TB cases in the country has steadily increased (World Health Organization, International Union Against Tuberculosis and Lung Disease 2004). A similar scenario with XDR-TB should be avoided, as 16.2% of South Africans between the ages of 15 and 49 are estimated to be HIV-positive (Shisana O *et al.* 2005) and the twin epidemics of HIV and TB makes the emergence of XDR-TB potentially disastrous for both local and international TB control.

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CHAPTER 7: Conclusions and Future Work

7.0 Conclusions and recommendations

South Africa has high burden of TB and MDR-TB and currently ranks 4th among 27 high burden MDR-TB countries worldwide and yet, very little is known about the population structure and transmission patterns of the MDR-TB strains in most of the country's provinces. Apart from two nationwide studies, both of which had limited samples of MDR-TB isolates from the Gauteng Province (Stavrum, 2009), no other information is available on the composition of MDR-TB genotypes in the province. The work from this PhD is the first study to characterize a significant sample of MDR-TB isolates from Johannesburg, the most populous of the country's cities.

The ability of *M. tuberculosis* strains to spread varies between strain types and different strains have different geographical specificities (Gagneux *et al.* 2006, Filliol *et al.* 2006). Molecular genotyping allows for tracking local epidemics and provides insight into the global migration and expansion of strains (Brudey *et al.* 2006, Sola *et al.* 2003). In the current study, the use of spoligotyping and MIRU-VNTR identified possible ongoing transmission of diverse of MDR-TB strains, comprising Beijing, LAM, EAI, T, S, H and X families. The spread of such drug-resistant strains poses a great challenge both local and global efforts to control TB. Strategies such as rapid diagnostic tests for MDR-TB, early identification of MDR-TB cases through an increase in DST in new and retreatment patients and efficient referrals to MDR-TB hospitals are urgently required in the region in order to minimize the ongoing transmission of MDR-TB strains.

Resource-limited, high disease burden countries like South Africa should aim to take advantage of rapid, accurate and reliable methods used in TB molecular epidemiological investigations. Spoligotyping and MIRU-VNTR typing are PCR-based methods that proved to be suitable for use in identifying and differentiating *M. tuberculosis* strains in the current study. Our experience, in the current study, with using IS*6110*-RFLP typing confirmed the reports regarding the challenges of this method (van Soolingen 2001, Mathema *et al.* 2006). The use of IS*6110*-RFLP analysis was attempted throughout the study but proved to be challenging, mostly because of the lack of sufficient quantities of DNA. Some of the cultures used in the study had been stored in MGIT tubes at 4°C for extended periods, and these either did not grow sufficiently when sub-cultured or did not provide sufficient DNA after extraction. Such technical difficulties can prove to be expensive and time consuming, especially in areas where resources are limited. Results from the study illustrate the effectiveness of MIRU-VNTR typing together with spoligotyping in epidemiological studies in the region.

The work from this PhD also describes treatment outcomes of MDR-TB from the referral hospital in Gauteng, South Africa. The results indicate periodic improvement in MDR-TB treatment success from 2004 – 2007. The short-comings in the referral of patients from health centres in Johannesburg to the MDR-TB treatment hospital were highlighted. Only 10% of patients identified in Johannesburg were found in the Sizwe Hospital patient records. Several studies from the country have recently been advocating a change in the system used to manage MDR and XDR-TB patients (Padayatchi *et al.* 2009, Heller *et al.* 2010, Brust *et al.* 2010). The high TB burden would benefit from a decentralization of TB treatment and the establishment of a community-based treatment approach that would target the two most dangerous diseases in

South Africa to date, TB and HIV/AIDS. Our study was initiated prior to the reports of XDR-TB strains in the KZN Province of South Africa and was already ongoing at the time of the KZN outbreak. The association of successful treatment outcomes with the year of initiation of treatment observed in the current study possibly reflects the changes in patient management implemented at Sizwe hospital subsequent to the XDR-TB outbreak in KZN. A better referral system, routine use of rapid diagnostic methods and DST as well as an integration of TB and HIV care are still required in order to effectively manage the drug-resistant TB epidemic.

In view of the high rates of additional resistance (resistance to INH and RIF together with other first-line anti-TB drugs) observed in the study we recommend that regular drug surveillance is essential in South Africa. The last national survey was done in 2001-2002 using a limited sample of drug-resistant strains from the various provinces in the country. Our findings indicate that among the MDR-TB isolates, more than 50% are resistant to all the first-line drugs. MDR-TB is a precursor to XDR-TB and poses a massive challenge to the TB control programmes in the country. Our findings are especially worrying since the MDR-TB strains seem to have become established and may be spreading as efficiently as drug-susceptible strains. In a country like South Africa, with an estimated co-infection rate of TB/HIV of 70% (Abdool Karim *et al.* 2009), the duel epidemics could result in further increases in mortality.

7.1 Future Prospects

The studies described in this thesis form the basis for further research in several ways. Firstly, the epidemiological database of drug-resistant TB in Gauteng allows for ongoing studies comparing drug-resistant TB isolates from the rest of the country as well as from other countries in Africa.

Such a database would help in monitoring strain dynamics which is essential for TB control over time. The establishment of molecular typing techniques in our department together with our collaboration with the NHLS TB referral laboratory opens up possibilities for multiple studies. For example, baseline information of the drug-susceptible and mono-resistant strains of TB that are circulating in the region is required in order to better understand TB disease.

Spoligotyping and MIRU-VNTR analysis offer reliable, fast methods for this analysis. Also, the spoligotyping and MIRU results are easily comparable with other laboratories and submission of such information to the international spoligotyping and MIRU-VNTR typing databases would improve on global knowledge of TB disease.

Clinicians are often faced with challenges when treating suspected cases of mixed *M. tuberculosis* strain infection. Several studies from South Africa have identified mixed infection in TB specimen (Richardson *et al.* 2002a, Stavrum *et al.* 2009). Stavrum and colleagues acknowledge that the contribution of mixed infections is not known in South Africa (Stavrum *et al.* 2009). The collaboration with the NHLS TB referral lab will allow for investigating this question by using MIRU-VNTR typing together with spoligotyping to characterize patients with changing drug susceptibility patterns or treatment failure. A recent study from KZN demonstrated the importance of exogenous re-infection in TB patients in an area of high TB incidence (Andrews *et al.* 2008). Spoligotyping was used to confirm this phenomenon.

The real and serious threat of XDR-TB outbreaks in our region necessitates studies into the mechanisms of resistance in *M. tuberculosis* strains with extensive resistance to anti-TB drugs.

Several studies worldwide have used mutational analysis to gain insight into the differences between MDR and XDR-TB strains of similar and different strain families. The current study has provided the department with a wealth of MDR and XDR-TB strains that will contribute in an understanding of the mechanisms conferring drug resistance in TB strains circulating to our region. As has been illustrated, molecular epidemiological methods can be used effectively to understand the dynamic of TB disease. These methods on their own however will not accomplish the end goal of increasing TB treatment success rates and controlling the development and spread of drug-resistant TB. Innovative rapid and affordable diagnostics are required as well as novel treatment regimens. Public health systems and National TB programmes also need to be strengthened and encouraged to integrate strategies such as combined treatment strategies of TB-HIV in clinics. Supervised community-based treatment programs for both TB and HIV/AIDS would also aid the over-burdened TB referral hospitals and result in greater distribution of the medication and care required by patients.

APPENDICES

Appendix 1: Ethical approval from the University of the Witwatersrand

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Khudzie Mlambo

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M050628**
PROJECT	Molecular & Epidemiological Characterization of Drug Resistant Mycobacterium TB Isolates in Johannesburg, South Africa
INVESTIGATORS	Ms C Khudzie Mlambo
DEPARTMENT	Clinical Micro. & Infectious Disease
DATE CONSIDERED	05.06.24
DECISION OF THE COMMITTEE*	Approved unconditionally

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

DATE 05.06.27 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr E Marais

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix 2: Permission for Access to Patient Files at Sizwe Hospital

24. AUG. 2005 12:28 NO. 657 P. 2

GAUTENG PROVINCIAL GOVERNMENT

Health Region A

Johannesburg and West Rand

Office of the Director: DR.S.GAELEJWE
ENQUIRIES: N. NYANDENI
DEPARTMENT OF HEALTH
Telephone: (011) 481 -5266
Facsimile: (011) 481 -5263
Gauteng Health Department
Private Bag X21
Johannesburg 2100

VISION
"Health for a Better Life"

MISSION
Ensure a Caring Climate for Service Users

THREE STRATEGIC PRIORITIES

To Improve the Health of the People of Gauteng

To Provide Better Health Care Services

To Secure Better Value For Money and Effective Organisation

TO : NATIONAL HEALTH LABORATORY SERVICE

ATTENTION : DR. ELSE MARAIS
SENIOR MEDICAL SCIENTIST.
TEL: (011) 489-8510
FAX: (011) 489-8530

CC : MRS. VAN STADEN

FROM : DR. S. GAELEJWE
DIRECTOR JHB METRO

DATE : 24 AUGUST 2005.

SUBJECT: PATIENT INFORMATION FROM SIZWE HOSPITAL.

This letter serves to inform you that permission is granted to collect the relevant information from Sizwe Hospital.

Johannesburg Metro Office would appreciate submission of findings, conclusion and recommendations.

Kind regards.

24/08/05

DR. S. GAELEJWE
DIRECTOR
JOHANNESBURG METRO AREA

Directors: Dr S.Gaelejwe - Metro Area (Johannesburg) Tel.: (011) 481-5262 Fax: (011) 481-5263
Mrs. J. More - Systems Support - Tel.: (011) 481-5259 Fax: (011) 481-5245
Mrs. N. Mekgwe District Council Area (West Rand) Tel.: 953-2152 Fax: 953-4519

Appendix 3: Solutions for Spoligotyping

Stock solutions

20 X SSPE
27.6g Sodium Hydrogen Phosphate (dihydrate),
175.3g Sodium Chloride
7.4g EDTA
deionised water to 1000ml (pH 7.4)
adjust pH with sodium hydroxide pellets

10% SDS (50g SDS, mix in Fume Hood)
deionised water to 500ml

0.5M EDTA
93g EDTA,
deionised water to 500ml

Working Solutions

2 X SSPE / 1% SDS
100ml 10x SSPE
5ml 10% SDS
deionised water to 500ml

2 X SSPE / 0.5% SDS
100ml 10x SSPE
25ml 10% SDS
deionised water to 500ml

1% SDS
20 ml 10% SDS
deionised water to 200ml

20Mm EDTA
20ml 0.5M EDTA
deionised water to 500ml

Appendix 4: Composition of MIRU-VNTR Typing Sets for 12, 15 and 24 loci

MIRU-VNTR loci	12 loci MIRU	15 loci MIRU	24 loci MIRU
MRU 2	*		*
MIRU 4	*	*	*
MIRU 10	*	*	*
MIRU 16	*	*	*
MIRU 20	*		*
MIRU 23	*		*
MIRU 24	*		*
MIRU 26	*	*	*
M-27	*		*
M-31	*	*	*
M-39	*		*
M-40	*	*	*
ETR-A		*	*
ETR-B			*
ETR-C		*	*
QUB - 11b		*	*
QUB - 26		*	*
QUB - 4156		*	*
Mtub 04		*	*
Mtub 21		*	*
Mtub 29			*
Mtub 34			*
Mtub 30		*	*
Mtub 39		*	*

Appendix 5: MIRU-VNTR PCR Primer Sequences

MIRU primer	Sequence	Reference
MIRU2-F	TGGACTTGCAGCAATGGACCAACT	(Supply *et al.* 2001b)
MIRU2-R	TACTCGGACGCCGGCTCAAAAT	(Supply *et al.* 2001b)
MIRU4-F	GCGCGAGAGCCCGAACTGC	(Supply *et al.* 2001b)
MIRU4-R	GCGCAGCAGAAACGTCAGC	(Supply *et al.* 2001b)
MIRU10-F	GTTCTTGACCAACTGCAGTCGTCC	(Supply *et al.* 2001b)
MIRU10-R	GCCACCTTGGTGATCAGCTACCT	(Supply *et al.* 2001c)
MIRU16-F	TCGGTGATCGGGTCCAGTCCAAGTA	(Supply *et al.* 2001c)
MIRU16-R	CCCGTCGTGACGCCCTGGTAC	(Supply *et al.* 2001c)
MIRU20-F	TCGGAGAGATGCCCTTCGAGTTAG	(Supply *et al.* 2001c)
MIRU20-R	GGAGACCGCGACCAGGTACTTGTA	(Supply *et al.* 2001c)
MIRU23-F	CTGTCGATGGCCGCAACAAAACG	(Supply *et al.* 2001b)
MIRU23-R	AGCTCAACGGGTTCGCCCTTTTGTC	(Supply *et al.* 2001c)
MIRU24-F	CGACCAAGATGTGCAGGAATACAT	(Supply *et al.* 2001c)
MIRU24-R	GGGCGAGTTGAGCTCACAGAA	(Supply *et al.* 2001c)
MIRU26-F	TAGGTCTACCGTCGAAATCTGTGAC	(Supply *et al.* 2001b)
MIRU26-R	CATAGGCGACCAGGCGAATAG	(Supply *et al.* 2001b)
MIRU27-F	TCGAAAGCCTCTGCGTGCCAGTAA	(Supply *et al.* 2001b)
MIRU27-R	GCGATGTGAGCGTGCCACTCAA	(Supply *et al.* 2001b)
MIRU31-F	ACTGATTGGCTTCATACGGCTTTA	(Supply *et al.* 2001b)
MIRU31-R	GTGCCGACGTGGTCTTGAT	(Supply *et al.* 2001b)
MIRU39-F	CGCATCGACAAACTGGAGCCAAAC	(Supply *et al.* 2001c)
MIRU39-R	CGGAAACGTCTACGCCCCACACAT	(Supply *et al.* 2001c)
MIRU40-F	GGGTTGCTGGATGACAACGTGT	(Supply *et al.* 2001b)
MIRU40-R	GGGTGATCTCGGCGAAATCAGATA	(Supply *et al.* 2001b)
ETRA-F	AAATCGGTCCCATCACCTTCTTAT	(Frothingham, Meeker-O'Connell 1998)
ETRA-R	CGAAGCCTGGGGTGCCCGCGATTT	(Frothingham, Meeker-O'Connell 1998)
ETRB-F	CGCGAACACCAGGACAGCATCATG	(Frothingham, Meeker-O'Connell 1998)
ETRB-R	CGGTGATCGAGTGGCTATACGCTC	(Frothingham, Meeker-O'Connell 1998)
ETRC-F	GTGAGTCGCTGCAGAACCTGCAG	(Frothingham, Meeker-O'Connell 1998)
ETRC-R	GGCGTCTTGACCTCCACGAGTG	(Frothingham, Meeker-O'Connell 1998)
Qub11b-F	CGTAAGGGGGATGCGGGAAATAGG	(Skuce *et al.* 2002)
Qub11b-R	CGAAGTGAATGGTGGCAT	(Skuce *et al.* 2002)
Qub26-F	AACGCTCAGCTGTCGGAT	(Skuce *et al.* 2002)
Qub26-R	GGCCAGGTCCTTCCCGAT	(Skuce *et al.* 2002)
Qub4156-F	CTGGTCGCTACGCATCGTG	(Roring *et al.* 2002b)
Qub4156-R	TGGTGGTCGACTTGCCGTTGG	(Roring *et al.* 2002b)

Continued on next page

Continued from previous page: MIRU-VNTR PCR Primer Sequences

MIRU primer	Sequence	Reference
Mtub04-F	CTTGGCCGGCATCAAGCGCATTATT	(Supply *et al.* 2006a)
Mtub04-R	GGCAGCAGAGCCCGGGATTCTTC	(Supply *et al.* 2006a)
Mtub21-F	AGATCCCAGTTGTCGTCGTC	(Supply *et al.* 2006a)
Mtub21-R	CCACATCGCCTGGTTCTGTA	(Supply *et al.* 2006a)
Mtub29-F	GCCAGCCGCCGTGCATAAACCT	(Supply *et al.* 2006a)
Mtub29-R	AGCCACCCGGTGTGCCTTGTATGAC	(Supply *et al.* 2006a)
Mtub30-F	CTTGAAGCCCCGGTCTCATCTGT	(Supply *et al.* 2006a)
Mtub30-R	ACTTGAACCCCCACGCCCATTAGTA	(Supply *et al.* 2006a)
Mtub34-F	GGTGCGCACCTGCTCCAGATAA	(Supply *et al.* 2006a)
Mtub34-R	GGCTCTCATTGCTGGAGGGTTGTAC	(Supply *et al.* 2006a)
Mtub39-F	CGGTGGAGGCGATGAACGTCTTC	(Supply *et al.* 2006a)
Mtub39-R	TAGAGCGGCACGGGGGAAAGCTTAG	(Supply *et al.* 2006a)

Appendix 6: Allelic table for 12 MIRU loci

Number of copies	MIRU 02	MIRU 04	H37rv Miru04	MIRU 10	MIRU 16	MIRU 20	MIRU 23	MIRU 24	MIRU 26	MIRU 27	MIRU 31	MIRU 39	MIRU 40
0	402	175	122	482	565	437	150	395	285	498	492	540	354
1	455	252	199	537	618	514	200	447	336	551	545	593	408
2	508	329	276	590	671	591	253	501	387	604	598	646	462
3	561	406	353	643	724	668	306	555	438	657	651	699	516
4	614	483		696	777	745	359	609	489	710	704	752	570
5	667	560		749	830	822	412	663	540	763	757	805	624
6	720	637		802	883	899	465	717	591	816	810	858	678
7	773	714		855	936	976	518	771	642	869	863	911	732
8	826	791		908	989	1053	571	825	693	922	916	964	786
9	879	868		961	1042	1130	624	879	744	975	969	1017	840
10	932	945		1014	1095	1207	677	933	795	1028	1022	1070	894
11	985	1022		1067	1148	1284	730	987	846	1081	1075	1123	948
12	1038	1099		1120	1201	1361	783	1041	897	1134	1128	1176	1002
13	1091	1176		1173	1254	1438	836	1095	948	1187	1181	1229	1056
14	1144	1253		1226	1307	1515	889	1149	999	1240	1234	1282	1110
15	1197	1330		1279	1360	1592	942	1203	1050	1293	1287	1335	1164
Box = H37 control													

Reference tables courtesy of Thierry Zozio, Pasteur Institute Guadeloupe & Supply *et al* (2006).

Appendix 7: Allelic tables for ETR and VNTR Loci

	Mtub 04	ETR-C	Mtub 21	QUB-11b	ETR-A	Mtub 29	Mtub 30	ETR-B	Mtub 34	Mtub 39	QUB 26	QUB 4156
0	537	171	92	77	195	335	247	347	326	272	187	563
1	588	208	149	146	270	392	305	404	380	330	298	622
2	639	266	206	215	345	449	363	461	434	388	409	681
3	690	324	263	284	420	506	421	518	488	446	520	740
4	741	382	320	353	495	563	479	575	542	504	631	799
5	792	440	377	422	570	620	537	632	596	562	742	858
6	843	498	434	491	645	677	595	689	650	620	853	917
7	894	556	491	560	720	734	653	746	704	678	964	976
8	945	614	548	629	795	791	711	803	758	736	1075	1035
9	996	672	605	698	870	848	769	860	812	794	1186	1094
10	1047	730	662	767	945	905	827	917	866	852	1297	1153
11	1098	788	719	836	1020	962	885	974	920	910	1408	1212
12	1149	846	776	905	1095	1019	943	1031	974	968	1519	1271
13	1200	904	833	974	1170	1076	1001	1088	1028	1026	1630	1330
14	1251	962	890	1043	1245	1133	1059	1145	1082	1084	1741	1389
15	1302	1020	947	1112	1320	1190	1117	1202	1136	1142	1852	1448

Reference tables courtesy of Thierry Zozio, Pasteur Institute Guadeloupe & Supply *et al* (2006).

