
CHARACTERIZATION AND GENE EXPRESSION OF
TRANSMISSIBLE *MYCOBACTERIUM TUBERCULOSIS* STRAINS IN
SOUTH AFRICA.

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University of Stellenbosch.



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DECLARATION

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

Signature:

Date:

SUMMARY

The *Mycobacterium tuberculosis* Beijing strain family is a dominant strain family in most countries world wide, including South Africa. It has been suggested that this strain family has unique properties. These include the ability to evade the protective effect of Bacillus Calmette-Guérin vaccination, spread more readily and the more frequent acquisition of drug resistance. These properties might be the reasons for the Beijing strain's successful transmission. Comparative genomics have suggested that strains from the Beijing family can be broadly grouped into typical and atypical strains according to the presence or absence of an *IS6110* insertion in the NTF region in the genome of *Mycobacterium tuberculosis*. Phylogenetic analysis showed that these two groups originated from a common progenitor. However, the atypical Beijing strain has only rarely been identified. The atypical Beijing strains are also not frequently associated with drug resistance, is attenuated and therefore do not spread readily. In contrast, by applying molecular epidemiological techniques, this study showed that an atypical Beijing strain acquired drug resistance and was spreading amongst tuberculosis re-treatment patients in the Eastern Cape province of South Africa. Further molecular analysis showed that this strain had a high fitness cost mutation in the *rpoB* gene, conferring rifampicin resistance. This correlates with *in vitro* generated *rpoB* mutants. The human immune deficiency virus/tuberculosis co-infection was found to be a significant co-factor, which allowed the atypical Beijing strain to be transmitted. Therefore, the attenuated atypical Beijing strain can overcome its fitness cost in high human immune deficiency virus burdened communities and may cause ongoing transmission. This raises concern for the spread of all drug-resistant strains in vulnerable populations.

By analysing a longitudinal reference database at the University of Stellenbosch, it has been observed that the strain dynamics within a strain family differs. There are large and small clusters in the Beijing strain family which is suggestive of more and less transmissible strains. Comparative

proteomic analysis by 2-D gel electrophoresis identified 64 protein spots which were different between a large and small cluster in the Beijing strain family. Similarly, 59 protein spots were found different between the attenuated atypical Beijing strain and the typical large Beijing cluster. By comparing the atypical Beijing strain to the small Beijing cluster it was found that 132 protein spots were different between the two strains. These results strongly suggest that differential expression of certain genes is associated with differential transmission of different Beijing sub-lineages. The same may be true for other *Mycobacterium tuberculosis* strain families. It is likely that the bacterial genomic background play a more dominant role in the differential transmission of certain *Mycobacterium tuberculosis* strains, than host or programmatic related factors. A more comprehensive study, which involves the bacterium, host, and the tuberculosis control program, is needed to prove this assumption.

OPSOMMING

Die *Mycobacterium tuberculosis* Beijing familie is 'n prominent in meeste lande wêreld wyd, insluitende Suid-Afrika. Bevindings toon dat hierdie familie unieke eienskappe besit. Dit sluit in die vermoë om die uitwerking van die Bacillus Calmette-Guérin vaksien te ontduik, maklik te versprei, en die vermoë om meer gereeld middel weerstandigheid te verkry, en daarom so suksesvol is. Vergelykbare genomika het getoon dat stamme wat aan die Beijing familie behoort, in twee sub-groepe verdeel kan word naamlik, tipies en atipies as gevolg van die aanwesigheid of afwesigheid van 'n spesifieke IS6110 invoeging in die NTF area van die *Mycobacterium tuberculosis* genoom. Filogenetiese analises het verder getoon dat die twee groepe 'n gemeenskaplike oorsprong het maar die atipiese Beijing sub-groep is meer skaars en word nie dikwels met middel weerstandigheid geassosieer nie, en versprei daarom nie so maklik nie. In teenstelling, deur die toepassing van molekulere epidemiologiese tegnieke, het hierdie studie getoon dat daar 'n atipiese Beijing stam in die Oos-Kaap provinsie van Suid-Afrika gevind is, wat wel middel weerstandig is en versprei het tussen tuberkulose pasiente wat weer op behandeling is. Verdere molekulere analises het getoon dat die atipiese Beijing stam 'n hoë fiksheid verlies mutasie in die *rpoB* geen het wat rifampisien weerstandigheid veroorsaak. Hierdie bevinding korreleer met in vitro gegenereerde *rpoB* mutante. Die studie het gevind dat menslike immuniteitsgebrek-virus/tuberkulose ko-infeksie 'n belangrike faktor was in die verspreiding van hierdie stam. Dus, die minder virulente atipiese Beijing stam kan fiksheid verlies oorkom in gemeenskappe wat belas is met menslike immuniteits virus, en kan dus voortdurende transmissie veroorsaak. Hierdie bevinding wek kommer oor die verspreiding van alle middel weerstandige *Mycobacterium tuberculosis* stamme in kwesbare gemeenskappe. *Mycobacterium tuberculosis*

Die ontleding van 'n aaneenlopende databasis van die Universiteit van Stellenbosch het getoon dat die dinamika van stamme binne 'n stam familie verskil. Daar kom groot en klein groepe in die

