Molecular Characterization of Drug resistant *Mycobacterium tuberculosis* isolates from different regions in South Africa

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Declaration

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

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Summary

Application of molecular fingerprinting highlights transmission as the driving force behind the drug resistant epidemic in South Africa. Different strains dominate within different geographical regions, which is a reflection of micro-epidemics of drug resistance in the different regions. Cluster analysis shows that strains within the same strain family are different. The Beijing drug resistant strain family is the most dominant strain family (31%) in the Western Cape and of particular concern is the highly transmissible Beijing cluster 220 strain in the Western Cape communities. This cluster is widespread in the region and was previously identified in a MDR outbreak in a high school in Cape Town. Results suggest that the spread of Beijing drug resistant cluster 220 in the community was due to a combination of acquisition of drug resistant markers and transmission. This study also indicate that atypical Beijing can acquire drug resistance and become fit amongst HIV infected individuals. This is contrary to believe that atypical Beijing strains are not frequently associated with drug resistance and are attenuated. This implies that HIV levels the playing field for all drug resistant strains.

Mechanisms leading to the evolution of MDR-TB and XDR-TB in a mine setting with a well-functioning TB control program which exceeds the target for cure rates set by the WHO were investigated. Despite the excellent control program, an alarming increase in the number of drug resistant cases was observed in 2003 and subsequent years. Phylogenetic analysis shows sequential acquisition of resistance to first and second-line anti-TB drugs leading to the development of MDR and XDR-TB. Contact tracing indicate extensive transmission of drug resistant TB in the shafts, hospital and place of residence. This study shows that despite

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exceeding the WHO cure rate target, it was not possible to control the spread and amplification of drug resistance. In summary, as a top priority, future TB control plans need to address diagnostic delay more vigorously.

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Opsomming

Molukulêre tegnieke toon transmissie as die hoofrede vir die toename in die anti-tuberkulose middelweerstandigheid epidemie in Suid-Afrika. Die verskillende Mikobakterium tuberkulose rasse wat domineer in verskillende areas is ‘n refleksie van middelweerstandige mikro-epidemies in verskillende gebiede. Analise van identiese rasgroepe demonstreer dat ras families bestaan uit verskillende rasse. Die Beijing middelweerstandige rasfamilie is die mees dominante familie in die Wes-Kaap (31% van monsters van middelweerstandige families) en van spesifieke belang is die hoogs oordraagbare Beijing 220 groep. Hierdie groep is die mees wydverspreide groep in die studie area en was voorheen geïdentifiseer tydens ‘n meervoudige middelweerstandige uitbreking in ‘n hoërskool in Kaapstad. Die resultate dui aan dat die Beijing middelweerstandige groep 220 in die gemeenskap versprei as gevolg van ‘n kombinasie van middelweerstand verwerwing en transmissie. Hierdie studie dui verder aan dat die atipiese Beijing ook middelweerstandigheid kan verwerf en hoogs geskik is vir infeksie veral in MIV geïnfekteerde individue. Hierdie data is in teenstelling met die algemene denke dat atipiese Beijing nie gereeld geassosieer word met middelweerstandigheid nie en dat dit dikwels geattenueer is. Dit beteken dat MIV die hoof faktor is wat alle middelweerstandige rasse kans gee om te versprei.

Hierdie studie het die meganisme wat lei tot die evolusie van middelweerstandigheid en “XDR-TB” in die myne ondersoek. Die myn besit ‘n goeie funksioneerde tuberkulose kontrole program wat alreeds die Wêreld Gesondheids Organisasie se mikpunt vir tuberkulose genesing oortref. Ten spyte van ‘n uitstekende tuberkulose kontrole program, is daar ‘n bekommerenswaardige toename in die aantal middelweerstandige tuberkulose gevalle waargeneem in 2003 en in die

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daaropvolgende jare. Filogenetiese analise wys dat opeenvolgende verwerwing van middelweerstandigheid teen eerste en tweede vlak anti-tuberkulose middels gelei het tot die ontwikkeling van meervoudige middelweerstandigheid en "XDR-TB". Die opsporing van kontakpersone om transmissie te bewys dui aan dat transmissie van middelweerstandige tuberkulose in die werk plek, hospitaal en woon plek plaasvind. Hierdie studie wys dat ongeag die feit dat die Wêreld Gesondheids Organisasie se genesings verwagtinge oortref is, dit steeds onmoontlik was om die verspreiding en amplifisering van middelweerstandigheid te beheer. 'n Top prioriteit vir tuberkulose kontrole planne in die toekoms behoort die vertraging van diagnose sterk aan te spreek.

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Table of Contents

Title Page i

Declaration ii

Summary iii

Opsomming v

Acknowledgements vii

Table of Contents viii

List of Abbreviations xiii

Chapter 1

Title: The role of IS*6110* in the evolution of *M.tuberculosis* 1

1. Introduction 2

1.1. Polymorphisms in *M.tuberculosis* genome used as genetic markers in TB epidemiology 3

1.2. Molecular Methods currently used in TB epidemiology

1.2.1 IS*6110* Restriction Fragment Length Polymorphism (RFLP) Analysis 4

1.2.2 PGRS-RFLP 6

1.2.3 Spoligotyping 6

1.2.4 MIRU-VNTR Analysis 8

1.2.5 Other PCR-based methods 9

1.3. Comparative Strain Analysis based upon DNA fingerprinting Methods 10

1.4. TB Control 11

Stellenbosch University http://scholar.sun.ac.za

1.5. Molecular Biological Methods used for detection of Drug Resistance 14

Tables and Figures 18-23

Problem Statement 24

Hypothesis

Aim of the study

Experimental Approach

References 25

Stellenbosch University http://scholar.sun.ac.za

Chapter 2

Title: Materials and Methods 37

2. Materials and Methods 37

2.1. Section 1 (Methodology) 38

A) IS6110 RFLP 38

Analysis of IS*6110* genotypes 48

B) Spoligotyping 50

C) MIRU-VNTR 52

D) Polymerase Chain Reaction Amplification of Drug Resistant Genes 56

E) Phylogenetic Analysis 56

2.2. Section 2 (Buffers and Solution used in the following methods) 57

A) IS*6110* RFLP 57

B) Spoligotyping 59

C) MIRU-VNTR 60

D) PCR Amplification of Drug Resistant Genes 60

References 61

Stellenbosch University http://scholar.sun.ac.za

Chapter 3

Title: Emergence of Extensively drug resistant tuberculosis in a well-functioning tuberculosis control program 94

Summary 95

3.1. Introduction 97

3.2. Materials and Methods 99

3.2.1. Study Setting and Population 99

3.2.2. Genotyping 101

3.3. Results 103

3.4. Discussion 106

Tables and Figures 109

Acknowledgements 115

References 116

Stellenbosch University http://scholar.sun.ac.za

Chapter 4

Title: The dynamics of drug resistant Mycobacterium tuberculosis strains in the Western and Southern Cape of South Africa 118

4.1. Introduction 119

4.2. Materials and Methods 121

4.2.1. Study Design and Treatment features 122

4.2.2. Study Settings 122

4.2.3. Definitions 126

4.2.4. IS*6110* RFLP Analysis 127

4.2.5. Quality Control 129

4.3. Results 131

4.3.1 Study 1 131

4.3.2 Study 2 134

4.3.3 Study 3 135

4.4. Discussion 136

References 140

Stellenbosch University http://scholar.sun.ac.za

List of Abbreviations

TB	Tuberculosis
MDR	Multi drug resistant
HIV	Human immunodeficiency syndrome
IS	Insertion sequence
RFLP	Restriction Fragment Length Polymorphism
LJ slant	Lowenstein Jensen slant
DR	Direct repeat
DVR	Direct variable repeat
PGRS	Polymorphic GC-rich Sequence
MIRU	Multiple interspersed repetitive units
VNTR	Variable number tandem repeats
PCR	Polymerase Chain Reaction
CDC	Centres for Disease Control
WHO	World Health Organization
DOTS	Directly observed treatment, short-course
INH	Isoniazid
RIF	Rifampicin
Emb	Ethambutol
Eto	Ethionamide
Z	Pyrazinamide
S	Streptomycin
K	Kanamycin
A	Amikacin
C	Capreomycin
XDR	Extreme drug resistance
SSCP	Single stranded conformational polymorphism
EDTA	Ethylenediaminetetraacetic acid Disodium salt Dihydrate
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
HCl	Hydrochloric acid
SDS	Sodium dodecyl sulphate

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- References of this thesis will be structured according to the instructions of Journal of Clinical Microbiology unless otherwise stated.
- The figures and tables for each chapter are sequentially listed.

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Chapter 1

The role of IS*6110* in the evolution of *Mycobacterium tuberculosis.*

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- My contributions

  AAF initiated the literature review and was actively part of the literature research as well as the manuscript preparation.

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1. Introduction

Mycobacterium tuberculosis (M.tuberculosis) is the causative agent of tuberculosis (TB), a respiratory disease responsible for over 2 million deaths annually, with an estimated one third of the world's population being latently infected (13). In industrialised countries the TB infection rate has decreased due to the availability of effective drugs as well as improved socio-economic conditions. However, poor public health systems in developing countries, the HIV/AIDS pandemic, and the emergence of multi-drug resistant (MDR) strains, have contributed to an ongoing increase in reported cases worldwide (19).

One of the main aspects of TB research is the epidemiology of *M.tuberculosis*, with the aim to document the disease dynamics in different groups of individuals (11). This will enable the epidemiologist to deduce the reason for the disease occurring in a particular setting. In recent years, molecular epidemiological methods have been used extensively in transmission studies of *M.tuberculosis*. With the release of the *M.tuberculosis* genome (12) our knowledge of this organism has increased significantly and we can now focus on its evolution and genetics. *M.tuberculosis* is one member of the *M.tuberculosis* complex (MTBC), which consists of *M.tuberculosis, M.bovis, M.africanum, M.pinnipedii, M.caprae, M.microti and M.canetti.* The members are closely related, demonstrated by the high (99.9%) sequence similarity on the nucleotide level (44), which may have resulted due to a recent evolutionary bottleneck. Although high genetic homogeneity between these members is evident, they all exhibit different phenotypes, induce different pathologies and demonstrate different host specificities. The exact evolutionary origin of

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M.tuberculosis is still unknown but previously it has been suggested that it derived from *M.bovis* (an organism infecting cattle) as a result of a cross-species jump (45). However, new evidence (27,39) suggest that *M.bovis* derived from *M.tuberculosis*.

1.1. Polymorphisms in *M.tuberculosis* genome used as genetic markers in TB epidemiology

Approximately a decade ago, the only molecular markers available to study the epidemiology of TB were drug susceptibility profiles and phage types. However, there were some limitations to these methods and in recent years, numerous genotyping methods have been developed based on DNA polymorphisms in the genome of the organism. The discovery of polymorphic regions within the genome has led to a whole new era of epidemiology based on molecular methods using these polymorphic regions as markers. These polymorphisms are generally found in non-coding regions with different frequencies demonstrated between different strain families. Thus, molecular epidemiology of *M.tuberculosis* utilizes specific genetic markers within the *M.tuberculosis* genome to study the distribution of *M.tuberculosis* strains as well as how the strain distribution changes over a period of time. Molecular methods are more informative than traditional methods and due to the rapidity of certain molecular methods, it is suitable for use in TB diagnosis.

For epidemiological studies, it is important that the marker chosen for a specific method must be suitable for the study setting. In the following pages some of the more common molecular methods used in the study of *M.tuberculosis* epidemiology are discussed.

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1.2. Molecular Methods currently used in TB epidemiology

1.2.1. IS*6110* RFLP Analysis

In 1990, Thierry et al (49) described a 1.36 kb insertion sequence (IS), which is only found within the MTBC. This sequence termed, (IS) *6110*, belongs to the IS3 family and is characterised by unique 28 bp imperfect terminal inverted repeats (TIRs). IS*6110* possesses a transposase that enable it to extract itself from the genome and re-insert into another genomic region and is also capable of copying itself for insertion at another position. This ability of the sequence to frequently 'jump' from one location to another result in IS*6110* displaying positional as well as numerical polymorphisms. Restriction fragment length polymorphism (RFLP) is a method based on this feature of IS*6110,* which is an extremely laborious and time-consuming technique. The start point for RFLP is bacterial growth on a specific medium, Lowenstein Jensen slant, for sufficient DNA to fingerprint. Restriction endonuclease, *PvuII*, cuts the DNA, which is then redissolved for gel electrophoresis to separate the cleaved DNA. This is followed by southern transfer of DNA to a specialized membrane followed by detection and visualization of RFLP patterns by autoradiography. The DNA bands represent the IS*6110* insertion elements within the genome of different isolates of *M.tuberculosis* (fig.1).

As a rule, transposition events of IS*6110* are common enough to allow differentiation between more distantly evolved strains but are still rare enough to show stability within more closely related strains. Therefore, this method does not only differentiate between

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strains but can determine whether epidemiological events were either recent (transmission) or distant (reactivation). Generally, recent transmission is expressed as a cluster (fig. 1, lanes 2 and 3), which is defined as two or more isolates demonstrating identical or related IS*6110* fingerprints. The varying rates at which different IS*6110* elements transpose may have important implications for the interpretation of epidemiological results and highlights the fact that a more thorough knowledge of the biology of IS*6110* and its relation to the emergence of specific *M. tuberculosis* strains is needed (16).

The use of this DNA fingerprinting method has proved to be crucial in distinguishing between endogenous reactivation and exogenous re-infection (25), detecting outbreaks in hospitals (14,20,38), in prisons (52), among health care workers (4,29) and within communities (55). Even though the standardized IS*6110* fingerprinting method (53) is the most widely used genotyping method in molecular epidemiological studies of *M. tuberculosis* due to its high discriminatory power, it has its shortcomings. This includes the laborious workload and the need for culture growth on Lowenstein-Jensen (LJ) slants of up to 4-6 weeks. Therefore, this method may not be suitable for diagnostic use since it may contribute to diagnostic delay and subsequently, treatment delay; however, it is suitable for an epidemiological research. Another shortcoming of this method is that discrimination is much lower for strains with <5 IS*6110* copy numbers (low copy number strains) than strains with >5 IS*6110* copy numbers (high copy number strains). Low copy number strains are evolutionary stable with regard to IS*6110* transposition and are less

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likely to exhibit evolving RFLP patterns. Thus, for low copy strains, other genotyping methods are preferred.

1.2.2. PGRS-RFLP

The polymorphic GC-rich Repetitive Sequence (PGRS) is a 96bp consensus sequence found in the *M.tuberculosis* genome. The technique used is similar to IS*6110* RFLP, thus it is time consuming and the workload is laborious. Like IS*6110* RFLP, it is also based on the number and position of these sequences in the genome and can be used to distinguish between low copy number strains (10). Unfortunately due to the complex fingerprints (as a result of the high frequency of PGRS copies in the genome), reproducibility and analysis of these fingerprints is extremely difficult (35). Therefore, the use of this method is restricted.

1.2.3. Spoligotyping

This DNA amplification-based typing technique is more rapid than RFLP and is also capable of detecting and typing *M.tuberculosis* in clinical specimens (24,31). A polymorphic region, containing numerous well-conserved 36-bp direct repeats (DRs) is unique in MTBC bacteria. These loci are interspersed with 34 - 41 bp long unique non-repetitive spacer sequences, which can be amplified through PCR using primers designed from the DR sequence (fig 2) (26). A single DR region along with the adjacent spacer is

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known as the Direct Variable Repeat (DVR). The first step in this methodology is PCR amplification of the DR region. This is followed by linkage of synthetic oligonucleotides, specific to each spacer, to an activated membrane in parallel lines. The PCR products are then hybridised perpendicular to the oligo lines, which can be visualised using chemoluminescence and autoradiography. Differentiation between *M.tuberculosis* isolates depend on the presence or absence of the spacer sequences (fig 3) (31).

It has been demonstrated that spoligotyping can aid in distinguishing whether a particular TB episode is due to relapse or re-infection (58). Except for the reproducibility of this method, other advantages of spoligotyping is its high throughput and that data can be easily compared between laboratories. The discriminative power of this method however, is much lower than RFLP (24), which means that the use of this method alone can lead to an overestimation of true transmission. An example is the Beijing/W lineage, which has earned a lot of attention based on its ability to frequently cause outbreaks (5). It consists of numerous families and can be subdivided into multiple strains using IS*6110* RFLP and MIRU-VNTR markers (40). However, the genotype of the Beijing strain remains identical when using the spoligotyping method.

According to the Centres of Disease Control (CDC) TB laboratory procedures, results of spoligotyping must be given as an octal code designation with a binary conversion as an intermediate (fig.4). This is to simplify the recording of spoligotypes and the report of results from genotyping laboratories to TB programs.

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1.2.4. MIRU-VNTR Analysis

Due to the time consuming factor of RFLP and the low discriminatory power of spoligotyping compared to RFLP, a new method has been developed, which may provide a viable alternative. Mycobacterial interspersed repetitive units (MIRUs) are 40-100 bp long minisatellite-like structures that were identified within the *M.tuberculosis* genome and were found to be located at 41 locations within the H37Rv genome (47). The polymorphisms within certain locations enable the differentiation of *M.tuberculosis* strains since MIRU copy number differences are evident between non-related *M.tuberculosis* isolates, therefore this genotyping method is based on the variable numbers of tandem repeats (VNTRs) of MIRUs (21,34). With this method, different sets of loci can be used for strain typing depending on the strain population. Currently, the system based on the 12 set loci is the most widely used (36,48). Other loci sets proposed for use in routine epidemiological analysis are the 15 loci set with discrimination equal to RFLP as well as the 9 loci set.

This technique is PCR based so it is rapid, it has a high throughput (2) and its output or the MIRU type is expressed as a 12 digit code (depending on the loci set used) (fig.5). This code can be analyzed and it facilitates exchange of data between laboratories (17).

MIRU-VNTR can be used as a tool to study the evolution of the *M.tuberculosis* genome (47). MIRUs are also a suitable marker for transmission studies in high incidence settings where clustering may be over represented.

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As described previously, a numbering system is used in the analysis of different MIRU strain types. Currently, these following 12 set loci are frequently used: 02, 04, 10, 16, 20, 23, 24, 26, 27, 31, 39 and 40. Each number in the 12 character designated code represents the number of repeats at each MIRU loci. If the number of repeats exceeds 9, a letter is incorporated in the code to avoid the use of double-digit numbers, e.g. 10 repeats = "a", 11 repeats = "b", etc. Occasionally, no repeats are present at any one of the MIRU loci, which is represented with a "0" and regions that are deleted are indicated by a dash (-).

1.2.5. Other PCR-based methods

Due to the limitations of IS*6110* RFLP, another rapid method based on PCR amplification was developed. Fast Ligation-Mediated PCR is an easily reproducible (33) method with discriminatory power that is slightly less than IS*6110* RFLP. However, the method is limited to genotyping high copy number strains as the same principle applied to IS*6110* RFLP is applied here. Clusters that are of epidemiological interest may contain specific polymorphisms that can be used for screening purposes by PCR, which is much more rapid than RFLP fingerprinting.

Recently, a study (30) documented transmission of the drug resistant Beijing cluster 220 strain in the Western Cape region. In this paper, a novel PCR based method (fig.6) was described, which amplified a genomic region that was specific to this cluster. When the results obtained from the PCR-based method were compared to the IS*6110* RFLP fingerprint patterns of cluster 220 patients, it was found that the results correlated well.

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Similarly, sequences that are specific to mycobacterium species can also be amplified through PCR as shown in figure 7 (57). The method is useful in studies showing the evolutionary pathway of mycobacterial species based on specific genomic deletions.

Today, molecular methods are increasingly applied to the control of TB and more methods are developed as more information on the *M.tuberculosis* genome becomes available. As a consequence, molecular methods have become an extremely valuable tool in the detection of drug resistance and in the understanding of the transmission dynamics of different *M.tuberculosis* strains including those that possess drug resistance.

1.3. Comparative Strain Analysis based upon DNA Fingerprinting Methods (Databases)

We have established a comprehensive RFLP database using the GelCompar II analysis software, which contain fingerprints of 4209 isolates taken from 2034 drug susceptible and drug resistant patients between January 1993-December 2004. During this time, 700 strains have been identified. With this program, it is possible to calculate the similarity between fingerprints of study samples based on mathematical algorithms, which will generate groupings of similar fingerprint patterns.

The international spoligotype database, SpolDB4, contain spoligopatterns of isolates collected globally (6). Our local spoligotype database is a collection of 7053 isolates obtained from various regions locally as well as globally. Strains that are new to the database (i.e. not previously identified) can be compared to strains within the SpolDB4 database and entered into the local database. As a result, these databases are updated

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regularly and are linked to a collective database containing clinical as well as socio-economic data of patients (treated as confidential) for research purposes

1.4. TB Control

In 1991, the World Health Organization (WHO) recommended an innovative strategy for the control of TB, called the Direct Observed Treatment Short-course (DOTS) program. In 1996, the DOTS program was implemented in South Africa and since then successful treatment rates have increased yet still remains low at 70% in 2004 (62). The DOTS strategy is built on the five following elements:

- Political commitment to effective TB control.
- Case detection by sputum smear microscopy among symptomatic people.
- Standardized treatment regimen of 6-8 months of short-course chemotherapy (SCC) with first-line anti-TB drugs, administered under proper case management conditions, including direct observation.
- Uninterrupted supply of all essential anti-TB drugs.
- Standardized recording and reporting system, allowing assessment of treatment results.