Appendix 8: Patient Data Collection Forms

Section A: Patient demographic information

1. Study identification number:
2. Admitting Hospital/Clinic:
3. Hospital/Clinic location:
4. Date of birth:
5. Age on admission: (Yrs)
6. Weight on admission: (Kg)
7. Ethnic group: ☐Black ☐White ☐Asian ☐Coloured
8. Gender: ☐Male ☐Female
9. Occupation: ☐ Employed _____
   ☐Unemployed
10. Area/Region residing for the past six months: Regions A-G _____
    Area _____
11. Nationality/Country of origin: _____
12. Has the person worked as a miner: Previously ☐Yes ☐No, when (Yr) _____
    Currently ☐Yes ☐No, where _____
13. Has the person been in prison : Previously ☐Yes ☐No, when (Yr) _____
    Currently ☐Yes ☐No, where _____

Section B: Laboratory Report (results also available from NHLS TB referral lab)

14. Referral Hospital/Clinic:
15. Clinic Code:
16. Region/ Area:
17. Specimen Type:
18. Date received:
19. Date processed:
20. Microscopy result:
21. Culture result:
22. Drug susceptibility test results:

Section C: Treatment History

23. TB contacts: ☐ Yes ☐No ☐ Not available
24. Previous TB treatment: ☐Yes ☐No ☐Not available

25. If Yes:
26. Year of diagnosis with TB: _____
27. the outcome of last treatment course

☐Cure ☐Treatment completed ☐Treatment default ☐Transfer out

Where was patient treated: ☐Hospital ☐Home ☐Other
When (Yr):
Length of treatment: ‹1 mth, 1-2 mths 3-4 mths, 4-6 mths ›6 mths

28. Year of Diagnosis with MDR-TB: _____
29. Localization of disease: ☐Pulmonary ☐Extra-pulmonary ☐Both
30. Management of MDR-TB soon after diagnosis
    ☐ In-patient ☐ Out-patient

31. Previous admission at Sizwe: ☐Yes ☐ No
32. If yes, date & duration of hospitalization:
33. Criteria for hospitalizing patient:

☐defaulting ☐persistency of positive sputum ☐severity of MDR-TB ☐Other

34. Underlying diseases

Diabetes ☐Yes ☐ No ☐Not available
Substance abuse ☐Yes ☐No
Silicosis ☐Yes ☐No
Smoker ☐Yes ☐ No
Asthma ☐Yes ☐ No
Hypertension ☐ Yes ☐ No
Other _____

HIV status: ☐ Reactive ☐ Non-reactive ☐ Not available

Section D: Treatment monitoring

A. Initial phase of treatment

35. Date of starting initial phase:

36. Initial phase treatment:

Drug	Dose	Freq	# of days/wk	duration
Kanamycin/Amikacin				
Pyrazinamide				
Ofloxacin/Ciprofloxacin				
Ethionamide				
Ethambutol				
Terizidone/Cycloserine				
Other				

Monthly smears and culture:

Month	Test	Date of Specimen	Result Negative/ Positive
First	Smear 1		
	Smear 2		
	Culture		
Second	Smear 1		
	Smear 2		
	Culture		
Third	Smear 1		
	Smear 2		
	Culture		
Fourth	Smear 1		
	Smear 2		
	Culture		

Fifth	Smear 1		
	Smear 2		
	Culture		
Sixth	Smear 1		
	Smear 2		
	Culture		

B. Continuation phase

37. Date of starting continuation phase:
38. Drugs used in continuation phase

Drug	Dose	Freq	# of days/wk	duration
Kanamycin/Amikacin				
Pyrazinamide				
Ofloxacin/Ciprofloxacin				
Ethionamide				
Ethambutol				
Terizidone/Cycloserine				
Other				

Monthly smears and culture

Month	Test	Date of Specimen	Result Negative/ Positive
First	Smear 1		
	Smear 2		
	Culture		
Second	Smear 1		
	Smear 2		
	Culture		
Third	Smear 1		
	Smear 2		
	Culture		
Fourth	Smear 1		
	Smear 2		
	Culture		
Fifth	Smear 1		
	Smear 2		
	Culture		
Sixth	Smear 1		
	Smear 2		
	Culture		

Seven	Smear 1		
	Smear 2		
	Culture		
Eight	Smear 1		
	Smear 2		
	Culture		
Nine	Smear 1		
	Smear 2		
	Culture		
Ten	Smear 1		
	Smear 2		
	Culture		
Eleven	Smear 1		
	Smear 2		
	Culture		
Twelve	Smear 1		
	Smear 2		
	Culture		

39. Other treatment monitoring practices: _____
40. Surgical intervention: ☐Yes ☐ No
41. Type of intervention:
    ☐Pneumonectomy ☐ Lobectomy ☐Intercostal drainage
    ☐ Other

Section E: Treatment outcomes

Outcome	Date	Discharged to clinic
Cure (successful)		
Treatment completed (successful)		
Treatment failure (poor)		
Death (poor)		
Treatment default		
Transfer out		

Appendix 9: Publication

INT J TUBERC LUNG DIS 12(1):99–104
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Genotypic diversity of extensively drug-resistant tuberculosis (XDR-TB) in South Africa

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SUMMARY

SETTING: The epidemiology of extensively drug-resistant tuberculosis (XDR-TB), an emerging threat to TB control, is not well understood.

OBJECTIVE: To gain insight into the genotypic population structure of XDR *Mycobacterium tuberculosis* strains in South Africa using a molecular approach and thereby determine whether XDR-TB is mainly acquired or transmitted.

DESIGN: Sputum isolates from patients with multidrug-resistant tuberculosis (MDR-TB) were submitted to the National Referral Laboratory for second-line drug susceptibility testing. The XDR-TB isolates were spoligotyped and these data were compared to the geographic origin of the isolate.

RESULTS: Of the 699 MDR-TB isolates submitted for testing between June 2005 and December 2006, 101 (17%) patients had a culture that was resistant to either ofloxacin or kanamycin, and 41 (6%) were resistant to both drugs (XDR-TB). Spoligotyping of the XDR-TB isolates identified 17 genotypes. As a result of the high genotypic diversity and geographical distribution, we estimate that between 63% and 75% of cases developed XDR-TB through acquisition.

CONCLUSION: Acquisition of extensive drug resistance appears to be the primary mechanism driving the XDR-TB epidemic in South Africa. This urgent TB control issue has to be addressed to prevent the spread of this potentially incurable disease.

KEY WORDS: *Mycobacterium tuberculosis*; MDR-TB; XDR-TB

THE DEVELOPMENT of resistance to anti-tuberculosis drugs is a serious threat to tuberculosis (TB) control, particularly in developing countries where health care resources are limited and the spread of resistant strains therefore more difficult to contain. This is especially true for multidrug-resistant (MDR) TB, which is defined as disease caused by strains of *Mycobacterium tuberculosis* that are resistant in vitro to both rifampicin and isoniazid, with or without resistance to other TB drugs.

South Africa has been identified as one of the high-burden countries for drug-resistant TB, with an estimated annual incidence of >3000 cases.[1] Treatment of MDR-TB is a difficult, lengthy process that can lead to patient non-adherence and the potential for the development of resistance to second-line drugs.[2] Drug resistance can also be acquired through inappropriate treatment, such as monotherapy, an unsuitable combination of drugs, poor quality drugs and malabsorption of the agents. Subsequent to the development of resistance, transmission of strains within the community or hospital/clinic setting is likely to play an important role in spreading the disease.[3–5]

Extensive drug resistance was first noted in the late 1980s and 1990s, and in 2004 the World Health Organization (WHO) and the US Centers for Disease Control and Prevention surveyed selected national reference laboratories to estimate the rates of XDR-TB.[6] It was found that between 2000 and 2004, 20% of the strains tested were MDR-TB and that 10% of these were XDR-TB, using the previous definition of XDR-TB (resistance to three of the six classes of second-line drugs). The data collected showed that rates of XDR-TB varied across the globe, with Asian and East European countries showing the highest rates. However, the true global rate of XDR-TB is unknown, as the methodologies to test for XDR-TB were not standardised and not all countries were represented in this survey. In October 2006, the WHO re-defined XDR-TB as MDR-TB with additional resistance to any fluoroquinolone (FQ) and to at least one of three injectable second-line anti-tuberculosis drugs used in

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treatment (capreomycin [CPM], kanamycin [KAN] or amikacin [AMK]).[7]

In 2006, a study of TB strains from patients at a rural district hospital in KwaZulu-Natal, South Africa, led to the identification of 53 XDR-TB cases, which represented 24% of the MDR-TB cases.[8] This XDR-TB prevalence rate and number of affected patients was of great concern, as was the fact that the median time period from diagnosis to death of XDR-TB patients was only 16 days. The cases in this study were also notable, as XDR-TB was primarily seen in patients with either no history of TB or where TB treatment had been successfully completed, and nosocomial transmission is believed to have occurred. Of the patients tested for human immunodeficiency virus (HIV) status, all were positive. The above report documented a localised event, and the true extent of XDR-TB disease in South Africa therefore remains to be determined.

Since the development of genotyping methods, molecular epidemiological investigations have provided new insights into the disease dynamics of both drug-sensitive and drug-resistant TB.[9] To date, the only published genotypic information on XDR-TB strains comes from an Iranian report of 12 patients, where a clear chain of transmission of Haarlem and East African Indian-3 strains could be shown.[10]

The purpose of the present study was to use a molecular epidemiological approach to gain an insight into the genotypic diversity of XDR-TB *M. tuberculosis* strains in South Africa and thereby to determine whether XDR-TB is mainly acquired (secondary resistance) or transmitted (primary resistance).

MATERIALS AND METHODS

Sampling

In South Africa, the national policy is to perform smear microscopy for TB diagnosis and monitoring of treatment. Mycobacterial culture is only performed for patients at high risk of developing MDR-TB (e.g., retreatment cases). Drug susceptibility testing (DST) for first- and second-line drugs is primarily performed on request by the attending clinician or according to a specific provincial policy.

In this study a convenience sample was used, representing all MDR-TB isolates submitted to the National Health Laboratory Services (NHLS) TB Referral Laboratory, Johannesburg, from June 2005 to December 2006. This laboratory is the primary government facility able to test for second-line resistance in the country. The indirect susceptibility procedure using BACTEC 460/BACTEC 12B medium (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) was used as per the manufacturer's instructions to test for resistance to second-line drugs. Second-line testing was performed using ofloxacin (OFX) (2.0 μg/ml), KAN (5.0 μg/ml) and ethionamide (ETH) (2.5 μg/ml). DST was not performed for all second-line drugs at the NHLS for several reasons: CPM has not been available in South Africa for treatment, AMK may exhibit cross-resistance with the other aminoglycoside agents (such as KAN), and there were no guidelines for testing para-aminosalicylic acid (PAS). Due to the difficulties associated with retrospective data collection in South Africa, the only patient information available was sex, age and clinic/hospital of sample origin.

Ethical clearance for the study was obtained from the University of the Witwatersrand Human Research Ethics Committee.

Spoligotyping

A heat-inactivated aliquot of the BACTEC 460/BACTEC 12B culture from each of the submitted MDR-TB isolates was obtained from the NHLS. Only the first heat-inactivated culture received from each patient was used for genotypic analysis; all follow-up heat-inactivated cultures were excluded from the analysis. Spoligotyping was performed according to the internationally standardised protocol[11] using commercially available reagents from Isogen Bioscience BV (Maarssen, The Netherlands). To assess whether laboratory contamination could have influenced the interpretation of the genotyping data, serial isolates from 13 patients were randomly sampled and spoligotyped. All of these results were concordant.

The SpolDB4 database* was used to assign genotype families to the spoligotypes obtained.[12] Culture material was unavailable for restriction fragment length polymorphism analysis.

RESULTS

During the period June 2005 to December 2006, MDR-TB isolates (n = 845) from 699 patients were submitted from seven of the nine provinces of South Africa for testing for resistance to second-line drugs (Figure 1). Resistance to a single second-line drug was observed in isolates from 146 (21%) patients, while resistance to two or three second-line drugs was observed in isolates from 113 patients (16%) (Figure 2). Forty-one patients (6%) had an isolate that was resistant to KAN and OFX, defining them as XDR-TB (Figure 2). These patients form the study cohort. The average age of the patients with XDR-TB was 37 years (median 36, range 18–54). Sixty-six per cent of these patients were male.

To determine the XDR-TB population structure, the 41 isolates were genotyped by spoligotyping. A total of 17 spoligotypes were identified (Figure 3). Thirty-one of the patients (76%) had an isolate that matched a previously described spoligotype. These spoligotypes represented seven of the internationally recognised genotype families (Figure 3). Of these, the

* http://www.pasteur-guadeloupe.fr/tb/spoldb4



Figure 1 Map of Southern Africa showing the South African provinces in which XDR-TB was found. The number of MDR-TB isolates submitted and the number of XDR-TB patients identified in each province is shown. XDR = extensively drug-resistant; TB = tuberculosis; MDR = multidrug-resistant.

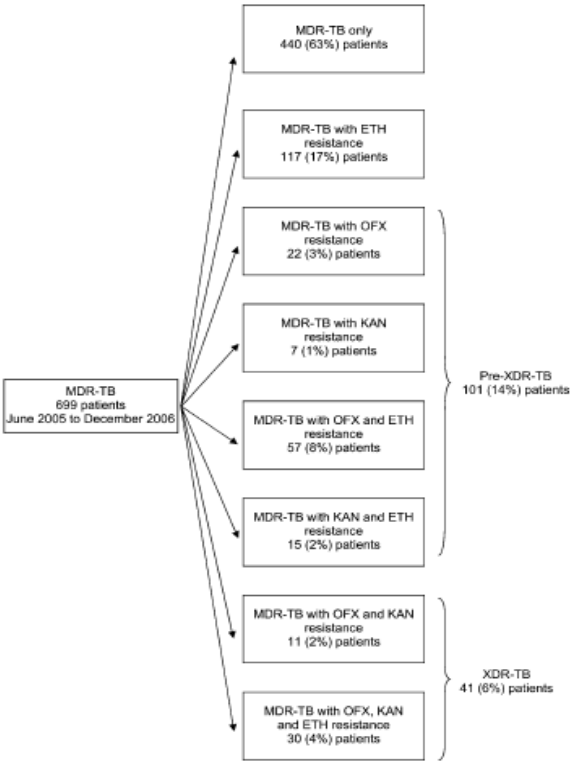


Figure 2 Second-line DST results of the 699 submitted MDR-TB isolates. MDR = multidrug-resistant; TB = tuberculosis; ETH = ethionamide; OFX = ofloxacin; KAN = kanamycin; XDR = extensively drug-resistant; DST = drug susceptibility testing.

Beijing genotype family formed the largest group, with 14 isolates (34%). The remaining isolates were distributed amongst the Latino-American-Mediterranean (LAM) family (LAM4 and LAM9 STs), the East-African-Indian (EAI1) family (EAI1_SOM ST48 and EAI_SOM ST 806), the T family (T1, T2 and T3), the H family (H1 and H3) and the S and X3 families (Figure 3). Ten of the patients (24%) had isolates with spoligotypes that did not match any of the spoligotype patterns deposited in the SpolDB4 database (Figure 3).

To determine whether XDR-TB was primarily transmitted or acquired, the genotype data were compared to the geographic origin of each isolate (Figure 3). The 41 XDR-TB patients were from 16 hospitals/clinics in four provinces. These hospitals/clinics are located in different geographical settings and service patients from their immediate surroundings. It is therefore unlikely that patients attending different hospitals/clinics are epidemiologically linked. Accordingly, 26 of the 41 patients (63%) had a strain that was unique to their geographical setting, suggesting acquisition of extensive drug resistance (Figure 3). Fifteen patients (37%) from five clinics/hospitals showed isolates with clustered spoligotypes, indicative of XDR-TB transmission (Figure 3). However, if it is assumed that that each cluster was initiated by a source case who acquired XDR-TB, then we estimate that 31 patients (75%) acquired XDR-TB within the study cohort.

DISCUSSION

This is the first study to demonstrate that a significant proportion of MDR-TB cases in South Africa have XDR-TB. The calculated proportion of XDR-TB may be an under-estimate, given that this study only analysed a convenience sample of isolates which were largely collected before the description of XDR-TB in South Africa.[8] In the absence of awareness, attending clinicians may not have submitted MDR-TB specimens for routine second-line DST.

This study also raises concerns over the significant proportion of MDR-TB cases (14%) who were resistant to a single marker of XDR-TB (either OFX or KAN). As drug resistance in *M. tuberculosis* is due to the accumulation of mutations in the genome, this pool of 'pre-XDR-TB' strains are at risk of developing extensive resistance if not managed appropriately. The higher proportion of OFX resistance as compared to KAN resistance seen in this study could be due to the widespread use of FQs to treat other ailments (such as diarrhoea and urinary tract infections).[13] In contrast, the clinical use of KAN to treat other infections is limited.

Genotyping of the isolates by spoligotyping showed that a large number of different strains had developed XDR-TB. These XDR-TB strains represented at least seven different genotype families, with the Beijing genotype making up 34% of the isolates. In previous

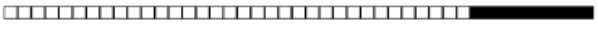
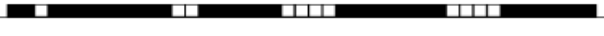
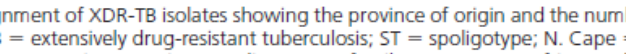
Spoligotype family	Sublineage/ST	*n* (%)	Spoligotype patterns	Province	Isolates *n*	Clinics *n*
Beijing	1	14 (34)		N. Cape	5	4
				N. West	4	3
				Limpopo	3	1
				Gauteng	2	1
LAM	LAM4/60	3 (7)		Gauteng	2	2
				Limpopo	1	1
	LAM9/42	2 (5)		Gauteng	2	2
EAI1	EAI1_SOM/48	2 (5)		N. West	1	1
				Limpopo	1	1
	EAI1_SOM/806	2 (5)		Gauteng	1	1
				N. West	1	1
T	T1/53	2 (5)		Gauteng	1	1
				N. West	1	1
	T2/52	1 (2)		Gauteng	1	1
	T3/37	1 (2)		N. West	1	1
H	H1/47	1 (2)		N. West	1	1
	H3/50	1 (2)		Gauteng	1	1
X	X3/92	1 (2)		N. West	1	1
S	71	1 (2)		N. West	1	1
Not in SpolDB4	Type A, possible H	6 (14)		N. West	6	1
		1 (2)		Gauteng	1	1
		1 (2)		Gauteng	1	1
	Possible LAM	1 (2)		Limpopo	1	1
	Possible LAM	1 (2)		Limpopo	1	1
Total		41			41	

Figure 3 Spoligotype family assignment of XDR-TB isolates showing the province of origin and the number of clinics in which each spoligotype was identified. XDR-TB = extensively drug-resistant tuberculosis; ST = spoligotype; N. Cape = Northern Cape Province; N. West = North West Province; LAM = Latino-American-Mediterranean family; EAI1 = East-African-Indian.

studies, the Beijing genotype has been associated with drug resistance[14] and has been found to be over-represented in children with drug-resistant TB in the Western Cape, South Africa.[15] The prevalence of the Beijing family in non-XDR-TB strains in the study provinces is not known, but data from an urban study group in the Western Cape showed a rate of 17%.[16] It is therefore likely that there is also an over-representation of Beijing genotypes in the XDR-TB population in this study.