Beijing stam familie voor wat bes moontlik op stamme wat met onderskeidelik 'n hoe en lae oordraaglikheid dui. Vergelykende proteomiese analise deur middle van 2-D elektroforese het 64 protein verskille opgelewer tussen 'n groot en klein stam van die Beijing stam familie. Netso is 59 protein verskille gevind toe die groot tipiese Beijing stam en die geattenueerde atipiese Beijing stam vergelyk word. 'n Vergelyking tussen die klein tipiese Beijing stam en die atipiese Beijing stam het 132 protein verskille getoon. Hierdie resultate laat 'n sterk vermoede dat differensiele uitdrukking van sekere gene geassosieer kan word met differensiele oordraag van verskillende Beijing stamme. Dieselfde mag ook geld vir ander *Mycobacterium tuberculosis* stam families. Dit is moontlik dat die genomiese agtergrond van die bakterium 'n meer dominante rol by die differensiele oordraag van sekere *Mycobacterium tuberculosis* stamme het as ander faktore rakende die draer van die tuberkulose infeksie, of die tuberkulose-beheerprogram. Om hierdie aanname te staaf sal 'n meer omvattende studie wat die *Mycobacterium tuberculosis* bakterium, die draer, en die *Mycobacterium tuberculosis* tuberkulose beheerprogram betrek, nodig wees.

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LIST OF ABBREVIATIONS

°C	:	degree Celsius
µl	:	microlitres
1D	:	one dimension
2D	:	two dimension
AcOH	:	Acetic acid
ADC	:	Albumin dextrose catalase
BCG	:	Bacillus Calmette-Guérin
BOKS	:	Boland Overberg Karoo Southern Cape
bp	:	base pairs
BSA	:	Bovine serum albumin
BSL3	:	Biosafety level 3
dH ₂ O	:	distilled water
DNA	:	Deoxyribonucleic acid
dNTP	:	deoxyribonucleotide triphosphate
DR	:	Direct repeat
DTT	:	DL-Dithiothreitol
EC	:	Eastern Cape
EDTA	:	Ethylene diamine tetra-acetic acid
EtBr	:	Ethidiumbromide
EtOH	:	Ethanol
g	:	grams
IEF	:	Isoelectric focusing
INH	:	isoniazid
LJ	:	Loewenstein Jensen

<i>M. tuberculosis</i>	:	<i>Mycobacterium tuberculosis</i>
MgCl ₂	:	Magnesium chloride
min.	:	Minute
MIRU	:	Mycobacterial interspersed repetitive units
ml	:	millilitres
mM	:	millimoles
mQH ₂ O	:	milliQ water
NaAc	:	Sodium acetate
Na ₂ CO ₃	:	Sodium carbonate
NaCl	:	Sodium chloride
NaOH	:	Sodium hydroxide
HNa ₂ PO ₄	:	Sodium hydrogen phosphate
Na ₂ S ₂ O ₃	:	Sodium thiosulfate
((NH ₄) ₂ SO ₄)	:	Ammonium sulfate
ng	:	nanograms
OD	:	optical density
PMSF	:	phenylmethylsulfonyl fluoride
PBS	:	Phosphate buffer saline
PCR	:	Polymerase chain reaction
PE	:	Port Elizabeth
KH ₂ PO ₄	:	Potassium dihydrogen phosphate
pM	:	picomoles
RFLP	:	Restriction Fragment Length Polymorphism
RIF	:	rifampicin
rpm	:	Revolutions per minute
RRDR	:	rifampicin resistance determining region

SA	:	South Africa
SDS	:	Sodium dodecyle sulphate
SDS-PAGE	:	Sodium dodecyle sulphate polyacrylamide gel electrophoresis
Spoligo	:	spacer oligo
SNP	:	Single nucleotide polymorphism
TB	:	tuberculosis
TBE	:	Tris/Borate/EDTA
TE	:	Tris/EDTA
Tm	:	melting temperature
TRIS	:	Trishydroxymethylaminomethane
U	:	Units
VNTR	:	Variable numbers of tandem repeats
WC	:	Western Cape
WCL	:	Whole cell lysate

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Mycobacterium tuberculosis (*M. tuberculosis*) is one of the most successful human pathogens. It is responsible for tuberculosis (TB) in one third of the world's population and cause many mortalities each year (30). For many years this pathogen has been studied to determine why it so successful. A great amount of knowledge has been obtained about *M. tuberculosis*, the host, and environment through different studies, but still not enough is known to stop the disease from causing epidemics and deaths. The good news is that TB is treatable with anti-TB drugs. However the bacterium has many protecting mechanisms, of which one is developing spontaneous mutations in chromosomal genes that are specific targets of the anti-TB drugs, causing the bacterium to become resistant to the drugs (2,7). These chromosomal mutations can also be selected for as a result of inadequate treatment or failure by the patient to comply with adequate treatment (29).