The DOTS strategy is the first line of defends against drug resistant TB. Patients that are drug susceptible can be cured in six to eight months with the first-line anti-TB drugs: isoniazid (INH), rifampicin (RIF), ethambutol (Emb), pyrazinamide (PZA) and

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streptomycin (Sm). Different levels of drug resistance exist, depending on the quantity and class of drugs to which an *M.tuberculosis* isolate demonstrates resistance. Multiple drug resistance implies *M.tuberculosis*, which shows resistance to more than one anti-TB drug. Multi-drug resistance (MDR) is defined as *M.tuberculosis* resistant to two of the most important first-line anti-TB drugs, RIF and INH. The standard MDR-TB treatment consists of second-line anti-TB drugs, since the first-line drugs, RIF and INH, are ineffective. Currently, it is estimated that more than 6000 new MDR-TB cases are diagnosed in the country each year (61).

Drug resistant TB is a man-made problem and is found globally (61). Drug resistance mainly develop through the improper use of antibiotics by drug-susceptible TB patients (60), which can then spread to secondary cases. This improper use includes, administration of improper treatment regimens by health care workers and failure to ensure that patients complete the whole course of treatment (15). The management initiative, DOTS-Plus is a strategy based upon the five elements of the DOTS program with the aim to prevent the further development and spread of MDR-TB. The DOTS-Plus strategy is only administered in selective regions where drug resistance has been observed with the priority to increase access to second-line anti-TB drugs especially in middle- and low- income countries.

The mechanism by which drug resistance emerges is through the sequential acquisition of mutations. Initially, a spontaneous mutation causes one organism to become naturally resistant to anti-tuberculosis drugs. Due to a selection process, such as a non-compliant

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patient, an overgrowth of the resistant population, containing the initial specific mutation, occurs. Subsequently, selection of sub-populations carrying these mutations conferring drug resistance may occur (43), which is known as “acquired” drug resistance. Therefore, the defining criterion of acquired drug resistance according to the WHO is that a patient with drug resistant TB has been treated one month or longer (1). In contrast, “primary resistance” is an indication of drug resistance in a new patient that had no previous treatment. Surveys have shown that drug resistance due to acquisition are more common than primary drug resistance (41). In the Western Cape however, we have found that most MDR epidemics was due to transmission (56). Van Rie et al (2000)(54) reported that clinical classification of drug resistant patients (patients that were previously treated) alone may be insufficient in interpreting drug resistant epidemics. With RFLP analysis, the study revealed that MDR strains were actually transmitted between patients that were previously treated. Therefore, it was suggested that acquired resistance be re-defined as “drug resistance in previously treated cases”, which would include cases in which drug resistance was truly acquired and cases through which drug resistance was transmitted.

Extreme Drug Resistant TB (XDR-TB) has recently emerged and has been defined as MDR-TB with additional resistance demonstrated to the second-line drugs; a Fluoroquinolone and one or more of the three injectable drugs Kanamycin (K), Amikacin (Am), and Capreomycin (C) (9). A joint global survey by the World Health Organization (WHO) and the Centres for Disease Control (CDC) identified XDR-TB in all regions of the world, thereby recognising this as a new threat to TB control (9). The first reported

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incidence of XDR-TB in South Africa was in Tugela Ferry, KwaZulu-Natal, where 52 immunocompromised TB patients died (23).

Despite efforts by DOTS-Plus program to treat and prevent the spread of drug resistance, the problem of MDR and XDR-TB has now become a global threat including in South Africa. The HIV epidemic exacerbates drug resistance since it leads to rapid disease progression in HIV-seropositive cases. Both MDR-TB (7) and XDR-TB (23), have been associated with HIV. However, there have also been reports demonstrating MDR-TB and XDR-TB being diagnosed among HIV-seronegative cases (55)(our unpublished data. "Emergence of XDR-TB in a South African gold mine"-Chapter 3). Therefore, as the precursor of XDR-TB, the management of MDR-TB must be re-evaluated since it is likely that wherever second-line anti-TB drugs are administered, XDR-TB may evolve.

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1.5. Molecular Biological Methods used for detection of Drug Resistance

In the past, susceptibility to anti-TB drugs relied on classical methods based on bacilli growth on culture media, often resulting in treatment delay. Therefore, rapid diagnosis of drug resistant as well as susceptible TB is an essential part in the control of TB. However, such methods must be cost effective especially for resource poor countries. PCR amplification based methods are fast and efficient and will shorten the delay period. Most cases of drug resistance are caused by known mutations in a specific gene that is associated with resistance to anti-TB drugs. This makes it relatively easy to detect these mutations by PCR amplification followed by sequencing (51). Indeed, Sekiguchi et al (2007) (42) demonstrated that the amount of time in which PCR followed by sequencing could be accomplished, was 6.5 hours. Real-time PCR is an easy and specific method used to screen for drug resistance. However, since probes are designed for specific mutations, some resistant isolates may be missed. This problem can be circumvented by using molecular methods in conjunction with classical culture methods (32).

Since MDR as well as XDR are important concerns for the clinician, early detection of drug resistance can be the most crucial tool in saving lives of those suspected of being infected. In settings with high MDR prevalence, detection of RIF resistance can be useful because in most instances RIF resistance is accompanied by INH resistance, i.e. it acts as a good indicator of MDR (22,50,59). Thus, early detection of RIF resistance as well as resistance to any other anti-TB drugs may be vital for a good TB control strategy. As mentioned above, amplification and sequencing of the gene, *rpoB*, which is associated

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with RIF resistance is a relatively easy and rapid method to use for detecting RIF resistance. Another, molecular test known as INNO-LiPA.Rif TB (Innogenetics, Belgium), rapidly detects RIF resistance within 2 days while simultaneously detecting *M.tuberculosis* in clinical specimens, therefore no culture is needed (51). This method has a high specificity however; the high cost of this test make it unsuitable for resource poor countries. The rapid diagnostic test, the Genotype MTBDR assay shows promise however, conventional drug susceptibility testing must be used in conjunction since not all mutations that confer resistance can be detected by this assay (28). The microscopic-observation drug susceptibility (MODS) assay detects both TB and MDR-TB directly from sputum with high specificity as well as more speed and reliability than identification using conventional methods (37).

Other method for detecting RIF resistance include heteroduplex analysis and single-stranded conformational polymorphism (SSCP) analysis but both these methods are cumbersome. One rapid, low cost method for detecting RIF resistant isolates is the microarray (8), which proved also useful as a screening tool for PZA resistance (18).

Genotyping methods are not used as a substitute for classical methods but rather these methods combined ensure rapid results and confirmation of drug susceptibility. Population-based genotyping studies indicate that some strains are more easily transmitted than other strains. Outbreak strains may thus have specific characteristics that make them more predisposed to transmit more frequently. With molecular epidemiological studies, it has been determined that most MDR-TB cases in the Western

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Cape region were due to transmission (56). Today, numerous micro-epidemics, as a result of MDR-TB transmission, are occurring throughout local communities (46,54,55), highlighting the need to be more vigilant in settings of high drug resistance and the need for better TB control strategies.

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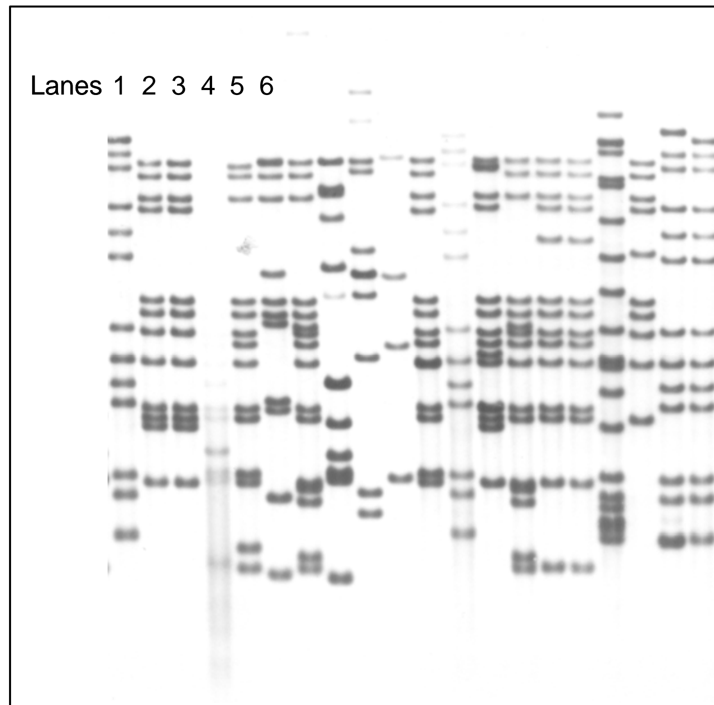

Fig 1.: Autoradiograph of IS*6110* DNA patterns of different *M.tuberculosis* isolates.

Legend: Each lane represent an *M.tuberculosis* isolate and each DNA band represents an IS*6110* copy. Lanes 2 and 3, from two different cases, are identical strains and are thus considered clustered. Unrelated strains will have different DNA patterns, for example, lanes 5 and 6. Lane 1 is the standard laboratory strain, *M.tuberculosis* 14323.

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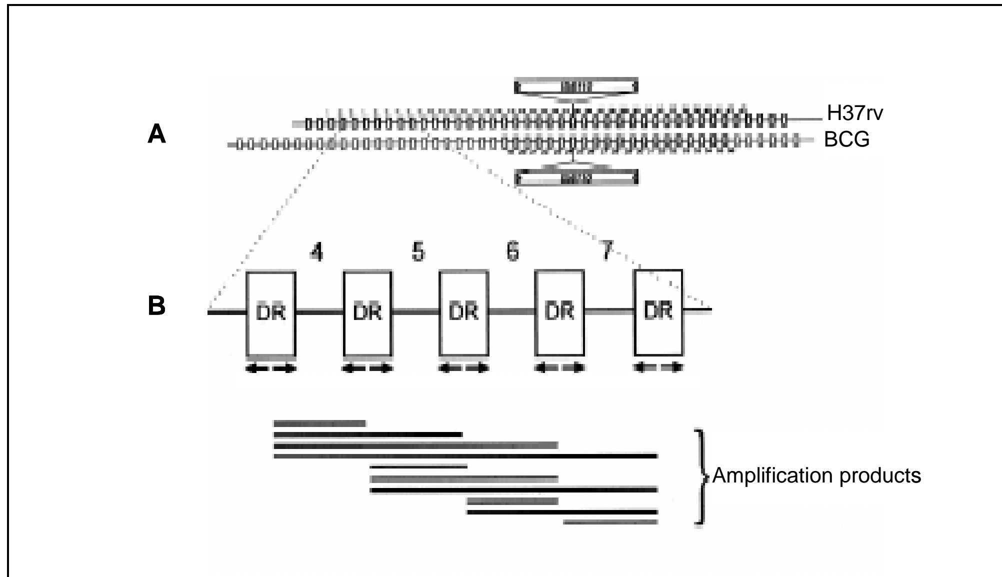

Fig.2: Schematic view of DR regions and spoligotyping.

Legend: A) The structure of the DR locus in genomes of H37rv (top) and BCG (bottom). B) In vitro amplification of DNA between the DR regions. Primers synthesized specifically for the DR regions, amplify the sequence spacers in between. The resultant PCR products differ in length based on two points 1) the product contains several spacers and the DRs in between, if the primers anneal to DR's not next to each other, and 2) the product itself can act as a primer, and become elongated with one or more DVR's.

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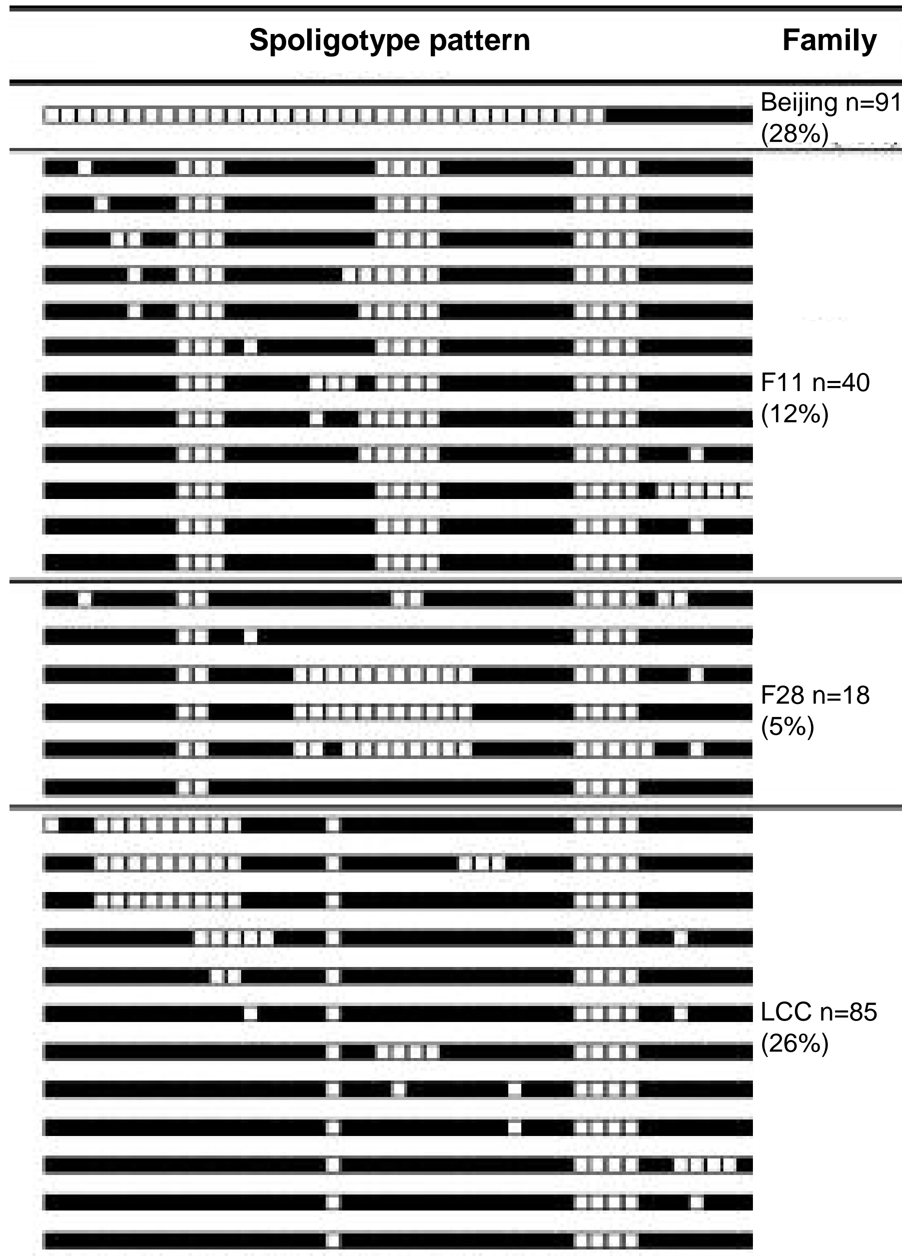

Fig 3.: Spoligotype patterns of drug resistant *M. tuberculosis* isolates from two health districts in the Western Cape, South Africa (46).

Legend: The black area within the fingerprint represents the presence of a spacer sequence and the blanks represent the absence of the spacers

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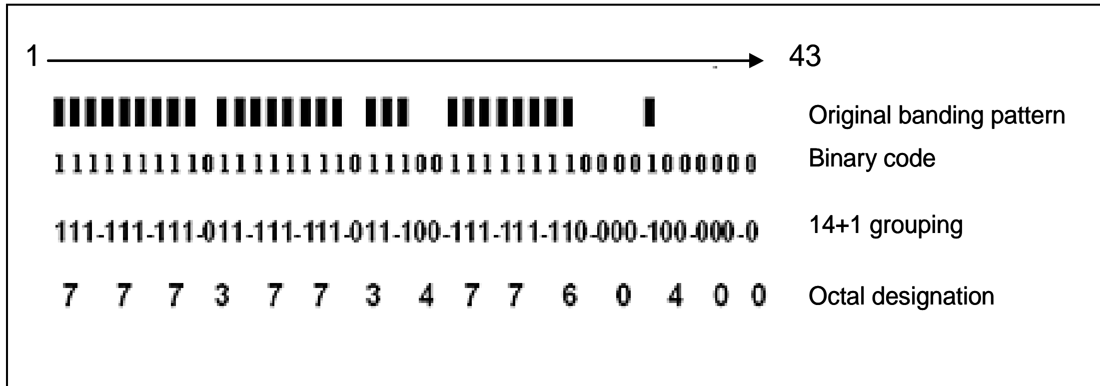

Fig 4.: Conversion of spacer sequences to an octal code.

Legend: The binary code is reverted to an octal designation by way of a two-step process. 1) The binary code is divided into 14 sets of three digits with an extra digit. 2) Each three digit code is converted to an octal code, while the extra digit remain as either 1 or 0. The octal designation for each different three digits is: 000 = 0; 001 = 1; 010 = 2; 011 = 3; 100 = 4; 101 = 5; 110 = 6; and 111 = 7.

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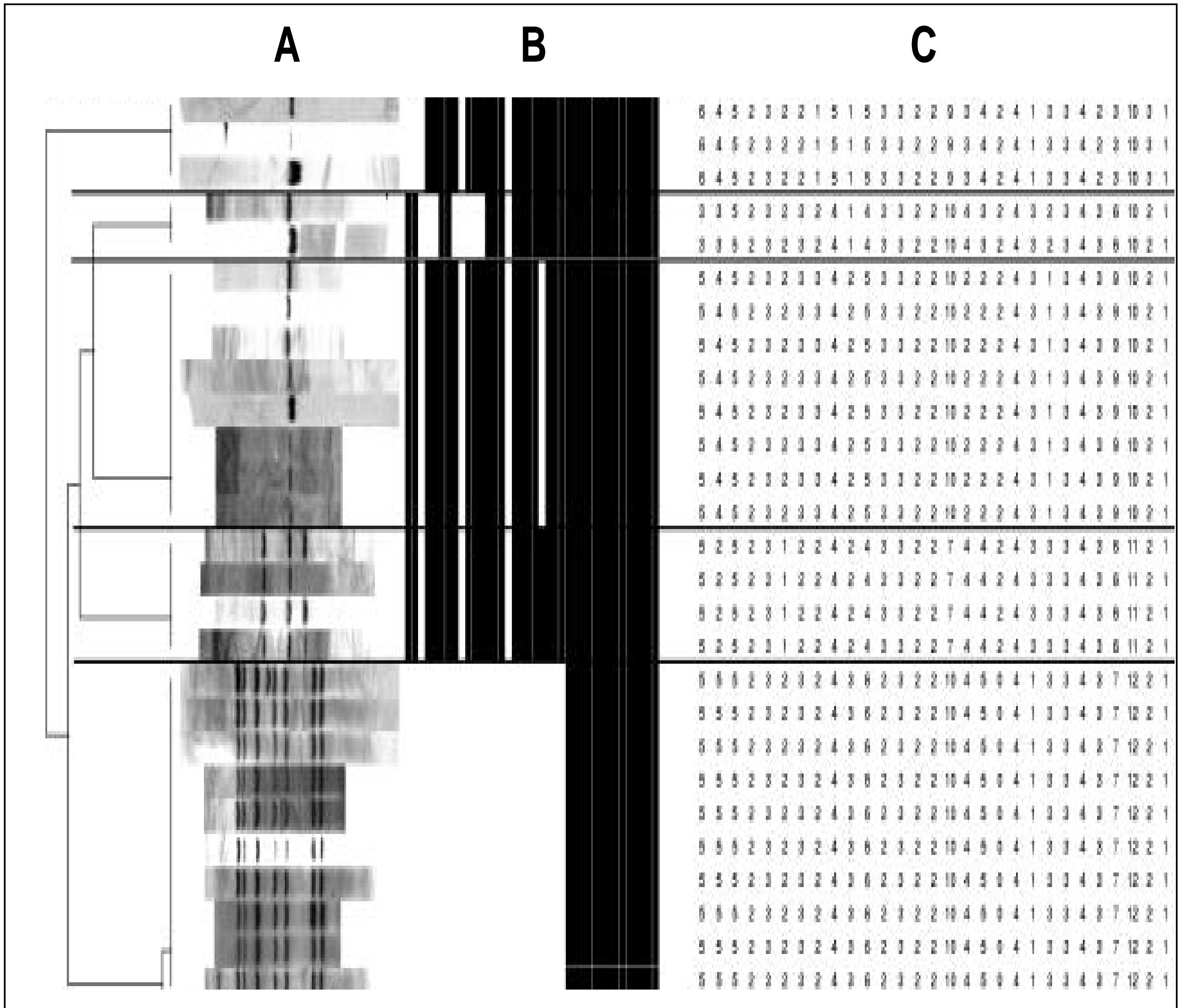

Fig 5.: Results of a study to investigate the validity of MIRU-VNTR in the classification of *M.bovis* using the 29 MIRU-VNTR loci set.

Legend: The three DNA fingerprint methods; A) IS*6110* RFLP, B) spoligotyping and C) MIRU-VNTR was used to analyze *M.bovis* isolates collected from cattle. UPGMA algorithm was used to build the dendogram on the extreme left, which is based upon the MIRU-VNTR profiles (on the extreme right). This dendogram show five distinct clusters among animals from different farms all with definite epidemiological links (3).

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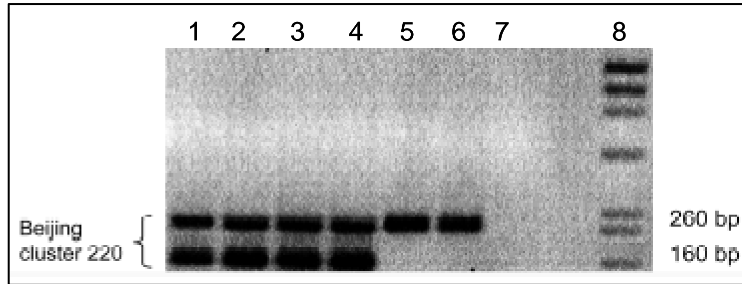

Fig 6.: *M.tuberculosis* strains belonging to the Beijing family amplified by PCR.