Analysis of the spoligotyping data suggest that the majority of XDR-TB cases (63%) in South Africa have developed extensive drug resistance through acquisition, as defined by the presence of a unique spoligotype within a defined geographical setting. This estimate may be as high as 75% if it is assumed that each cluster (within a defined geographical setting) was initiated by a source case who acquired extensive drug resistance.[17] One reason for this high level of acquisition could be the high number of MDR-TB patients who default from treatment in South Africa. A recent study showed that 30% of patients defaulted within the first 6 months of treatment, and even more at later stages.[2] Acquisition of resistance reflects negatively on the success of the National Tuberculosis Programme in the country and is an issue that has to be addressed to prevent the spread of this potentially incurable disease.[18]

This study also raises the concern that XDR-TB may be transmitted between patients in the community and/or hospitals and clinics. A likely instance of transmission was observed where six patients admitted to a hospital in the North West Province presented with XDR-TB with an identical *M. tuberculosis* strain (previously undescribed spoligotype [type A]). Another possible case of transmission in the North West Province was noted where two patients from the same health care facility had isolates with the Beijing spoligotype. All of the XDR-TB isolates analysed from this province originated from gold mining hospitals or health care facilities in mining areas. It therefore appears that transmission may play an important role in this setting, which is consistent with an increased risk of transmission expected in the crowded working and residential conditions typically associated with mines. Identical XDR-TB strains (with identical spoligotypes) were isolated from two or more patients attending the same hospital/clinic in three additional instances. However, we acknowledge that the discriminatory power of spoligotyping may not be able to accurately define transmission in all instances, particularly in the case of strains with the Beijing genotype. In future studies, it will be important to use high resolution genotyping methods (IS*6110* DNA fingerprinting or mycobacterial interspersed repetitive unit typing) to accurately quantify the contribution of transmission to the XDR-TB epidemic.

The first MDR-TB case in South Africa was recorded in 1985.[19] Since then the number of MDR-TB cases in the country has steadily increased.[1] A similar scenario with XDR-TB should be avoided, as 16.2% of South Africans between the ages of 15 and 49 are estimated to be HIV-positive,[20] and the twin epidemics of HIV and TB makes the emergence of XDR-TB potentially disastrous for both local and international TB control. Improved infection control is essential to contain the spread of resistant disease, as patients with MDR-TB/XDR-TB are often hospitalised for extended periods and come into contact with other patients and

health care workers. As seen in KwaZulu Natal, this can lead to nosocomial infections.[8] Undiagnosed patients with resistant TB also pose a risk in the community. Rapid diagnostics, standardised and accurate DST, molecular analysis of strains for epidemiological studies and improved treatment of all forms of TB are critical elements in battling this new form of the epidemic.

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References

1 World Health Organization, International Union Against Tuberculosis and Lung Disease. Anti-tuberculosis drug resistance in the world. Geneva, Switzerland: WHO, 2004. http://www.who.int/tb/publications/who_htm_tb_2004_343/en/ Accessed August 2007.
2 Holtz T H, Lancaster J, Laserson K F, et al. Risk factors associated with default from multidrug-resistant tuberculosis treatment, South Africa, 1999–2001. Int J Tuberc Lung Dis 2006; 10: 649–655.
3 Bifani P J, Mathema B, Liu Z, et al. Identification of a W variant outbreak of *Mycobacterium tuberculosis* via population-based molecular epidemiology. JAMA 1999; 282: 2321–2327.
4 van Rie A, Warren R M, Beyers N, et al. Transmission of a multidrug-resistant *Mycobacterium tuberculosis* strain resembling 'strain W' among noninstitutionalized, human immunodeficiency virus-seronegative patients. J Infect Dis 1999; 180: 1608–1615.
5 Toungoussova O S, Mariandyshev A, Bjune G, et al. Molecular epidemiology and drug resistance of *Mycobacterium tuberculosis* isolates in the Archangel prison in Russia: predominance of the W-Beijing clone family. Clin Infect Dis 2003; 37: 665–672.
6 Centers for Disease Control and Prevention. Emergence of *Mycobacterium tuberculosis* with extensive resistance to second-line drugs—worldwide, 2000–2004. MMWR 2006; 55: 301–305.
7 World Health Organization. WHO media report. WHO Global Task Force outlines measures to combat XDR-TB worldwide. Geneva, Switzerland: WHO, 2006. http://www.who.int/mediacentre/news/notes/2006/np29/en/index.html Accessed August 2007.
8 Gandhi N R, Moll A, Sturm A W, et al. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet 2006; 368: 1575–1580.
9 Mathema B, Kurepina N E, Bifani P J, et al. Molecular epidemiology of tuberculosis: current insights. Clin Microbiol Rev 2006; 19: 658–685.
10 Masjedi M R, Farnia P, Sorooch S, et al. Extensively drug-resistant tuberculosis: 2 years of surveillance in Iran. Clin Infect Dis 2006; 43: 841–847.
11 Kamerbeek J, Schouls L, Kolk A, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 1997; 35: 907–914.
12 Brudey K, Driscoll J R, Rigouts L, et al. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol 2006; 6: 23.
13 Grimaldo E R, Tupasi T E, Rivera A B, et al. Increased resistance to ciprofloxacin and ofloxacin in multidrug-resistant mycobacterium tuberculosis isolates from patients seen at a tertiary hospital in the Philippines. Int J Tuberc Lung Dis 2001; 5: 546–550.
14 Centers for Disease Control and Prevention. European concerted action on new generation genetic markers and techniques for the epidemiology and control of tuberculosis. Beijing/W genotype *Mycobacterium tuberculosis* and drug resistance. Emerg Infect Dis 2006; 12: 736–743.
15 Marais B J, Victor T C, Hesseling A C, et al. Beijing and Haarlem genotypes are overrepresented among children with drug-resistant tuberculosis in the Western Cape Province of South Africa. J Clin Microbiol 2006; 44: 3539–3543.
16 Richardson M, van Lill S W, van der Spuy G D, et al. Historic and recent events contribute to the disease dynamics of Beijing-like *Mycobacterium tuberculosis* isolates in a high incidence region. Int J Tuberc Lung Dis 2002; 6: 1001–1011.
17 Small P M, Hopewell P C, Singh S P, et al. The epidemiology of tuberculosis in San Francisco. A population-based study using conventional and molecular methods. N Engl J Med 1994; 330: 1703–1709.
18 van Rie A, Enarson D. XDR tuberculosis: an indicator of public-health negligence. Lancet 2006; 368: 1554–1556.
19 Weyer K, Groenewald P, Zwarenstein M, et al. Tuberculosis drug resistance in the Western Cape. S Afr Med J 1995; 85: 499–504.
20 Shisana O, Rehle T, Simbayi L C, et al. South African National HIV Prevalence, HIV Incidence, Behaviour and Communication Survey, 2005. Cape Town, South Africa: Human Sciences Research Council Press, 2005.

RÉSUMÉ

CONTEXTE : On ne comprend pas bien jusqu'ici l'épidémiologie des tuberculoses à résistance élargie aux médicaments (TB-XDR), une menace émergente pour la lutte antituberculeuse.

OBJECTIF : Utilisant une approche moléculaire, chercher à obtenir des données sur la structure génotypique des populations des souches de *Mycobacterium tuberculosis* XDR en Afrique du Sud et déterminer ainsi si la TB-XDR est le plus souvent acquise au cours du traitement ou le résultat d'une transmission.

SCHÉMA : Les isolats de crachats provenant de patients atteints de tuberculose à germes multirésistants (TB-MDR) ont été envoyés au Laboratoire National de Référence pour des tests de sensibilité aux médicaments de deuxième ligne. On a pratiqué le spoligotypage des isolats de TB-XDR et ces données ont été comparées en fonction de l'origine géographique de l'isolat.

RÉSULTATS : Sur les 699 isolats de TB-MDR soumis au test entre juin 2005 et décembre 2006, la culture des isolats de 101 patients (17%) s'est avérée résistante soit à l'ofloxacine soit à la kanamycine, et s'est avérée résistante aux deux médicaments (TB-XDR) chez 41 patients (6%). Le spoligotypage des isolats TB-XDR a identifié 17 génotypes. Par suite de la grande diversité génotypique et de la grande dispersion géographique, nous estimons que le pourcentage de cas ayant développé une TB-XDR

sous forme de maladie acquise en cours de traitement va de 63% à 75%.
CONCLUSION : Le développement d'une résistance médicamenteuse élargie en cours de traitement semble être le mécanisme principal qui sous-tend l'épidémie de TB-XDR en Afrique du Sud. Ce problème urgent de lutte antituberculeuse doit être pris en compte afin de prévenir la diffusion de cette maladie potentiellement incurable.

RESUMEN

MARCO DE REFERENCIA : No se conocen a cabalidad las características epidemiológicas de la tuberculosis (TB) extremadamente drogorresistente (XDR), un reto de aparición reciente en el control de la TB.
OBJETIVO : Aplicando un método molecular, investigar la estructura genotípica de una población de cepas XDR de *Mycobacterium tuberculosis* en Sudáfrica y determinar con ello, si este tipo de TB es principalmente adquirida o transmitida.
MÉTODO : Los aislados provenientes de muestras de esputo de pacientes con TB multidrogorresistente (MDR) se sometieron a pruebas de sensibilidad a medicamentos antituberculosos de segunda línea en el Laboratorio Nacional de Referencia. Se practicó la tipificación con oligonucleótidos que reconocen las secuencias espaciadoras (spoligotyping) y estos datos se pusieron en relación con el origen geográfico del aislado.
RESULTADOS : De los 699 aislados de TB-MDR recibidos entre junio de 2005 y diciembre de 2006, 101 (17%) presentaron un cultivo resistente a ofloxacino o a kanamicina y 41 (6 %) fueron resistentes a ambos medicamentos (TB-XDR). Con la genotipificación de los aislados XDR se definieron 17 genotipos. Considerando la gran diversidad genotípica y la distribución geográfica, se estima que entre 63% y 75% de los casos de TB-XDR fueron adquiridos.
CONCLUSIÓN : La adquisición de drogorresistencia extensa pareciera el mecanismo primario de diseminación de la epidemia de TB-XDR en Sudáfrica. Es preciso considerar este aspecto urgente del control de la TB, con el fin de prevenir la diseminación de esta enfermedad potencialmente incurable.

REFERENCES

Abadia, E., Sequera, M., Ortega, D., Mendez, M.V., Escalona, A., Da Mata, O., Izarra, E., Rojas, Y., Jaspe, R., Motiwala, A.S., Alland, D., de Waard, J. & Takiff, H.E. (2009). *Mycobacterium tuberculosis* ecology in Venezuela: epidemiologic correlates of common spoligotypes and a large clonal cluster defined by MIRU-VNTR-24. *BMC Infectious Diseases,* **9:** 122.

Abdool Karim, S.S., Churchyard, G.J., Abdool Karim, Q. & Lawn, S.D. (2009). HIV infection and tuberculosis in South Africa: an urgent need to escalate the public health response. *Lancet,* **374:** 921-933.

Abebe, F. & Bjune, G. (2006). The emergence of Beijing family genotypes of *Mycobacterium tuberculosis* and low-level protection by bacille Calmette-Guerin (BCG) vaccines: is there a link?. *Clinical and Experimental Immunology,* **3:** 389-397.

Aktas, E., Zozio, T., Comert, F.B., Kulah, C., Aydin, O., Rastogi, N. & Sola, C. (2008). A first insight into the genetic diversity and population structure of *Mycobacterium tuberculosis* in Zonguldak, Turkey. *Clinical Microbiology and Infection,* **1:** 55-59.

Alangaden, G.J., Kreiswirth, B.N., Aouad, A., Khetarpal, M., Igno, F.R., Moghazeh, S.L., Manavathu, E.K. & Lerner, S.A. (1998). Mechanism of resistance to amikacin and kanamycin in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy,* **5:** 1295-1297.

Al-Hajoj, S.A., Zozio, T., Al-Rabiah, F., Mohammad, V., Al-Nasser, M., Sola, C. & Rastogi, N. (2007). First insight into the population structure of *Mycobacterium tuberculosis* in Saudi Arabia. *Journal of Clinical Microbiology,* **8:** 2467-2473.

Ali, A., Hasan, Z., Tanveer, M., Siddiqui, A.R., Ghebremichael, S., Kallenius, G. & Hasan, R. (2007). Characterization of *Mycobacterium tuberculosis* Central Asian Strain 1 using mycobacterial interspersed repetitive unit genotyping. *BMC Microbiology*, **7:** 76.

Allix, C., Supply, P. & Fauville-Dufaux, M. (2004). Utility of fast mycobacterial interspersed repetitive unit-variable number tandem repeat genotyping in clinical mycobacteriological analysis. *Clinical Infectious Diseases,* **39:** 783-789.

Allix-Beguec, C., Fauville-Dufaux, M. & Supply, P. (2008). Three-year population-based evaluation of standardized mycobacterial interspersed repetitive-unit-variable-number tandem-repeat typing of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology,* **46:** 1398-1406.

Allix-Beguec, C., Harmsen, D., Weniger, T., Supply, P. & Niemann, S. (2008). Evaluation and strategy for use of MIRU-VNTRplus, a multifunctional database for online analysis of genotyping data and phylogenetic identification of *Mycobacterium tuberculosis* complex isolates. *Journal of Clinical Microbiology,* **46:** 2692-2699.

Almeida, D., Rodrigues, C., Ashavaid, T.F., Lalvani, A., Udwadia, Z.F. & Mehta, A. (2005). High incidence of the Beijing genotype among multidrug-resistant isolates of *Mycobacterium tuberculosis* in a tertiary care center in Mumbai, India. *Clinical Infectious Diseases,* **40:** 881-886.

Alonso-Rodriguez, N., Martinez-Lirola, M., Herranz, M., Sanchez-Benitez, M., Barroso, P., INDAL-TB group, Bouza, E. & Garcia de Viedma, D. (2008). Evaluation of the new advanced 15-loci MIRU-VNTR genotyping tool in *Mycobacterium tuberculosis* molecular epidemiology studies. *BMC Microbiology,* **8:** 34.

Alonso-Rodriguez, N., Martinez-Lirola, M., Sanchez, M.L., Herranz, M., Penafiel, T., Bonillo Mdel, C., Gonzalez-Rivera, M., Martinez, J., Cabezas, T., Diez-Garcia, L.F., Bouza, E. & Garcia de Viedma, D. (2009). Prospective universal application of mycobacterial interspersed repetitive-unit-variable-number tandem-repeat genotyping to characterize *Mycobacterium tuberculosis* isolates for fast identification of clustered and orphan cases. *Journal of Clinical Microbiology,* **47:** 2026-2032.

Andrews, J.R., Gandhi, N.R., Moodley, P., Shah, N.S., Bohlken, L., Moll, A.P., Pillay, M., Friedland, G., Sturm, A.W. & Tugela Ferry Care and Research Collaboration. (2008). Exogenous reinfection as a cause of multidrug-resistant and extensively drug-resistant tuberculosis in rural South Africa. *The Journal of Infectious Diseases,* **198:** 1582-1589.

Aranaz, A., Cousins, D., Mateos, A. & Dominguez, L. (2003.) Elevation of *Mycobacterium tuberculosis subsp. caprae* to species rank as *Mycobacterium caprae* comb. nov., sp. nov. *International Journal of Systematic and Evolutionary Microbiology,* **53:** 1785-1789.

Arias, M., Mello, F.C., Pavon, A., Marsico, A.G., Alvarado-Galvez, C., Rosales, S., Pessoa, C.L., Perez, M., Andrade, M.K., Kritski, A.L., Fonseca, L.S., Chaisson, R.E., Kimerling, M.E. & Dorman, S.E. (2007). Clinical evaluation of the microscopic-observation drug-susceptibility assay for detection of tuberculosis. *Clinical Infectious Diseases,* **44:** 674-680.

Asiimwe, B.B., Ghebremichael, S., Kallenius, G., Koivula, T. & Joloba, M.L. (2008). *Mycobacterium tuberculosis* spoligotypes and drug susceptibility pattern of isolates from tuberculosis patients in peri-urban Kampala, Uganda. *BMC Infectious Diseases,* **8:** 101.

Baboolal, S., Millet, J., Akpaka, P.E., Ramoutar, D. & Rastogi, N. (2009). First insight into *Mycobacterium tuberculosis* epidemiology and genetic diversity in Trinidad and Tobago. *Journal of Clinical Microbiology,* **47:** 1911-1914.

Bandera, A., Gori, A., Catozzi, L., Degli Esposti, A., Marchetti, G., Molteni, C., Ferrario, G., Codecasa, L., Penati, V., Matteelli, A. & Franzetti, F. (2001). Molecular epidemiology study of exogenous reinfection in an area with a low incidence of tuberculosis. *Journal of Clinical Microbiology,* **39:** 2213-2218.

Bang, D., Lillebaek, T., Thomsen, V.O. & Andersen, A.B. (2010). Multidrug-resistant tuberculosis: treatment outcome in Denmark, 1992-2007. *Scandinavian Journal of Infectious Diseases,* **42:** 288-293.

Barnard, M., Albert, H., Coetzee, G., O'Brien, R. & Bosman, M.E. (2008). Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa. *American Journal of Respiratory and Critical Care,* **177:** 787-792.

Bauer, J., Andersen, A.B., Kremer, K. & Miorner, H. (1999). Usefulness of spoligotyping to discriminate IS*6110* low-copy-number *Mycobacterium tuberculosis* complex strains cultured in Denmark. *Journal of Clinical Microbiology,* **37:** 2602-2606.

Beggs, M.L., Eisenach, K.D. & Cave, M.D. (2000). Mapping of IS*6110* insertion sites in two epidemic strains of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology,* **38:** 2923-2928.

Ben Amor, Y., Nemser, B., Singh, A., Sankin, A. & Schluger, N. (2008). Underreported threat of multidrug-resistant tuberculosis in Africa, *Emerging Infectious Diseases,* **14:** 1345-1352.

Bhanu, N.V., van Soolingen, D., van Embden, J.D., Dar, L., Pandey, R.M. & Seth, P. (2002). Predominace of a novel *Mycobacterium tuberculosis* genotype in the Delhi region of India. *Tuberculosis,* **82:** 105-112.

Bifani, P.J., Mathema, B., Kurepina, N.E. & Kreiswirth, B.N. (2002). Global dissemination of the *Mycobacterium tuberculosis* W-Beijing family strains. *Trends in Microbiology,* **10:** 45-52.

Bifani, P.J., Mathema, B., Liu, Z., Moghazeh, S.L., Shopsin, B., Tempalski, B., Driscol, J., Frothingham, R., Musser, J.M., Alcabes, P. & Kreiswirth, B.N. (1999). Identification of a W variant outbreak of *Mycobacterium tuberculosis* via population-based molecular epidemiology. *Journal of the American Medical Association,* **282:** 2321-2327.

Bifani, P.J., Plikaytis, B.B., Kapur, V., Stockbauer, K., Pan, X., Lutfey, M.L., Moghazeh, S.L., Eisner, W., Daniel, T.M., Kaplan, M.H., Crawford, J.T., Musser, J.M. & Kreiswirth, B.N. (1996). Origin and interstate spread of a New York City multidrug-resistant *Mycobacterium tuberculosis* clone family. *Journal of the American Medical Association,* **275:** 452-457.

Bock, N.N., Jensen, P.A., Miller, B. & Nardell, E. (2007).Tuberculosis infection control in resource-limited settings in the era of expanding HIV care and treatment. *The Journal of Infectious Diseases,* **196:** S108-S113.

Braden, C.R., Morlock, G.P., Woodley, C.L., Johnson, K.R., Colombel, A.C., Cave, M.D., Yang, Z., Valway, S.E., Onorato, I.M. & Crawford, J.T. (2001). Simultaneous infection with multiple strains of *Mycobacterium tuberculosis*. *Clinical Infectious Diseases,* **33:** 42-47.

Braden, C.R., Templeton, G.L., Cave, M.D., Valway, S., Onorato, I.M., Castro, K.G., Moers, D., Yang, Z., Stead, W.W. & Bates, J.H. (1997). Interpretation of restriction fragment length polymorphism analysis of *Mycobacterium tuberculosis* isolates from a state with a large rural population. *The Journal of Infectious Diseases,* **175:** 1446-1452.