TB drug resistance is a major problem worldwide. Treatment of patients infected with drug-resistant *M. tuberculosis* strains, has to be prolonged and less effective second-line drugs that are more toxic and more expensive, have to be used (5). *M. tuberculosis* strains become multi-drug resistant (MDR) when they develop resistance to at least two of the most effective first-line anti-TB drugs, isoniazid (INH) and rifampicin (RIF) (14) but in addition may also be resistant to any other anti-TB drugs. Recently, the TB drug resistance problem has been amplified greatly by the discovery of extensively drug-resistant (XDR) TB strains. These are *M. tuberculosis* strains that have developed

MDR as well as resistance to any fluoroquinolone and also one of the three injectable second-line drugs (capreomycin, kanamycin and amikacin) (5).

There are many factors that contribute to the spread of drug-resistant strains which include, non-compliance of the patients to their anti-TB treatment therapy (29); the quality of TB control programs (3); the relative fitness of drug-resistant *M. tuberculosis* strains ; as well as their genetic backgrounds (9). Fitness, virulence, pathogenicity, and transmission are tightly linked as demonstrated diagrammatically in Figure1. The more fit the bacterium, the more virulent it is and the more it can be transmitted. The fitness of the bacterium can therefore be defined as a combined measure of the bacterium's ability to survive, reproduce, and to be transmitted to other individuals under certain environmental conditions which then cause disease or pathogenesis in the newly infected individual (6,15). In other words the bacterium's ability to infect, persist, and proliferate, causing disease and then transmitting to a secondary host. Environmental factors such as poverty, malnutrition, stress, overcrowding, and exposure to environmental mycobacteria, might also play a role (8,11,12,17). In addition, poor TB control programs may lack the ability to contain the spread of certain fit *M. tuberculosis* strains.

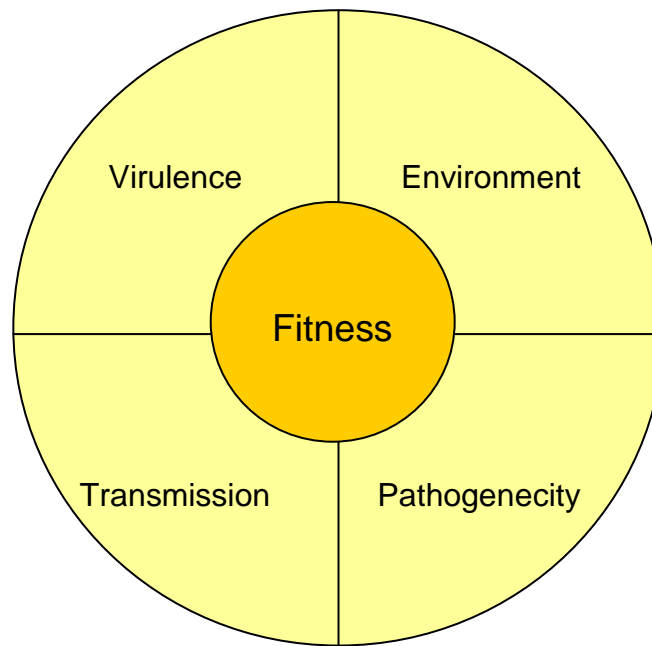


Figure1. The diagram illustrates the relationship between the fitness, virulence, pathogenicity, and transmission of *M. tuberculosis* and as well as environmental factors.

Drug-resistant *M. tuberculosis* strains are often associated with a reduced competitive ability when compared to drug-sensitive *M. tuberculosis* strains (6,9,15). Certain studies showed that the fitness of a drug-resistant bacterium is reduced compared to that of a drug-sensitive bacterium; it was therefore concluded that there is a cost to being drug-resistant (1,9). This fitness cost depends on specific drug resistance conferring mutations. The degree to which the mutations affect the fitness of the bacterium varies with the specific drug resistance conferring mutation, the environment, and the genetic background of the strain (9). Several studies have found that some bacteria obtained secondary mutations which seemed to reduce the fitness cost of the first mutations (2,6,9,15,19). Therefore the bacterium can adapt to this fitness cost by gaining secondary mutations that can compensate for the cost of drug resistance.

RIF is one of the most important first-line anti-TB drugs and is a very good marker for the detection of MDR-TB (4). This drug interacts with the β -subunit of the RNA polymerase encoded by the *rpoB* gene in *M. tuberculosis*, causing inhibition of the early steps of transcription (13,16). In addition to the early bactericidal effect on metabolically active bacteria, RIF also exhibits late sterilizing action on semi-dormant bacteria undergoing short bursts of metabolic activity (22). Among clinical isolates RIF-resistance is almost exclusively due to mutations in an 81 base pair region called the RIF resistance-determining region (RRDR) in the *rpoB* gene (15,18,24). The fitness cost of drug resistance has been clearly demonstrated during the evolution of RIF-resistance in clinical isolates and *in vitro* experiments, where it has been shown that different mutations conferring RIF-resistance occur at different rates (2,9,13,16) and the frequency at which mutations are observed correlates directly with their fitness cost. It has been shown *in vitro* and in clinical isolates that mutations at codon 531 of *rpoB* exhibited the lowest or nearly no fitness cost, which explains why this specific mutation is so frequently observed in *in vitro* generated *rpoB* mutants as well as clinical isolates (2,6,9,15). In contrast, mutations at codons 511, 516, 519 and 529 of *rpoB*, are examples of high fitness cost mutations, conferring RIF-resistance (21). Accordingly, the molecular epidemiology of drug-resistant strains of *M. tuberculosis* should correspond to fitness cost and overall strain fitness.