Legend: Cluster 220; lanes 1-4. Cluster 208; lane 5. H37Rv control; lane 6. Molecular marker; lane 8 (30).

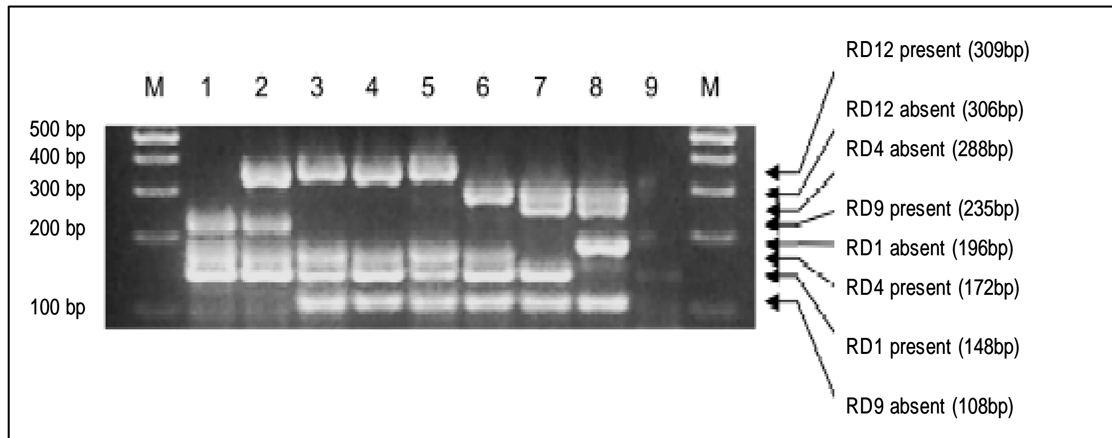

Fig 7.: Species-specific sequences differentiate between species and are often used to phylogenetically group them together.

Legend: Lanes 1 to 8 are *M.canetti*, *M.tuberculosis, M.africanum, M.microti, M.pinnepedii, M.caprae, M.bovis, M.bovis* BCG and lane 9, negative control. Lane M is the molecular weight marker. Within lane 1, no band for RD12 is visible since this region is deleted in *M.canetti* (57).

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Problem Statement

Little is known about the population structure of drug resistant *Mycobacterium tuberculosis* strains in South Africa.

Hypothesis

Application of molecular epidemiological methods will aid to understand what drives the drug resistant epidemic in South Africa.

Aim of the study

To use molecular methods to investigate the population structure of drug resistant *M.tuberculosis* isolates from different regions in South Africa.

Experimental Approach

Sputum specimens will be collected from different geographical regions in South Africa. These isolates will be characterized by IS*6110* restriction fragment length polymorphism (RFLP), spoligotyping and/or MIRU typing as well as drug resistant genotyping (only if stated). Strain comparison using the international SpolDB4 database will be used to identify new spoligopatterns whereas IS*6110* RFLP patterns will be entered into a local Gelcompar II database for analysis. IS*6110* RFLP will be used as a secondary typing method to determine the clonal spread of certain strains thereby investigating transmission dynamics of drug resistant *M.tuberculosis*.

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Reference List

1. 1998. Guidelines for surveillance of drug resistance in tuberculosis. WHO Geneva/IUATLD Paris. International Union Against Tuberculosis and Lung Disease. Int.J.Tuberc.Lung Dis. 2:72-89.

2. Allix, C., P. Supply, and M. Fauville-Dufaux. 2004. Utility of fast mycobacterial interspersed repetitive unit-variable number tandem repeat genotyping in clinical mycobacteriological analysis. Clin.Infect.Dis. 39:783-789.

3. Allix, C., K. Walravens, Saegerman c, J. Godfroid, P. Supply, and M. Fauville-Dufaux. 2006. Evaluation of the epidemiological relevance of variable-number tandem-repeat genotyping of Mycobacterium bovis and comparison of the method with IS6110 restriction fragment length polymorphism analysis and spoligotyping. J.Clin.Microbiol. 44:1951-1962.

4. Beck-Sague, C., S. W. Dooley, M. D. Hutton, J. Otten, A. Breeden, J. T. Crawford, A. E. Pitchenik, C. Woodley, G. Cauthen, and W. R. Jarvis. 1992. Hospital outbreak of multidrug-resistant Mycobacterium tuberculosis infections. Factors in transmission to staff and HIV-infected patients. JAMA 268:1280-1286.

5. Bifani, P. J., B. Mathema, N. E. Kurepina, and B. N. Kreiswirth. 2002. Global dissemination of the Mycobacterium tuberculosis W-Beijing family strains. Trends Microbiol. 10:45-52.

Stellenbosch University http://scholar.sun.ac.za

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

7. Campos, P. E., P. G. Suarez, J. Sanchez, D. Zavala, J. Arevalo, E. Ticona, C. M. Nolan, T. M. Hooton, and K. K. Holmes. 2003. Multidrug-resistant Mycobacterium tuberculosis in HIV-infected persons, Peru. Emerg.Infect.Dis. 9:1571-1578.

8. Caoili, J. C., A. Mayorova, D. Sikes, L. Hickman, B. B. Plikaytis, and T. M. Shinnick. 2006. Evaluation of the TB-Biochip oligonucleotide microarray system for rapid detection of rifampin resistance in Mycobacterium tuberculosis. J.Clin.Microbiol. 44:2378-2381.

Stellenbosch University http://scholar.sun.ac.za

9. CDC. 2006. Emergence of Mycobacterium tuberculosis with extensive resistance to second-line drugs--worldwide, 2000-2004. MMWR Morb.Mortal.Wkly.Rep. 55:301-305.

10. Chaves, F., Z. Yang, H. el Hajj, M. Alonso, W. J. Burman, K. D. Eisenach, F. Dronda, J. H. Bates, and M. D. Cave. 1996. Usefulness of the secondary probe pTBN12 in DNA fingerprinting of Mycobacterium tuberculosis. J.Clin.Microbiol. 34:1118-1123.

11. Coggon, G., G. Rose, and D. Barker. 1997. Epidemiology for the uninitated.

12. Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, B. G. Barrell, and a. et. 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 393:537-544.

13. Corbett, E. L., C. J. Watt, N. Walker, D. Maher, B. G. Williams, M. C. Raviglione, and C. Dye. 2003. The Growing Burden of Tuberculosis: Global Trends and Interactions With the HIV Epidemic. Arch.Intern.Med. 163:1009-1021.

14. Coronado, V. G., C. M. Beck-Sague, M. D. Hutton, B. J. Davis, P. Nicholas, C. Villareal, C. L. Woodley, J. O. Kilburn, J. T. Crawford, T. R. Frieden, and . 1993. Transmission of multidrug-resistant Mycobacterium tuberculosis among persons with

Stellenbosch University http://scholar.sun.ac.za

human immunodeficiency virus infection in an urban hospital: epidemiologic and restriction fragment length polymorphism analysis. J.Infect.Dis. 168:1052-1055.

15. Crofton, J., P. Chaulet, and D. Maher. 1997. Guidelines for the Management of drug resistant tuberculosis.

16. Dale, J. W., T. H. Tang, S. Wall, Z. F. Zainuddin, and B. Plikaytis. 1997. Conservation of IS6110 sequence in strains of Mycobacterium tuberculosis with single and multiple copies. Tuber.Lung Dis. 78:225-227.

17. de Viedma, D. G., N. A. Rodriguez, S. Andres, M. M. Lirola, M. J. Ruiz-Serrano, and E. Bouza. 2006. Evaluation of alternatives to RFLP for the analysis of clustered cases of tuberculosis. Int.J.Tuberc.Lung Dis. 10:454-459.

18. Denkin, S., D. Volokhov, V. Chizhikov, and Y. Zhang. 2005. Microarray-based pncA genotyping of pyrazinamide-resistant strains of Mycobacterium tuberculosis. J.Med.Microbiol. 54:1127-1131.

19. Dye, C., S. Scheele, P. Dolin, V. Pathania, and M. C. Raviglione. 1999. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. JAMA 282:677-686.

20. Edlin, B. R., J. I. Tokars, M. H. Grieco, J. T. Crawford, J. Williams, E. M. Sordillo, K. R. Ong, J. O. Kilburn, S. W. Dooley, K. G. Castro, and . 1992. An outbreak of multidrug-resistant tuberculosis among hospitalized patients with the acquired immunodeficiency syndrome. N.Engl.J.Med. 326:1514-1521.

Stellenbosch University http://scholar.sun.ac.za

21. Frothingham, R. and W. A. Meeker-O'Connell. 1998. Genetic diversity in the Mycobacterium tuberculosis complex based on variable numbers of tandem DNA repeats. Microbiology 144 (Pt 5):1189-1196.

22. Gamboa, F., P. J. Cardona, J. M. Manterola, J. Lonca, L. Matas, E. Padilla, J. R. Manzano, and V. Ausina. 1998. Evaluation of a commercial probe assay for detection of rifampin resistance in Mycobacterium tuberculosis directly from respiratory and nonrespiratory clinical samples. Eur.J.Clin.Microbiol.Infect.Dis. 17:189-192.

23. Gandhi, N. R., A. Moll, A. W. Sturm, R. Pawinski, T. Govender, U. Lalloo, K. Zeller, J. Andrews, and G. Friedland. 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.

24. Goguet de la Salmoniere YO, H. M. Li, G. Torrea, A. Bunschoten, J. Van Embden, and B. Gicquel. 1997. Evaluation of spoligotyping in a study of the transmission of Mycobacterium tuberculosis. J.Clin.Microbiol. 35:2210-2214.

25. Heldal, E., H. Docker, D. A. Caugant, and A. Tverdal. 2000. Pulmonary tuberculosis in Norwegian patients. The role of reactivation, re-infection and primary infection assessed by previous mass screening data and restriction fragment length polymorphism analysis. Int.J.Tuberc.Lung Dis. 4:300-307.

26. Hermans, P. W., D. van Soolingen, E. M. Bik, P. E. de Haas, J. W. Dale, and J. D. van Embden. 1991. Insertion element IS987 from Mycobacterium bovis BCG is

Stellenbosch University http://scholar.sun.ac.za

located in a hot-spot integration region for insertion elements in Mycobacterium tuberculosis complex strains. Infect.Immun. 59:2695-2705.

27. Hewinson, R. G., H. M. Vordermeier, N. H. Smith, and S. V. Gordon. 2006. Recent advances in our knowledge of Mycobacterium bovis: a feeling for the organism. Vet.Microbiol. 112:127-139.

28. Hillemann, D., S. Rusch-Gerdes, and E. Richter. 2006. Application of the Genotype MTBDR assay directly on sputum specimens. Int.J.Tuberc.Lung Dis. 10:1057-1059.

29. Jereb, J. A., R. M. Klevens, T. D. Privett, P. J. Smith, J. T. Crawford, V. L. Sharp, B. J. Davis, W. R. Jarvis, and S. W. Dooley. 1995. Tuberculosis in health care workers at a hospital with an outbreak of multidrug-resistant Mycobacterium tuberculosis. Arch.Intern.Med. 155:854-859.

30. Johnson, R., R. M. Warren, O. J. Strauss, A. Jordaan, A. A. Falmer, N. Beyers, H. S. Schaaf, M. Murray, K. Cloete, P. D. van Helden, and T. C. Victor. 2006. An outbreak of drug resistant Tuberculosis caused by a Beijing strain in the Western Cape, South Africa. Int.J.Tuberc.Lung Dis. 10:1412-1414.

31. Kamerbeek, J., L. Schouls, A. Kolk, M. van Agterveld, D. van Soolingen, S. Kuijper, A. Bunschoten, H. Molhuizen, R. Shaw, M. Goyal, and J. Van Embden. 1997. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. J.Clin.Microbiol. 35:907-914.

Stellenbosch University http://scholar.sun.ac.za

32. Kocagoz, T., Z. Saribas, and A. Alp. 2005. Rapid determination of rifampin resistance in clinical isolates of Mycobacterium tuberculosis by real-time PCR. J.Clin.Microbiol. 43:6015-6019.

33. Kremer, K., C. Arnold, A. Cataldi, M. C. Gutierrez, W. H. Haas, S. Panaiotov, R. A. Skuce, P. Supply, A. G. van der Zanden, and S. D. Van. 2005. Discriminatory power and reproducibility of novel DNA typing methods for Mycobacterium tuberculosis complex strains. J.Clin.Microbiol. 43:5628-5638.

34. Le Fleche, P., M. Fabre, F. Denoeud, J. L. Koeck, and G. Vergnaud. 2002. High resolution, on-line identification of strains from the Mycobacterium tuberculosis complex based on tandem repeat typing. BMC.Microbiol. 2:37.

35. Mathema, B., Kurepina, N., Bifani, P., and Kreiswirth, B. Molecular Epidemiology of Tuberculosis: Current Insights. Clin.Microbiol.Rev. 19[4], 658-684. 2006.
Ref Type: Journal (Full)

36. Mazars, E., S. Lesjean, A. L. Banuls, M. Gilbert, V. Vincent, B. Gicquel, M. Tibayrenc, C. Locht, and P. Supply. 2001. High-resolution minisatellite-based typing as a portable approach to global analysis of Mycobacterium tuberculosis molecular epidemiology. Proc.Natl.Acad.Sci.U.S.A 98:1901-1906.

37. Moore, D. A., Evans, C. A., and Gilman, R. H. Microscopic-Observation Drug-Susceptibility Assay for the diagnosis of TB. New England Journal of Medicine 355[15], 1539-1550. 12-10-2006.
Ref Type: Journal (Full)

Stellenbosch University http://scholar.sun.ac.za

38. Moro, M. L., A. Gori, I. Errante, A. Infuso, F. Franzetti, L. Sodano, and E. Iemoli. 1998. An outbreak of multidrug-resistant tuberculosis involving HIV-infected patients of two hospitals in Milan, Italy. Italian Multidrug-Resistant Tuberculosis Outbreak Study Group. AIDS 12:1095-1102.

39. Mostowy, S., D. Cousins, J. Brinkman, A. Aranaz, and M. A. Behr. 2002. Genomic deletions suggest a phylogeny for the Mycobacterium tuberculosis complex. J.Infect.Dis. 186:74-80.

40. Nikolayevskyy, V., K. Gopaul, Y. Balabanova, T. Brown, I. Fedorin, and F. Drobniewski. 2006. Differentiation of tuberculosis strains in a population with mainly Beijing-family strains. Emerg.Infect.Dis. 12:1406-1413.

41. Pablos-Mendez, A., M. C. Raviglione, A. Laszlo, N. Binkin, H. L. Rieder, F. Bustreo, D. L. Cohn, C. S. Lambregts-van Weezenbeek, S. J. Kim, P. Chaulet, and P. Nunn. 1998. Global surveillance for antituberculosis-drug resistance, 1994-1997. World Health Organization-International Union against Tuberculosis and Lung Disease Working Group on Anti-Tuberculosis Drug Resistance Surveillance. N.Engl.J.Med. 338:1641-1649.

42. Sekiguchi, J., T. Miyoshi-Akiyama, E. ugustynowicz-Kopec, Z. Zwolska, F. Kirikae, E. Toyota, I. Kobayashi, K. Morita, K. Kudo, S. Kato, T. Kuratsuji, T. Mori, and T. Kirikae. 2007. Detection of multidrug resistance in Mycobacterium tuberculosis. J.Clin.Microbiol. 45:179-192.

Stellenbosch University http://scholar.sun.ac.za

43. Somoskovi, A., L. M. Parsons, and M. Salfinger. 2001. The molecular basis of resistance to isoniazid, rifampin, and pyrazinamide in Mycobacterium tuberculosis. Respir.Res. 2:164-168.

44. Sreevatsan, S., X. Pan, K. E. Stockbauer, N. D. Connell, B. N. Kreiswirth, T. S. Whittam, and J. M. Musser. 1997. Restricted structural gene polymorphism in the Mycobacterium tuberculosis complex indicates evolutionarily recent global dissemination. Proc.Natl.Acad.Sci.U.S.A 94:9869-9874.

45. Stead, W. W., K. D. Eisenach, M. D. Cave, M. L. Beggs, G. L. Templeton, C. O. Thoen, and J. H. Bates. 1995. When did Mycobacterium tuberculosis infection first occur in the New World? An important question with public health implications. Am.J.Respir.Crit Care Med. 151:1267-1268.

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

47. Supply, P., J. Magdalena, S. Himpens, and C. Locht. 1997. Identification of novel intergenic repetitive units in a mycobacterial two-component system operon. Mol Microbiol 26:99-1003.

48. Supply, P., E. Mazars, S. Lesjean, V. Vincent, B. Gicquel, and C. Locht. 2000. Variable human minisatellite-like regions in the Mycobacterium tuberculosis genome. Mol.Microbiol. 36:762-771.

Stellenbosch University http://scholar.sun.ac.za

49. Thierry, D., A. Brisson-Noel, V. Vincent-Levy-Frebault, S. Nguyen, J. L. Guesdon, and B. Gicquel. 1990. Characterization of a Mycobacterium tuberculosis insertion sequence, IS6110, and its application in diagnosis. J.Clin.Microbiol. 28:2668-2673.

50. Traore, H., K. Fissette, I. Bastian, M. Devleeschouwer, and F. Portaels. 2000. Detection of rifampicin resistance in Mycobacterium tuberculosis isolates from diverse countries by a commercial line probe assay as an initial indicator of multidrug resistance. Int.J.Tuberc.Lung Dis. 4:481-484.

51. Traore, H., A. Van Deun, I. C. Shamputa, L. Rigouts, and F. Portaels. 2006. Direct detection of Mycobacterium tuberculosis complex DNA and rifampin resistance in clinical specimens from tuberculosis patients by line probe assay. J.Clin.Microbiol. 44:4384-4388.

52. Valway, S. E., S. B. Richards, J. Kovacovich, R. B. Greifinger, J. T. Crawford, and S. W. Dooley. 1994. Outbreak of multi-drug-resistant tuberculosis in a New York State prison, 1991. Am.J.Epidemiol. 140:113-122.

53. van Embden, J. D., M. D. Cave, J. T. Crawford, J. W. Dale, K. D. Eisenach, B. Gicquel, P. Hermans, C. Martin, R. McAdam, and T. M. Shinnick. 1993. Strain identification of Mycobacterium tuberculosis by DNA fingerprinting: recommendations for a standardized methodology [see comments]. J.Clin.Microbiol. 31:406-409.

Stellenbosch University http://scholar.sun.ac.za

54. van Rie, A., R. Warren, M. Richardson, R. P. Gie, D. A. Enarson, N. Beyers, and P. D. van Helden. 2000. Classification of drug-resistant tuberculosis in an epidemic area. Lancet 356:22-25.

55. van Rie, A., R. M. Warren, N. Beyers, R. P. Gie, C. N. Classen, M. Richardson, S. L. Sampson, T. C. Victor, and P. D. van Helden. 1999. Transmission of a multidrug-resistant Mycobacterium tuberculosis strain resembling "strain W" among noninstitutionalized, human immunodeficiency virus-seronegative patients. J.Infect.Dis. 180:1608-1615.

56. Victor, T. C., E. M. Streicher, C. Kewley, A. M. Jordaan, G. D. van der Spuy, M. Bosman, H. Louw, M. Murray, D. Young, P. D. van Helden, and R. M. Warren. 2007. Spread of an emerging Mycobacterium tuberculosis drug-resistant strain in the western Cape of South Africa. Int.J.Tuberc.Lung Dis. 11:195-201.

57. Warren, R. M., N. C. Gey Van Pittius, M. Barnard, A. Hesseling, E. Engelke, K. M. de, M. C. Gutierrez, G. K. Chege, T. C. Victor, E. G. Hoal, and P. D. van Helden. 2006. Differentiation of Mycobacterium tuberculosis complex by PCR amplification of genomic regions of difference. Int.J.Tuberc.Lung Dis. 10:818-822.

58. Warren, R. M., E. M. Streicher, S. Charalambous, G. Churchyard, G. D. van der Spuy, A. D. Grant, P. D. van Helden, and T. C. Victor. 2002. Use of spoligotyping for accurate classification of recurrent tuberculosis. J.Clin.Microbiol. 40:3851-3853.

Stellenbosch University http://scholar.sun.ac.za

59. Watterson, S. A., S. M. Wilson, M. D. Yates, and F. A. Drobniewski. 1998. Comparison of three molecular assays for rapid detection of rifampin resistance in Mycobacterium tuberculosis. J.Clin.Microbiol. 36:1969-1973.

60. Weyer K. Survey of Tuberculosis drug resistance, 2001-2002, Western Cape. http://www.sahealthinfo.org/tb/tbdrugresistance.htm . 2001.
Ref Type: Electronic Citation

61. WHO. 2004. Anti-TB drug resistance in the world:WHO/ IUATLD global project on Anti-Tuberculosis drug resistance surveillance, 1999-2002, Third Report .

62. World Health Organization. 2007. Global Tuberculosis Control: surveillance, planning, financing: WHO Report 2007.

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Chapter 2

Materials and Methods

(Methods will be given in detail in Section 1, whereas buffers and solutions will be given in Section 2 of this chapter.)