Brennan, P.J. & Nikaido, H. (1995). The envelope of mycobacteria. *Annual Review of Biochemistry,* **64:** 29-63.

Brudey, K., Driscoll, J.R., Rigouts, L., Prodinger, W.M., Gori, A., Al-Hajoj, S.A., Allix, C., Aristimuno, L., Arora, J., Baumanis, V., Binder, L., Cafrune, P., Cataldi, A., Cheong, S., Diel, R., Ellermeier, C., Evans, J.T., Fauville-Dufaux, M., Ferdinand, S., Garcia de Viedma, D., Garzelli, C., Gazzola, L., Gomes, H.M., Guttierez, M.C., Hawkey, P.M., van Helden, P.D., Kadival, G.V., Kreiswirth, B.N., Kremer, K., Kubin, M., Kulkarni, S.P., Liens, B., Lillebaek, T., Ho, M.L., Martin, C., Martin, C., Mokrousov, I., Narvskaia, O., Ngeow, Y.F., Naumann, L., Niemann, S., Parwati, I., Rahim, Z., Rasolofo-Razanamparany, V., Rasolonavalona, T., Rossetti, M.L., Rusch-Gerdes, S., Sajduda, A., Samper, S., Shemyakin, I.G., Singh, U.B., Somoskovi, A., Skuce, R.A., van Soolingen, D., Streicher, E.M., Suffys, P.N., Tortoli, E., Tracevska, T., Vincent, V., Victor, T.C., Warren, R.M., Yap, S.F., Zaman, K., Portaels, F., Rastogi, N. & Sola, C. (2006). *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. *BMC Microbiology,* **6:** 23.

Brust, J.C., Gandhi, N.R., Carrara, H., Osburn, G. & Padayatchi, N. (2010). High treatment failure and default rates for patients with multidrug-resistant tuberculosis in KwaZulu-Natal, South Africa, 2000-2003. *International Journal of Tuberculosis and Lung Disease,* **14:** 413-419.

Calver, A.D., Falmer, A.A., Murray, M., Strauss, O.J., Streicher, E.M., Hanekom, M., Liversage, T., Masibi, M., van Helden, P.D., Warren, R.M. & Victor, T.C. (2010). Emergence of increased resistance and extensively drug-resistant tuberculosis despite treatment adherence, South Africa. *Emerging Infectious Diseases,* **16:** 264-271.

Caminero, J.A. (2010). Multidrug-resistant tuberculosis: epidemiology, risk factors and case finding. *International Journal of Tuberculosis and Lung Disease.* **14:** 382-390.

Candia, N., Lopez, B., Zozio, T., Carrivale, M., Diaz, C., Russomando, G., de Romero, N.J., Jara, J.C., Barrera, L., Rastogi, N. & Ritacco, V. (2007). First insight into *Mycobacterium tuberculosis* genetic diversity in Paraguay. *BMC Microbiology,* **7:** 75

Cardoso Leao, S. & Portaels, F. (2007). *History in Tuberculosis, From Basic Science to Patient Care.*, eds. J.C. Palomino, S. Cardoso Leao & V. Ritacco. (book on line). www.tuberculosistextbook.com, 25-48. Accessed December 2009.

Caviedes, L., Lee, T.S., Gilman, R.H., Sheen, P., Spellman, E., Lee, E.H., Berg, D.E. & Montenegro-James, S. (2000). Rapid, efficient detection and drug susceptibility testing of *Mycobacterium tuberculosis* in sputum by microscopic observation of broth cultures. The Tuberculosis Working Group in Peru. *Journal of Clinical Microbiology,* **38:** 1203-1208.

Centers for Disease Control (CDC) .(1991). Nosocomial transmission of multidrug-resistant tuberculosis among HIV-infected persons- Florida and New York, 1988-1991. *Morbidity and Mortality Weekly Report,* **40:** 585-591.

Centers for Disease Control and Prevention (CDC). (2006). Emergence of *Mycobacterium tuberculosis* with extensive resistance to second-line drugs-worldwide, 2000-2004. *Morbidity and Mortality Weekly Report,* **55:** 301-305.

Chan, E.D. & Iseman, M.D. (2008). Multidrug-resistant and extensively drug-resistant tuberculosis: a review. *Current Opinion in Infectious Diseases,* **21:** 587-595.

Chan, E.D., Laurel, V., Strand, M.J., Chan, J.F., Huynh, M.L., Goble, M. & Iseman, M.D. (2004). Treatment and outcome analysis of 205 patients with multidrug-resistant tuberculosis. *American Journal of Respiratory and Critical Care,* **169:** 1103-1109.

Charalambous, S., Grant, A.D., Moloi, V., Warren, R., Day, J.H., van Helden, P., Hayes, R.J., Fielding, K.L., De Cock, K.M., Chaisson, R.E. & Churchyard, G.J. (2008). Contribution of reinfection to recurrent tuberculosis in South African gold miners. *International Journal of Tuberculosis and Lung Disease,* **12:** 942-948.

Chiang, C.Y., Enarson, D.A., Yu, M.C., Bai, K.J., Huang, R.M., Hsu, C.J., Suo, J. & Lin, T.P. (2006). Outcome of pulmonary multidrug-resistant tuberculosis: a 6-yr follow-up study. *European Respiratory Journal,* **28:** 980-985.

Chihota, V., Apers, L., Mungofa, S., Kasongo, W., Nyoni, I.M., Tembwe, R., Mbulo, G., Tembo, M., Streicher, E.M., van der Spuy, G.D., Victor, T.C., van Helden, P. & Warren, R.M. (2007). Predominance of a single genotype of *Mycobacterium tuberculosis* in regions of Southern Africa. *International Journal of Tuberculosis and Lung Disease,* **11:** 311-318.

Chuang, P.C., Liu, H., Sola, C., Chen, Y.M. & Jou, R. (2008). Spoligotypes of *Mycobacterium tuberculosis* isolates of a high tuberculosis burden aboriginal township in Taiwan. *Infection, Genetics and Evolution,* **8:** 553-557.

Ciglenecki, I., Glynn, J.R., Mwinga, A., Ngwira, B., Zumla, A., Fine, P.E. & Nunn, A. (2007). Population differences in death rates in HIV-positive patients with tuberculosis. *International Journal of Tuberculosis and Lung Disease,* **11:**1121-1128.

Cohn, D.L. & O'Brien, R.J. (1998). The use of restriction fragment length polymorphism (RFLP) analysis for epidemiological studies of tuberculosis in developing countries. *International Journal of Tuberculosis and Lung Disease,* **2:** 16-26.

Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E.,3rd, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M.A., Rajandream, M.A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J.E., Taylor, K., Whitehead, S. & Barrell, B.G. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature,* **393**: 537-544.

Comstock, G.W. (1982). Epidemiology of tuberculosis. *The American Review of Respiratory Disease,* **125:** 8-15.

Corbett, E.L., Watt, C.J., Walker, N., Maher, D., Williams, B.G., Raviglione, M.C. & Dye, C. (2003). The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. *Archives of Internal Medicine*, **163:** 1009-1021.

Cousins, D.V., Bastida, R., Cataldi, A., Quse, V., Redrobe, S., Dow, S., Duignan, P., Murray, A., Dupont, C., Ahmed, N., Collins, D.M., Butler, W.R., Dawson, D., Rodriguez, D., Loureiro, J., Romano, M.I., Alito, A., Zumarraga, M. & Bernardelli, A. (2003). Tuberculosis in seals caused by a novel member of the *Mycobacterium tuberculosis* complex: *Mycobacterium pinnipedii* sp. nov. *International Journal of Systematic and Evolutionary Microbiology,* **53:** 1305-1314.

Cowan, L.S., Diem, L., Monson, T., Wand, P., Temporado, D., Oemig, T.V. & Crawford, J.T. (2005). Evaluation of a two-step approach for large-scale, prospective genotyping of *Mycobacterium tuberculosis* isolates in the United States. *Journal of Clinical Microbiology*, **43:** 688-695.

Cowan, L.S., Mosher, L., Diem, L., Massey, J.P. & Crawford, J.T. (2002). Variable-number tandem repeat typing of *Mycobacterium tuberculosis* isolates with low copy numbers of IS*6110* by using mycobacterial interspersed repetitive units. *Journal of Clinical Microbiology,* **40:** 1592-1602.

Cowie, R.L. (1995). Silicotuberculosis: long-term outcome after short-course chemotherapy, *Tubercle and Lung Disease,* **76:** 39-42.

Cowie, R.L. (1994). The epidemiology of tuberculosis in gold miners with silicosis. *American Journal of Respiratory and Critical Care Medicine*, **150:** 1460-1462.

Cox, H.S., Kalon, S., Allamuratova, S., Sizaire, V., Tigay, Z.N., Rusch-Gerdes, S., Karimovich, H.A., Kebede, Y. & Mills, C. (2007). Multidrug-resistant tuberculosis treatment outcomes in Karakalpakstan, Uzbekistan: treatment complexity and XDR-TB among treatment failures. *PloS One,* **2:** e1126.

Cox, H.S., Kubica, T., Doshetov, D., Kebede, Y., Rusch-Gerdess, S. & Niemann, S. (2005). The Beijing genotype and drug-resistant tuberculosis in the Aral Sea region of Central Asia. *Respiratory Research,* **6:** 134.

Cox, H.S., Sibilia, K., Feuerriegel, S., Kalon, S., Polonsky, J., Khamraev, A.K., Rusch-Gerdes, S., Mills, C. & Niemann, S. (2008). Emergence of extensive drug resistance during treatment for multidrug-resistant tuberculosis. *The New England Journal of Medicine,* **359:** 2398-2400.

Cruciani, F., Santolamazza, P., Shen, P., Macaulay, V., Moral, P., Olckers, A., Modiano, D., Holmes, S., Destro-Bisol, G., Coia, V., Wallace, D.C., Oefner, P.J., Torroni, A., Cavalli-Sforza, L.L., Scozzari, R. & Underhill, P.A. (2002). A back migration from Asia to sub-Saharan Africa is supported by high-resolution analysis of human Y-chromosome haplotypes. *American Journal of Human Genetics,* **70:** 1197-1214.

Dale, J.W., Brittain, D., Cataldi, A.A., Cousins, D., Crawford, J.T., Driscoll, J., Heersma, H., Lillebaek, T., Quitugua, T., Rastogi, N., Skuce, R.A., Sola, C., Van Soolingen, D. & Vincent, V. (2001). Spacer oligonucleotide typing of bacteria of the *Mycobacterium tuberculosis* complex: recommendations for standardised nomenclature. *International Journal of Tuberculosis and Lung Disease,* **5:** 216-219.

Daley, C.L., Small, P.M., Schecter, G.F., Schoolnik, G.K., McAdam, R.A., Jacobs, W.R.,Jr & Hopewell, P.C. (1992). An outbreak of tuberculosis with accelerated progression among persons infected with the human immunodeficiency virus. An analysis using restriction-fragment-length polymorphisms. *The New England Journal of Medicine,* **326:** 231-235.

Daniel, T.M. (2006). The history of tuberculosis. *Respiratory Medicine,* **100:** 1862-1870.

Dannenberg, A.M.,Jr. (1989). Immune mechanisms in the pathogenesis of pulmonary tuberculosis. *Reviews of Infectious Diseases,* S369-S378.

Davies, G.R., Pillay, M., Sturm, A.W. & Wilkinson, D. (1999). Emergence of multidrug-resistant tuberculosis in a community-based directly observed treatment programme in rural South Africa. *International Journal of Tuberculosis and Lung Disease,* **3:** 799-804.

de Boer, A.S., Borgdorff, M.W., de Haas, P.E., Nagelkerke, N.J., van Embden, J.D. & van Soolingen, D. (1999). Analysis of rate of change of IS*6110* RFLP patterns of *Mycobacterium tuberculosis* based on serial patient isolates. *The Journal of Infectious Diseases,* **180**: 1238-1244.

De Cock, K.M., Soro, B., Coulibaly, I.M. & Lucas, S.B. (1992).Tuberculosis and HIV infection in sub-Saharan Africa. *Journal of the American Medical Association,* **268:** 1581-1587.

Dean, G.L., Edwards, S.G., Ives, N.J., Matthews, G., Fox, E.F., Navaratne, L., Fisher, M., Taylor, G.P., Miller, R., Taylor, C.B., de Ruiter, A. & Pozniak, A.L. (2002). Treatment of tuberculosis in HIV-infected persons in the era of highly active antiretroviral therapy. *AIDS,* **16:** 75-83.

Dheda, K., Lampe, F.C., Johnson, M.A. & Lipman, M.C. (2004). Outcome of HIV-associated tuberculosis in the era of highly active antiretroviral therapy. *The Journal of Infectious Diseases,* **190:** 1670-1676.

Dheda, K., Shean, K., Zumla, A., Badri, M., Streicher E.M., Page-Shipp, L., Willcox, P., John, MA., Reubenson, G., Govindasamy, D., Wong, D., Padanilam, X., Dziwiecki, A., van Helden, P.D., Siwendu, S., Jarand, J., Menezes, C.N., Burns, A., Victor, T., Warren, R., Grobusch, M.P., van der Walt, M & Kvasnovsky, C. (2010). Early treatment outcomes and HIV status of patients with extensively drug-resistant tuberculosis in South Africa: a retrospective cohort study. *Lancet*, **22:** 1798-1807.

Donoghue, H.D., Spigelman, M., Greenblatt, C.L., Lev-Maor, G., Bar-Gal, G.K., Matheson, C., Vernon, K., Nerlich, A.G. & Zink, A.R. (2004). Tuberculosis: from prehistory to Robert Koch, as revealed by ancient DNA. *The Lancet Infectious Diseases,* **4:** 584-592.

Dou, H.Y., Lu, J.J., Lin, C.W., Chang, J.R., Sun, J.R. & Su, I.J. (2009). Utility and evaluation of new variable-number tandem-repeat systems for genotyping mycobacterial tuberculosis isolates. *Journal of Microbiological Methods,* **77:** 127-129.

Dou, H.Y., Tseng, F.C., Lin, C.W., Chang, J.R., Sun, J.R., Tsai, W.S., Lee, S.Y., Su, I.J. & Lu, J.J. (2008a). Molecular epidemiology and evolutionary genetics of *Mycobacterium tuberculosis* in Taipei. *BMC Infectious Diseases,* **8:**170.

Dou, H.Y., Tseng, F.C., Lu, J.J., Jou, R., Tsai, S.F., Chang, J.R., Lin, C.W., Miu, W.C. & Su, I.J. (2008b). Associations of *Mycobacterium tuberculosis* genotypes with different ethnic and migratory populations in Taiwan. *Infection. Genetics and Evolution,* **8:** 323-330.

Douglas, J.T., Qian, L., Montoya, J.C., Musser, J.M., Van Embden, J.D., van Soolingen, D. & Kremer, K. (2003). Characterization of the Manila family of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology,* **41:** 2723-2726.

Ducati, R.G., Ruffino-Netto, A., Basso, L.A. & Santos, D.S. (2006). The resumption of consumption - a review on tuberculosis. *Memorias Instituto Oswaldo Cruz,* **101:** 697-714.

Durmaz, R., Zozio, T., Gunal, S., Allix, C., Fauville-Dufaux, M. & Rastogi, N. (2007). Population-based molecular epidemiological study of tuberculosis in Malatya, Turkey. *Journal of Clinical Microbiology,* **45:** 4027-4035.

Dye, C. & Williams, B.G. (2000). Criteria for the control of drug-resistant tuberculosis. *Proceedings of the National Academy of Sciences of the United States of America,* **97:** 8180-8185.

Easterbrook, P.J., Gibson, A., Murad, S., Lamprecht, D., Ives, N., Ferguson, A., Lowe, O., Mason, P., Ndudzo, A., Taziwa, A., Makombe, R., Mbengeranwa, L., Sola, C., Rastogi, N. & Drobniewski, F. (2004). High rates of clustering of strains causing tuberculosis in Harare, Zimbabwe: a molecular epidemiological study. *Journal of Clinical Microbiology,* **42:** 4536-4544.

Ebrahimi-Rad, M., Bifani, P., Martin, C., Kremer, K., Samper, S., Rauzier, J., Kreiswirth, B., Blazquez, J., Jouan, M., van Soolingen, D. & Gicquel, B. (2003). Mutations in putative mutator genes of *Mycobacterium tuberculosis* strains of the W-Beijing family. *Emerging Infectious Diseases,* **9:** 838-845.

Edlin, B.R., Tokars, J.I., Grieco, M.H., Crawford, J.T., Williams, J., Sordillo, E.M., Ong, K.R., Kilburn, J.O., Dooley, S.W. & Castro, K.G. (1992). An outbreak of multidrug-resistant tuberculosis among hospitalized patients with the acquired immunodeficiency syndrome. *New England Journal of Medicine,* **326:** 1514-1521.

Eker, B., Ortmann, J., Migliori, G.B., Sotgiu, G., Muetterlein, R., Centis, R., Hoffmann, H., Kirsten, D., Schaberg, T., Ruesch-Gerdes, S., Lange, C. & German TBNET Group. (2008). Multidrug and extensively drug-resistant tuberculosis, Germany. *Emerging Infectious Diseases,* **14:** 1700-1706.

Eldholm, V., Matee, M., Mfinanga, S.G., Heun, M. & Dahle, U.R. (2006). A first insight into the genetic diversity of *Mycobacterium tuberculosis* in Dar es Salaam, Tanzania, assessed by spoligotyping. *BMC Microbiology,* **6:** 76.

Escombe, A.R., Oeser, C.C., Gilman, R.H., Navincopa, M., Ticona, E., Pan, W., Martinez, C., Chacaltana, J., Rodriguez, R., Moore, D.A., Friedland, J.S. & Evans, C.A. (2007). Natural ventilation for the prevention of airborne contagion. *PLoS Medicine,* **4:** e68.

Espinal, M.A., Kim, S.J., Suarez, P.G., Kam, K.M., Khomenko, A.G., Migliori, G.B., Baez, J., Kochi, A., Dye, C. & Raviglione, M.C. (2000). Standard short-course chemotherapy for drug-resistant tuberculosis: treatment outcomes in 6 countries. *Journal of the American Medical Association,* **283:** 2537-2545.

European Concerted Action on New Generation Genetic Markers and Techniques for the Epidemiology and Control of Tuberculosis (2006). Beijing/W genotype *Mycobacterium tuberculosis* and drug resistance. *Emerging Infectious Diseases*, **12:** 736-743.

Faustini, A., Hall, A.J. & Perucci, C.A. (2006). Risk factors for multidrug-resistant tuberculosis in Europe: a systematic review. *Thorax*, **61:** 158-163.

Ferdinand, S., Valetudie, G., Sola, C. & Rastogi, N. (2004). Data mining of *Mycobacterium tuberculosis* complex genotyping results using mycobacterial interspersed repetitive units validates the clonal structure of spoligotyping-defined families. *Research in Microbiology,* **155:** 647-654.

Ferrara, G., Richeldi, L., Bugiani, M., Cirillo, D., Besozzi, G., Nutini, S., Casali, L., Fiorentini, F., Codecasa, L.R. & Migliori, G.B. (2005). Management of multidrug-resistant tuberculosis in Italy. *International Journal of Tuberculosis and Lung Disease,* **9:** 507-513.