Scientists in the Division of Molecular and Cellular Biology, Stellenbosch University, SA, have studied TB for the last decade and all the data gathered from those studies were deposited into a longitudinal reference database. The database contains phenotypic and genotypic data of different *M. tuberculosis* strains, as well as clinical and demographic information from the patients infected with these strains, from different regions in SA as well as from a few other countries in Africa. Upon analysing the database we made a number of interesting findings related to this study. We have observed that the TB epidemic in SA is driven predominantly by transmission of drug susceptible- as well as drug-resistant *M. tuberculosis* strains (10,20,23,25-28). Large drug-resistant

strain families (defined as strains with closely related DNA fingerprints) have been observed (28) but what we have noticed is that within these large strain families are certain clusters that are more dominant than others (Figure 1.2) implying that they are transmitted more and are therefore more fit. These dominant clusters will be referred to in this thesis as large clusters (defined by more than 10 isolates with identical or closely related DNA fingerprints and genotypic characteristics). There are also clusters within these large strain families that consist of only a few isolates (2-5 isolates with identical or closely related DNA fingerprints), implying that they are less transmitted and therefore less fit. They will be referred to as small clusters.

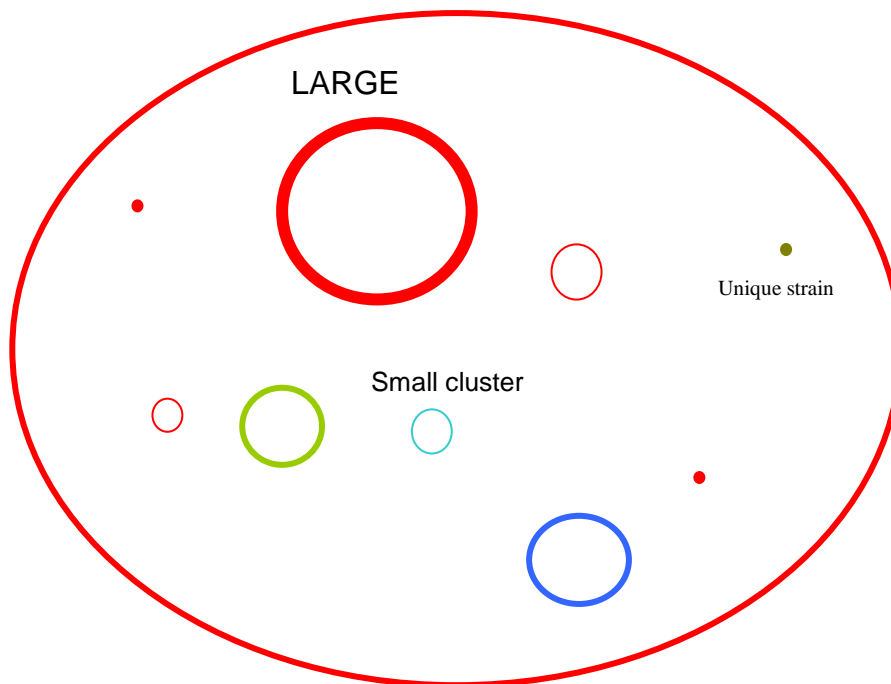


Figure1.2. A representation of a large *M. tuberculosis* strain family consisting of large and small clusters, as well as strains with unique DNA fingerprints.

Some of the questions that we will attempt to answer in this study are, why some strains within the same strain family are more transmitted than others? Are they more fit and what makes them more fit? By using different molecular methods, we attempted to answer these questions.

1.2 PROBLEM STATEMENT

Drug resistance in *M. tuberculosis* is a major problem worldwide as well as in SA. This form of the bacterium is able to overcome the host defences, TB control programme efforts, as well as possible evolutionary costs. The study was designed to try and understand on genomic and proteomic level mechanisms that might give certain *M. tuberculosis* strains an advantage over others, in their ability to cause ongoing drug-resistant TB.

1.3 HYPOTHESIS

Transmission of drug resistant strains is due to a combination of (1) strain fitness, (2) drug tolerance and (3) short comings in the TB control program, which include the social anthropology of patients.

The main focus of this study is on strain FITNESS.

1.4 AIMS

- (1) To test the hypothesis that high fitness cost mutations would not be found in the Beijing strain family and that the attenuated form of Beijing strains would not actively spread.
- (2) To discover the proteins that make large clusters of drug-resistant Beijing isolates more fit than small clusters of drug-resistant Beijing isolates.

1.5 EXPERIMENTAL APPROACH

The experimental approach of this study was to make use of existing data on drug-resistant *M. tuberculosis* strains from different settings in SA, and to use different molecular methods to get a better understanding of the drug-resistant TB epidemic in SA. Genomic methods such as DNA sequencing were used to characterise *M. tuberculosis* isolates. Proteomic methods such as 2-Dimensional Gel Electrophoresis were used to identify new proteins that might play an important role in the virulence/fitness of drug-resistant *M. tuberculosis* strains.