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Section 1

Methodology

A. IS*6110* RFLP

Extraction of DNA

1. A confluent Lowenstein-Jansen (LJ) slant culture of *M. tuberculosis* is required for a good DNA preparation.
2. Each LJ slant culture were heat inactivated at 80ºC for 1hour.
3. Labelled (both on the lid and the tube) 50 ml polypropylene tubes with the corresponding isolate number were used.
4. All work from now onwards was done in a P2 category laminar flow cabinet. This was done to protect against contamination of samples and also to protect the person doing the extraction against live bacteria.
5. Once the culture has cooled down (5 to 10 minutes), extraction of DNA can start. To each slant, 3ml of Extraction buffer was added to aid in the separation of clumps of bacteria and enables easier removal of the colonies from the slants. The buffer contains Tris, which is a pH buffer and EDTA, which is a chelating agent. The colonies were carefully scraped (without scraping too much media off) off the slant with a sterile 10µl loop. The extraction buffer was poured into a labelled sterile 50 ml polypropylene tube, which contained approximately 30 X 4mm glass balls (B&M Scientific, CGBE0004) to separate colonies during vortex, forming single cell suspension. A further 3 ml of extraction buffer was added to the slant. Any remaining bacterial colonies were scraped off and the buffer was swirled around in the bottle to wash off any bacterial matter on the side of the bottle. This was then also poured into the 50 ml tube. The tube was vigorously shaken and vortexed for approximately 2 minutes.
6. Proteinase K (10mg/ml) (Roche Molecular Biochemicals, 3115801), RNAse A (10mg/ml) (Roche Molecular Biochemicals, 109169) [pre-heat RNAse A at 100°C for 10 minutes to remove DNAse activity] was put on ice to thaw.

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7. Lysozyme (50mg/ml) (Roche Molecular Biochemicals, 837059) was left at room temperature to thaw.
8. Lysozyme with a volume of 500µl and 2.5µl of RNAse A were added to each tube.
9. Each tube was gently inverted to mix and then incubated at 37ºC for 2 hours.
10. After incubation, 600µl of 10x Proteinase K buffer and 150µl Proteinase K was added. Each sample was gently mixed and incubated overnight at 45ºC.
11. The following steps were done in a standard fume hood.
12. Phenol/Chloroform (Phenol equilibrated, Sigma Aldrich, P4557/ Chloroform, MERCK, UN1888) was used to extract DNA. A volume of 5ml of Phenol/Chloroform/Isoamyl-alcohol (25/24/1) was added to each bacterial preparation and the mixture was gently shaken intermittently for 30 minutes for a period of at least one hour. The Phenol/Chloroform/Isoamylalcohol was prepared by mixing 480ml chloroform with 20ml of isoamyl-alcohol, after which 400ml of this mixture was added to 400ml of phenol. Each sample was centrifuged at 3000rpm for 20 minutes. The supernatant was carefully aspirated using a P5000 (without any interface) pipette and added to a new "labelled" 50ml tube containing 5ml chloroform/isoamyl-alcohol. Each tube was gently mixed and centrifuged at 3000rpm for 20 minutes. The supernatant was again carefully aspirated and added to a new "labelled" 50 ml tube containing 600µl 3M Sodium-Acetate pH 5.5.
13. DNA was precipitated by adding 7ml ice-cold (-20ºC) Iso-propanol (Merck, 5075040LC). Each tube was gently inverted displaying precipitated DNA. With a "labelled" pasteur pipette, of which the front end has been melted closed, DNA was removed from 50ml tube. The glass pipette with DNA was placed into a 1.5ml eppendorf tube containing 1ml of 70% ethanol (absolute ethanol, Merck, 1.00983.2500) for approximately 1 minute to remove any salt. The glass pipette with DNA sticking to it was transferred to a new "labelled" 1.5ml eppendorf tube. DNA was left at room temperature until dry. Once dry, an aliquot (300 to 600 µl) of TE was added and the pellet was allowed to re-hydrate, after which it was gently shaken off the glass rod. DNA was allowed to dissolve by incubation at 60ºC or at 4ºC overnight.

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Extremely important: If the amount of DNA, after the precipitation step, was insufficient, the 50ml tube was placed in the freezer at -20ºC overnight. This was followed by centrifugation at 3000rmp for 20 minutes at 4ºC. The supernatant was carefully poured out (taking care not to pour the sediment out). To remove the salts 10ml of ice cold 70% ethanol was added. The sample was centrifuged under the same conditions as above for 10 minutes. The ethanol was poured out and tube was inverted on a blotting paper or paper towel. The tube was placed in 65ºC oven for about 10 minutes or at 37º*C* until dry. The pellet was re-hydrated and dissolved with TE pH 8.0 (300 to 600µl).

14. If DNA does not dissolve after extended incubation then it was re-purified as follows: For re-purification of DNA, 0.1 x volume Protenase K buffer and 1/40 x volume Protenase K were added to the mixture and gently mixed. DNA was incubated at 45 ºC overnight. Phenol/chloroform extraction was done, by adding half volume of Phenol/Chloroform to the bacterial preparation and the mixture was shaken well and often for about 20 minutes. Half the volume of chloroform/isoamyl-alcohol was aliquoted into a sterile 1.5ml eppendorf tube and 1/60 x volume 3M Sodium-Acetate (to adjust the pH) into another tube. The sample was centrifuged at 3000rpm for 20 minutes. The aqueous phase was carefully aspirated off, using a P1000 pipette (if the aqueous phase was very viscous, the front of the pipette tip was cut off), ensuring that none of the inter-phase or phenol-phase was taken. The aqueous phase was added to the chloroform/isoamyl-alcohol and the tube was marked. After thorough mixing, the sample was centrifuged again at 3000rpm for 20 minutes. The aqueous phase was collected and added to the Na-acetate.
15. The DNA was stored at –20 ºC.

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Determining the concentration of DNA

1. DNA was measured using the Nanodrop (ND-1000 Spectrophotometer, Inqaba biotec, S.A) according to the manufacturer's instructions. These measurements were then used to calculate DNA concentrations.
2. At least 2µl DNA of each sample was used (the optical density (OD) of 1 OD at 260nm is equivalent to 50µg/µl of double-stranded DNA).

Restriction Enzyme Digestion of the DNA

1. Usually 6 µg of *M. tuberculosis* DNA is used per digest with the restriction enzymes *Pvu*II (Laboratory Specialist Services, R0151-L). The reason for the 6µg is to do 2 reactions of 3µg each. Each eppendorf tube was clearly marked with the sample number and the restriction enzyme used.
2. The prescribed buffer for the enzyme was allowed to thaw.
3. DNA was allowed to thaw and dissolved uniformly by incubating at 65ºC for 30 minutes and followed by gentle mixing.
4. The digestion was done in a total volume of 100µl and the order of addition was as follows: 10 µl of 10x Restriction Buffer

   µl H_2O (accordingtocalculation)

   µl DNA (according to calculation)

   Vortex

   2.5 µl Restriction Enzyme (if the activity is 12u per µl, otherwise adapt to use 5u per g of DNA). The normal standard: 1U/µg = 1µg/µl DNA for plasmid DNA and 5µg/µl DNA for chromosomal DNA. The total volume of the digest mixture should be 100µl.

   Vortex

   (The digestion components should be added in this order otherwise the digestion process would not be optimal. When the restriction enzyme is added before the DNA, it will denature because the conditions for the functioning of the enzyme are not optimal.)

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5. The digestion mix was mixed just before and just after the addition of the enzyme. (Mixing of the components are important so that the components can be distributed evenly throughout the tube otherwise the digestion will not be optimal.) The digestion mixture was incubated overnight at 37ºC (minimum 3 hours).
6. After digestion, the reaction was incubated at 65ºC for 10 minutes to inactivate any remaining enzyme activity. (Inactivating the enzyme with heat won't interfere with $MgCl_2$ concentration. Since EDTA is a chelating agent, it will bind to the divalent cation (Mg^{2+}) and remove it. The Mg^{2+} is important for enzyme function if digestion was to be repeated.)

Gel Electrophoresis of Restricted DNA (Test gel)

1. To test whether DNA was digested, 4µl of 6x Loading Buffer and 8µl of the digested sample was mixed in a 0.5ml eppendorf tube. The mixture was loaded (8µl) onto a 1% agarose gel (Whitehead Scientific, D1-LE) dissolved in TBE. No marker was loaded, because only the amount of digested DNA were judged, not the size of the bands. The amount was judged according to the presence of a smear, which is evenly spread, and if digestion did not occur there will not be a smear. The test gel for *PvuII* is run overnight at 40 Volts and 19 Amps. The voltage is increased if the gel is run for 3- 4 hours (100-110 volts). The gel chamber is 20cm wide by 25 cm long.
2. The gel was stained with 50µl ethidium bromide (interchelating agent) in 500ml of TBE running buffer with constant shaking for 30 minutes.
3. DNA was visualized by UV light (245 nm). (The gel picture was used to calculate the volume in which each sample DNA should be re-dissolved in before the fingerprinting gel is prepared.)

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Gel Electrophoresis of Restricted DNA (fingerprinting gel)

1. If DNA was digested, it was precipitated by adding 10µl 3M Sodium Acetate (pH 5.2) and 330µl of 100% Ethanol.
2. It was mixed well by vortexing and incubated at –20ºC overnight.
3. To pellet DNA, each sample was centrifuged at 10000xg for 30 minutes at 4 ºC.
4. The supernatant was carefully aspirated off without disturbing the pellet. Approximately 50ul was left behind in the tube. To each tube, 500µl 70% ethanol was added to wash the sodium acetate salt out of the DNA pellet.
5. Centrifugation of DNA was repeated at 10000xg for 30 minutes at 4 ºC.
6. DNA pellet was left to dry at room temperature and in moist instances, overnight.
7. Dried DNA pellets were re-dissolved in a specific volume of bromophenol blue buffer (standard 6x application buffer containing marker X) according to the ethidium bromide intensity of the test gel. The volume of the buffer added to each sample should be chosen relative to a chosen reference band intensity and volume. Samples with higher intensity bands will need a relatively higher volume of the buffer than the reference band and the lower intensity bands will require relatively lower volume of the buffer than the reference bands. (The volume of the reference band should be standard for each laboratory in all the analysis done. In our case 20µl was used as the reference volume and the volume added to each sample adjusted according to the band intensity as described above). The buffer was made as follows; 4ml of 6X loading buffer, 12ml Tris -EDTA buffer, 13.2µl Marker X (250ng/µl), which was aliquoted into 1.5ml eppendorf tubes and stored at -20ºC. DNA was allowed to dissolve in the buffer by incubation at 65ºC.
8. DNA (10 µl) that was re-dissolved was electrophoretically fractionated in a 0.8% agarose gel with TBE pH8.3 at 65 Volts overnight (until the bromophenol blue has migrated approximately 23 cm).
9. The fractionated DNA in the agarose gel was stained by incubating the agarose gel in 500ml TBE containing 50µl (10mg/ml ethidium bromide) for 30 minutes.
10. The gel was then photographed to determine the resolution of fractionation and the loading intensity.

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Southern Transfer of the Fingerprinting gel

1. DNA from a fingerprinting gel was transferred from the agarose onto a charged Nylon membrane (HybondN+, AEC Amersham, RPN 203B) by Southern Transfer.
2. Gloves were worn whenever handling the gel and nylon membrane.
3. After the gel was photographed, it was inverted into a plastic dish, using the gel tray. DNA was denatured in the gel by covering with denaturing buffer and gently shaken for 20 – 25 minutes. The denaturing solution was then sucked off the gel with a Venturi pump and the gel was covered with neutralizing buffer. The gel was again gently shaken for 20 – 25 minutes.
4. The membrane was labelled with a black ballpoint pen to allow future recognition.
5. Orientation marks were spotted onto the membrane to allow future alignment of resultant autoradiographs. Aliquots of 0,2µl were spotted in six positions on the membrane on the same side onto which the DNA had to be transferred.
6. The membrane was hydrated in H_2O and then transferred to a dish with 20x SSPE solution. The transfer of DNA was as described in "Molecular Cloning" (Sambrook, Fritsch, Maniatis).
7. The gel was carefully placed, face down onto a blotting tray covered with Whatman (3MM[Merck, 3030917]) blotting paper. The wells of the gel were removed by cutting with a surgical blade and trimmed at the bottom (if necessary). Air bubbles were removed under the gel by rolling over the gel with a 10ml pipette. The correctly pre-treated (see above) labelled membrane was placed (spotted side on the side containing the labelling, i.e. downwards) on top of its corresponding gel and air bubbles were removed if any were present.
8. The exposed portions or sides of the wet blot block (gel + membrane) were covered with strips of parafilm to ensure that the fluid flows through the gel rather than short-circuiting the gel.
9. Whatman blotting papers were cut to the size of the blot block and individually hydrated first in H_2O and then in 20 × SSPE. The papers (x2) were placed on the blot blocks. All air bubbles, if any, were removed.

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10. One pack of paper towels (2 inches high) was placed on top of the Whatman papers (i.e. one blot block = one pack paper towels).
11. A plastic gel tray with a 1000ml weight was placed on top of the towels. The blotting tray was filled with 20 × SSPE to within ½ inch from the top of the wet blot blocks.
12. The transfer was done overnight.
13. After transfer, the membrane was removed and baked at 80ºC for 2 hours (between 2 sheets of Whatman blotting paper). After baking, the membrane was sealed in a plastic sleeve and stored at 4ºC until further use.

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ECL labelling and hybridisation

It was done according to the manufactures instructions

1. Pre-hybridisation and hybridisation was done in a flat-bottomed plastic dish in a shaking water-bath (rpm). The hybridisation sleeve was kept in place by a sealed plastic bag, containing 500ml water, which also aid in the even distribution of the buffer over the membrane.
2. The membrane was sealed in a plastic bag with 40ml gold buffer, which acts as a blocking agent since it contains the blocking agent gelatin and NaCl, which determines the stringency of hybridisation. It is crucial that all air bubbles were removed. Pre-hybridised was done at 42ºC for a minimum of 1 hour.
3. The ECL labelling and detection kit (AEC Amersham, RPN3001) was used. The probe (*3'* or X [DNA Molecular Weight Marker X, Roche Molecular Biochemicals, 1498037001]) was made up to 15µl in a 0.5ml eppendorf tube.

PCR reaction for constructing *3'* probe

- The probe was amplified by PCR from the plasmid-DNA.
- Reaction mixture contained the following:

  195,5 µl of dH_2O

  100 µl Q buffer

  50 µl 10X buffer

  40 µl $MgCl_2$

  80 µl dNTP

  10 µl Forward primer - 5'-TCG GTC TTG TAT AGG CCG TTG-3' : 50pmol/mL.

  10 µl Reverse primer - 5'-ATG GCG AAC TCA AGG AGC AC-3' : 50 pmol/mL.

  2,5 µl Taq polymerase

  2 µl plasmid DNA pIS986

- This was mixed and amplified by PCR using the following cycle:

  Denaturation :95ºC for 15 minutes

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Annealing :94ºC for 1 minute, 62ºC for 1minutesute, 72ºC for 1 minute

Elongation :72ºC for 10 minutes

Amplification was confirmed by fractionation on 2% agarose gel after staining with ethidium bromide.

The amplified fragment was purified using the SV kit from Promega (Whitehead Scientific, #A9281). An equal volume of membrane binding buffer was added to the PCR reaction and mixed well. Solution was transferred to membrane and allowed to bind for 1 minute followed by gentle removal of solution through the membrane. The membrane was washed twice with 700ul wash buffer followed with 500ul wash buffer. The membrane cup was centrifuged for 5 minutes at 13000 rpm to remove excess wash buffer. Amplified DNA was eluted by adding 50ul H_2O (preheated to 65ºC). Amplicon concentration was determined as described above and stored at -20ºC.

4. Water (13µl) was added to 200ng DNA (did not use TE since labelling will not take place as N in Tris will compete with N-groups on DNA for cross-linking of the Horse Radish Peroxidase).
5. The probe was denatured at 100ºC for 5 minutes and immediately put on ice for a further 5 minutes.
6. Of the Labelling Mix (Horse Radish Peroxidase), 15µl of was added and mixed.
7. Of the Glutaraldehyde solution, 15µl of was added, mixed and incubated at 37ºC for 10 minutes.
8. The hybridisation bag was taken from the water-bath and cut open at one end. An automatic pipette was used to put the probe into the bag and the bag was sealed immediately. A pipette was rolled over the membrane in the bag to mix the labelled probe and the hybridisation buffer. The bag was sealed after excluding all air bubbles and put back in the plastic dish under the weight and incubated overnight at 42ºC in the shaking water-bath (72 rpm).
9. After hybridisation, the membrane was removed from the hybridisation bag for the washing steps. The membrane was washed twice at 42°C for 20 minutes each

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in a plastic dish with 400ml primary buffer. The second wash was recycled as the first wash of another membrane.

10. This was followed by 2 washes, 5 minutes each, at room temperature in 400ml 2 × SSC.
11. The membrane was then placed in a new plastic sleeve and all excess liquid removed.
12. Instead of 4ml as mentioned in the original protocol, 3ml of the two detection reagents were mixed in a McCartney tube and mixed.
13. These reagents were poured onto the membrane in the plastic sleeve. The solution was gently spread, by rolling with a pipette over the membrane for a period of 90 seconds and then pushed out of the bag.
14. The bag was sealed and exposed to X-ray film for an optimum period of time (varying between 1 minute to 2 hours). The film was initially exposed for a shorter period to assess the strength of the signal.
15. If membranes were needed to be repeated, stripping of the membranes consisted of a single wash with 100ºC 0.1% SDS until cool.

Analysis of IS*6110* genotypes

IS*6110* genotypes were analysed using Gelcompar II (Version 4.0, Applied Maths, BVBA, Kortrijk, Belgium). The unweighted pair group method with arithmetic mean (UPGMA), based on the Dice coefficient was used for cluster analysis with band-matching tolerance parameters allowing for a 5% shift in each pattern as a whole and a 0.5% variance in individual band positions. Strain families were classified according to a similarity index of 65%. Isolates were grouped into clusters (strains with identical IS*6110* genotype) and uniques (strains demonstrating IS*6110* genotype patterns that are not identical to any other IS*6110* genotype pattern). For quality control of DNA band assignment, additional analysis by colleagues was done.

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B. Spoligotyping

Table 1: Spoligotyping Polymerase Chain Reaction mix

PCR MIX	50µl
H_2O	23µl
10× buffer	5µl
$MgCl_2$	5µl
dNTPs (0.4 mM)	4µl
Dra (Isogen)*	4µl
DRb (Isogen)*	4µl
Tag polymerase (Promega)	0.5µl

*Isogen LIFE SCIENCE, Lagedijk Noord 18, 3401 VA IJsselstein.

PCR Cycles

95°C for 3 minutes

94°C for 1 minute

55°C for 1 minute

72°C for 30 seconds

72°C for 10 minutes

Cycles of ×30 but can vary from 28-35 cycles

1 After PCR amplification, 20µl PCR products were added to 150µl 2xSSPE/0.1%SDS.
2 The diluted PCR products were heat-denatured for 10 minutes at 99ºC and cooled on ice immediately.
3 The membranes (Isogen LIFE SCIENCE, Lagedijk Noord 18, 3401 VA IJsselstein) were washed at 57ºC in 2xSSPE/0.1%SDS for 5 minutes.
4 The membranes were placed on a support cushion in a miniblotter (Immunetics, Cambridge, MA 02139) with slots perpendicular to the line pattern.
5 The residual fluid was removed from the slots by aspiration.

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6. The slots were carefully filled with diluted denatured PCR products to avoid air bubbles.
7. Hybridisation occurred on a horizontal surface for 60 minutes at 57ºC.
8. The samples were removed from the blotter by aspiration.
9. The membranes were washed twice in 2xSSPE/0.5%SDS for 5-10 minutes at 57ºC.
10. Exactly 40ml 2xSSPE/0.5%SDS (42 ºC) was added to 10µl Strepavidine-peroxidase conjugate (500U/ml) (Isogen LIFE SCIENCE, Lagedijk Noord 18, 3401 VA IJsselstein), which was incubated at 42ºC for 45-60 minutes.
11. The membranes were washed twice with 2xSSPE/0.5%SDS at 42ºC for 5-10 minutes.
12. This was followed by two washes with 2xSSPE for 5 minutes at room temperature.
13. The membranes were then incubated in 20ml (10ml solution 1 + 10ml solution 2) ECL mix for 1.5 minutes.
14. Visualization of spacer sequences follows after exposure to film for 5-20 minutes.
15. For repeated use of membranes, the membranes were washed twice in 1% SDS at 80 ºC for 30 minutes to 1 hour
16. The membranes were then stored at 4 ºC in 20mM EDTA pH8 (overnight).

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C. MIRU-VNTR

Reaction mixture for Polymerase Chain Reaction amplification:
For the reaction to take place, 0.2µg template DNA was used. Of boiled cultures, 1µl DNA was used and if DNA was purified, 1µl of a 1 in 40 dilution was used. The following reagents were used in the reaction:
5 µl Q-Buffer, 2.5µl 10 x Buffer, 2µl 25 mM $MgCl_2$, 4µl 10 mM dNTP's, 1µl of each primer (50 pmol/µl) (for VNTR: dilute primers to 1 in 16), 0.125 µl HotStarTaq DNA polymerase (Qiagen, Germany) made up to 25 µl with H_2O.

In a separate demarcated room (no bacterial DNA was allowed in this room), all of the above reagents were added except for the DNA, into an eppendorf tube.