Filliol, I., Driscoll, J.R., Van Soolingen, D., Kreiswirth, B.N., Kremer, K., Valetudie, G., Anh, D.D., Barlow, R., Banerjee, D., Bifani, P.J., Brudey, K., Cataldi, A., Cooksey, R.C., Cousins, D.V., Dale, J.W., Dellagostin, O.A., Drobniewski, F., Engelmann, G., Ferdinand, S., Gascoyne-Binzi, D., Gordon, M., Gutierrez, M.C., Haas, W.H., Heersma, H., Kallenius, G., Kassa-Kelembho, E., Koivula, T., Ly, H.M., Makristathis, A., Mammina, C., Martin, G., Mostrom, P., Mokrousov, I., Narbonne, V., Narvskaya, O., Nastasi, A., Niobe-Eyangoh, S.N., Pape, J.W., Rasolofo-Razanamparany, V., Ridell, M., Rossetti, M.L., Stauffer, F., Suffys, P.N., Takiff, H., Texier-Maugein, J., Vincent, V., De Waard, J.H., Sola, C. & Rastogi, N. (2002). Global distribution of *Mycobacterium tuberculosis* spoligotypes. *Emerging Infectious Diseases,* **8:** 1347-1349.

Filliol, I., Driscoll, J.R., van Soolingen, D., Kreiswirth, B.N., Kremer, K., Valetudie, G., Dang, D.A., Barlow, R., Banerjee, D., Bifani, P.J., Brudey, K., Cataldi, A., Cooksey, R.C., Cousins, D.V., Dale, J.W., Dellagostin, O.A., Drobniewski, F., Engelmann, G., Ferdinand, S., Gascoyne-Binzi, D., Gordon, M., Gutierrez, M.C., Haas, W.H., Heersma, H., Kassa-Kelembho, E., Ho, M.L., Makristathis, A., Mammina, C., Martin, G., Mostrom, P., Mokrousov, I., Narbonne, V., Narvskaya, O., Nastasi, A., Niobe-Eyangoh, S.N., Pape, J.W., Rasolofo-Razanamparany, V., Ridell, M., Rossetti, M.L., Stauffer, F., Suffys, P.N., Takiff, H., Texier-Maugein, J., Vincent, V., de Waard, J.H., Sola, C. & Rastogi, N. (2003). Snapshot of moving and expanding clones of *Mycobacterium tuberculosis* and their global distribution assessed by spoligotyping in an international study. *Journal of Clinical Microbiology,* **41:** 1963-1970.

Filliol, I., Motiwala, A.S., Cavatore, M., Qi, W., Hazbon, M.H., Bobadilla del Valle, M., Fyfe, J., Garcia-Garcia, L., Rastogi, N., Sola, C., Zozio, T., Guerrero, M.I., Leon, C.I., Crabtree, J., Angiuoli, S., Eisenach, K.D., Durmaz, R., Joloba, M.L., Rendon, A., Sifuentes-Osornio, J., Ponce de Leon, A., Cave, M.D., Fleischmann, R., Whittam, T.S. & Alland, D. (2006). Global phylogeny of *Mycobacterium tuberculosis* based on single nucleotide polymorphism (SNP) analysis: insights into tuberculosis evolution, phylogenetic accuracy of other DNA fingerprinting systems, and recommendations for a minimal standard SNP set. *Journal of Bacteriology,* **188:** 759-772.

Flament-Saillour, M., Robert, J., Jarlier, V. & Grosset, J. (1999). Outcome of multi-drug-resistant tuberculosis in France: a nationwide case-control study. *American Journal of Respiratory and Critical Care,* **160:** 587-593.

Frieden, T.R., Sherman, L.F., Maw, K.L., Fujiwara, P.I., Crawford, J.T., Nivin, B., Sharp, V., D., H.,Jr, Brudney, K., Alland, D. & Kreisworth, B.N. (1996). A multi-institutional outbreak of highly drug-resistant tuberculosis: epidemiology and clinical outcomes. *Journal of the American Medical Association,* **276:** 1229-1235.

Frieden, T.R., Sterling, T.R., Munsiff, S.S., Watt, C.J. & Dye, C. (2003). Tuberculosis. *Lancet,* **362:** 887-899.

Friedland, G., Harries, A. & Coetzee, D. (2007). Implementation issues in tuberculosis/HIV program collaboration and integration: 3 case studies. *The Journal of Infectious Diseases*, **196:** S114-S123.

Frothingham, R. & Meeker-O'Connell, W.A. (1998). Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. *Microbiology,* **144:** 1189-1196.

Gagneux, S., DeRiemer, K., Van, T., Kato-Maeda, M., de Jong, B.C., Narayanan, S., Nicol, M., Niemann, S., Kremer, K., Gutierrez, M.C., Hilty, M., Hopewell, P.C. & Small, P.M. 2006, Variable host-pathogen compatibility in *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America,* **103:** 2869-2873.

Gandhi, N.R., Moll, A., Sturm, A.W., Pawinski, R., Govender, T., Lalloo, U., Zeller, K., Andrews, J. & Friedland, G. (2006). Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet,* **368:** 1575-1580.

Gandhi, N.R., Shah, N.S., Andrews, J.R., Vella, V., Moll, A.P., Scott, M., Weissman, D., Marra, C., Lalloo, U.G., Friedland, G.H. & Tugela Ferry Care and Research (TF CARES) Collaboration (2010). HIV coinfection in multidrug- and extensively drug-resistant tuberculosis results in high early mortality. *American Journal of Respiratory and Critical Care Medicine,* **181:** 80-86.

Garcia de Viedma, D., Alonso Rodriguez, N., Andres, S., Ruiz Serrano, M.J. & Bouza, E. (2005). Characterization of clonal complexity in tuberculosis by mycobacterial interspersed repetitive unit-variable-number tandem repeat typing, *Journal of Clinical Microbiology,* **43:** 5660-5664.

Geng, E., Kreiswirth, B., Burzynski, J. & Schluger, N.W. (2005). Clinical and radiographic correlates of primary and reactivation tuberculosis: a molecular epidemiology study. *Journal of the American Medical Association,* **293:** 2740-2745.

Githui, W.A., Jordaan, A.M., Juma, E.S., Kinyanjui, P., Karimi, F.G., Kimwomi, J., Meme, H., Mumbi, P., Streicher, E.M., Warren, R., van Helden, P.D. & Victor, T.C. (2004). Identification of MDR-TB Beijing/W and other *Mycobacterium tuberculosis* genotypes in Nairobi, Kenya. *International Journal of Tuberculosis and Lung Disease*, **8:** 352-360.

Glynn, J.R., Bauer, J., de Boer, A.S., Borgdorff, M.W., Fine, P.E., Godfrey-Faussett, P. & Vynnycky, E. (1999). Interpreting DNA fingerprint clusters of *Mycobacterium tuberculosis*. European Concerted Action on Molecular Epidemiology and Control of Tuberculosis. *International Journal of Tuberculosis and Lung Disease,* **3:** 1055-1060.

Glynn, J.R., Crampin, A.C., Traore, H., Yates, M.D., Mwaungulu, F.D., Ngwira, B.M., Chaguluka, S.D., Mwafulirwa, D.T., Floyd, S., Murphy, C., Drobniewski, F.A. & Fine, P.E. (2005). *Mycobacterium tuberculosis* Beijing genotype, northern Malawi. *Emerging Infectious Diseases,* **11:** 150-153.

Glynn, J.R., Murray, J., Bester, A., Nelson, G., Shearer, S. & Sonnenberg, P. (2008). Effects of duration of HIV infection and secondary tuberculosis transmission on tuberculosis incidence in the South African gold mines. *AIDS,* **14:** 1859-1867.

Glynn, J.R., Whiteley, J., Bifani, P.J., Kremer, K. & van Soolingen, D. (2002). Worldwide occurrence of Beijing/W strains of *Mycobacterium tuberculosis*: a systematic review. *Emerging Infectious Diseases,* **8:** 843-849.

Goble, M., Iseman, M.D., Madsen, L.A., Waite, D., Ackerson, L. & R., H.C.,Jr. (1993). Treatment of 171 patients with pulmonary tuberculosis resistant to isoniazid and rifampin. *New England Journal of Medicine,* **328:** 527-532.

Godfrey-Faussett, P., Sonnenberg, P., Shearer, S.C., Bruce, M.C., Mee, C., Morris, L. & Murray, J. (2000). Tuberculosis control and molecular epidemiology in a South African gold-mining community. *Lancet,* **356:** 1066-1071.

Godreuil, S., Torrea, G., Terru, D., Chevenet, F., Diagbouga, S., Supply, P., Van de Perre, P., Carriere, C. & Banuls, A.L. (2007). First molecular epidemiology study of *Mycobacterium tuberculosis* in Burkina Faso. *Journal of Clinical Microbiology*, **45:** 921-927.

Goguet de la Salmoniere, Y.O., Li, H.M., Torrea, G., Bunschoten, A., van Embden, J. & Gicquel, B. (1997). Evaluation of spoligotyping in a study of the transmission of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology,* **35:** 2210-2214.

Goletti, D., Weissman, D., Jackson, R.W., Graham, N.M., Vlahov, D., Klein, R.S., Munsiff, S.S., Ortona, L., Cauda, R. & Fauci, A.S. (1996). Effect of *Mycobacterium tuberculosis* on HIV replication. Role of immune activation. *Journal of Immunology*, **157:** 1271-1278.

Gori, A., Bandera, A., Marchetti, G., Degli Esposti, A., Catozzi, L., Nardi, G.P., Gazzola, L., Ferrario, G., van Embden, J.D., van Soolingen, D., Moroni, M. & Franzetti, F. (2005). Spoligotyping and *Mycobacterium tuberculosis. Emerging Infectious Diseases,* **11:** 1242-1248.

Goyal, M., Saunders, N.A., van Embden, J.D., Young, D.B. & Shaw, R.J. (1997). Differentiation of *Mycobacterium tuberculosis* isolates by spoligotyping and IS*6110* restriction fragment length polymorphism, *Journal of Clinical Microbiology,* **35:** 647-651.

Grimaldo, E.R., Tupasi, T.E., Rivera, A.B., Quelapio, M.I., Cardano, R.C., Derilo, J.O. & Belen, V.A. (2001). Increased resistance to ciprofloxacin and ofloxacin in multidrug-resistant *Mycobacterium tuberculosis* isolates from patients seen at a tertiary hospital in the Philippines. *International Journal of Tuberculosis and Lung Disease,* **5:** 546-550.

Groenen, P.M., Bunschoten, A.E., van Soolingen, D. & van Embden, J.D. (1993). Nature of DNA polymorphism in the direct repeat cluster of *Mycobacterium tuberculosis*; application for strain differentiation by a novel typing method. *Molecular Microbiology,* **10:** 1057-1065.

Gutierrez, M.C., Brisse, S., Brosch, R., Fabre, M., Omais, B., Marmiesse, M., Supply, P. & Vincent, V. (2005). Ancient origin and gene mosaicism of the progenitor of *Mycobacterium tuberculosis*. *PLoS Pathogens,* **1:** e5.

Gutierrez, M.C., Supply, P. & Brosch, R. (2009). Pathogenomics of Mycobacteria. *Genome Dynamics,* **6:** 198-210.

Haar, C.H., Cobelens, F.G., Kalisvaart, N.A., van der Have, J.J., van Gerven, P.J. & van Soolingen, D. (2007). Tuberculosis drug resistance and HIV infection, the Netherlands. *Emerging Infectious Diseases,* **13:** 776-778.

Han, H., Wang, F., Xiao, Y., Ren, Y., Chao, Y., Guo, A. & Ye, L. (2007). Utility of mycobacterial interspersed repetitive unit typing for differentiating *Mycobacterium tuberculosis* isolates in Wuhan, China. *Journal of Medical Microbiology,* **56:** 1219-1223.

Hanekom, M., van der Spuy, G.D., Gey van Pittius, N.C., McEvoy, C.R., Hoek, K.G., Ndabambi, S.L., Jordaan, A.M., Victor, T.C., van Helden, P.D. & Warren, R.M. (2008). Discordance between mycobacterial interspersed repetitive-unit-variable-number tandem-repeat typing and IS*6110* restriction fragment length polymorphism genotyping for analysis of *Mycobacterium tuberculosis* Beijing strains in a setting of high incidence of tuberculosis. *Journal of Clinical Microbiology,* **46:** 3338-3345.

Hanekom, M., van der Spuy, G.D., Gey van Pittius, N.C., McEvoy, C.R., Ndabambi, S.L., Victor, T.C., Hoal, E.G., van Helden, P.D. & Warren, R.M. (2007a). Evidence that the spread of *Mycobacterium tuberculosis* strains with the Beijing genotype is human population dependent. *Journal of Clinical Microbiology,* **45:** 2263-2266.

Hanekom, M., van der Spuy, G.D., Streicher, E., Ndabambi, S.L., McEvoy, C.R., Kidd, M., Beyers, N., Victor, T.C., van Helden, P.D. & Warren, R.M. (2007b). A recently evolved sublineage of the *Mycobacterium tuberculosis* Beijing strain family is associated with an increased ability to spread and cause disease. *Journal of Clinical Microbiology,* **45:** 1483-1490.

Havlir, D.V. & Barnes, P.F. (1999). Tuberculosis in patients with human immunodeficiency virus infection. *New England Journal of Medicine,* **340:** 367-373.

Heersma, H.F., Kremer, K. & van Embden, J.D. (1998). Computer analysis of IS*6110* RFLP patterns of *Mycobacterium tuberculosis*. *Methods in Molecular Biology,* **101:** 395-422.

Heller, T., Lessells, R.J., Wallrauch, C.G., Barnighausen, T., Cooke, G.S., Mhlongo, L., Master, I. & Newell, M.L. (2010). Community-based treatment for multidrug-resistant tuberculosis in rural KwaZulu-Natal, South Africa. *International Journal of Tuberculosis and Lung Disease*, **14:** 420-426.

Hermans, P.W., Messadi, F., Guebrexabher, H., van Soolingen, D., de Haas, P.E., Heersma, H., de Neeling, H., Ayoub, A., Portaels, F. & Frommel, D. (1995). Analysis of the population structure of *Mycobacterium tuberculosis* in Ethiopia, Tunisia, and The Netherlands: usefulness of DNA typing for global tuberculosis epidemiology. *The Journal of Infectious Diseases*, **171:** 1504-1513.

Hermans, P.W., van Soolingen, D., Dale, J.W., Schuitema, A.R., McAdam, R.A., Catty, D. & van Embden, J.D. (1990). Insertion element IS*986* from *Mycobacterium tuberculosis*: a useful tool for diagnosis and epidemiology of tuberculosis. *Journal of Clinical Microbiology*, **28:** 2051-2058.

Hermans, P.W.M., van Soolingen, D., Bik, E.M., Haas, P.E.W., Dale, J.W & van Embden, J.D (1991). Insertion element IS*987* from *Mycobacterium bovis* BCG is located in a hot-spot integration region for insertion elements in *Mycobacterium tuberculosis* complex strains, *Infection and Immunology*, 59: 2695-2705.

Hermans, P.W., van Soolingen, D. & van Embden, J.D. (1992). Characterization of a major polymorphic tandem repeat in *Mycobacterium tuberculosis* and its potential use in the epidemiology of *Mycobacterium kansasii* and *Mycobacterium gordonae*. *Journal of Bacteriology*, **174**: 4157-4165.

Herzog, H. (1998). History of tuberculosis. *Respiration,* **65:** 5-15.

Heym, B., Honore, N., Truffot-Pernot, C., Banerjee, A., Schurra, C., R., J.W.,Jr, van Embden, J.D., Grosset, J.H. & Cole, S.T. (1994). Implications of multidrug resistance for the future of short-course chemotherapy of tuberculosis: a molecular study. *Lancet,* **344:** 293-298.

Hirsh, A.E., Tsolaki, A.G., DeRiemer, K., Feldman, M.W. & Small, P.M. (2004). Stable association between strains of *Mycobacterium tuberculosis* and their human host populations. *Proceedings of the National Academy of Sciences of the United States of America*, **101:** 4871-4876.

Holtz, T.H., Lancaster, J., Laserson, K.F., Wells, C.D., Thorpe, L. & Weyer, K. (2006). Risk factors associated with default from multidrug-resistant tuberculosis treatment, South Africa, 1999-2001. *International Journal of Tuberculosis and Lung Disease,* **10:** 649-655.

Homolka, S., Post, E., Oberhauser, B., George, A.G., Westman, L., Dafae, F., Rusch-Gerdes, S. & Niemann, S. (2008). High genetic diversity among *Mycobacterium tuberculosis* complex strains from Sierra Leone. *BMC Microbiology,* **8:** 103.

Honer zu Bentrup, K. & Russell, D.G. (2001). Mycobacterial persistence: adaptation to a changing environment. *Trends Microbiology,* **9:** 597-605.

Honore, N. & Cole, S.T. (1994). Streptomycin resistance in mycobacteria. *Antimicrobial Agents and Chemotherapy,* **38:** 238-242.

Hunter, P.R. & Gaston, M.A. (1988). Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *Journal of Clinical Microbiology,* **26:** 2465-2466.

Ioerger, T.R., Koo, S., No, E.G., Chen, X., Larsen, M.H., Jacobs, W.R.,Jr, Pillay, M., Sturm, A.W. & Sacchettini, J.C. (2009). Genome analysis of multi and extensively-drug-resistant tuberculosis from KwaZulu Natal, South Africa. *PloS One,* **4:** e7778.

Iwamoto, T., Yoshida, S., Suzuki, K., Tomita, M., Fujiyama, R., Tanaka, N., Kawakami, Y. & Ito, M. (2007). Hypervariable loci that enhance the discriminatory ability of newly proposed 15-loci and 24-loci variable-number tandem repeat typing method on *Mycobacterium tuberculosis* strains predominated by the Beijing family. *FEMS Microbiology Letters,* **270:** 67-74.

Jarlier, V. & Nikaido, H. (1990). Permeability barrier to hydrophilic solutes in *Mycobacterium chelonei*. *Journal of Bacteriology,* **172:** 1418-1423.

Jawahar, M.S. (2004). Current trends in chemotherapy of tuberculosis. *Indian Journal of Medical Research,* **120:** 398-417.

Jiao, W.W., Mokrousov, I., Sun, G.Z., Guo, Y.J., Vyazovaya, A., Narvskaya, O. & Shen, A.D. (2008) Evaluation of new variable-number tandem-repeat systems for typing *Mycobacterium tuberculosis* with Beijing genotype isolates from Beijing, China. *Journal of Clinical Microbiology,* **46:** 1045-1049.

Johnson, R., Warren, R.M., van der Spuy, G.D., Gey van Pittius, N.C., Theron, D., Streicher, E.M., Bosman, M., Coetzee, G.J., van Helden, P.D. & Victor, T.C. (2010). Drug-resistant tuberculosis epidemic in the Western Cape driven by a virulent Beijing genotype strain. *International Journal of Tuberculosis and Lung Disease,* **14:** 119-121.

Jones, W.D.,Jr & Greenberg, J. (1978). Modification of methods used in bacteriophage typing of *Mycobacterium tuberculosis* isolates. *Journal of Clinical Microbiology,* **7:** 467-469.

Kallenius, G., Hoffner, S.E., Miorner, H. & Svenson, S.B. (1994). Novel approaches to the diagnosis of mycobacterial infections. *The European Respiratory Journal,* **7:** 1921-1924.

Kam, K.M., Yip, C.W., Tse, L.W., Leung, K.L., Wong, K.L., Ko, W.M. & Wong, W.S. (2006). Optimization of variable number tandem repeat typing set for differentiating *Mycobacterium tuberculosis* strains in the Beijing family. *FEMS Microbiology Letters,* **256:** 258-265.

Kam, K.M., Yip, C.W., Tse, L.W., Wong, K.L., Lam, T.K., Kremer, K., Au, B.K. & van Soolingen, D. (2005). Utility of mycobacterial interspersed repetitive unit typing for differentiating multidrug-resistant *Mycobacterium tuberculosis* isolates of the Beijing family. *Journal of Clinical Microbiology,* **43:** 306-313.

Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M. & van Embden, J. (1997). Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *Journal of Clinical Microbiology,* **35**: 907-914.