This thesis was structured according to the instructions of the Journal of Clinical Microbiology. References, figures and tables relevant to each chapter will be given in each chapter.

1.6

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CHAPTER 2

LITERATURE REVIEW

METHODOLOGIES FOR STUDYING VIRULENCE OF *MYCOBACTERIUM TUBERCULOSIS*.

MY CONTRIBUTION:

Literature search

Writing of literature review

2.1 Background

The tuberculosis (TB) disease burden has reach frightening proportions in certain countries and is cause for much concern worldwide (94). *Mycobacterium tuberculosis* (*M. tuberculosis*), the causative organism of TB, is responsible for 2-3 million deaths annually and one-third of the world's population is infected with this bacterium (94). Only a small proportion (5%) of those infected develop primary TB (94). TB is a disease that is treatable with chemotherapy using anti-TB drugs but the emergence of drug-resistant *M. tuberculosis* strains, which include multi drug-resistant (MDR) strains (strains that are resistant to at least two of the most effective front-line anti-TB drugs, isoniazid (INH) and rifampicin (RIF)) (45) as well as extreme drug-resistant (XDR) strains (strains that are resistant to most of the anti-TB drugs available) (49,96) has made it very difficult to treat and cure TB patients leading to the high mortality rates seen today.

For any bacteria to cause disease there must be an interaction between the bacterium and the host. Some bacteria are more aggressive in their ability to cause disease and those bacteria are usually referred to as being more virulent. Many bacteria produce “virulence factors” such as spores or toxins to assist them in causing disease, but for *M. tuberculosis*, no clear virulence factor could be identified yet. Defining virulence in tuberculosis (TB) is complicated and ill defined. *M. tuberculosis* virulence can be divided into four different components that include infection, pathogenicity, transmission, and active disease. Infection, pathogenicity and transmission are tightly linked in causing active disease and the different components are experimentally difficult to study. As a result there are quite a number of definitions for virulence in TB. The most common definitions used include mortality, which is defined as the percentage of infected host that die (72), it has also been defined as the time that it took the host to die after being infected (72). Other definitions include, the capacity to produce disease, and disease severity (55); and the ability to cause progressive pathology in the lungs (22,60).

Transmission is considered a key component of virulence in *M. tuberculosis*. Results from molecular epidemiological studies suggest that some *M. tuberculosis* strains are more dominant in certain regions because of their ability to transmit (32,40,88,90,92), and it is suggested that these strains are more virulent, which can be seen as a reflection of the fitness of the strain . The transmissibility or fitness of a bacterium is determined by the bacterium's ability to infect a susceptible host, to persist and proliferate in that host and than causing disease in such a manner so that it can be transmitted to a secondary host (18).

Humans cannot be used as a model to study *M. tuberculosis* pathogenesis. Therefore, alternative, but appropriate in vivo and in vitro experimental models that mimic the specific environments of the natural host are required to identify the determinants of *M. tuberculosis* virulence in humans. It is necessary to have good models for studying the mechanisms and determinants of virulence in *M. tuberculosis* since this will help to understand this extremely successful pathogen.

This review describes some of the major methodologies and models that are currently used to study virulence in *M. tuberculosis*. These models are used to study the molecular and physiological mechanisms of pathogenesis, pathology, and immunology of the disease, thus helping us to gain insight into the pathogen–host interaction in an attempt to understand how the bacterium evade and survive host defences and cause disease. The first part of the review will focus on the major models that are used and the second part on the molecular methodologies that are used to study virulence in conjunction with the experimental models.

2.2 TISSUE CULTURE MODELS

TB infection begins when *M. tuberculosis* reach the pulmonary alveoli where they invade and replicate within alveolar macrophages (53). The bacteria are then picked up by dendritic cells, which can transport the bacilli to local lymph nodes (10). From there the bacteria get into the bloodstream and are transported further to other tissues and organs. Progression from TB infection to TB disease occurs when the TB bacilli overcome the immune system defences and begin to multiply.

Although some animals acquire disease in appropriate tissues and organs when infected with human pathogens, the immune and other physiological responses encountered in an animal model may be different from those that the bacteria would engage during human disease (65). In those instances model systems containing human cells may be more appropriate (7). These tissue culture models may be mono-layered or multiple-layered (7). They are much easier to work with than animal models and results are obtained much faster. Tissue culture models may include macrophages (53), dendritic cells (DCs) (10), or pneumocytes (53).

2.2.1 Macrophage models

Macrophage models are very useful for studying virulence of *M. tuberculosis* since these are the cells that are primarily infected by *M. tuberculosis* (20). Human- or mice macrophages can be used, but human macrophages are difficult to obtain (72). Macrophages can be obtained as primary cultures or immobilised cell lines, and although primary human macrophages are the models of choice since they are more representative of the actual *in vivo* situation, they are not so readily obtainable than mice macrophages and are also more variable (72).