Reagents were added in the following order:

1. H_2O
2. Q-Buffer
3. 10 x Buffer
4. $MgCl_2$
5. dNTP's
6. primer (see below)

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Primers for MIRU loci

Alleles	loci	Primer sequence (5'-3')
MIRU 2F MIRU 2R	154	TGG ACT TGC AGC AAT GGA CCA ACT TAC TCG GAC GCC GGC TCA AAA T
MIRU 4F MIRU 4R	580	GTC AAA CAG GTC ACA ACG AGA GGA A CCT CCA CAA TCA ACA CAC TGG TCA T
MIRU 10F MIRU 10R	960	GTT CTT GAC CAA CTG CAG TCG TCC GCC ACC TTG GTG ATC AGC TAC CT
MIRU 16F MIRU 16R	1644	TCG GTG ATC GGG TCC AGT CCA AGT A CCC GTC GTG CAG CCC TGG TAC
MIRU 20F MIRU 20R	2059	TCG GAG AGA TGC CCT TCG AGT TAG GGA GAC CGC GAC CAG GTA CTT GTA
MIRU 23F MIRU 23R	2531	CTG TCG ATG GCC GCA ACA AAA CG AGC TCA ACG GGT TCG CCC TTT TGT C
MIRU 24F MIRU 24R	2687	CGA CCA AGA TGT GCA GGA ATA CAT GGG CGA GTT GAG CTC ACA GAA
MIRU 26F MIRU 26R	2996	TAG GTC TAC CGT CGA AAT CTG TGA C CAT AGG CGA CCA GGC GAA TAG
MIRU 27F MIRU 27R	3007	TCG AAA GCC TCT GCG TGC CAG TAA GCG ATG TGA GCG TGC CAC TCA A
MIRU 31F MIRU 31R	3192	ACT GAT TGG CTT CAT ACG GCT TTA GTG CCG ACG TGG TCT TGA T
MIRU 39F MIRU 39R	3192	CGC ATC GAC AAA CTG GAG CCA AAC CGG AAA CGT CTA CGC CCC ACA CAT
MIRU 40F MIRU 40R	802	GGG TTG CTG GAT GAC AAC GTG T GGG TGA TCT CGG CGA AAT CAG ATA

The mixture was vortexed followed by the addition of:

7. HotStarTaq DNA polymerase

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The mixture was vortexed again followed by addition of DNA in another room (should be free of PCR amplicons). After DNA has been added to the reaction mixture, samples were placed in vortex, ready for amplification.

Amplification:

1. incubation at 95°C for 15 minutes
2. 35 - 45 cycles at 94°C for 1 minute
3. annealing temperature for 1 minute,
4. 72°C for 1 minute
5. After the last cycle, the samples were incubated at 72°C for 10 minutes.

Primers and polymerase chain reaction conditions for VNTR's

MIRU loci	**Primer dilutions (normal primer stock = (50 pmol/µl)**	**12-loci set**	**TM**	**Cycles**
Miru 2	1/16	✔	62	35
Miru 4	1/16	✔	62	35
Miru 10	1/16	✔	62	35
Miru 16	1/16	✔	62	35
Miru 20	1/16	✔	62	35
Miru 23	1/16	✔	62	35
Miru 24	1/16	✔	62	35
Miru 26	1/16	✔	62	35
Miru 27	1/16	✔	62	35
Miru 31	1/16	✔	62	35
Miru 39	1/16	✔	62	35
Miru 40	1/16	✔	62	35

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After amplification, the PCR amplification products were electrophoretically fractionated in 3.0% agarose in 1xTBE pH 8.3 at 6V/cm for 4 hours. To each sample, 5ul of loading dye was added, mixed and 8µl of each mixture was added in a slot on the gel. After 4 hours, DNA were visualized by staining with ethidium bromide. The existence of an event was determined by the presence or absence and the size of the respective PCR product.

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D. PCR Amplification of Drug Resistant Genes

A PCR amplification based method was used for the detection of mutations followed by sequencing of amplified products with an ABI PRISM (model 377, Perkin Elmer, Foster City, CA, USA). Table 2 describes the primers designed for the mutations associated with resistance to various drugs. Mutations were screened in *katG* (INH), *inhA* promoter (INH), *rpoB* (RIF), *pncA* (PZA), *emb* (Emb) and *gyrA* (Ofx) genes.

Table 2: Primer sequences targeting specific genes

Gene	Primer name	Primer sequence	Size	Tm	Reference
katG	RTB 59 RTB 38	TGGCCGCGGCGGTCGACATT GGTCAGTGGCCAGCATCGTC	419	62	Victor et al 1999(3)
inhA	inhA P5 InhA P3	CGCAGCCAGGGCCTCGCTG CTCCGGTAACCAGGACTGA	246	55	Victor et al 1999(3)
rpoB	rpoB For rpoB Rev	TGGTCCGCTTGCACGAGGGTCAGA CTCAGGGGTTTCGATCGGGCACAT	437	78 76	Victor et al 1999(3)
pncA	pncA F pncA R	AGTCGCCCGAACGTATGGTG CAACAGTTCATCCCGGTTCG	615	62	Louw et al 2006(2)
embB	emb151 emb131	CGGCATGCGCCGGCTGATTC TCCACAGACTGGCGTCGCTG	260	64	Johnson et al 2006(1)
gyrA	gyrA R gyrA F	CTGGCGAGCCGAAGTTG GAGACCATGGGCAACTACCA	124	62	Current

Amplified PCR products were run on 1% gel in 1× TBE buffer at 100 V for 2 hours.
PCR products cleaned using ExoSAP-IT (AEC Amersham, 78201) followed by sequencing.

E. Phylogenetic Analysis

The non-synonymous single nucleotide polymorphisms (nsSNPs) were used to construct a phylogenetic tree. Analysis was done using the neighbour joining algorithm (PAUP 4.0*; Phylogenetic Analysis Using Parsimony (*Other Methods) Version 4b10. Sinauer Associates, Sunderland, Massachusetts) with bootstrapping to determine the statistical

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support for nodes within each phylogenetic reconstruction. The program, contree (PAUP 4.0*) was used to generate a consensus tree combined with the majority rule formula.

Section 2

Buffers and solutions

A. IS*6110* RFLP

Extraction buffer (1 litre)	50g Mono Sodium Glutamic Acid (MSG) 6.06g Tris 9.3g EDTA pH 7.4 with HCl
10× Proteinase K buffer (500ml)	6.05g Tris 9.3g EDTA 25g SDS
3M Sodium Acetate (3M Na-Ac) (100ml)	40.82g Na-Ac-3H_2O pH 5.5 with Glacial Acetic Acid
70% ethanol	700ml of absolute ethanol 300ml distilled H_2O
TE (1 litre)	1.211g Tris 0.372g EDTA pH 8.0
Application buffer (100ml)	30 ml 100% Glycerol Bromo-Phenol Blue 0.6g SDS TE to make up to 100ml

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10× TBE	216g Tris
(2 litre)	110g Boric Acid
	14.88g EDTA
1% Test Gel	3g DLE agar
	300ml 1× TBE
Blue Running Buffer with Marker X	6.6µl Marker X (Boeringer Mannheim)
	(To give total of 2ng per well)
	8 ml of 1:3 dilution of Running buffer
0.8% Proper Gel	2.4g SEAKEM agar
	300ml 1× TBE
Orientation Marker "Spotter"	2µl of 0.25µg/µl Marker X
	20µl of 1.25 µg/µl MTB DNA (H37Rv)
	(Total of 2.5µg DNA)
	23µl TE
	Total of 45µl
	Add 45µl 0.8M NaOH
Denaturing buffer	87.66g NaCl
(1 litre)	20g NaOH
Neutralization buffer	87.66g NaCl
(1 litre)	60.5g Tris
20× SSPE	175.3g NaCl
(1 litre)	27.6g $NaH_2PO_4.H_2O$
	7.4g EDTA
	pH 7.4 with NaOH pellets (approx. 8g)

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Nucleic Acid Transfer Buffer (20× SSC)	350.6g NaCl
(2 litre)	176.4g Tri-Sodium Citrate (hydrous)/
	Tri-Na Citrate (anhydrous)
	PH 7.0
Primary Wash for ECL Blots	720g Urea
(2 litres)	8g SDS
	25ml 20× SSC
Stripping buffer (0.1% SDS)	20ml 10% SDS
	Distilled H_2O up to 2 litres

B. Spoligotyping

10× SSPE	13.7g Sodiumhydrogen Phosphate 100mM
(1 litre)	105.19g Sodium Chloride -18M
	3.36g EDTA – 10mM
	pH 7.4
10% SDS	50g SDS
(500ml)	
0.5 M EDTA	93g EDTA
(500ml)	

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Working stock solutions

500ml 2×SSPE/0.1% SDS	100ml 10× SSPE
	5ml 10% SDS
1 litre 2×SSPE/0.5% SDS	200ml 10×SSPE
	50ml 10% SDS
1 litre 1% SDS	100ml 10% SDS
500ml 20mM EDTA	20ml 0.5M EDTA

C. MIRU-VNTR

3% PCR Gel	3g agarose
	300ml 1× TBE pH 8.3

D. PCR Amplification of Drug Resistant Genes

PCR gel	1.25g DLE agarose
	100ml 1× TBE pH 8.3

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Reference List

1. **Johnson, R., A. M. Jordaan, L. Pretorius, E. Engelke, S. G. van der, C. Kewley, M. Bosman, P. D. van Helden, R. Warren, and T. C. Victor**. 2006. Ethambutol resistance testing by mutation detection. Int.J.Tuberc.Lung Dis. **10**:68-73.

2. **Louw, G. E., Warren, R. M., Donald, P. R., Murray, M. B., Bosman, M, van Helden, P. D., Young, D. B., and Victor, T. C.** 2006. Frequency and implications of Pyrazinamide resistance in managing previously treated tuberculosis patients. Int.J.Tuberc.Lung Dis.
Ref Type: In Press

3. **Victor, T. C., A. M. Jordaan, A. van Rie, G. D. van der Spuy, M. Richardson, P. D. van Helden, and R. Warren**. 1999. Detection of mutations in drug resistance genes of Mycobacterium tuberculosis by a dot-blot hybridization strategy. Tuber.Lung Dis. **79**:343-348.

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Chapter 3

Emergence of Extensively Drug Resistant Tuberculosis despite a good control program

(Draft manuscript written according to the specifications of the journal Lancet.)

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AAF contributed to the study design and was directly responsible for the genotypic analysis of all of the isolates. AAF also contributed to the drug resistance genotyping and was responsible for the collation of both the clinical and genotypic data. AAF contributed to the writing of the manuscript.

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Summary

Background: Despite an excellent tuberculosis (TB) control program, extensively drug resistant TB (XDR-TB) has emerged in a gold mine located in the North West province of South Africa. We investigated the mechanisms leading to the development of MDR and XDR-TB in this setting.

Methods: TB control was in strict accordance to the WHO DOTS and DOTS-Plus guidelines and TB was proactively diagnosed by chest x-ray, smear and culture in this setting. The study cohort consists of TB cases infected with drug-resistant *Mycobacterium tuberculosis* strains. Demographic data were obtained from all cases and cultures were subjected to drug-susceptibility testing and genotyping by standard procedures.

Findings: Between 2003-2005, 128 cases were diagnosed with drug-resistant TB, of which 10% were isoniazid mono-resistant, 5% were multiple drug resistant and 85% were multi drug resistant TB (MDR-TB). Of the MDR-TB isolates, 5% were XDR-TB. The drug-resistant epidemic resulted from importation, acquisition and transmission. MDR-TB was primarily transmitted, however, risk factors for transmission were over-whelmed by the incidence of HIV co-infection. Contrary to previous findings, a large proportion of HIV co-infected cases were smear positive, perpetuating the epidemic. Phylogenetic analysis of the largest cluster showed how drug-resistance was sequentially acquired possibly as a direct consequence of treatment guidelines.

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Interpretation: TB control measures over and above those recommended by the WHO are necessary to control the spread of drug-resistant TB in a setting with a high incidence of HIV. In the study setting, efforts made by the TB control programme were compromised by an influx of disease, emphasising the need to ensure well functioning TB control programs in all settings. Failure to rapidly diagnose drug-resistant TB lead to transmission thereby highlighting the need to address clinical as well as laboratory diagnostic delay more vigorously. The absence of rapid diagnostics promoted the acquisition of additional resistance through inappropriate treatment regimes, culminating in the evolution of XDR-TB.

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3.1. Introduction

Failure to implement proper tuberculosis (TB) control programs and correctly manage TB cases has been ascribed as the primary reason for the emergence of drug resistant TB (1). Drug resistance evolves through the sequential acquisition of mutations in specific genes of the bacterium, *Mycobacterium tuberculosis (M. tuberculosis)*, and the subsequent selection of sub-populations carrying mutations conferring drug resistance (2). Resistance to the two first-line anti-TB drugs, isoniazid (INH) and rifampicin (RIF) with or without resistance to other first-line drugs has been termed Multi Drug Resistant TB (MDR-TB) and is of particular concern to TB control due to the cost and duration of treatment. Globally, an estimate of 424,203 MDR-TB cases are diagnosed each year (3), while in South Africa it is estimated that more than 6000 new MDR-TB cases are diagnosed each year (4). Together, this demonstrates the inability of TB control strategies and programmes to ensure adherence and completion of TB treatment regimens to restrict the development and spread of this difficult-to-treat form of TB disease.

More recently, concern at a global level has been raised over the emergence of Extensively Drug Resistant TB (XDR-TB) (5). XDR-TB has been defined as MDR-TB with additional resistance to a Fluoroquinolone and one or more of the injectable drugs Kanamycin (K), Amikacin (A), and Capreomycin (C) (6). A joint global survey by the World Health Organization (WHO) and the Centres for Disease Control (CDC) identified XDR-TB in all regions of the world, thereby recognising this as a new threat to TB control (7). In South

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Africa, an XDR-TB outbreak involving fifty-three cases (8), raised concern because of the high mortality of this disease among HIV co-infected patients.

Drug resistance surveys in South Africa have suggested that acquired resistance was the main cause of MDR-TB and thereby implied poor patient compliance (9). In contrast, molecular epidemiological studies have demonstrated that MDR-TB may be primarily transmitted (10), possibly as a result of diagnostic delay. In 2003, an increase in the number of MDR-TB cases was noted at a mine hospital supplying comprehensive health care to a number of mines in the region. However, despite a Directly Observed Therapy Short-course (DOTS) based TB control program that achieves WHO targets for successful outcomes for new sputum smear positive TB (averaging 89%), the incidence of TB and drug resistant TB continued to increase.

In this study, we used a molecular epidemiological approach together with clinical and epidemiological data to understand how sequential acquisition of resistance could lead to the evolution of MDR-TB and XDR-TB within a well-functioning TB control program.

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3.2. Materials & Methods

This study was approved by the ethics committee (IRB) of Stellenbosch University, Tygerberg, South Africa (Ref. 2002/C118).

3.2.1. Study Setting and Population

This study was done in a gold mine (between the period of December 2002 to November 2005) in the North West province, South Africa. The TB control program in this setting has been modelled on the WHO recommendations for TB programs, the South African National TB Control Program and the Guidelines for TB Control in the Mining Industry of the Department of Mineral and Energy Affairs of South Africa. All employees are subjected to a 6 monthly chest x-ray for occupational lung disease screening. The bacteriological diagnosis of Pulmonary TB (PTB) was made by Auramine-O fluorescent stain microscopy of 4 sputum smears and 2 TB cultures using Mycobacterial Growth Indicator Tube (MGIT) (Bectin Dickenson, USA). Drug susceptibility testing (DST) for INH and RIF was done, using the MGIT technique, on all cultures that provided a positive growth and were identified as *M. tuberculosis*, using the Gen/Probe kit at the in-hospital laboratory. DST for streptomycin (S) and ethambutol (Emb) were routinely performed on all isolates resistant to INH or RIF. INH and RIF resistant cultures (MDR-TB) were sent to the National Health Laboratory Service (NHLS) for second-line DST using Middlebrook 7H10 agar containing critical concentrations of 20 μg/mL ethionamide (Eto), 2 μg/mL ofloxacin (Ofx) and 5 μg/mL kanamycin (K). Pyrazinamide (PZA) DST was done

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according to the BACTEC manual (BD Diagnostic Systems, Franklin Lakes, NJ, USA) (11).

All bacteriologically confirmed TB cases were treated using DOTS. Confirmed new cases were treated using a 6 month course of therapy comprising 2 months HRZE and 4 months HR. Re-treatment cases were treated with an 8 month course of therapy comprising 2 months HRZES, 1 month HRZE and 5 months HRE according to the WHO/IUATLD guidelines. All cases were initially hospitalised where they received daily supervised treatment. Cases were discharged from hospital when their sputum smears were negative or scanty positive and negative (2 successive sputum specimens collected 24 hours apart, checked weekly). Thereafter these cases were treated as outpatients where they received supervised treatment, 5 days per week.

Cases identified with MDR-TB were hospitalised and treated according to the DOTS-Plus strategy using Guidelines for the treatment of MDR-TB from the South African National TB control program. Drugs utilised for the treatment of MDR-TB cases included, Ofloxacin (Ofx), Ethionamide (Eto), Pyrizinamide (Z), either Ethambutol (Emb) or Terizidone (Tz), one of the injectable aminoglycosides Streptomycin (S), Kanamycin (K), or Amikacin (A), with the addition of Clarythromycin (Clr). The respective drugs were used in dosages appropriate to the weight and clinical condition of the individual patient with higher dose Clr being used for cases on anti-retroviral therapy (ARV) therapy. Treatment was administered 7 days per week, with the injectable drugs being reduced to 3 times per week when monthly sputum cultures became negative or the patient developed side effects. The injectable drug was stopped when cultures were negative for at least 2

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successive months or side effects or complications necessitated discontinuance. Cases were discharged to outpatient therapy when their sputum cultures were negative for at least 3 successive months. Providing there were no contraindications, all oral medication was continued for at least twelve months after the first negative culture had been obtained, with a minimum total length of 18 months treatment.

The data from the cases were collected retrospectively using standard data collecting instruments. This included age, sex, case type, sputum smear results, case definition (new or re-treatment), outcome, and HIV status (if documented). Also collected was the demographic data pertaining to the place of residence of cases at the mine, as well as, the details of all hospital admissions. The confidentiality of individual case information was protected and only the clinician in charge of the cases had access to their identities.

3.2.2. Genotyping

All drug-resistant TB isolates were genotyped by IS*6110* restriction fragment length polymorphism (RFLP) (refer to method section 2.1-A and 2.2-A), spoligotyping (refer to method section 2.1-B and 2.2-B) and MIRU-typing (refer to method section 2.1-C and 2.2-C). IS*6110* genotypes were analysed using Gelcompar II (Version 4.0, Applied Maths, BVBA, Kortrijk, Belgium). The unweighted pair group method with arithmetic mean (UPGMA), based on the Dice coefficient was used for cluster analysis with band-matching tolerance parameters allowing for a 5% shift in each pattern as a whole and a 0.5% variance in individual band positions.

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Strains with an identical genotype were grouped into clusters reflecting ongoing transmission, while strains with unique genotypes were interpreted to reflect either acquisition of drug resistance in re-treatment cases or primary resistance in new cases (12).

The *katG*, *rpoB*, *pncA* ,*embB* and *gyrA* genes were sequenced using the ABI PRISM DNA sequencer to identify non-synonymous single nucleotide polymorphisms (nsSNPs) conferring resistance. Genotypic data was subjected to phylogenetic analysis using the neighbour joining algorithm. Bootstrapping was done to establish a degree of statistical support for nodes within each phylogenetic reconstruction. The phylogenetic tree was constructed using genotypic data since phenotyping is less accurate and showed high variability.

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3.3. Results

During 2003 a marked increase (140%) in the number of cases with drug-resistant TB was noted on a South African gold mine, prompting a molecular epidemiological investigation to identify the factors exacerbating this form of disease. All of the drug-resistant TB cases (n=128) diagnosed during the period from December 2002 to November 2005 were included in the study cohort. Routine DST showed that 13 (10%) cases were diagnosed with INH mono-resistant TB, 7 (5%) cases were diagnosed with multiple-drug-resistant TB and 108 (85%) cases were diagnosed with MDR-TB. Review of the case folders showed that the majority (97%) were male and that the HIV sero-prevalence rate was at least 65% (Table 1). Despite excellent treatment adherence (treatment interruption = 0%), 34% of the cases died while on treatment, often as a result of AIDS related causes. Five of the MDR-TB cases were diagnosed with XDR-TB.

To determine the *M. tuberculosis* population structure of the drug-resistant epidemic the first available isolate from each case was genotypically classified by MIRU typing and/or spoligotyping and IS*6110* DNA fingerprinting. Isolates cultured from cases with mono- and multiple-drug-resistant TB showed unique genotypes. Of these, 11 (55%) were new cases, suggesting primary resistance. A total of 33 different genotypes were identified among isolates from MDR-TB cases. Twenty-seven of these genotypes were unique, of which 11 (41%) were cultured from new cases implying primary MDR-TB. The remaining 81 MDR-TB isolates could be grouped into 6 clusters, containing between 2-47 cases per cluster. Assuming that each cluster was initiated by a case that acquired MDR-TB, it was

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estimated that at least 75 of the MDR-TB cases were recently infected with an MDR-TB strain through ongoing transmission. Risk factors for ongoing transmission were not evident, possibly due to the over-whelming incidence of HIV co-infection. However, review of the clinical data showed that a large proportion of recently infected MDR-TB cases (62%) had a documented previous episode of TB suggesting re-infection with a circulating MDR-TB strain (Table 2). Many of these clustered cases (76%) had a CD4+ count of <200, suggesting that cases with a depressed immunity were at a high risk of disease progression after recent infection.

This study showed that a large proportion of HIV co-infected cases (66%) were smear positive, thereby suggesting that these individuals contributed to the infectious pool. However, no association could be found among smear positivity and clustering, implying that the pathogenicity of the different bacterial strains may have contributed to the transmission of defined strains.

To determine the natural history of the MDR-TB epidemic in this setting, strains from the largest cluster (n = 47 patients) were analysed in more detail. A phylogenetic reconstruction based on the nsSNPs conferring resistance to INH, RIF, Emb, PZA and Ofx showed that the progenitor MDR strain had acquired resistance to PZA on two separate occasions (Figure 2). These two these strains were subsequently transmitted. Thereafter, Emb resistance evolved independently in a number of different cases. Following transmission, these strains independently evolved resistance to Ofx, ultimately culminating with the evolution of three cases of XDR-TB. Contact tracing showed that 41/45 of the

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cases (91%) had had contact at either their place of residence, place of work and during hospitalisation thereby supporting the notion of ongoing transmission (Figure 2).