Kanduma, E., McHugh, T.D. & Gillespie, S.H. (2003). Molecular methods for *Mycobacterium tuberculosi*s strain typing: a users guide. *Journal of Applied Microbiology,* **94:** 781-791.

Kaufmann, S.H. & Schaible, U.E. (2005). 100th anniversary of Robert Koch's Nobel Prize for the discovery of the tubercle bacillus. *Trends Microbiology,* **13:** 469-475.

Kibiki, G.S., Mulder, B., Dolmans, W.M., de Beer, J.L., Boeree, M., Sam, N., van Soolingen, D., Sola, C. & van der Zanden, A.G. (2007). *M. tuberculosis* genotypic diversity and drug susceptibility pattern in HIV-infected and non-HIV-infected patients in northern Tanzania. *BMC Microbiology*, **7:** 51.

Kim, D.H., Kim, H.J., Park, S.K., Kong, S.J., Kim, Y.S., Kim, T.H., Kim, E.K., Lee, K.M., Lee, S.S., Park, J.S., Koh, W.J., Lee, C.H., Kim, J.Y. & Shim, T.S. (2008). Treatment outcomes and long-term survival in patients with extensively drug-resistant tuberculosis. *American Journal of Respiratory and Critical Care Medicine,* **178:** 1075-1082.

Kim, H.R., Hwang, S.S., Kim, H.J., Lee, S.M., Yoo, C.G., Kim, Y.W., Han, S.K., Shim, Y.S. & Yim, J.J. (2007). Impact of extensive drug resistance on treatment outcomes in non-HIV-infected patients with multidrug-resistant tuberculosis. *Clinical Infectious Diseases,* **45:** 1290-1295.

Kliiman, K. & Altraja, A. (2009). Predictors of poor treatment outcome in multi- and extensively drug-resistant pulmonary TB. *The European Respiratory Journal,* **33:** 1085-1094.

Kremer, K., Arnold, C., Cataldi, A., Gutierrez, M.C., Haas, W.H., Panaiotov, S., Skuce, R.A., Supply, P., van der Zanden, A.G. & van Soolingen, D. (2005). Discriminatory power and reproducibility of novel DNA typing methods for *Mycobacterium tuberculosis* complex strains. *Journal of Clinical Microbiology,* **43:** 5628-5638.

Kremer, K., Glynn, J.R., Lillebaek, T., Niemann, S., Kurepina, N.E., Kreiswirth, B.N., Bifani, P.J. & van Soolingen, D. (2004). Definition of the Beijing/W lineage of *Mycobacterium tuberculosis* on the basis of genetic markers. *Journal of Clinical Microbiology,* **42:** 4040-4049.

Kremer, K., van Soolingen, D., Frothingham, R., Haas, W.H., Hermans, P.W., Martin, C., Palittapongarnpim, P., Plikaytis, B.B., Riley, L.W., Yakrus, M.A., Musser, J.M. & van Embden, J.D. (1999). Comparison of methods based on different molecular epidemiological markers for typing of *Mycobacterium tuberculosis* complex strains: interlaboratory study of discriminatory power and reproducibility, *Journal of Clinical Microbiology,* **37:** 2607-2618.

Kruuner, A., Hoffner, S.E., Sillastu, H., Danilovits, M., Levina, K., Svenson, S.B., Ghebremichael, S., Koivula, T. & Kallenius, G. (2001). Spread of drug-resistant pulmonary tuberculosis in Estonia. *Journal of Clinical Microbiology,* **39:** 3339-3345.

Kruuner, A., Pehme, L., Ghebremichael, S., Koivula, T., Hoffner, S.E. & Mikelsaar, M. (2002). Use of molecular techniques to distinguish between treatment failure and exogenous reinfection with *Mycobacterium tuberculosis*. *Clinical Infectious Diseases,* **35:** 146-155.

Kurepina, N.E., Sreevatsan, S., Plikaytis, B.B., Bifani, P.J., Connell, N.D., Donnelly, R.J., van Sooligen, D., Musser, J.M. & Kreiswirth, B.N. (1998). Characterization of the phylogenetic distribution and chromosomal insertion sites of five IS*6110* elements in *Mycobacterium tuberculosis*: non-random integration in the dnaA-dnaN region. *Tubercle and Lung Disease,* **79:** 31-42.

Kwara, A., Schiro, R., Cowan, L.S., Hyslop, N.E., Wiser, M.F., Roahen Harrison, S., Kissinger, P., Diem, L. & Crawford, J.T. (2003). Evaluation of the epidemiologic utility of secondary typing methods for differentiation of *Mycobacterium tuberculosis* isolates. *Journal of Clinical Microbiology,* **41:** 2683-2685.

Lai, C.C., Tan, C.K., Lin, S.H., Liao, C.H., Huang, Y.T., Chou, C.H., Hsu, H.L., Wang, C.Y., Lin, H.I. & Hsueh, P.R. (2010). Clinical and genotypic characteristics of extensively drug-resistant and multidrug-resistant tuberculosis. *European Journal of Clinical Microbiology & infectious diseases,* **29:** 597-600.

Lari, N., Rindi, L., Bonnani D., Rastogi, N., Sola, C., Tortoli, E & Garzelli, C. (2007). A three year longitudinal study of the genotypes of *Mycbacterium tuberculosis* isolates in Tuscany, Italy. *Journal of Microbiology*, **45**: 1851-1857.

Laserson, K.F., Thorpe, L.E., Leimane, V., Weyer, K., Mitnick, C.D., Riekstina, V., Zarovska, E., Rich, M.L., Fraser, H.S., Alarcon, E., Cegielski, J.P., Grzemska, M., Gupta, R. & Espinal, M. (2005). Speaking the same language: treatment outcome definitions for multidrug-resistant tuberculosis. *International Journal of Tuberculosis and Lung Disease*, **9:** 640-645.

Lee, A.S., Tang, L.L., Lim, I.H., Bellamy, R. & Wong, S.Y. 2002, Discrimination of single-copy IS*6110* DNA fingerprints of *Mycobacterium tuberculosis* isolates by high-resolution minisatellite-based typing. *Journal of Clinical Microbiology,* **40:** 657-659.

Leimane, V., Riekstina, V., Holtz, T.H., Zarovska, E., Skripconoka, V., Thorpe, L.E., Laserson, K.F. & Wells, C.D. (2005). Clinical outcome of individualised treatment of multidrug-resistant tuberculosis in Latvia: a retrospective cohort study. *Lancet*, **365:** 318-326.

Li, X.Z. & Nikaido, H. (2004). Efflux-mediated drug resistance in bacteria. *Drugs*, **64:** 159-204.

Lockman, S., Sheppard, J.D., Braden, C.R., Mwasekaga, M.J., Woodley, C.L., Kenyon, T.A., Binkin, N.J., Steinman, M., Montsho, F., Kesupile-Reed, M., Hirschfeldt, C., Notha, M., Moeti, T. & Tappero, J.W. (2001). Molecular and conventional epidemiology of *Mycobacterium tuberculosis* in Botswana: a population-based prospective study of 301 pulmonary tuberculosis patients. *Journal of Clinical Microbiology*, **39:** 1042-1047.

Lopez, B., Aguilar, D., Orozco, H., Burger, M., Espitia, C., Ritacco, V., Barrera, L., Kremer, K., Hernandez-Pando, R., Huygen, K. & van Soolingen, D. (2003). A marked difference in pathogenesis and immune response induced by different *Mycobacterium tuberculosis* genotypes. *Clinical and Experimental Immunology*, **133:** 30-37.

Madariaga, M.G., Lalloo, U.G. & Swindells, S. (2008). Extensively drug-resistant tuberculosis. *The American Journal of Medicine*, **121:** 835-844.

Maes, M., Kremer, K., van Soolingen, D., Takiff, H. & de Waard, J.H. (2008). 24-locus MIRU-VNTR genotyping is a useful tool to study the molecular epidemiology of tuberculosis among Warao Amerindians in Venezuela. *Tuberculosis*, **88:** 490-494.

Magdalena, J., Vachee, A., Supply, P. & Locht, C. (1998). Identification of a new DNA region specific for members of *Mycobacterium tuberculosis* complex. *Journal of Clinical Microbiology,* **36:** 937-943.

Malik, A.N. & Godfrey-Faussett, P. (2005). Effects of genetic variability of *Mycobacterium tuberculosis* strains on the presentation of disease. *Lancet Infectious Diseases*, **5:** 174-183.

Manca, C., Tsenova, L., Freeman, S., Barczak, A.K., Tovey, M., Murray, P.J., Barry, C. & Kaplan, G. (2005). Hypervirulent *M. tuberculosis* W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway. *Journal of Interferon & Cytokine Research,* **25:** 694-701.

Marais, B.J., Hesseling, A.C., Schaaf, H.S., Gie, R.P., van Helden, P.D. & Warren, R.M. (2009). *Mycobacterium tuberculosis* transmission is not related to household genotype in a setting of high endemicity. *Journal of Clinical Microbiology*, **47:** 1338-1343.

Marais, B.J., Victor, T.C., Hesseling, A.C., Barnard, M., Jordaan, A., Brittle, W., Reuter, H., Beyers, N., van Helden, P.D., Warren, R.M. & Schaaf, H.S. (2006). Beijing and Haarlem

genotypes are overrepresented among children with drug-resistant tuberculosis in the Western Cape Province of South Africa. *Journal of Clinical Microbiology,* **44**: 3539-3543.

Mardassi, H., Namouchi, A., Haltiti, R., Zarrouk, M., Mhenni, B., Karboul, A., Khabouchi, N., Gey van Pittius, N.C., Streicher, E.M., Rauzier, J., Gicquel, B. & Dellagi, K. (2005). Tuberculosis due to resistant Haarlem strain, Tunisia. *Emerging Infectious Diseases,* **6:** 957-961.

Mariani, F., Goletti, D., Ciaramella, A., Martino, A., Colizzi, V. & Fraziano, M. (2001). Macrophage response to *Mycobacterium tuberculosis* during HIV infection: relationships between macrophage activation and apoptosis. *Current Molecular Medicine,* **1:** 209-216.

Martin, A., Portaels, F. & Palomino, J.C. (2007). Colorimetric redox-indicator methods for the rapid detection of multidrug resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. *Journal of Antimicrobial Chemotherapy,* **59:** 175-183.

Martinez, M., Garcia de Viedma, D., Alonso, M., Andres, S., Bouza, E., Cabezas, T., Cabeza, I., Reyes, A., Sanchez-Yebra, W., Rodriguez, M., Sanchez, M.I., Rogado, M.C., Fernandez, R., Penafiel, T., Martinez, J., Barroso, P., Lucerna, M.A., Diez, L.F. & Gutierrez, C. (2006). Impact of laboratory cross-contamination on molecular epidemiology studies of tuberculosis. *Journal of Clinical Microbiology,* **44:** 2967-2969.

Masjedi, M.R., Farnia, P., Sorooch, S., Pooramiri, M.V., Mansoori, S.D., Zarifi, A.Z., Akbarvelayati, A. & Hoffner, S. (2006). Extensively drug-resistant tuberculosis: 2 years of surveillance in Iran. *Clinical Infectious Diseases,* **43:** 841-847.

Mathema, B., Kurepina, N.E., Bifani, P.J. & Kreiswirth, B.N. (2006). Molecular epidemiology of tuberculosis: current insights. *Clinical Microbiology Reviews,* **19:** 658-685.

Mathuria, J.P., Sharma, P., Prakash, P., Samaria, J.K., Katoch, V.M. & Anupurba, S. (2008). Role of spoligotyping and IS*6110*-RFLP in assessing genetic diversity of *Mycobacterium tuberculosis* in India. *Infection, Genetics and Evolution,* **8:** 346-351.

Maus, C.E., Plikaytis, B.B. & Shinnick, T.M. (2005). Mutation of tlyA confers capreomycin resistance in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy,* **49:** 571-577.

Mazars, E., Lesjean, S., Banuls, A.L., Gilbert, M., Vincent, V., Gicquel, B., Tibayrenc, M., Locht, C. & Supply, P. (2001). High-resolution minisatellite-based typing as a portable approach to global analysis of *Mycobacterium tuberculosis* molecular epidemiology. *Proceedings of the National Academy of Sciences of the United States of America,* **98:** 1901-1906.

McAdam, R.A., Hermans, P.W., van Soolingen, D., Zainuddin, Z.F., Catty, D., van Embden, J.D. & Dale, J.W. (1990). Characterization of a *Mycobacterium tuberculosis* insertion sequence belonging to the IS3 family. *Molecular Microbiology,* **4:** 1607-1613.

Mendez, M.P., Landon, M.E., McCloud, M.K., Davidson, P. & Christensen, P.J. (2009). Co-infection with pansensitive and multidrug-resistant strains of *Mycobacterium tuberculosis*. *Emerging Infectious Diseases,* **15:** 578-580.

Metcalf, C. (1991). A history of tuberculosis: in *A Century of Tuberculosis, South African Pespectives*, eds. H.M. Coovadia & S.R. Benatar, First edn, Oxford University Press, Cape Town, 1-28.

Middelkoop, K., Bekker, L.G., Mathema, B., Shashkina, E., Kurepina, N., Whitelaw, A., Fallows, D., Morrow, C., Kreiswirth, B., Kaplan, G. & Wood, R. (2009). Molecular epidemiology of *Mycobacterium tuberculosis* in a South African community with high HIV prevalence. *The Journal of Infectious Diseases*, **200:** 1207-1211.

Migliori, G.B., Besozzi, G., Girardi, E., Kliiman, K., Lange, C., Toungoussova, O.S., Ferrara, G., Cirillo, D.M., Gori, A., Matteelli, A., Spanevello, A., Codecasa, L.R. & Raviglione, M.C. (2007). Clinical and operational value of the extensively drug-resistant tuberculosis definition. *European Respiratory Journal,* **30:** 623-626.

Migliori, G.B., Lange, C., Centis, R., Sotgiu, G., Mutterlein, R., Hoffmann, H., Kliiman, K., De Iaco, G., Lauria, F.N., Richardson, M.D., Spanevello, A., Cirillo, D.M. & TBNET Study Group (2008a). Resistance to second-line injectables and treatment outcomes in multidrug-resistant and extensively drug-resistant tuberculosis cases. *The European Respiratory Journal,* **31:** 1155-1159.

Migliori, G.B., Lange, C., Girardi, E., Centis, R., Besozzi, G., Kliiman, K., Ortmann, J., Matteelli, A., Spanevello, A. & Cirillo, D.M. (2008b). Extensively drug-resistant tuberculosis is worse than multidrug-resistant tuberculosis: different methodology and settings, same results. *Clinical Infectious Diseases,* **46:** 958-959.

Migliori, G.B., Ortmann, J., Girardi, E., Besozzi, G., Lange, C., Cirillo, D.M., Ferrarese, M., De Iaco, G., Gori, A., Raviglione, M.C. & SMIRA/TBNET Study Group (2007). Extensively drug-resistant tuberculosis, Italy and Germany. *Emerging Infectious Diseases*, **13:** 780-782.

Migliori, G.B., Sotgiu, G., D'Arcy Richardson, M., Centis, R., Facchini, A., Guenther, G., Spanevello, A., Lange, C. & TBNET (2009). MDR-TB and XDR-TB: drug resistance and treatment outcomes. *The European Respiratory Journal,* **34:** 778-779.

Millet, J., Miyagi-Shiohira, C., Yamane, N., Sola, C. & Rastogi, N. (2007). Assessment of mycobacterial interspersed repetitive unit-QUB markers to further discriminate the Beijing genotype in a population-based study of the genetic diversity of *Mycobacterium tuberculosis* clinical isolates from Okinawa, Ryukyu Islands, Japan. *Journal of Clinical Microbiology,* **45:** 3606-3015.

Mitchison, D.A. (2005). The control of tuberculosis: progress & prospect. *The Indian Journal of Medical Research,* **121:** 137-139.

Mitchison, D.A. (1985). The action of antituberculosis drugs in short-course chemotherapy. *Tubercle,* **66:** 219-225.

Mitnick, C., Bayona, J., Palacios, E., Shin, S., Furin, J., Alcantara, F., Sanchez, E., Sarria, M., Becerra, M., Fawzi, M.C., Kapiga, S., Neuberg, D., Maguire, J.H., Kim, J.Y. & Farmer, P. (2003). Community-based therapy for multidrug-resistant tuberculosis in Lima, Peru. *New England Journal of Medicine,* **348:** 119-128.

Mitnick, C.D., Shin, S.S., Seung, K.J., Rich, M.L., Atwood, S.S., Furin, J.J., Fitzmaurice, G.M., Alcantara Viru, F.A., Appleton, S.C., Bayona, J.N., Bonilla, C.A., Chalco, K., Choi, S., Franke, M.F., Fraser, H.S., Guerra, D., Hurtado, R.M., Jazayeri, D., Joseph, K., Llaro, K., Mestanza, L., Mukherjee, J.S., Munoz, M., Palacios, E., Sanchez, E., Sloutsky, A. & Becerra, M.C. (2008). Comprehensive treatment of extensively drug-resistant tuberculosis. *The New England Journal of Medicine,* **359:** 563-574.

Mlambo, C.K., Warren, R.M., Poswa, X., Victor, T.C., Duse, A.G. & Marais, E. (2008). Genotypic diversity of extensively drug-resistant tuberculosis (XDR-TB) in South Africa. *International Journal of Tuberculosis and Lung Disease,* **12:** 99-104.

Mokrousov, I., Narvskaya, O., Limeschenko, E., Vyazovaya, A., Otten, T. & Vyshnevskiy, B. (2004). Analysis of the allelic diversity of the mycobacterial interspersed repetitive units in *Mycobacterium tuberculosis* strains of the Beijing family: practical implications and evolutionary considerations. *Journal of Clinical Microbiology,* **42:** 2438-2444.

Monot, M., Honore, N., Garnier, T., Araoz, R., Coppee, J.Y., Lacroix, C., Sow, S., Spencer, J.S., Truman, R.W., Williams, D.L., Gelber, R., Virmond, M., Flageul, B., Cho, S.N., Ji, B., Paniz-Mondolfi, A., Convit, J., Young, S., Fine, P.E., Rasolofo, V., Brennan, P.J. & Cole, S.T. (2005). On the origin of leprosy. *Science,* **308:** 1040-1042.

Moore, D.A., Evans, C.A., Gilman, R.H., Caviedes, L., Coronel, J., Vivar, A., Sanchez, E., Pinedo, Y., Saravia, J.C., Salazar, C., Oberhelman, R., Hollm-Delgado, M.G., LaChira, D., Escombe, A.R. & Friedland, J.S. (2006). Microscopic-observation drug-susceptibility assay for the diagnosis of TB. *New England Journal of Medicine,* **355:** 1539-1550.

Morozova, I., Riekstina, V., Sture, G., Wells, C. & Leimane, V. (2003). Impact of the growing HIV-1 epidemic on multidrug-resistant tuberculosis control in Latvia. *International Journal of Tuberculosis and Lung Disease,* **7:** 903-906.

Mostowy, S., Cousins, D. & Behr, M.A. (2004). Genomic Interrogation of the dassie bacillus reveals it is a unique RD1 mutant within the Mycobacterium tuberculosis complex. *Journal of Bacteriology,* **186:** 104-109.

Mukherjee, J.S., Rich, M.L., Socci, A.R., Joseph, J.K., Viru, F.A., Shin, S.S., Furin, J.J., Becerra, M.C., Barry, D.J., Kim, J.Y., Bayona, J., Farmer, P., Smith Fawzi, M.C. & Seung, K.J. (2004). Programmes and principles in treatment of multidrug-resistant tuberculosis. *Lancet,* **363:** 474-481.

Murdoch, D.M., Venter, W.D., Feldman, C. & Van Rie, A. (2008). Incidence and risk factors for the immune reconstitution inflammatory syndrome in HIV patients in South Africa: a prospective study. *AIDS,* **22:** 601-610.