2.2.2 Dendritic cells

Dendritic cells (DCs) produce numerous amounts of cytokines involved in host defence mechanism and therefore play a critical role in innate immunity as well as in the initiation of an adaptive immune response (10,39,54). These cells are considered to be better antigen processors and presenters than macrophages. They can also capture antigens against which immunity is normally avoided. They are also migratory and therefore may play an important role the dissemination of *M. tuberculosis*. They can be derived from human peripheral blood or mouse bone marrow, and are considered to be the most potent antigen presenting cells (10).

2.2.3 Pneumocyte models

Several *in vitro* studies have shown that *M. tuberculosis* enter and replicate in pneumocytes, but the interaction is short lived because the cells proceed rapidly to death releasing a cascade of inflammatory chemokines and cytokines (70). Other studies, where they made use of transcytosis assays conducted with pneumocyte monolayers, indicated that pneumocytes internalized the bacteria, but with low efficiency, the bacteria is then exocytosed at the basolateral surface of the cell (70). It was therefore concluded that the pneumocytes might play a role in the rapid dissemination of the bacteria to other tissues and organs (70).

2.3 ANIMAL MODELS

The major animal models used to study *M. tuberculosis* virulence include mice, guinea pigs, rabbits (72), and to a much lesser extent the non-human primates (15,46,51). A number of aerosol delivery systems have been developed for the infection of animal models (17). In some of the experiments, the animals are exposed to aerosols of *M. tuberculosis* that are deposited directly into the alveolar spaces of the lungs. This is done using aerosol exposure chambers (Figure 1.1) that have been designed so as to produce uniform clouds of droplet nuclei, which result in pulmonary infection of the animals simultaneously. The animals mentioned above, develop disease that exhibits many of the important features of human TB (52). These include the development of granulomas in the lung and other tissues, the onset of a strong immune response mediated by CD4 and CD8 T cells, temporary control of the accumulation of bacilli in the lungs and other organs, and depending on the animal species, persistent infection that remains under control for many months, as well as the eventual (continuing bacterial proliferation leading to disease progression and death) increase and uncontrollable infection followed by death. In addition, these models are appropriate and very useful for the screening of new anti-TB drugs and vaccines since it was found that oral therapy with first-line anti-TB drugs as well as vaccination with BCG results in significant protection in all of the models (52).

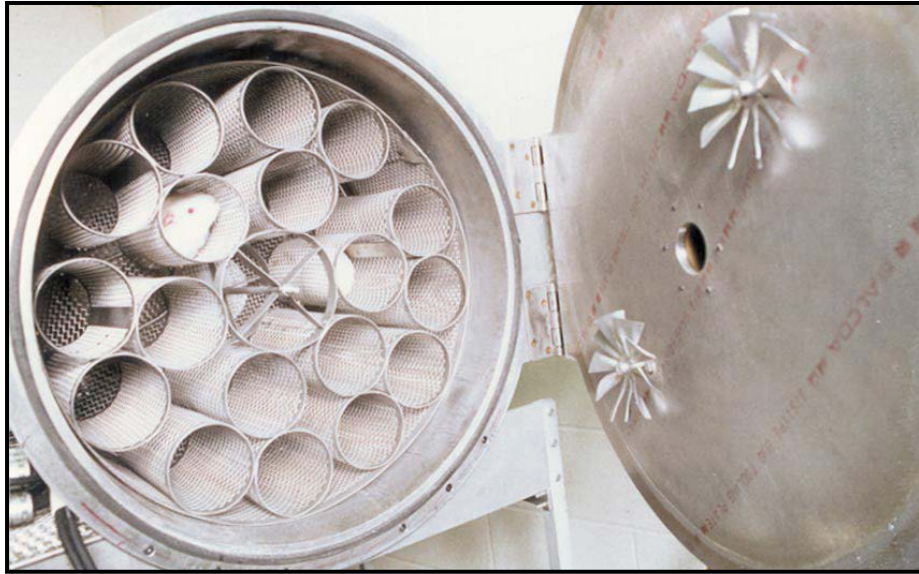


Figure1.1. Custom-made aerosol exposure chamber, in which small experimental animals can be infected by the reproducible deposition of very small numbers of virulent *Mycobacterium tuberculosis* directly into the alveolar spaces of the lung (52).

2.3.1 Mouse model

There are many reasons why this model is the most used to study TB. The mouse model has very well studied genetics (31,52,62). The mouse immune system is very well characterised and many immunological reagents for the mouse model are commercially available. The cost of purchasing and maintaining mice is low, and this animal can easily be housed under BSL3 conditions. The genetic manipulation of the mouse is also highly advanced. However, there are certain limitations that this model cannot overcome. The disease process is significantly different than that of humans (24,52). The granulomas do not progress to necrosis, caseation and liquefaction (24). The mouse can sustain very high levels of *M. tuberculosis* without progressing to disease for months and it has increased pathology due to high bacterial numbers (52).