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3.4. Discussion

In 1999 the WHO put forward guidelines, known as the DOTS-Plus strategy to control the emergence and spread of drug-resistant TB (13). These guidelines were designed to complement a well functioning DOTS program and were strictly adhered to in the study setting from 2001. To improve TB control, a policy of biannual chest x-ray screening was included in the TB control program to actively identify TB cases. Successful treatment outcome targets (set by the WHO) were exceeded for 2.5 years prior to the initiation of this study. Despite these efforts, an alarming increase in the number of cases with drug-resistant TB and the emergence of XDR-TB was observed. The increase in the number of cases with drug-resistant TB would have been described in classical epidemiological terms as an outbreak, possibly as a consequence of the high prevalence of HIV infection, with silicosis as a possible additional risk factor or the combination thereof. Using a combination of clinical, epidemiological and molecular data we showed that the drug resistant TB epidemic occurred as a result of three factors. Firstly, the identification of drug resistant strains with unique genotypes in cases with no previous documented history of treatment with anti-TB drugs, suggested primary drug resistance as a result of infection outside of the study setting. Secondly, the identification of drug resistant strains with unique genotypes in cases with a previous documented history of treatment with anti-TB drugs, suggested acquisition of drug resistance despite excellent treatment adherence rates. However, in these cases we cannot exclude the possibility of re-infection outside of the study setting. Lastly, clustering of strains with identical genotypes showed that the MDR-TB epidemic was primarily driven by recent transmission of already drug resistant strains. From the data available it is not possible to determine whether these MDR-TB strains

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evolved within the study setting or were imported into the study setting. If MDR-TB was evolving, despite excellent treatment adherence, the mechanism leading to the evolution of resistance in these adherent cases warrants further investigation.

In this setting, risk factors for transmission were not obviously evident due to the high incidence of HIV co-infection. However, despite this limitation this study demonstrated that a large proportion of patients who had a previous documented episode of TB were re-infected with a circulating MDR-TB strain. A previous study has suggested that a prior episode of TB may increase the risk of re-infection in a population with a low HIV prevalence (14). This may be particular true in HIV co-infected patients given the fact that TB accelerates immune suppression (15) and many of these patients had a CD4+ count of <200.

Our findings that a large proportion of the smear positive cases were also HIV+ appeared counter intuitive, given that immune suppression has been associated with smear negative disease (16). A possible explanation for our observation was the fact that the diagnostic algorithm required 4 sputum samples, thereby raising the chance of detecting O-Auramine stained bacilli. Most importantly, this finding demonstrated that immune suppressed TB cases might contribute to ongoing transmission. This has raised concern over the efficacy of the TB control programme, which was unable to identify infectious TB cases prior to spreading MDR-TB to their close and susceptible contacts.

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Our phylogenetic reconstruction of the largest outbreak strain demonstrated sequential acquisition of resistance markers, ultimately leading to the evolution of XDR-TB. This may be explained by the fact that all new TB cases were placed on regimen I (HREZ) while all re-treatment TB cases were placed on regimen II treatment (HREZS) until drug susceptibility tests were available for H and R. During this diagnostic delay period, TB patients with undiagnosed MDR-TB were receiving inappropriate therapy leading to the consequential acquisition of additional resistance markers. Furthermore, these patients could have come in contact with susceptible individuals who were hospitalised for illnesses other than TB thereby unintentionally perpetuating the drug-resistance epidemic. These results emphasise the importance of the development and implementation of rapid diagnostics to minimise the risk of inadvertently placing the patient at risk of developing more severe forms of drug resistant disease.

In conclusion this study suggests that TB control measures over and above those recommended by the WHO are necessary to control the spread of drug resistant TB in a setting with a high incidence of HIV. Efforts made by the TB control programme in the study setting were compromised by an influx of disease thereby emphasising the need to ensure well functioning TB control programs in all settings. A top priority in future TB control plans should address clinical and laboratory diagnostic delay more vigorously.

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Table 1: Patient and clinical data of entire cohort.

		HIV+		HIV-		HIV Unk		Total	
		N	AVG	N	AVG	N	AVG	N	AVG
AVERAGE AGE	YEARS	84	43.0	7	43.0	37	42	128	42
		N	%	N	%	N	%	N	%
SEX	MALE	82	97.6%	8	100.0%	37	94.6%	124	96.9%
	FEMALE	2	2.2%	0	0.0%	2	5.5%	4	2.9%
CASE TYPE	NEW	38	45.2%	0	0.0%	18	48.6%	56	43.8%
	RE TREATMENT	49	54.8%	7	100.0%	21	51.4%	72	56.3%
SPUTUM SMEAR	POSITIVE	59	70.2%	4	57.1%	31	83.8%	94	73.4%
	NEGATIVE	24	28.6%	3	42.9%	5	13.5%	32	25.0%
	OTHER	1	1.2%	0	0.0%	1	2.7%	2	1.6%
OUTCOMES	RC	19	22.6%	3	42.9%	8	21.6%	30	23.4%
	RF	1	1.2%	0	0.0%	0	0.0%	1	0.8%
	D	31	36.9%	1	14.3%	12	32.4%	44	34.4%
	TO	19	22.6%	0	0.0%	4	10.8%	23	18.0%
	A	6	7.1%	0	0.0%	4	10.8%	10	7.8%
ON TREATMENT		8	9.1%	3	37.5%	9	24.3%	20	15.6%

*International spoligotypes (17)
Fig. 1.: DNA fingerprint patterns (IS*6110* RFLP, Spoligotype, MIRU type) of drug resistant isolates (n=128).

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Other = Bacteriological confirmation obtained from lymph node, abscess or bone marrow aspiration.
HIV = Human Immunodeficiency virus, HIV Unk = HIV status unknown
RC = Treatment completed, bacteriological cure proven, RT = Treatment completed bacteriological cure not proven (n=0),
RI = Treatment interruption (n=0), RF = Treatment failure, D = Patient died, TO = Transferred Out to other facilities,
A = Absconded

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Table 2: Association between clinical and genotypic data of entire cohort.

	Category	HIV status	CD4+ count	Genotype: Unique		Genotype: Clustered
				H mono /Multiple	MDR	MDR
Treatment History	New	HIV+		6	7	20
		HIV-/Unknown		5	4	8
	Re-treatment	HIV+		1	9	30
		HIV-/Unknown		2	7	13
	New	HIV+	<200	2	3	16
		HIV+	>200	-	-	2
	Re-treatment	HIV+	<200	1	3	19
		HIV+	>200	-	-	9
Smear	Positive	HIV+		3	10	38
		HIV-/Unknown		6	11	14
	Negative/Extra-pulmonary	HIV+		4	6	12
		HIV-/Unknown		1	-	7
	Positive	HIV+	<200	2	4	28
		HIV+	>200	-	2	8
	Negative/Extra-pulmonary	HIV+	<200	1	2	7
		HIV+	>200	2	1	3
Deaths				1	8	29

HIV = Human Immunodeficiency syndrome, HIV+ = HIV sero positive, HIV - = HIV sero negative, Unknown = HIV status unknown

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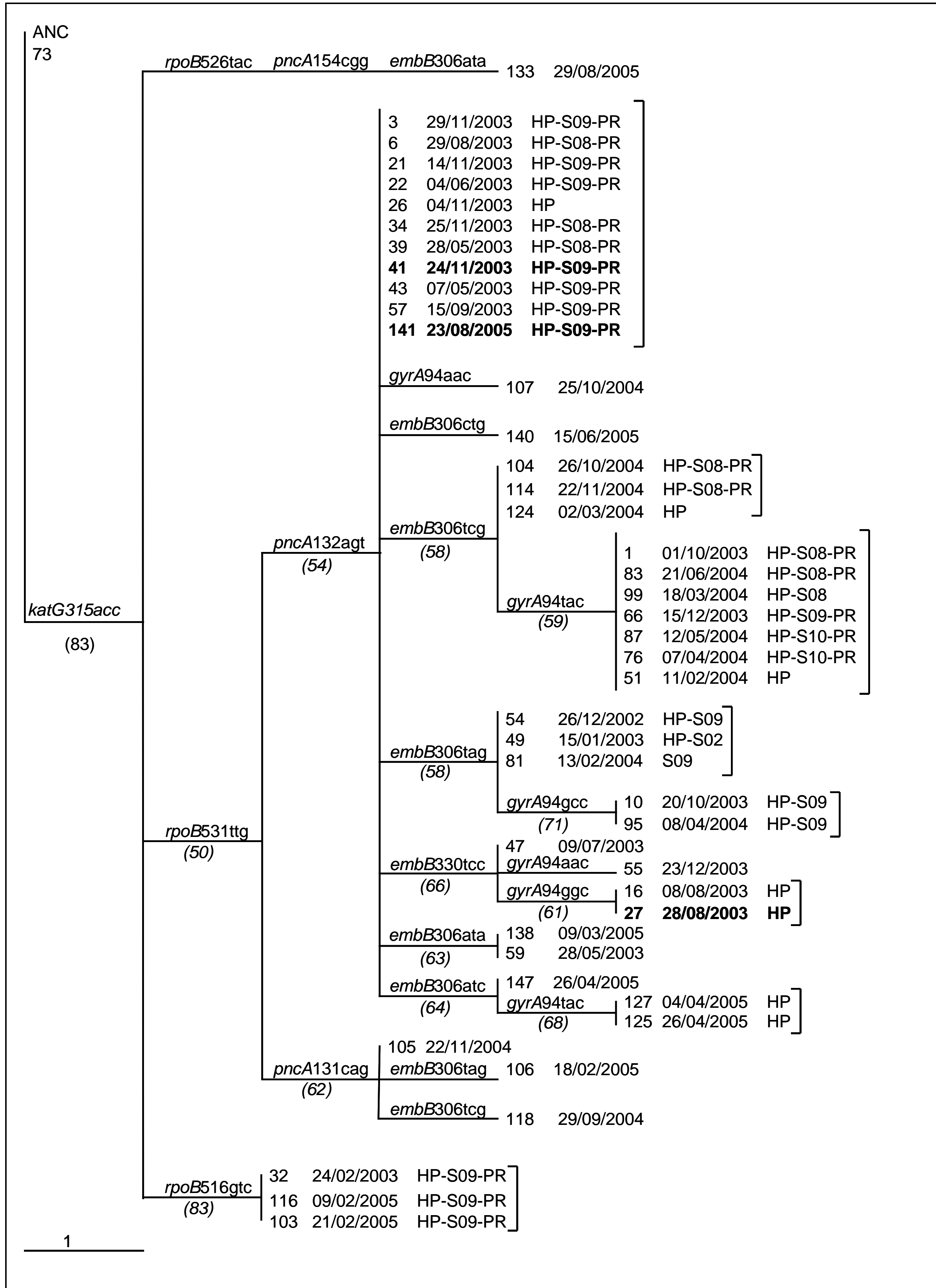

Fig.2.: Phylogenetic history of the largest outbreak MDR-TB cluster.

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Legend: The phylogentic tree was constructed using the neighbour joining algorithm (18). The gene and the mutation conferring resistance are indicated at the node where they occurred. Bootstrap values are shown at internal nodes in brackets. Each case is represented by a number at the end of each branch with the corresponding dates representing the time of MDR-TB diagnosis. Contact tracing data is indicated for each case where PR indicates sharing the same residence, S08 denotes the mine shaft were the case worked and HP indicates whether the case was hospitalised at the same time as another member of the cluster was being treated from MDR-TB. The three XDR-TB cases are indicated in bold. Genetic data for two cases was not available.

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Reference List

(1) Crofton, J, Chaulet, P., and Maher, D. Guidelines for the Management of drug resistant tuberculosis. 1997.

(2) Somoskovi A, Parsons LM, Salfinger M. The molecular basis of resistance to isoniazid, rifampin, and pyrazinamide in Mycobacterium tuberculosis. Respir Res 2001;2(3):164-8.

(3) Zignol M, Hosseini MS, Wright A, Weezenbeek CL, Nunn P, Watt CJ et al. Global incidence of multidrug-resistant tuberculosis. J Infect Dis 2006 August 15;194(4):479-85.

(4) World Health Organization. WHO Report 2004: Global Tuberculosis Control. 2004.

(5) Centers for Disease control and Prevention (CDC). Emergence of Mycobacterium tuberculosis with extensive resistance to second-line drugs--worldwide, 2000-2004. MMWR Morb Mortal Wkly Rep 2006 March 24;55(11):301-5.

(6) Centers for Disease control and Prevention (CDC). Emergence of Mycobacterium tuberculosis with extensive resistance to second-line drugs--worldwide, 2000-2004. MMWR Morb Mortal Wkly Rep 2006 March 24;55(11):301-5.

(7) Centers for Disease control and Prevention (CDC). Emergence of Mycobacterium tuberculosis with extensive resistance to second-line drugs--worldwide, 2000-2004. MMWR Morb Mortal Wkly Rep 2006 March 24;55(11):301-5.

(8) Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U 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 November 4;368(9547):1575-80.

(9) Weyer K. **Survey of Tuberculosis drug resistance, 2001-2002, Western Cape**. http://www sahealthinfo org/tb/tbdrugresistance htm 2001;

(10) Centers for Disease control and Prevention (CDC). Emergence of Mycobacterium tuberculosis with extensive resistance to second-line drugs--worldwide, 2000-2004. MMWR Morb Mortal Wkly Rep 2006 March 24;55(11):301-5.

(11) Louw GE, Warren RM, Donald PR, Murray MB, Bosman M, van Helden PD et al. Frequency and implications of Pyrazinamide resistance in managing previously treated tuberculosis patients. Int J Tuberc Lung Dis. In press 2006.

(12) van Rie A, Warren R, Richardson M, Gie RP, Enarson DA, Beyers N et al. Classification of drug-resistant tuberculosis in an epidemic area. Lancet 2000 July 1;356(9223):22-5.

Stellenbosch University http://scholar.sun.ac.za

(13) WHO. Stop TB working group on DOTS-Plus for MDR-TB: Strategic Plan 2006-2015. 2006.

(14) Verver S, Warren RM, Beyers N, Richardson M, van der Spuy GD, Borgdorff MW et al. Rate of reinfection tuberculosis after successful treatment is higher than rate of new tuberculosis. Am J Respir Crit Care Med 2005 June 15;171(12):1430-5.

(15) Whalen C, Horsburgh CR, Hom D, Lahart C, Simberkoff M, Ellner J. Accelerated course of Human immunodeficiency virus after Tuberculosis infection. Am.J.Respir.Crit Care Med. 151[1], 129-135. 1995.
Ref Type: Journal (Full)

(16) Corbett EL, Bandason T, Cheung YB. Epidemiology of Tuberculosis in a high HIV prevalence population provided with enhanced diagnosis of symptomatic disease. PLoS Med 2007 January;4(1):e22.

(17) Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al Hajoj SA et al. Mycobacterium tuberculosis complex genetic diversity : mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol 2006 March 6;6(1):23.

(18) Hanekom M, van der Spuy GD, Streicher E, Ndabambi SL, McEvoy CR, Kidd M et al. A recently evolved sublineage of the Mycobacterium tuberculosis Beijing strain family was associated with an increased ability to spread and cause disease. J Clin Microbiol 2007 March 14.

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Chapter 4

The dynamics of drug resistant *Mycobacterium tuberculosis* strains in the Western and Southern Cape of South Africa

My contributions:

- Study design.
- Genotyping by IS*6110* RFLP.
- Interpretation of data.
- This chapter includes data that formed part of a published paper: An outbreak of drug resistant Tuberculosis caused by a Beijing strain in the Western Cape, South Africa.2006. International Journal of Tuberculosis and Lung Disease. R. Johnson, R.M. Warren, O. Strauss, A.M. Jordaan, A.A. Falmer, N. Beyers, H.S. Schaaf, M. Murray, K. Cloete, P.D. van Helden, T.C. Victor.
- This chapter includes data that was included in a manuscript, submitted for peer review in October 2007 to Journal of Clinical Microbiology: Spread of a low-fitness drug resistant *M.tuberculosis* strain in a setting of a high HIV prevalence. O. Strauss, R.M. Warren, A.M. Jordaan, E.M. Streicher, M. Hanekom, A.A. Falmer, H. Albert, A. Trollip, E. Hoosain, P.D. van Helden, T.C. Victor.

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4.1. Introduction

South Africa is currently listed as one of the high-burden countries for drug resistant tuberculosis (TB) (25). MDR-TB was first identified in the Western Cape in 1982, subsequently rates have increased but has remained lower compared to other provinces in the country (24). The concern over MDR-TB is particularly valid especially with the emergence of extreme drug resistant TB (XDR-TB) (6). Based on strains that were stored since 2005 at the South African National Health Laboratory Services (NHLS), XDR-TB was already present in all nine provinces (11). Currently, it is estimated that 600 cases per year will be diagnosed with XDR-TB in South Africa (26). The province with the highest number of XDR-TB cases is Kwa-Zulu Natal, possibly as a result of high HIV prevalence and/or due to low TB cure rates. In the Western Cape TB cure rates have exceeded 70%, and is currently one of the provinces with the lowest number of XDR-TB cases.

The application of molecular studies can aid in unravelling and understanding the dynamics of drug resistant TB strains circulating within communities. The polymorphic GC-rich sequence (PGRS) (3), multiple interspersed repetitive units (MIRU) (15), spoligotyping (8), IS*6110* restriction fragment length polymorphism (RFLP) (17), and other PCR-based methods (9) are examples of various genotyping methods that have been developed as molecular epidemiological tools. Even though each method has its limitations, these methods provide information based on the type of study and the question that is asked. RFLP and MIRU-VNTR typing are the most appropriate

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genotyping methods for discriminating between strains such as the Beijing family, while MIRU analysis can also be used to determine the phylogenetic relationship of strains within different Beijing families as well as low copy clade families (10). Since the spoligopatterns of low copy IS*6110* strains are less stable than the IS*6110* RFLP patterns, spoligotyping can be used as a secondary typing method for these low copy clade strains (2). However, RFLP still remains the gold standard, and was central in the identification of drug resistant *M.tuberculosis* outbreaks in hospitals (5,13), prisons (16) and communities (18,19,22).

The aim in this study is to investigate the dynamics of drug resistant strains in three different regions in South Africa.

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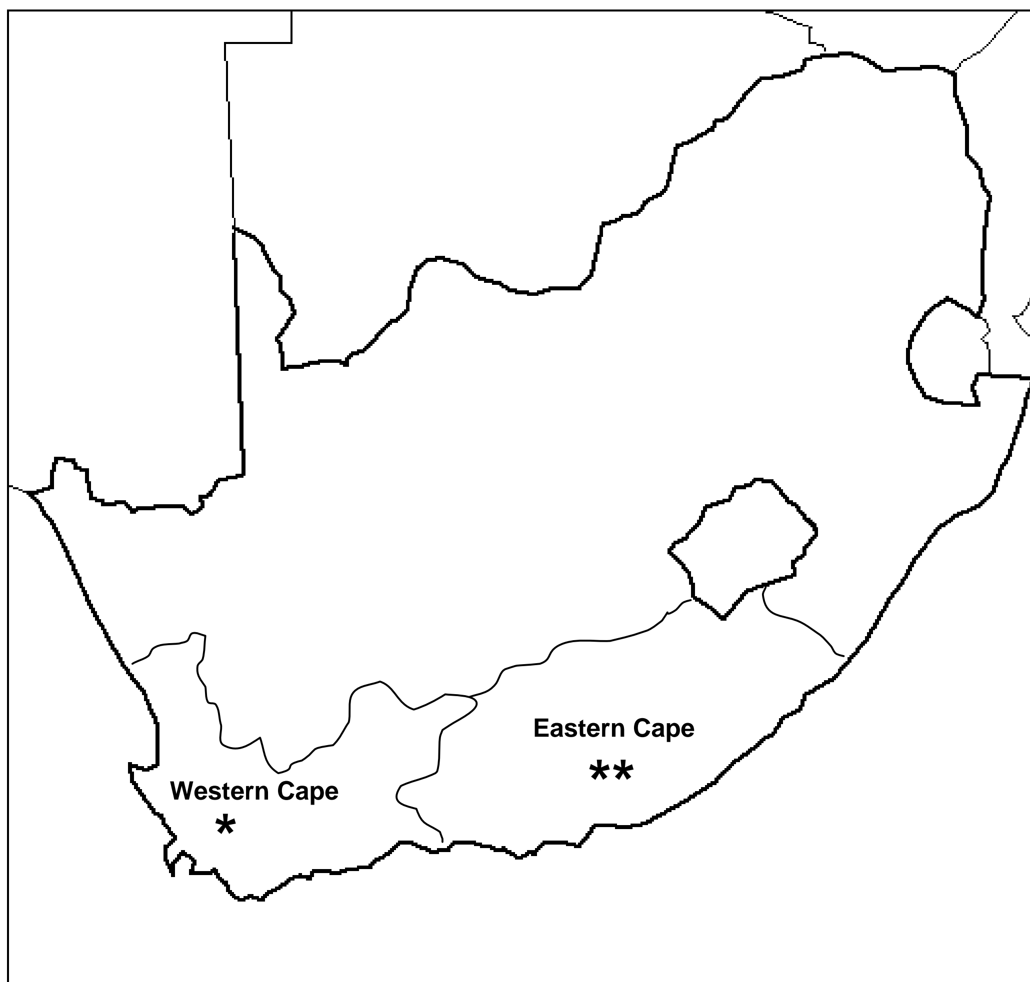
4.2. Materials and Methods



* Western Cape: TB incidence = 1030.7/100 000 pa[1] ; HIV prevalence = 10%[2]

** Eastern Cape: TB incidence = 705/100 000 pa[1] ; HIV prevalence = 30%[2]

[1][DOH TB] Department of Health (TB section). Pretoria. http://www.doh.gov.za/tb/

[2][HIV Household Survey 2002] Shisana O, Principal Investigator. Nelson Mandela/HSRC Study of HIV/AIDS - South African National HIV Prevalence, Behavioural Risks and Mass Media Household Survey 2002. Cape Town: Human Sciences Research Council; 2002. http://www.hsrcpublishers.co.za/hiv.html hh

Fig.1.: Map showing the incidence of TB as well as the HIV prevalence in the total population during 2006.