Nair, J., Rouse, D.A., Bai, G.H. & Morris, S.L. (1993). The rpsL gene and streptomycin resistance in single and multiple drug-resistant strains of *Mycobacterium tuberculosis*. *Molecular Microbiology,* **10:** 521-527.

Namouchi, A., Karboul, A., Mhenni, B., Khabouchi, N., Haltiti, R., Ben Hassine, R., Louzir, B., Chabbou, A. & Mardassi, H. (2008). Genetic profiling of *Mycobacterium tuberculosis* in Tunisia: predominance and evidence for the establishment of a few genotypes. *Journal of Medical Microbiology,* **57:** 864-872.

Nardell, E.A. (2004). Catching droplet nuclei: toward a better understanding of tuberculosis transmission. *American Journal of Respiratory and Critical Care Medicine*, **169:** 553-554.

Narita, M., Kellman, M., Franchini, D.L., McMillan, M.E., Hollender, E.S. & Ashkin, D. (2002). Short-course rifamycin and pyrazinamide treatment for latent tuberculosis infection in patients with HIV infection: the 2-year experience of a comprehensive community-based program in Broward County, Florida. *Chest,* **122:** 1292-1298.

Nathanson, E., Lambregts-van Weezenbeek, C., Rich, M.L., Gupta, R., Bayona, J., Blondal, K., Caminero, J.A., Cegielski, J.P., Danilovits, M., Espinal, M.A., Hollo, V., Jaramillo, E., Leimane, V., Mitnick, C.D., Mukherjee, J.S., Nunn, P., Pasechnikov, A., Tupasi, T., Wells, C. & Raviglione, M.C. (2006). Multidrug-resistant tuberculosis management in resource-limited settings. *Emerging Infectious Diseases,* **12:** 1389-1397.

Nicol, M.P., Sola, C., February, B., Rastogi, N., Steyn, L. & Wilkinson, R.J. (2005). Distribution of strain families of *Mycobacterium tuberculosis* causing pulmonary and extrapulmonary disease in hospitalized children in Cape Town, South Africa. *Journal of Clinical Microbiology,* **43:** 5779-5781.

Nikolayevskyy, V., Gopaul, K., Balabanova, Y., Brown, T., Fedorin, I. & Drobniewski, F. (2006). Differentiation of tuberculosis strains in a population with mainly Beijing-family strains. *Emerging Infectious Diseases,* **12:** 1406-1413.

Niobe-Eyangoh, S.N., Kuaban, C., Sorlin, P., Cunin, P., Thonnon, J., Sola, C., Rastogi, N., Vincent, V. & Gutierrez, M.C. (2003). Genetic biodiversity of *Mycobacterium tuberculosis* complex strains from patients with pulmonary tuberculosis in Cameroon. *Journal of Clinical Microbiology,* **41:** 2547-2553.

Niobe-Eyangoh, S.N., Kuaban, C., Sorlin, P., Thonnon, J., Vincent, V. & Gutierrez, M.C. (2004). Molecular characteristics of strains of the Cameroon family, the major group of *Mycobacterium tuberculosis* in a country with a high prevalence of tuberculosis. *Journal of Clinical Microbiology,* **42:** 5029-5035.

O'Donnell, M.R., Padayatchi, N., Master, I., Osburn, G. & Horsburgh, C.R. (2009). Improved early results for patients with extensively drug-resistant tuberculosis and HIV in South Africa. *International Journal of Tuberculosis and Lung Disease,* **13:** 855-861.

Oelemann, M.C., Diel, R., Vatin, V., Haas, W., Rusch-Gerdes, S., Locht, C., Niemann, S. & Supply, P. (2007). Assessment of an optimized mycobacterial interspersed repetitive- unit-variable-number tandem-repeat typing system combined with spoligotyping for population-based molecular epidemiology studies of tuberculosis. *Journal of Clinical Microbiology*, **45:** 691-697.

Orenstein, E.W., Basu, S., Shah, N.S., Andrews, J.R., Friedland, G.H., Moll, A.P., Gandhi, N.R. & Galvani, A.P. (2009). Treatment outcomes among patients with multidrug-resistant tuberculosis: systematic review and meta-analysis. *Lancet Infectious Diseases,* **9:** 153-161.

Padayatchi, N. & Friedland, G. (2008). Decentralised management of drug-resistant tuberculosis (MDR and XDR-TB) in South Africa: an alternative model of care. *International Journal of Tuberculosis and Lung Disease*, **12:** 978-980.

Padayatchi, N., Stiefvater, E., Naidoo, K., Naidoo, K., Ndung'u, T. & Abdool Karim, Q. (2009). Expanding HIV surveillance to include TB patients in resource-limited settings with a generalized epidemic. *International Journal of Tuberculosis and Lung Disease,* **13:** 1447-1449.

Palmero, D.J., Ambroggi, M., Brea, A., De Lucas, M., Fulgenzi, A., Martinez, D., Mosca, C., Musella, R., Natiello, M., Gonzalez, C. & Abbate, E. (2004). Treatment and follow-up of HIV-negative multidrug-resistant tuberculosis patients in an infectious diseases reference hospital, Buenos Aires, Argentina. *International Journal of Tuberculosis and Lung Disease,* **8:** 778-784.

Palomino, J.C., Martin, A. & Portaels, F. (2007). Rapid drug resistance detection in *Mycobacterium tuberculosis*: a review of colourimetric methods. *Clinical Microbiology and Infection,* **13:** 754-762.

Park, H.H., Girdler-Brown, B.V., Churchyard, G.J., White, N.W. & Ehrlich, R.I. (2009). Incidence of tuberculosis and HIV and progression of silicosis and lung function impairment among former Basotho gold miners. *American Journal of Industrial Medicine,* **52:** 901-908.

Park, S.K., Lee, W.C., Lee, D.H., Mitnick, C.D., Han, L. & Seung, K.J. (2004). Self-administered, standardized regimens for multidrug-resistant tuberculosis in South Korea. *International Journal of Tuberculosis and Lung Disease,* **8:** 361-368.

Park, Y.K., Shin, S., Ryu, S., Cho, S.N., Koh, W.J., Kwon, O.J., Shim, Y.S., Lew, W.J. & Bai, G.H. (2005). Comparison of drug resistance genotypes between Beijing and non-Beijing family strains of *Mycobacterium tuberculosis* in Korea. *Journal of Microbiological Methods*, **63:** 165-172.

Parwati, I., van Crevel, R. & van Soolingen, D. (2010). Possible underlying mechanisms for successful emergence of the *Mycobacterium tuberculosis* Beijing genotype strains. *Lancet Infectious Diseases,* **10:** 103-111.

Pasca, M.R., Guglierame, P., Arcesi, F., Bellinzoni, M., De Rossi, E. & Riccardi, G. (2004). Rv2686c-Rv2687c-Rv2688c, an ABC fluoroquinolone efflux pump in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy*, **48:** 3175-3178.

Perkins, M.D. & Cunningham, J. (2007). Facing the crisis: improving the diagnosis of tuberculosis in the HIV era. *The Journal of Infectious Diseases,* **196:** S15-S27.

Pfyffer, G.E., Auckenthaler, R., van Embden, J.D. & van Soolingen, D. (1998). *Mycobacterium canettii*, the smooth variant of *M. tuberculosis*, isolated from a Swiss patient exposed in Africa. *Emerging Infectious Diseases,* **4:** 631-634.

Phyu, S., Stavrum, R., Lwin, T., Svendsen, O.S., Ti, T. & Grewal, H.M. (2009). Predominance of *Mycobacterium tuberculosis* EAI and Beijing lineages in Yangon, Myanmar. *Journal of Clinical Microbiology,* **47:** 335-344.

Pillay, M. & Sturm, A.W. (2010). Nosocomial transmission of the F15/LAM4/KZN genotype of *Mycobacterium tuberculosis* in patients on tuberculosis treatment. *International Journal of Tuberculosis and Lung Disease,* **14:** 223-230.

Pillay, M. & Sturm, A.W. (2007). Evolution of the extensively drug-resistant F15/LAM4/KZN strain of *Mycobacterium tuberculosis* in KwaZulu-Natal, South Africa. *Clinical Infectious Diseases,* **45:** 1409-1414.

Qian, L., Van Embden, J.D., Van Der Zanden, A.G., Weltevreden, E.F., Duanmu, H. & Douglas, J.T. (1999). Retrospective analysis of the Beijing family of *Mycobacterium tuberculosis* in preserved lung tissues. *Journal of Clinical Microbiology,* **37:** 471-474.

Reed, M.B., Domenech, P., Manca, C., Su, H., Barczak, A.K., Kreiswirth, B.N., Kaplan, G. & Barry, C.E. (2004). A glycolipid of hypervirulent tuberculosis strains that inhibits the innate immune response. *Nature,* **431:** 84-87.

Richardson, M., Carroll, N.M., Engelke, E., van Der Spuy, G.D., Salker, F., Munch, Z., Gie, R.P., Warren, R.M., Beyers, N. & Van Helden, P.D. (2002a). Multiple *Mycobacterium tuberculosis* strains in early cultures from patients in a high-incidence community setting. *Journal of Clinical Microbiology,* **40:** 2750-2754.

Richardson, M., van Lill, S.W., van der Spuy, G.D., Munch, Z., Booysen, C.N., Beyers, N., van Helden, P.D. & Warren, R.M. (2002b). Historic and recent events contribute to the disease dynamics of Beijing-like *Mycobacterium tuberculosis* isolates in a high incidence region. *International Journal of Tuberculosis and Lung Disease,* **6:** 1001-1011.

Riska, P.F., Jacobs, W.R.,Jr & Alland, D. (2000). Molecular determinants of drug resistance in tuberculosis. *International Journal of Tuberculosis and Lung Disease,* **4:** S4-S10.

Rocha-Ramirez, L.M., Estrada-Garcia, I., Lopez-Marin, L.M., Segura-Salinas, E., Mendez-Aragon, P., Van Soolingen, D., Torres-Gonzalez, R., Chacon-Salinas, R., Estrada-Parra, S., Maldonado-Bernal, C., Lopez-Macias, C. & Isibasi, A. (2008). *Mycobacterium tuberculosis* lipids regulate cytokines, TLR-2/4 and MHC class II expression in human macrophages. *Tuberculosis,* **88:** 212-220.

Roring, S., Scott, A., Brittain, D., Walker, I., Hewinson, G., Neill, S. & Skuce, R. (2002). Development of variable-number tandem repeat typing of *Mycobacterium bovis*: comparison of results with those obtained by using existing exact tandem repeats and spoligotyping. *Journal of Clinical Microbiology,* **40:** 2126-2133.

Sampson. S., Warren. R., Richardson. M., van der Spuy. G. & van Helden. (2001). IS*6110* insertions in *Mycobacterium tuberculosis*: predominantly into coding regions. *Journal of Clinical Microbiology*, **39**: 3423-3424.

Saraceni, V., King, B.S., Cavalcante, S.C., Golub, J.E., Lauria, L.M., Moulton, L.H., Chaisson, R.E. & Durovni, B. (2008). Tuberculosis as primary cause of death among AIDS cases in Rio de Janeiro, Brazil. *International Journal of Tuberculosis and Lung Disease,* **12:** 769-772.

Savine, E., Warren, R.M., van der Spuy, G.D., Beyers, N., van Helden, P.D., Locht, C. & Supply, P. (2002). Stability of variable-number tandem repeats of mycobacterial interspersed repetitive units from 12 loci in serial isolates of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology,* **40:** 4561-4566.

Scorpio, A. & Zhang, Y. (1996). Mutations in pncA, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. *Nature Medicine*, **2:** 662-667.

Sebban, M., Mokrousov, I., Rastogi, N. & Sola, C. (2002). A data-mining approach to spacer oligonucleotide typing of *Mycobacterium tuberculosis*. *Bioinformatics,* **18:** 235-243.

Seung, K.J., Omatayo, D.B., Keshavjee, S., Furin, J.J., Farmer, P.E. & Satti, H. (2009). Early outcomes of MDR-TB treatment in a high HIV-prevalence setting in Southern Africa. *PloS One*, **4:** e7186.

Sharma, P., Chauhan, D.S., Upadhyay, P., Faujdar, J., Lavania, M., Sachan, S., Katoch, K. & Katoch, V.M. (2008). Molecular typing of *Mycobacterium tuberculosis* isolates from a rural area of Kanpur by spoligotyping and mycobacterial interspersed repetitive units (MIRUs) typing. *Infection, Genetics and Evolution* **8:** 621-626.

Shean, K.P., Willcox, P.A., Siwendu, S.N., Laserson, K.F., Gross, L., Kammerer, S., Wells, C.D. & Holtz, T.H. (2008). Treatment outcome and follow-up of multidrug-resistant tuberculosis patients, West Coast/Winelands, South Africa, 1992-2002. *International Journal of Tuberculosis and Lung Disease,* **12:** 1182-1189.

Shin, S.S., Furin, J.J., Alcantara, F., Bayona, J., Sanchez, E. & Mitnick, C.D. (2006). Long-term follow-up for multidrug-resistant tuberculosis. *Emerging Infectious Diseases,* **12:** 687-688.

Shiraishi, Y., Katsuragi, N., Kita, H., Toishi, M. & Onda, T. (2008). Experience with pulmonary resection for extensively drug-resistant tuberculosis. *Interactive Cardiovascular and Thoracic Surgery,* **7:** 1075-1078.

Shisana O, Rehle T & Simbayi L.C (2005). South African national HIV prevalence, HIV incidence, behaviour and communication survey, 2005. *Human Sciences Research Council Press*, Cape Town.

Singh, J.A., Upshur, R. & Padayatchi, N. (2007). XDR-TB in South Africa: no time for denial or complacency. *PLoS Medicine,* **4:** e50.

Singh, S., Sankar, M.M. & Gopinath, K. (2007). High rate of extensively drug-resistant tuberculosis in Indian AIDS patients. *AIDS,* **21:** 2345-2347.

Singh, U.B., Arora, J., Suresh, N., Pant, H., Rana, T., Sola, C., Rastogi, N. & Pande, J.N. (2007). Genetic biodiversity of *Mycobacterium tuberculosis* isolates from patients with pulmonary tuberculosis in India. *Infection, Genetics and Evolution* **7:** 441-448.

Singh, U.B., Suresh, N., Bhanu, N.V., Arora, J., Pant, H., Sinha, S., Aggarwal, R.C., Singh, S., Pande, J.N., Sola, C., Rastogi, N. & Seth, P. (2004). Predominant tuberculosis spoligotypes, Delhi, India. *Emerging Infectious Diseases,* **10:** 1138-1142.

Skuce, R.A., McCorry, T.P., McCarroll, J.F., Roring, S.M., Scott, A.N., Brittain, D., Hughes, S.L., Hewinson, R.G. & Neill, S.D. (2002). Discrimination of *Mycobacterium tuberculosis* complex bacteria using novel VNTR-PCR targets. *Microbiology,* **148:** 519-528.

Small, P.M., Hopewell, P.C., Singh, S.P., Paz, A., Parsonnet, J., Ruston, D.C., Schecter, G.F., Daley, C.L. & Schoolnik, G.K. (1994). The epidemiology of tuberculosis in San Francisco.

A population-based study using conventional and molecular methods. *New England Journal of Medicine,* **330:** 1703-1709.

Small, P.M., Shafer, R.W., Hopewell, P.C., Singh, S.P., Murphy, M.J., Desmond, E., Sierra, M.F. & Schoolnik, G.K. (1993). Exogenous reinfection with multidrug-resistant *Mycobacterium tuberculosis* in patients with advanced HIV infection. *New England Journal of Medicine*, **328:** 1137-1144.

Smith, N.H., Hewinson, R.G., Kremer, K., Brosch, R. & Gordon, S.V. (2009). Myths and misconceptions: the origin and evolution of *Mycobacterium tuberculosis*. *Nature Reviews, Microbiology,* **7:** 537-544.

Soini, H., Pan, X., Teeter, L., Musser, J.M. & Graviss, E.A. (2001). Transmission dynamics and molecular characterization of *Mycobacterium tuberculosis* isolates with low copy numbers of IS*6110*. *Journal of Clinical Microbiology,* **39:** 217-221.

Sola, C., Filliol, I., Gutierrez, M.C., Mokrousov, I., Vincent, V. & Rastogi, N. (2001). Spoligotype database of *Mycobacterium tuberculosis*: biogeographic distribution of shared types and epidemiologic and phylogenetic perspectives. *Emerging Infectious Diseases,* **7:** 390-396.

Sola, C., Filliol, I., Legrand, E., Mokrousov, I. & Rastogi, N. (2001). *Mycobacterium tuberculosis* phylogeny reconstruction based on combined numerical analysis with IS1081, IS6110, VNTR and DR-based spoligotyping suggests the existence of two new phylogeographical clades. *Journal of Molecular Evolution,* **53**: 680-689.

Sola, C., Filliol, I., Legrand, E., Lesjean, S., Locht, C., Supply, P. & Rastogi, N. (2003). Genotyping of the *Mycobacterium tuberculosis* complex using MIRUs: association with VNTR and spoligotyping for molecular epidemiology and evolutionary genetics. *Infection, Genetics and Evolution,* **3:** 125-133.

Sreevatsan, S., Stockbauer, K.E., Pan, X., Kreiswirth, B.N., Moghazeh, S.L., Jacobs, W.R.,Jr, Telenti, A. & Musser, J.M. (1997). Ethambutol resistance in *Mycobacterium tuberculosis*: critical role of embB mutations. *Antimicrobial Agents and Chemotherapy,* **41:** 1677-1681.

Statistics South Africa (2009). *Mid-year population estimates 2009*. Statistics South Africa. Available at: www.statssa.gov.za/pulications/P0302/P03022009.pdf. Accessed January 2010.

Stavrum, R., Mphahlele, M., Ovreas, K., Muthivhi, T., Fourie, P.B., Weyer, K. & Grewal, H.M. (2009). High diversity of *Mycobacterium tuberculosis* genotypes in South Africa and preponderance of mixed infections among ST53 isolates. *Journal of Clinical Microbiology,* vol. **47:** 1848-1856.

Streicher, E.M., Victor, T.C., van der Spuy, G., Sola, C., Rastogi, N., van Helden, P.D. & Warren, R.M. (2007). Spoligotype signatures in the *Mycobacterium tuberculosis* complex. *Journal of Clinical Microbiology,* **45:** 237-240.

Streicher, E.M., Warren, R.M., Kewley, C., Simpson, J., Rastogi, N., Sola, C., van der Spuy, G.D., van Helden, P.D. & Victor, T.C. (2004). Genotypic and phenotypic characterization of drug-resistant *Mycobacterium tuberculosis* isolates from rural districts of the Western Cape Province of South Africa. *Journal of Clinical Microbiology,* **42:** 891-894.

Sun, Z., Chao, Y., Zhang, X., Zhang, J., Li, Y., Qiu, Y., Liu, Y., Nie, L., Guo, A. & Li, C. (2008). Characterization of extensively drug-resistant *Mycobacterium tuberculosis* clinical isolates in China. *Journal of Clinical Microbiology*, **46:** 4075-4077.

Supply, P., Allix, C., Lesjean, S., Cardoso-Oelemann, M., Rusch-Gerdes, S., Willery, E., Savine, E., de Haas, P., van Deutekom, H., Roring, S., Bifani, P., Kurepina, N., Kreiswirth, B., Sola, C., Rastogi, N., Vatin, V., Gutierrez, M.C., Fauville, M., Niemann, S., Skuce, R., Kremer, K., Locht, C. & van Soolingen, D. (2006). Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology,* **44:** 4498-4510.

Supply, P., Lesjean, S., Savine, E., Kremer, K., van Soolingen, D. & Locht, C. (2001). Automated high-throughput genotyping for study of global epidemiology of *Mycobacterium tuberculosis* based on mycobacterial interspersed repetitive units. *Journal of Clinical Microbiology,* **39:** 3563-3571.