Over the years quite a number of mouse models have been developed which include immunodeficient mouse models, gene-disrupted and transgenic mouse models, as well as immunosenescent mouse models (31). These models were very helpful to define the pattern of TB

disease (31), to investigate the strong immune response to TB that mice show, as a cost effective model for the evaluation of drugs, and to study virulence of *M. tuberculosis* (31). Some of the inbred mouse strains used are grouped as highly susceptible models (CBA, DBA/2, C3H, and 129/SvJ) and highly resistant models (e.g. BALB/c and C57BL/6) (16).

The mouse model was very important in certain virulence studies, where *M. tuberculosis* strains that were found to be more virulent than others, in humans, were also more virulent in mice. In contrast, other studies showed that strains that were found more virulent in humans were not necessarily more virulent in mice (67). In another study, it was found that a strain of *M. tuberculosis* that was believed to be virulent, only have the ability to induced a stronger immune response, therefore caused more tuberculin skin test conversions than other strains, but was actually less virulent (60).

2.3.2 Guinea pig model

The guinea pig model is considered the most susceptible animal model of TB. Impressive caseous necrosis, very similar to that in humans, develops in the lungs. Chronic progressive disease develops after very low-dose infection. This model has many significant similarities to humans. They are immunologically and hormonally closer to humans than mice. There are significant similarities in the physiology of the pulmonary tract, especially the response lung to inflammatory stimuli. They respond well to anti-TB drugs and can be successfully protected by BCG and some experimental vaccines. Several cytokine and chemokine genes of the guinea pig have also been cloned. This model is relatively inexpensive and easy to house under BSL3 conditions compared to larger animal models.

To study virulence of *M. tuberculosis*, this model has been used to compare different *M. tuberculosis* strains (grown under different conditions) to determine whether there is a difference in the ability to cause infection in the lung, and in the dissemination of the bacterium to other

organs. In a study done by Williams et al. (95), female Dunkin-Hartley guinea pigs were used and they made use of a 3-jet Collison nebulizer together with a Henderson apparatus (this apparatus allows the delivery of aerosols directly to the snout of the animal without contamination of the fur or eyes) to infect the animals with *M. tuberculosis* (17). The growth conditions used for these experiments must be relevant to the host environment. Two *M. tuberculosis* strains were compared under stress conditions and they found that infectivity as well as dissemination increased under these conditions (17). In another study done by Russell K. Karls *et al.* (42), guinea pigs were used to identify sigma factors that may be important regulators of virulence. Secondary sigma factors sense specific stress signals and coordinate expression of genes encoding functions that facilitate bacterial adaptation to those particular stresses. In this study, a Madison aerosol chamber was used to deliver *M. tuberculosis* into the lungs of the guinea pigs (42). Again, female Dunkin-Hartley guinea pigs were used. They found that sigma factor C (sigC) is an important regulator of virulence, because sigC deficient *M. tuberculosis* resulted in fewer and smaller lung and spleen granulomas (42). SigC is responsible for mediating adaptive survival of *M. tuberculosis* upon entering the host environment (42).

Recently, the guinea pig model has been advanced with the establishment of an airborne infection research (AIR) facility in South Africa in partnership between the South African Medical Research Council, CDC and USA Harvard School of public health (73). The facility includes a wing of an MDR-TB referral hospital converted into an experimental facility consisting of a) a clinical unit, providing human-source infectious MDR-TB aerosols or aerosol of bacteria through a nebulizer, b) separate guinea pig rooms coupled to each of the clinical units, and c) a dedicated specialist TB laboratory. Air from the clinical unit is conveyed to the animal exposure chamber under controlled conditions. Guinea pigs are tested regularly for infection and sacrificed at predetermined times for additional experiments. This facility currently does not measure animal-to-animal transmission but can measure infection and pathogenicity after aerosolizing bacteria in the clinical room. There are

no published results on the experimental validation of the facility to measure transmission of bacteria through aerosolization and the facility is extremely expensive to use.

2.3.3 Rabbit model

The rabbit model is useful for comparison studies between virulent *M. tuberculosis* isolates (47,83), as well as modelling tuberculous meningitis (84). Rabbits can be infected by aerosol by using a nose-only system. The aerosols are generated in a class 3-biosafety glove box cabinet under negative pressure or in a completely contained biosafety level 4 air-locked area. The biosafety level 3-exposure chamber is a 16-liter Plexiglas box with one side containing a circular latex dam with a cut-out into which the snout (nose and mouth) of the rabbit is inserted (8,47).

A spectrum of disease that represents many of the specific stages of human disease develops in these models, which is an advantage over both mouse and guinea pig models (47). They are relatively resistant to *M. tuberculosis* because they are able to contain disease caused by virulent *M. tuberculosis* isolates. Lung granulomas closely resemble the human granuloma, with caseous necrosis as well as cavity formation (47). However, these animals are difficult to house under BSL3 conditions, therefore increasing the cost of this model (24).

In 1999, Bishai *et al.* (8) made use of Lurie's tubercle count method to investigate the virulence of H37Rv compared to that of CDC1551. This method is based on the hypothesis that the more virulent the bacterium, the greater its resistance to destruction by both alveolar macrophages and the host immune response (8). The bacterium that is more virulent will therefore produce more grossly visible tubercles. Apart from the number of visible tubercles in the lung, the size of the tubercle as well as the number of bacilli culturable from the tubercles is important when determining virulence (8). For the experiment, Bishai *et al.* used 12 rabbits, 6 were infected with H37Rv and the other 6 were infected with strain CDC1551 by using an aerosol exposure chamber.