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4.2.1. Study Design and Treatment features

Re-treatment cases are more likely to develop drug resistance and MDR-TB, which makes this group a very high-risk TB group. Sputum were routinely collected from this high-risk group and sent to the NHLS for drug susceptibility testing. All drug resistant isolates were then referred to Stellenbosch University for further molecular analysis. Genotypic data were compiled in a longitudinal reference database along with clinical and socio-economic information of all of the cases. The results presented in this study only reflect the fingerprint data and not a comparison between clinical and socio-economical factors.

4.2.2. Study Settings

Study 1: Boland-Overberg and Southern Cape-Karoo (BOKS)

The TB incidence rate in the Western Cape region for 2006 was 1 030/100 000 per year and the HIV prevalence for the total population in this province was 10%. *M.tuberculosis* isolates were collected from re-treatment and treatment failure cases from 72 clinics within two of the four health districts; Boland-Overberg and Southern Cape-Karoo (collectively called BOKS) region between January 2001 to February 2006 in the Western Cape province. Initially, spoligotyping was used to genotype only the first isolate taken from cases having drug resistant TB (n=1159) and thereby group these isolates into the different strain families (14). To further investigate the distribution of the selected *M.tuberculosis* genotypes, IS*6110* RFLP (for full description of method, refer to method section 2.1 A and 2.2 A) was done.

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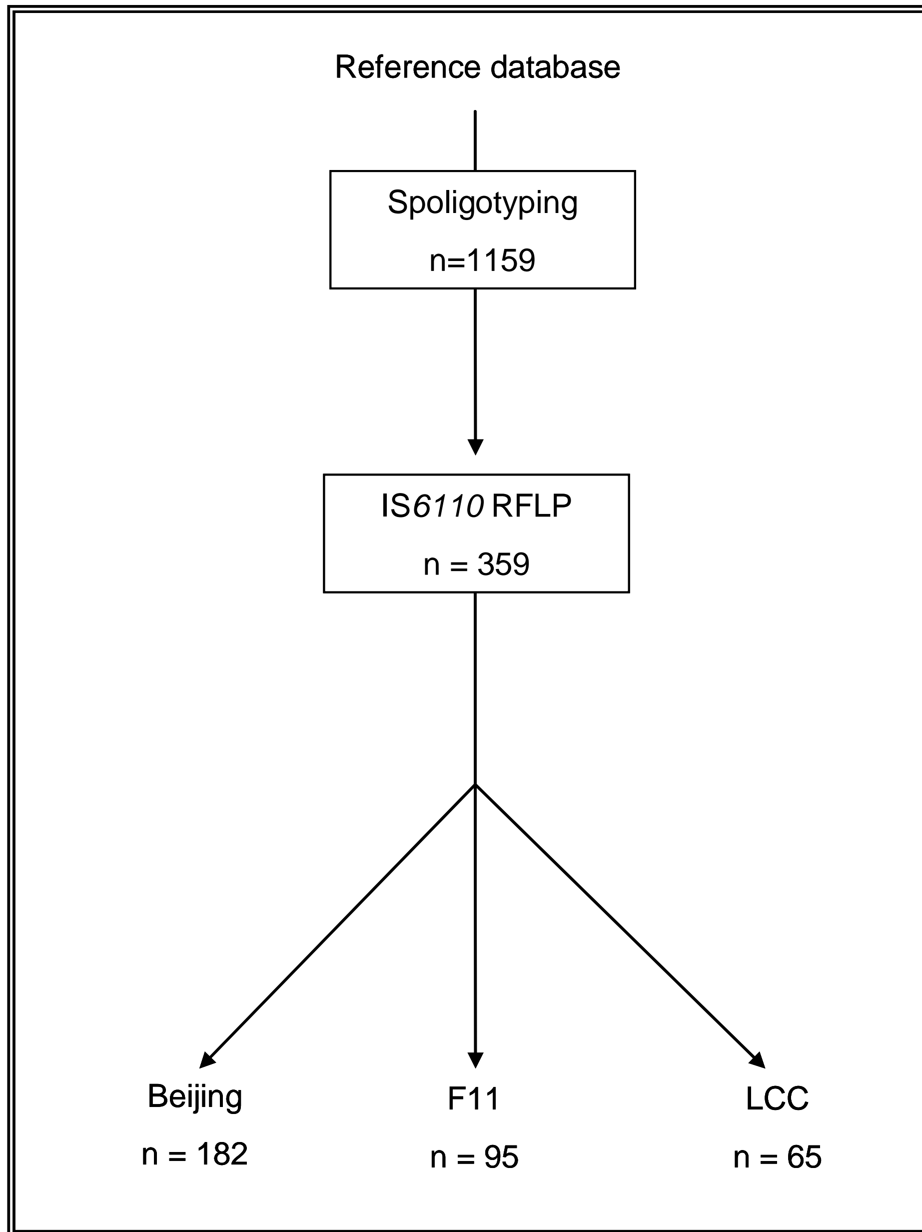

Fig.2.: Diagram of sample allocations.

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Study 2: XDR and Pre-XDR isolates

The second study involved 143 isolates collected (between November 2006-August 2007) from cases that were diagnosed with pre-XDR (MDR with additional resistance to either a flouroquinolone or one of the three injectable drugs: amikacin, kanamycin and capreomycin) and XDR-TB (MDR with additional resistance to a fluoroquinolone and one or more of the three injectable drugs: amikacin, kanamycin and capreomycin) in the Western Cape. Positive cultures needed for RFLP was available for only 139 isolates.

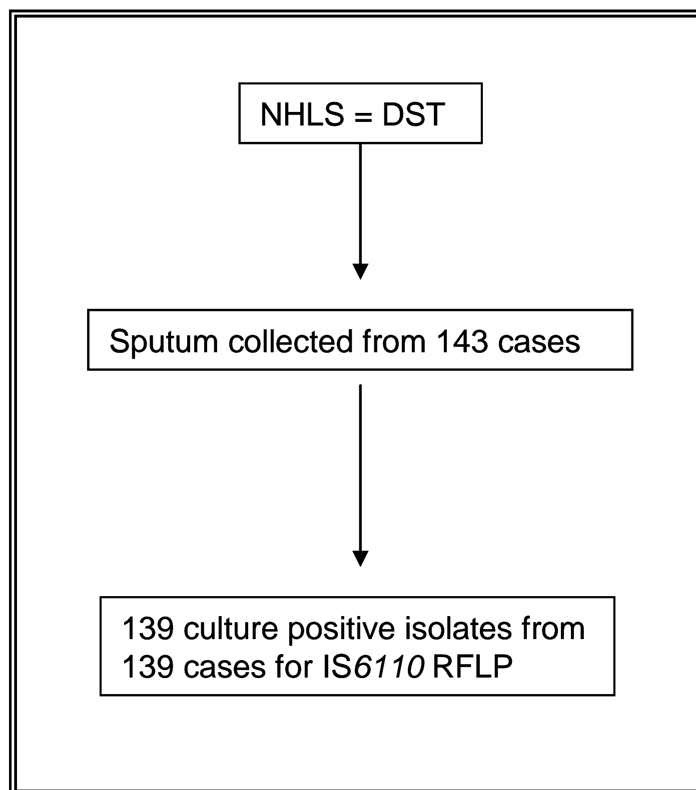

Fig.3: Diagram of sample allocations.

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Study 3: Eastern Cape

Sputum were collected from 127 re-treatment cases at 6 different clinics in the Port Elizabeth and Uitenhage Metropolitan region of the Eastern Cape. Routine culture drug susceptibility testing was done on all isolates. A total of 127 culture positive isolates were referred for IS*6110* RFLP analysis. Only 121 isolates had completed fingerprinting as well as drug resistant mutation data available for further analysis.

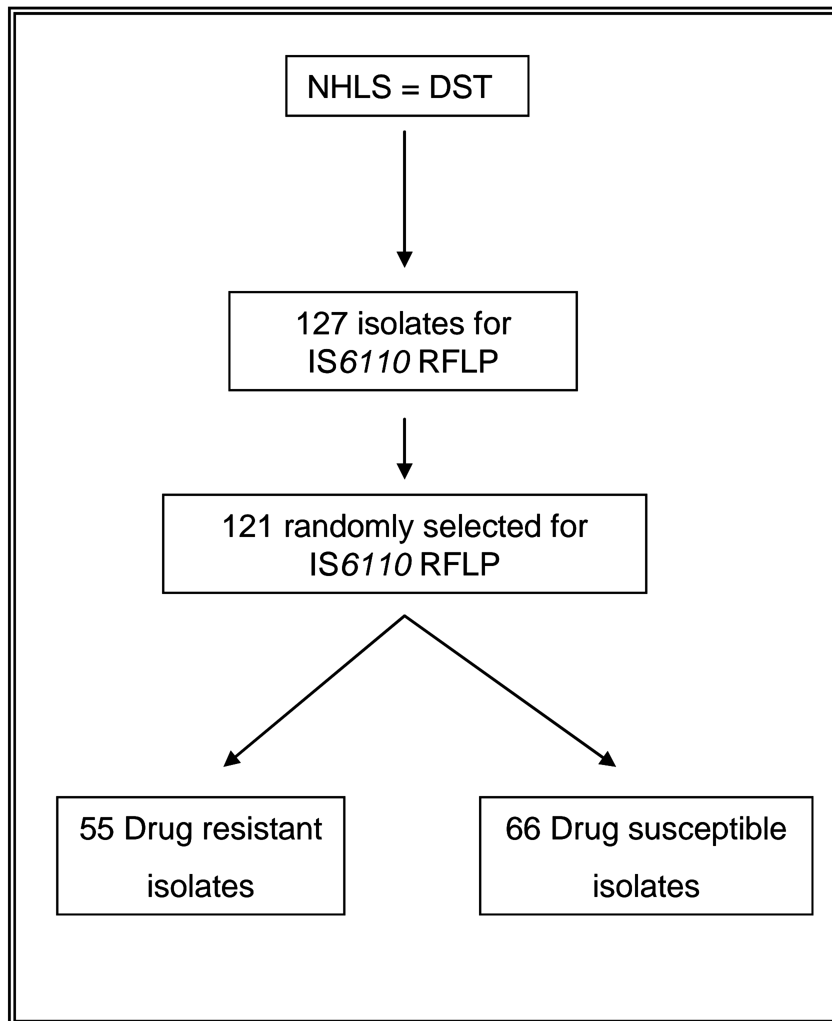

Fig.4: Diagram of sample allocations.

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4.2.3. Definitions based on IS*6110* RFLP

Genotype	: Genetic composition of strain.
Family	: Group of strains displaying similar genotypes, which derives from a common progenitor.
Super family/clade	: Internationally assigned according to the classification of groups of strain families based on genetic similarities.
Cluster	: A group of two or more TB cases with isolates displaying identical IS*6110* RFLP genotypes that have been in recent contact.
Small cluster	: Group of <10 cases with isolates that have identical RFLP genotypes.
Large cluster	: Group of >10 cases with isolates that have identical RFLP genotypes.
Unique	: A strain with a specific genotype resembling no other genotype within a setting.
Outbreak	: Is a sudden increase of drug resistant strains above the expected baseline for a specific region (fig.5).

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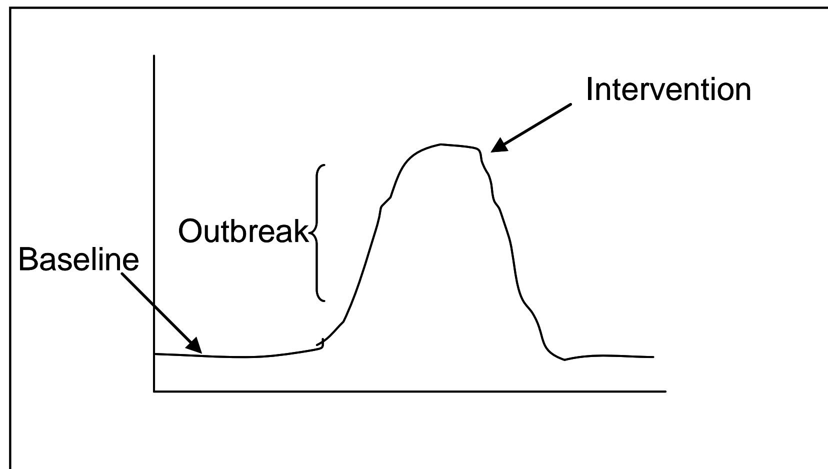

Fig.5.: A schematic view of an outbreak.

4.2.4. IS*6110* RFLP Analysis

The analysis software, Gelcompar is suited for analyzing DNA fingerprints and storage of these fingerprints in a database. Figure 6 is an example of TB strains characterized by IS*6110* RFLP analysis. The similarity between different IS*6110* RFLP patterns are calculated by the Dice coefficient with 5% tolerance parameters for band matching in each pattern as a whole and 0.5% variance in individual band positions. These patterns are then grouped according to their similarities by the unweighted pair group method with arithmetic mean (UPMGA) in the form of a dendogram with similarity matrix on the far left. Each isolate is indicated according to their corresponding isolate number for easy identification. Along with the isolate number, the 'Family' as well as 'Cluster' number are assigned. Strain family names are usually depicted with an "F" for family followed by the name assigned to the family. Based on the IS*6110* insertion sequence, different groups of strains exist within the same strain family (e.g. Beijing), which are also given a

name for easy identification. This is known as the IS*6110* number but it is usually referred to as the cluster name.

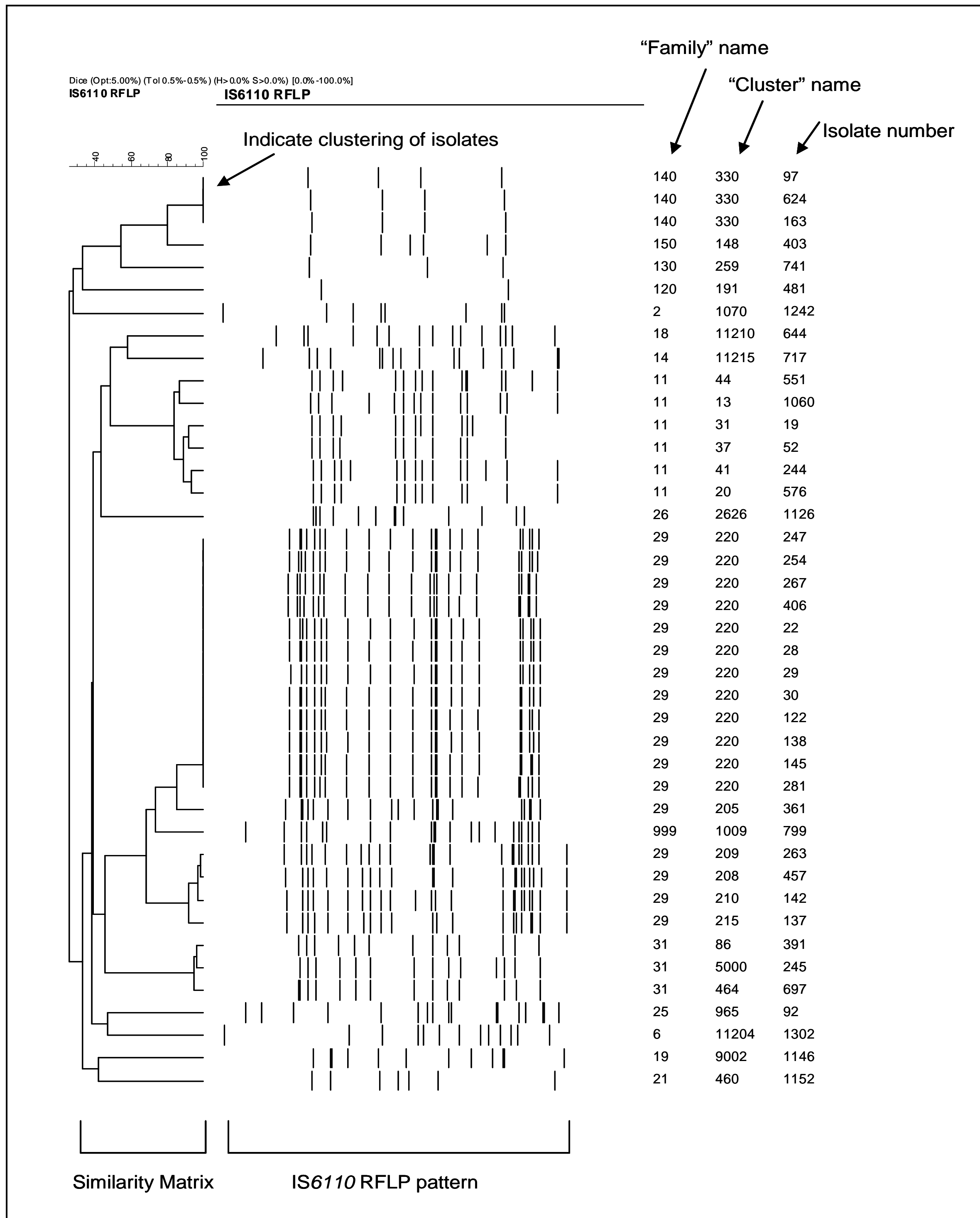

Fig.6.: Gelcompar (Applied Maths, Kortrijk, Belgium)-generated dendrogram of percentage similarity as calculated by Dice coefficient.

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Legend: A dendogram was constructed using the UPGMA algorithm, which is based on the Dice coefficient. Family assignments as well as cluster analysis were according to tolerance parameters (refer to chapter 2). On the extreme right, the "family" and "cluster" names are usually inserted. Within the dendogram, a cluster is usually presented as a single line i.e. F140 with cluster 330 (n=3).

Table 1 is an example of different strain families that are members of the same super family. The super family known as "X1" consist of all low copy clade (LCC) strains with <5 IS*6110* insertions (table 1).

Table 1: Summary of different strain families and examples of clusters within the strain families.

local Family name	Super family*
F29, F31	Beijing
F11, F9, F13	Latin-American-Mediterranean (LAM)
F110, F120, F140, F150	X1

*International recognized family

4.2.5. Quality Control

Parameters that allow for band shifting have a significant influence on fingerprinting analysis. As observed in figure 7, an arrow indicates the difference between two DNA patterns of two different typical Beijing isolates by one band shift. Therefore, it is important to apply parameters to ensure optimal strain analysis as well as quality control of the analysis.

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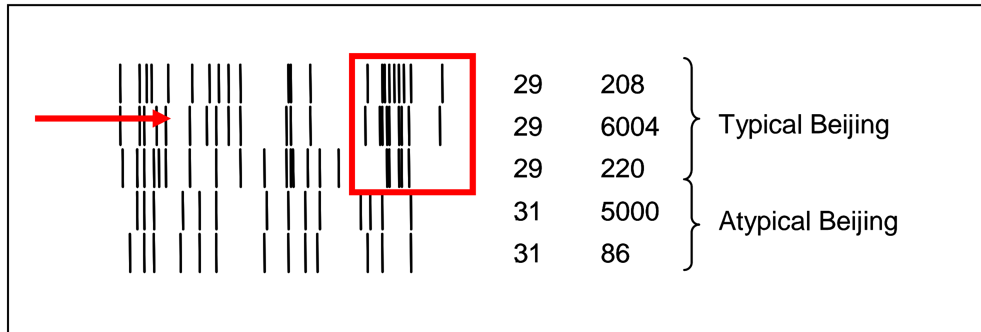

Fig.7.: Differences between IS*6110* patterns of a typical and atypical Beijing genotype.

Legend: The red box indicates the characteristic DNA bands, which is generally not present in the atypical Beijing strains. Also, note the two clusters; 208 and 6004, which are almost identical except for a single band shift (arrow).

With IS*6110* RFLP, two different strain lineages can be identified within the Beijing family. These strains can be differentiated by the characteristic IS*6110* DNA patterns (fig.7) or by the presence (typical) or absence (atypical) of an IS*6110* insertion within the NTF region. Phylogenetic analysis shows that the atypical Beijing strains are more ancient than the typical strains, which have recently evolved.

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4.3. Results

(Results from each study will be reported separately)

4.3.1. Study site 1: Boland/Overberg-Southern Cape/Karoo (BOKS)

Among the spoligotype patterns (n = 1159), three dominant strain families were present (fig. 8) of which the Beijing strain family was the most dominant drug resistant family. Based on the spoligotype data, a significant increase [p=0.0116] in the Beijing strain family was observed during this study period (fig.9).

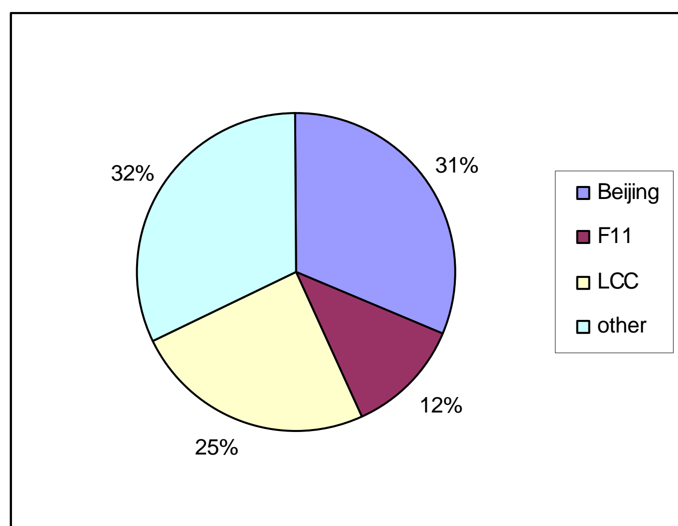

Fig. 8.: The three dominant drug resistant strain families according to spoligotyping.