Supply, P., Mazars, E., Lesjean, S., Vincent, V., Gicquel, B. & Locht, C. (2000). Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. *Molecular Microbiology,* **36:** 762-771.

Supply, P., Warren, R.M., Banuls, A.L., Lesjean, S., Van Der Spuy, G.D., Lewis, L.A., Tibayrenc, M., Van Helden, P.D. & Locht, C. (2003). Linkage disequilibrium between minisatellite loci supports clonal evolution of *Mycobacterium tuberculosis* in a high tuberculosis incidence area. *Molecular Microbiology,* **47:** 529-538.

Tahaoglu, K., Torun, T., Sevim, T., Atac, G., Kir, A., Karasulu, L., Ozmen, I. & Kapakli, N. (2001). The treatment of multidrug-resistant tuberculosis in Turkey. *New England Journal of Medicine,* **345:** 170-174.

Talay, F., Kumbetli, S. & Altin, S. (2008). Factors associated with treatment success for tuberculosis patients: a single center's experience in Turkey. *Japan Journal of Infectious Diseases,* **61:** 25-30.

Tang, J.W., Li, Y., Eames, I., Chan, P.K. & Ridgway, G.L. (2006). Factors involved in the aerosol transmission of infection and control of ventilation in healthcare premises. *The Journal of Hospital Infection,* **64:** 100-114.

Tanveer, M., Hasan, Z., Siddiqui, A.R., Ali, A., Kanji, A., Ghebremicheal, S. & Hasan, R. (2008). Genotyping and drug resistance patterns of *M. tuberculosis* strains in Pakistan. *BMC Infectious Diseases,* **8:** 171.

Tazi, L., Reintjes, R. & Banuls, A.L. (2007). Tuberculosis transmission in a high incidence area: a retrospective molecular epidemiological study of *Mycobacterium tuberculosis* in Casablanca, Morocco. *Infection, Genetics and Evolution* **7:** 636-644.

Telenti, A., Philipp, W.J., Sreevatsan, S., Bernasconi, C., Stockbauer, K.E., Wieles, B., Musser, J.M. & Jacobs, W.R.,Jr. (1997). The emb operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol. *Nature Medicine,* **3:** 567-570.

Thiam, S., LeFevre, A.M., Hane, F., Ndiaye, A., Ba, F., Fielding, K.L., Ndir, M. & Lienhardt, C. (2007). Effectiveness of a strategy to improve adherence to tuberculosis treatment in a resource-poor setting: a cluster randomized controlled trial. *Journal of the American Medical Association,* **297:** 380-386.

Thierry, D., Brisson-Noel, A., Vincent-Levy-Frebault, V., Nguyen, S., Guesdon, J.L. & Gicquel, B. (1990). Characterization of a *Mycobacterium tuberculosis* insertion sequence, IS*6110*, and its application in diagnosis. *Journal of Clinical Microbiology*, **28:** 2668-2673.

Tortoli, E. & Marcelli, F. (2007). Use of the INNO LiPA Rif.TB for detection of *Mycobacterium tuberculosis* DNA directly in clinical specimens and for simultaneous determination of rifampin susceptibility. *European Journal of Clinical Microbiology & Infectious Diseases* **26:** 51-55.

Torun, T., Tahaoglu, K., Ozmen, I., Sevim, T., Atac, G., Kir, A., Gungor, G., Bolukbasi, Y. & Maden, E. (2007). The role of surgery and fluoroquinolones in the treatment of multidrug-resistant tuberculosis. *International Journal of Tuberculosis and Lung Disease,* **11:** 979-985.

Toungoussova, O.S., Caugant, D.A., Sandven, P., Mariandyshev, A.O. & Bjune, G. (2004). Impact of drug resistance on fitness of *Mycobacterium tuberculosis* strains of the W-Beijing genotype. *FEMS Immunology and Medical Microbiology,* **42:** 281-290.

Toungoussova, O.S., Sandven, P., Mariandyshev, A.O., Nizovtseva, N.I., Bjune, G. & Caugant, D.A. (2002). Spread of drug-resistant *Mycobacterium tuberculosis* strains of the Beijing genotype in the Archangel Oblast, Russia. *Journal of Clinical Microbiology,* **40:** 1930-1937.

Tsenova, L., Ellison, E., Harbacheuski, R., Moreira, A.L., Kurepina, N., Reed, M.B., Mathema, B., Barry, C.E., & Kaplan, G. (2005). Virulence of selected *Mycobacterium tuberculosis*

clinical isolates in the rabbit model of meningitis is dependent on phenolic glycolipid produced by the bacilli. *The Journal of Infectious Diseases,* **192:** 98-106.

Tudo, G., Gonzalez-Martin, J., Obama, R., Rodriguez, J.M., Franco, J.R., Espasa, M., Simarro, P.P., Escaramis, G., Ascaso, C., Garcia, A. & Jimenez De Anta, M.T. (2004). Molecular epidemiology of tuberculosis in the Bata and Malabo districts of Equatorial Guinea. *International Journal of Tuberculosis and Lung Disease,* **8:** 1458-1463.

Ulrichs, T. & Kaufmann, S.H. (2002). Mycobacterial persistence and immunity. *Frontiers in Bioscience,* **7:** 458-469.

Valcheva, V., Mokrousov, I., Narvskaya, O., Rastogi, N. & Markova, N. (2008a). Utility of new 24-locus variable-number tandem-repeat typing for discriminating *Mycobacterium tuberculosis* clinical isolates collected in Bulgaria. *Journal of Clinical Microbiology,* **46:** 3005-3011.

Valcheva, V., Mokrousov, I., Rastogi, N., Narvskaya, O. & Markova, N. (2008). Molecular characterization of *Mycobacterium tuberculosis* isolates from different regions of Bulgaria. *Journal of Clinical Microbiology,* **46:** 1014-1018.

van der Spuy, G.D., Warren, R.M., Richardson, M., Beyers, N., Behr, M.A. & van Helden, P.D. (2003). Use of genetic distance as a measure of ongoing transmission of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology,* **41:** 5640-5644.

van der Spuy, G.D., Warren, R.M. & van Helden, P.D. (2009). The role of molecular epidemiology in low-income, high-burden countries. *International Journal of Tuberculosis and Lung Disease,* **13:** 419-420.

van Deun, A., Salim, M.A., Das, A.P., Bastian, I. & Portaels, F. (2004). Results of a standardised regimen for multidrug-resistant tuberculosis in Bangladesh. *International Journal of Tuberculosis and Lung Disease,* **8:** 560-567.

van Deutekom, H., Supply, P., de Haas, P.E., Willery, E., Hoijng, S.P., Locht, C., Coutinho, R.A. & van Soolingen, D. (2005). Molecular typing of *Mycobacterium tuberculosis* by mycobacterial interspersed repetitive unit-variable-number tandem repeat analysis, a more accurate method for identifying epidemiological links between patients with tuberculosis. *Journal of Clinical Microbiology,* **43:** 4473-4479.

van Embden, J.D., Cave, M.D., Crawford, J.T., Dale, J.W., Eisenach, K.D., Gicquel, B., Hermans, P., Martin, C., McAdam, R. & Shinnick, T.M. (1993). Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *Journal of Clinical Microbiology,* **31:** 406-409.

van Embden, J.D., van Gorkom, T., Kremer, K., Jansen, R., van Der Zeijst, B.A. & Schouls, L.M. (2000). Genetic variation and evolutionary origin of the direct repeat locus of *Mycobacterium tuberculosis* complex bacteria. *Journal of Bacteriology,* **182:** 2393-2401.

van Rie, A. & Enarson, D. (2006). XDR tuberculosis: an indicator of public-health negligence. *Lancet,* **368:** 1554-1556.

van Rie, A., Warren, R., Richardson, M., Victor, T.C., Gie, R.P., Enarson, D.A., Beyers, N. & van Helden, P.D. (1999). Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment. *New England Journal of Medicine,* **341:** 1174-1179.

van Soolingen, D. (2001). Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. *Journal of Internal Medicine,* **249:** 1-26.

van Soolingen, D., Hermans, P.W., de Haas, P.E., Soll, D.R. & van Embden, J.D. (1991). Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. *Journal of Clinical Microbiology,* **29:** 2578-2586.

van Soolingen, D., Hoogenboezem, T., de Haas, P.E., Hermans, P.W., Koedam, M.A., Teppema, K.S., Brennan, P.J., Besra, G.S., Portaels, F., Top, J., Schouls, L.M. & van Embden, J.D. (1997). A novel pathogenic taxon of the *Mycobacterium tuberculosis* complex, Canetti: characterization of an exceptional isolate from Africa. *International Journal of Systematic Bacteriology*, **47:** 1236-1245.

van Soolingen, D., Qian, L., de Haas, P.E., Douglas, J.T., Traore, H., Portaels, F., Qing, H.Z., Enkhsaikan, D., Nymadawa, P. & van Embden, J.D. (1995). Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of east Asia. *Journal of Clinical Microbiology,* **33:** 3234-3238.

van Soolingen, D., van der Zanden, A.G., de Haas, P.E., Noordhoek, G.T., Kiers, A., Foudraine, N.A., Portaels, F., Kolk, A.H., Kremer, K. & van Embden, J.D. (1998). Diagnosis of *Mycobacterium microti* infections among humans by using novel genetic markers. *Journal of Clinical Microbiology,* **36:** 1840-1845.

Vareldzis, B.P., Grosset, J., de Kantor, I., Crofton, J., Laszlo, A., Felten, M., Raviglione, M.C. & Kochi, A. (1994). Drug-resistant tuberculosis: laboratory issues. World Health Organization recommendations. *Tubercle and Lung Disease,* **75:** 1-7.

Veen, J., Raviglione, M., Rieder, H.L., Migliori, G.B., Graf, P., Grzemska, M. & Zalesky, R. (1998). Standardized tuberculosis treatment outcome monitoring in Europe. Recommendations of a Working Group of the World Health Organization (WHO) and the European Region of the International Union Against Tuberculosis and Lung Disease (IUATLD) for uniform reporting by cohort analysis of treatment outcome in tuberculosis patients. *The European Respiratory Journal,* **12:** 505-510.

Velayati, A.A., Farnia, P., Masjedi, M.R., Ibrahim, T.A., Tabarsi, P., Haroun, R.Z., Kuan, H.O., Ghanavi, J., Farnia, P. & Varahram, M. (2009). Totally drug-resistant tuberculosis strains:

evidence of adaptation at the cellular level. *The European Respiratory Journal,* **34**: 1202-1203.

Verver, S., Warren, R.M., Munch, Z., Vynnycky, E., van Helden, P.D., Richardson, M., van der Spuy, G.D., Enarson, D.A., Borgdorff, M.W., Behr, M.A. & Beyers, N. (2004). Transmission of tuberculosis in a high incidence urban community in South Africa. *International Journal of Epidemiology,* **33:** 351-357.

Victor, T.C., de Haas, P.E., Jordaan, A.M., van der Spuy, G.D., Richardson, M., van Soolingen, D., van Helden, P.D. & Warren, R. (2004). Molecular characteristics and global spread of *Mycobacterium tuberculosis* with a western cape F11 genotype, *Journal of Clinical Microbiology,* **42:** 769-772.

Victor, T.C., Streicher, E.M., Kewley, C., Jordaan, A.M., van der Spuy, G.D., Bosman, M., Louw, H., Murray, M., Young, D., van Helden, P.D. & Warren, R.M. (2007). Spread of an emerging *Mycobacterium tuberculosis* drug-resistant strain in the Western Cape of South Africa. *International Journal of Tuberculosis and Lung Disease,* **11:** 195-201.

Victor, T.C., Warren, R., Beyers, N. & van Helden, P.D. (1997). Transmission of multidrug-resistant strains of *Mycobacterium tuberculosis* in a high incidence community. *European Journal of Clinical Microbiology and Infectious Diseases,* **16:** 548-549.

Wada, M. (2007). Anti-tuberculosis chemotherapy, *Kekkaku,* **82:** 771-781.

Wada, T., Maeda, S., Hase, A. & Kobayashi, K. (2007). Evaluation of variable numbers of tandem repeat as molecular epidemiological markers of *Mycobacterium tuberculosis* in Japan. *Journal of Medical Microbiology,* **56:** 1052-1057.

Warren, R., Hauman, J., Beyers, N., Richardson, M., Schaaf, H.S., Donald, P. & van Helden, P. (1996). Unexpectedly high strain diversity of *Mycobacterium tuberculosis* in a high-incidence community. *South African Medical Journal,* **86:** 45-49.

Warren, R.M., Richardson, M., Sampson, S.L., van der Spuy, G.D., Bourn, W., Hauman, J.H., Heersma, H., Hide, W., Beyers, N. & van Helden, P.D. (2001). Molecular evolution of *Mycobacterium tuberculosis*: phylogenetic reconstruction of clonal expansion. *Tuberculosis,* **81:** 291-302.

Warren, R.M., Victor, T.C., Streicher, E.M., Sampson, S.L., van Der Spuy, G.D., Richardson, M., Nguyen, D., Behr, M.A., Vctor, T.C. & van Helden, P.D. (2002). Microevolution of the direct repeat region of *Mycobacterium tuberculosis*: implications for interpretation of spoligotyping data. *Journal of Clinical Microbiology,* **40**: 4457-4465

Warren, R.M., Victor, T.C., Streicher, E.M., Richardson, M., Beyers, N., Gey van Pittius, N.C. & van Helden, P.D. (2004). Patients with active tuberculosis often have different strains in the same sputum specimen. *American Journal of Respiratory and Critical Care,* **169**: 610-614.

Wells, C.D., Cegielski, J.P., Nelson, L.J., Laserson, K.F., Holtz, T.H., Finlay, A., Castro, K.G. & Weyer, K. (2007). HIV infection and multidrug-resistant tuberculosis: the perfect storm. *Journal of Infectious Diseases,* **196:** S86-S107.

Weyer, K. (2004). *DOTs-Plus for* standardised management of multidrug-resistant tuberculosis in South Africa. Policy Guidelines., Medical Research Council, Pretoria.

Weyer, K., Lancaster, J., Brand, J., van der Walt, M. & Levin, J. (2004). *Survey of Tuberculosis Drug Resistance in South Africa 2001-2002,* MRC, Pretoria, South Africa.

Weyer, K., Groenewald, P., Zwarenstein, M. & Lombard, C.J. 1995, Tuberculosis drug resistance in the Western Cape, *South African Medical Journal,* **85:** 499-504.

WHO .(2009a). *Global Tuberculosis Control: a short update to the 2009 report. WHO/HTM/TB/2009.426,* WHO press, Geneva, Switzerland.

WHO .(2009b). *Global tuberculosis control: Epidemiology, strategy and financing.* WHO Press, Geneva Switzerland.

WHO .(2008a). *Anti-tuberculosis drug resistance in the world. Fourth global report. The WHO/IUATLD global project on anti-tuberculosis drug resistance surveillance, 2002 - 2007,* WHO Press, Geneva Switzerland.

WHO .(2008b). *Guidelines to the programmatic management of drug-resistant tuberculosis. Emergency update 2008. WHO/HTM/TB/2008.402,* WHO Press, Geneva Switzerland.

WHO .(2008c). *WHO three Is meeting. Report of a joint WHO HIV/AIDS and TB Department Meeting.*Available:http://www.who.int/hiv/pub/meetingreports/WHO_3Is_meeting_report.pdf [2010, May].

WHO .(2005). *Global tuberculosis control: Surveillance, Planning, Financing. WHO report 2005.,* WHO Press, Geneva Switzerland.

WHO .(2004). *Anti-tuberculosis drug resistance in the world. Report no3. WHO/HTM/TB/2004.343,*WHO Press, Geneva Switzerland.

Wilkinson, R.J., Llewelyn, M., Toossi, Z., Patel, P., Pasvol, G., Lalvani, A., Wright, D., Latif, M. & Davidson, R.N. (2000). Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case-control study, *Lancet,* **355:** 618-621.

Wilson, S.M., al-Suwaidi, Z., McNerney, R., Porter, J. & Drobniewski, F. (1997). Evaluation of a new rapid bacteriophage-based method for the drug susceptibility testing of *Mycobacterium tuberculosis. Nature Medicine,* **3:** 465-468.

Wirth, T., Hildebrand, F., Allix-Beguec, C., Wolbeling, F., Kubica, T., Kremer, K., van Soolingen, D., Rusch-Gerdes, S., Locht, C., Brisse, S., Meyer, A., Supply, P. & Niemann, S. (2008), Origin, spread and demography of the *Mycobacterium tuberculosis* complex. *PLoS Pathogens,* **4:** e1000160.

World Health Organization, International Union Against Tuberculosis and Lung Disease (2004). *Anti-tuberculosis drug resistance in the world*. Available:http://www.who. int/tb/publications/who_htm_tb_2004_343/en/ [2007, August 2007].

Yan, J.J., Jou, R., Ko, W.C., Wu, J.J., Yang, M.L. & Chen, H.M. (2005). The use of variable-number tandem-repeat mycobacterial interspersed repetitive unit typing to identify laboratory cross-contamination with *Mycobacterium tuberculosis*. *Diagnostic Microbiology and Infectious Disease*, **52:** 21-28.

Yeh, R.W., Hopewell, P.C. & Daley, C.L. (1999). Simultaneous infection with two strains of Mycobacterium tuberculosis identified by restriction fragment length polymorphism analysis. *International Journal of Tuberculosis and Lung Disease,* **3:** 537-539.

Yeh, R.W., Ponce de Leon, A., Agasino, C.B., Hahn, J.A., Daley, C.L., Hopewell, P.C. & Small, P.M. (1998). Stability of *Mycobacterium tuberculosis* DNA genotypes, *The Journal of Infectious Diseases,* **177:** 1107-1111.

Yew, W.W., Chan, C.K., Chau, C.H., Tam, C.M., Leung, C.C., Wong, P.C. & Lee, J. (2000). Outcomes of patients with multidrug-resistant pulmonary tuberculosis treated with ofloxacin/levofloxacin-containing regimens, *Chest,* **117:** 744-751.

Yu, J.K., Bong, C.N., Chen, S.C., Dzimadzi, R., Lu, D.Y., Makombe, S.D., Schouten, E.J., Kamoto, K. & Harries, A.D. (2008). Outcomes in HIV- infected patients who develop tuberculosis after starting antiretroviral treatment in Malawi. *International Journal of Tuberculosis and Lung Disease*, **12:** 692-694.

Zhang, L.X., Tu, D.H. & Enarson, D.A. (2000).The impact of directly-observed treatment on the epidemiology of tuberculosis in Beijing. *International Journal of Tuberculosis and Lung Disease,* **4:** 904-910.

Zhang, M., Gong, J., Yang, Z., Samten, B., Cave, M.D. & Barnes, P.F. (1999). Enhanced capacity of a widespread strain of *Mycobacterium tuberculosis* to grow in human macrophages. *The Journal of Infectious Diseases,* **179:** 1213-1217.

Zink, A.R., Grabner, W., Reischl, U., Wolf, H. & Nerlich, A.G. (2003). Molecular study on human tuberculosis in three geographically distinct and time delineated populations from ancient Egypt. *Epidemiology and Infection,* **130:** 239-249.

Zozio, T., Allix, C., Gunal, S., Saribas, Z., Alp, A., Durmaz, R., Fauville-Dufaux, M., Rastogi, N. & Sola, C. (2005). Genotyping of *Mycobacterium tuberculosis* clinical isolates in two

cities of Turkey: description of a new family of genotypes that is phylogeographically specific for Asia Minor. *BMC Microbiology,* **5:** 44.

Zuber, B., Chami, M., Houssin, C., Dubochet, J., Griffiths, G. & Daffe, M. (2008). Direct visualization of the outer membrane of mycobacteria and corynebacteria in their native state. *Journal of Bacteriology*, **190:** 5672-5680.