Legend: Three drug resistant strain families were prominent but Beijing was characterized among the majority of drug resistant cases. The group labelled as “other” consist of unique drug resistant strains as well as drug resistant families that are in the minority of the total isolates.

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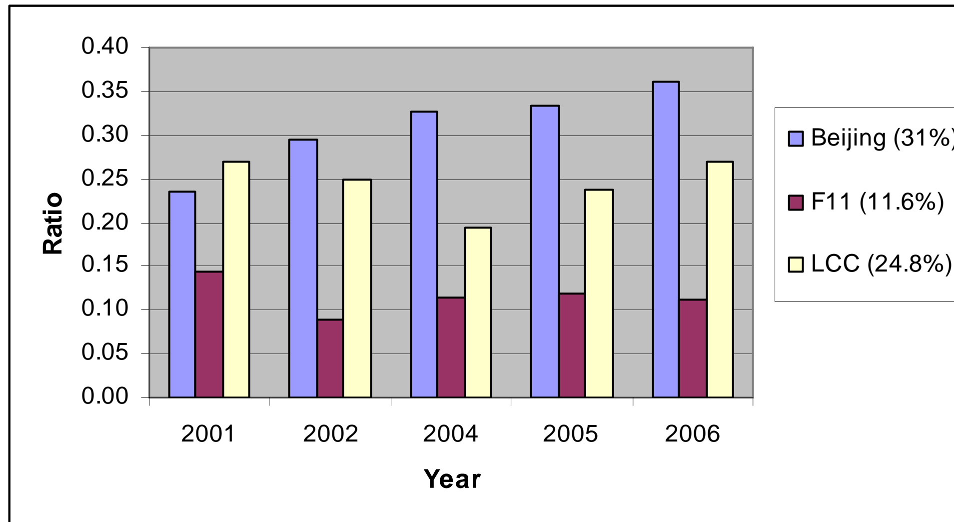

Fig.9.: Ratio of the three drug resistant strain families.

Legend: The Beijing strain increased over the years and remained dominant during this study period.

To further understand the reason responsible for the increase in the Beijing strain family, IS*6110* RFLP was used to type randomly selected Beijing isolates (n=182). The results showed that 92% were identified as typical Beijing and 7.7% were atypical Beijing strains. Fourteen clusters were identified among the typical Beijing isolates of which cluster 220 (57/182[31%]) was overrepresented (fig.10). During this study period, an MDR outbreak of cluster 220 was identified among children attending a high school in Cape Town (7). During that outbreak, a rapid PCR-based method was developed to uniquely identify cluster 220 isolates. This method was used by R. Johnson (from the Division of Molecular Biology and Human Genetics, Faculty of Health Science, Stellenbosch University) to identify cluster 220 strains amongst all the Beijing isolates (n=364) characterized by spoligotyping in this study. The results revealed that during 2001-2006, cluster 220 (147/364[40%]) strains increased and it was widespread in the region. Samples collected in 2003 were excluded from analysis due to under sampling as well as 2007 since sample collection is not completed. DST of cluster 220 isolates

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revealed that 36% (53/147) were INH mono resistant and 63% (93/147) were MDR, indicating that the incidence of this strain was due to a combination of acquisition and transmission. Thus far no drug susceptible cluster 220 has been found in the region (A manuscript concerning cluster 220 is currently in preparation by R Johnson).

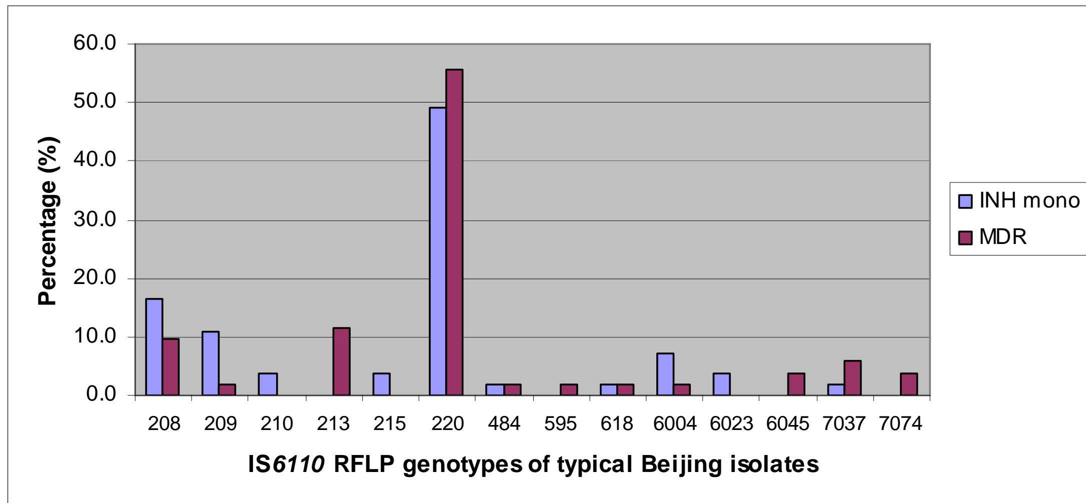

Fig.10.: Clusters within the typical Beijing drug resistant strain family.

Legend: Each cluster is represented by the percentage of isolates that are either INH mono resistant or MDR. Cluster 220 is the most prevalent among all typical Beijing clusters.

Of the limited number of randomly selected LCC isolates, 88% were clustered in this region whereas most of the F11 strains were identified as uniques (60%).

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4.3.2. Study site 2: Pre-XDR and XDR study

Of the 139 culture positive isolates, RFLP data of 49 (62% XDR and 38% Pre-XDR) isolates was available for analysis. However, Similar to study site 1, the typical Beijing genotype was dominant (49%[24/49]) among these isolates. None of the cases infected with atypical Beijing strains were diagnosed with pre-XDR-TB, however pre-XDR was predominantly diagnosed in cases infected with typical Beijing strains (fig.11). Among the typical Beijing group of strain, four genotypes were shared between 57% of cases.

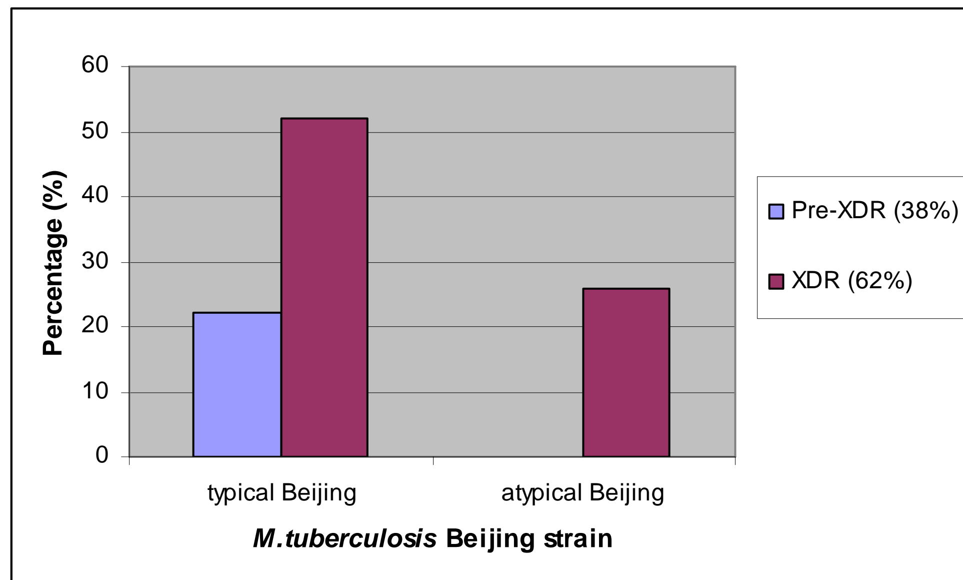

Fig.11.: Distribution of Pre-XDR and XDR strains among Beijing isolates

Legend: Pre-XDR and XDR isolates are grouped according to the IS*6110* family.

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4.3.3. Study site 3: Eastern Cape

One hundred and twenty-one randomly selected isolates were fingerprinted by IS*6110* RFLP of which 55 isolates were drug resistant and 50 of these isolates were MDR-TB. Atypical Beijing genotypes were characterized among 62% (34/55) of the drug resistant isolates and were mostly clustered, indicating ongoing transmission (Fig.12). Additional sequence analysis of the *rpoB* gene of the atypical strains indicated that the high fitness cost $rpoB_{516}$ mutation was overrepresented in the atypical strains and that cases infected with atypical strains are associated with HIV co-infection.

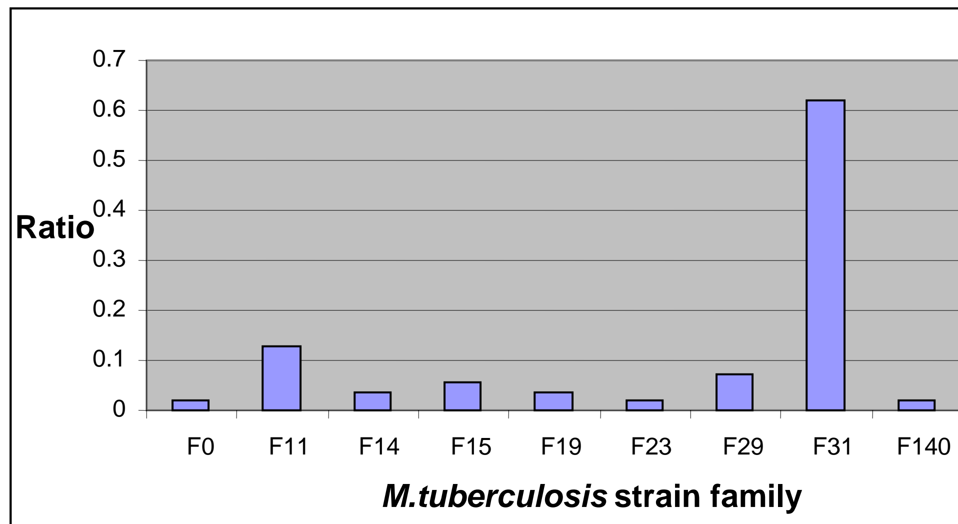

Fig.12.: Strain distribution in the Eastern Cape setting

Legend: Most isolates had the atypical Beijing genotype, which consisted of 3 distinct clusters with sizes varying from 3 – 16 cases. *M.tuberculosis* strain families are shown as F0, F11, F14, F15, F19, F23, F29, F31, F140 of which F29 and F31 are typical Beijing and atypical Beijing strains, respectively.

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4.4. Discussion

In recent years, molecular epidemiological methods have been applied to numerous studies to document the transmission dynamics of both drug susceptible and resistant *M.tuberculosis* strains within a given population, thereby providing new insights into TB epidemiology (20). This study indicates that transmission was largely the cause of the drug resistant epidemic in all three settings. This is in contrast to the findings obtained by drug resistance surveillance studies, which reported that acquired resistance is more common than primary (transmitted) drug resistance (4).

It is important to note that the 3 different studies cannot be compared due to the following reasons: 1) each of these studies was done during different study periods and 2) at least two years of study is necessary to obtain significant epidemiological data. Our conclusions therefore, are based on observations in each separate study setting. However, it must be noted that the aim of this study was not to report on the incidence of drug resistance but rather what factors drive the epidemic. We believe that a longitudinal study would be more informative than drug surveillance studies in a high incidence setting where most drug resistant cases are due to transmission. Therefore, cases of transmission would not be underestimated. The contribution of transmission as well as acquisition of drug resistance to the epidemic will aid in the subsequent selection of an appropriate TB control strategy designed to reduce the MDR-TB rates. Indeed, molecular epidemiology may provide more information about an epidemic therefore aiding in developing a TB control strategy suited for the epidemic.

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Of the three dominant drug resistant strains that we identified in the BOKS region during 2001 to 2006 (14), the Beijing family remained the dominant genotype. F11 is a prominent member of the LAM family, which has been previously identified in the Western Cape (21,23). The dominance of certain strain families in different geographical regions might be due to certain properties that allow these groups of strain families to be more virulent than others. Alternatively, an evolutionary investigation of the strain families might reveal that these families were present in the setting over a long period, which explains their high frequency of cases.

From earlier studies, there is evidence of hyper-virulent strains that are highly transmissible such as the drug resistant Beijing cluster 220 (7). Conclusive evidence from our data indicated that the hyper-virulent cluster 220 strain was responsible for the increasing Beijing frequency in the Boland-Overberg and Southern Cape-Karoo regions. The contribution of cluster 220 to the MDR-TB rates within these communities is significant and therefore cluster 220 is a huge problem and cannot be contained by the TB control program. Since the genome of *M.tuberculosis* has been sequenced, it is now possible to uncover genomic differences in these drug resistant strains that might lead to hyper- virulence. Additional methods include whole genome sequencing, proteomics and microarray analysis to identify these genomic properties.

The prevalence of Beijing in the Eastern Cape was due to an atypical Beijing genotype, which contradicts the perception that atypical Beijing genotypes are assumed to have a

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lower fitness than typical Beijing genotypes. Atypical Beijing strains are generally attenuated and usually not associated with transmission (12). Interestingly in this study setting, the majority of drug resistant atypical isolates showed the presence of the high cost *rpoB*$_{516}$ mutation, conferring rifampicin resistance. We suggest that HIV co-infection posed as a risk factor for the spread of atypical Beijing genotypes in the Eastern Cape. This was supported by our observations in the Western Cape where the HIV prevalence is low and the frequency of cases infected with the atypical Beijing genotype was much lower (7.7% among Beijing strains).

It is interesting to note however, that XDR cases in the Western Cape province (43% infected with Beijing strain) and in a mine setting in the northern province (F1 : n=2 ; F11 : n=1 ; F13 : n=1; F140 : n=3) were infected with strains that were different to the XDR-TB KZN strain that caused the deaths of 52 immunocompromised patients at Tugela Ferry (6). Therefore different strains have the ability to evolve into XDR-TB. We do not know, as yet, why only certain strains acquire more resistance readily than others, especially in the presence of an excellent TB control program, which exceeds the TB cure rate target set by the WHO.

It is possible that geographic evolution may have contributed to the genotypic diversity of *M.tuberculosis* strains within different geographic settings. Ahmed et al (1) suggested that specific genomic deletions and synonymous substitutions that were selected against host and environmental stresses, may have been the cause for such changes. We acknowledge that the evolutionary clock of the molecular markers have a significant

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impact on our interpretation of molecular epidemiology. Therefore, our interpretation of our data is based on the molecular methods, which was selected for analysis.

This study highlights the importance of drug susceptibility testing in all cases particularly in vulnerable populations. To prevent transmission of MDR-TB, better infection control need to be established. However, prevention of acquiring drug resistance might be the main root to the problem, thereby indicating the need to improve control programs so that they include rapid detection of drug resistance.

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Reference List

1. **Ahmed, N., M. Alam, K. R. Rao, F. Kauser, N. A. Kumar, N. N. Qazi, V. Sangal, V. D. Sharma, R. Das, V. M. Katoch, K. J. Murthy, S. Suneetha, S. K. Sharma, L. A. Sechi, R. H. Gilman, and S. E. Hasnain**. 2004. Molecular genotyping of a large, multicentric collection of tubercle bacilli indicates geographical partitioning of strain variation and has implications for global epidemiology of Mycobacterium tuberculosis. J.Clin.Microbiol. **42**:3240-3247.

2. **Bauer, J., A. B. Andersen, K. Kremer, and H. Miorner**. 1999. Usefulness of spoligotyping to discriminate IS6110 low-copy-number Mycobacterium tuberculosis complex strains cultured in Denmark. J.Clin.Microbiol. **37**:2602-2606.

3. **Chaves, F., Z. Yang, H. el Hajj, M. Alonso, W. J. Burman, K. D. Eisenach, F. Dronda, J. H. Bates, and M. D. Cave**. 1996. Usefulness of the secondary probe pTBN12 in DNA fingerprinting of Mycobacterium tuberculosis. J.Clin.Microbiol. **34**:1118-1123.

4. **Cohn, D. L., F. Bustreo, and M. C. Raviglione**. 1997. Drug-resistant tuberculosis: review of the worldwide situation and the WHO/IUATLD Global Surveillance Project. International Union Against Tuberculosis and Lung Disease. Clin.Infect.Dis. **24 Suppl 1**:S121-S130.

5. **Edlin, B. R., J. I. Tokars, M. H. Grieco, J. T. Crawford, J. Williams, E. M. Sordillo, K. R. Ong, J. O. Kilburn, S. W. Dooley, K. G. Castro, and .** 1992. An outbreak of multidrug-resistant tuberculosis among hospitalized patients with the acquired immunodeficiency syndrome. N.Engl.J.Med. **326**:1514-1521.

6. **Gandhi, N. R., A. Moll, A. W. Sturm, R. Pawinski, T. Govender, U. Lalloo, K. Zeller, J. Andrews, and G. Friedland**. 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.

7. **Johnson, R., R. M. Warren, O. J. Strauss, A. Jordaan, A. A. Falmer, N. Beyers, H. S. Schaaf, M. Murray, K. Cloete, P. D. van Helden, and T. C. Victor**. 2006. An outbreak of drug resistant Tuberculosis caused by a Beijing strain in the Western Cape, South Africa. Int.J.Tuberc.Lung Dis. **10**:1412-1414.

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

9. **Kremer, K., C. Arnold, A. Cataldi, M. C. Gutierrez, W. H. Haas, S. Panaiotov, R. A. Skuce, P. Supply, A. G. van der Zanden, and S. D. Van**. 2005.

Stellenbosch University http://scholar.sun.ac.za

Discriminatory power and reproducibility of novel DNA typing methods for Mycobacterium tuberculosis complex strains. J.Clin.Microbiol. **43**:5628-5638.

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

11. M, Mattheyse. Extensively drug resistant Tuberculosis. 15-3-2007.
Ref Type: Pamphlet

12. **Mokrousov, I., O. Narvskaya, T. Otten, A. Vyazovaya, E. Limeschenko, L. Steklova, and B. Vyshnevskyi**. 2002. Phylogenetic reconstruction within Mycobacterium tuberculosis Beijing genotype in northwestern Russia. Res.Microbiol. **153**:629-637.

13. **Moro, M. L., A. Gori, I. Errante, A. Infuso, F. Franzetti, L. Sodano, and E. Iemoli**. 1998. An outbreak of multidrug-resistant tuberculosis involving HIV-infected patients of two hospitals in Milan, Italy. Italian Multidrug-Resistant Tuberculosis Outbreak Study Group. AIDS **12**:1095-1102.

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

15. **Supply, P., J. Magdalena, S. Himpens, and C. Locht**. 1997. Identification of novel intergenic repetitive units in a mycobacterial two-component system operon. Mol Microbiol **26**:99-1003.

16. **Valway, S. E., S. B. Richards, J. Kovacovich, R. B. Greifinger, J. T. Crawford, and S. W. Dooley**. 1994. Outbreak of multi-drug-resistant tuberculosis in a New York State prison, 1991. Am.J.Epidemiol. **140**:113-122.

17. **van Embden, J. D., M. D. Cave, J. T. Crawford, J. W. Dale, K. D. Eisenach, B. Gicquel, P. Hermans, C. Martin, R. McAdam, and T. M. Shinnick**. 1993. Strain identification of Mycobacterium tuberculosis by DNA fingerprinting: recommendations for a standardized methodology [see comments]. J.Clin.Microbiol. **31**:406-409.

18. **van Rie, A., R. Warren, M. Richardson, R. P. Gie, D. A. Enarson, N. Beyers, and P. D. van Helden**. 2000. Classification of drug-resistant tuberculosis in an epidemic area. Lancet **356**:22-25.

Stellenbosch University http://scholar.sun.ac.za

19. **van Rie, A., R. M. Warren, N. Beyers, R. P. Gie, C. N. Classen, M. Richardson, S. L. Sampson, T. C. Victor, and P. D. van Helden**. 1999. Transmission of a multidrug-resistant Mycobacterium tuberculosis strain resembling "strain W" among noninstitutionalized, human immunodeficiency virus-seronegative patients. J.Infect.Dis. **180**:1608-1615.

20. **van Soolingen, D.** 2001. Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. J.Intern.Med. **249**:1-26.

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

22. **Victor, T. C., E. M. Streicher, C. Kewley, A. M. Jordaan, G. D. van der Spuy, M. Bosman, H. Louw, M. Murray, D. Young, P. D. van Helden, and R. M. Warren**. 2007. Spread of an emerging Mycobacterium tuberculosis drug-resistant strain in the western Cape of South Africa. Int.J.Tuberc.Lung Dis. **11**:195-201.

23. **Warren, R. M., S. L. Sampson, M. Richardson, G. D. van der Spuy, C. J. Lombard, T. C. Victor, and P. D. van Helden**. 2000. Mapping of IS*6110* flanking regions in clinical isolates of *M. tuberculosis* demonstrates genome plasticity. Mol.Microbiol. **37**:1405-1416.

24. **Weyer, K., P. Groenewald, M. Zwarenstein, and C. J. Lombard**. 1995. Tuberculosis drug resistance in the Western Cape. S.Afr.Med.J. **85**:499-504.

25. **WHO**. 2004. Anti-TB drug resistance in the world:WHO/ IUATLD global project on Anti-Tuberculosis drug resistance surveillance, 1999-2002, Third Report .

26. WHO. Fast action urge to halt deadly TB. Bulletin of the World Health Organization 85[5], 328-329. 2007.
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