They found that the rabbits infected with the different strains produced equal numbers of grossly visible tubercles, but the tubercles produced by CDC1551 were smaller and contained fewer bacilli. They therefore concluded that CDC1551 was less virulent in rabbits than H37Rv (8).

Another study done Manabe *et al.* (47) investigated tuberculosis infection in rabbits with 3 different strain which include *M. tuberculosis*, CDC1551 and Erdman. The rabbits were also exposed to the bacteria via an aerosol exposure chamber. They found that fewer inhaled bacilli of the Erdman strain than that of H37Rv were required to produce a visible tubercle/lesion in the rabbits at 5 weeks post infection (47). The rabbits infected with H37Rv had lesions that healed in 4 to 6 months whereas lesions in half of the rabbits infected with the Erdman strain had healed at that time. In this study the concluded that the Erdman strain is more virulent than H37Rv (47). They decided to do a H37Rv-based microarray to investigate this further and found that a gene called Rv3428c in RD6 was absent in Erdman (47). RD6 is also known to be deleted in CDC1551as well as in many strains of *M. bovis* (12). They concluded that the deletion of gene Rv3428c might in part be the reason for the different patterns of disease produced by the strains in the rabbit.

2.3.4 Non-human primate model

Several species of monkeys are susceptible to infection with *M. tuberculosis*, but the two species most used in studies, is the Cynomolgus monkey, *Macaca fascicularis* (15,26,46) and the Rhesus monkey, *Macaca mulatta* (51), which are referred to as Old World monkeys. These animals are closely related to humans and are quite susceptible to infection with *M. tuberculosis* (51). They can also be infected with very low doses of virulent *M. tuberculosis* via the respiratory route resulting in disease, which closely resembles the human disease (25). They exhibit antigen-induced T-lymphocyte activity and can be successfully protected by BCG. They represent by far the most closely related conditions found in humans than any of the other animal models (46). However, monkeys are very expensive to maintain, and are difficult to handle and house under BSL3

conditions (25). Much less research has been done on this model; therefore literature available on this model is very limited.

2.4 MOLECULAR METHODOLOGIES THAT CAN BE USED TO STUDY VIRULENCE OF *M. TUBERCULOSIS*.

Transmission is considered a key component of virulence in *M. tuberculosis*. The transmissibility of a bacterium is determined by the bacterium's ability to infect a susceptible host, to persist and proliferate in that host and then causing disease in such a manner so that it can be transmitted to a secondary host (18). Transmission events of *M. tuberculosis* to new hosts are difficult to study in any animal model other than humans. Studying transmissible clinical isolates, as measured by molecular epidemiological studies, has been the approach to this problem. Several studies using molecular epidemiological methods, that document transmission of drug susceptible and drug resistant *M. tuberculosis* strains in specific settings/populations, have been documented (11,14,28,48,71,81,89,90). Results from these studies suggest that some *M. tuberculosis* strains are more dominant in certain regions because of their ability to transmit, and it is suggested that these strains are more virulent which is then a reflection of the fitness of the strains (29).

Some of the molecular epidemiological methods currently used include IS6110 restriction fragment length polymorphism (RFLP) analysis (87), spoligotyping (41), MIRU-VNTR (58), SNP analysis (66), and genomic deletion analysis (36,43,57,85,86). The advantages and disadvantages of these methods are summarised in Table 2.1.

IS6110 RFLP analysis

IS6110 RFLP is based on the detection of the insertion sequence IS6110 which is present in different copy numbers (between 0 and 25 copies) in the *M. tuberculosis* complex and is integrated at various chromosomal sites. A pattern can be generated according to the IS6110 insertion sequences present in a particular *M. tuberculosis* strain. DNA is extracted, purified and digested with the restriction enzyme *Pvu*II which cleaves the IS6110 insertion sequence at a single site. The digested DNA

fragments are separated overnight on an agarose gel after which it is transferred to a DNA membrane. The hybridizing digested fragments are detected by a chemilluminescence reaction that is initiated by two substrates. The RFLP patterns can be detected on a light-sensitive film (50,87).

Spoligotyping

Spoligotyping is a technique that is based on the detection of DNA polymorphisms in the direct repeat (DR) region in the *M. tuberculosis* genome. The DR region contains a variable number of short direct repeats interspersed with non-repetitive spacer sequences (34). *M. tuberculosis* strains vary in the number of DRs and in the presence or absence of particular spacer sequences (30). DRs are very well conserved among *M. tuberculosis* strains and are used as targets for *in vitro* DNA amplification in which the variation in the spacers is used to obtain different hybridization patterns of the amplified DNA with multiple synthetic spacer oligonucleotides, which are covalently bound to a membrane. The spacer sequences are first amplified by PCR and then hybridized to a membrane containing the synthetic spacer oligonucleotides. Hybridisation is detected by streptavidine-peroxidase conjugate and a substrate which results in a chemilluminescence reaction that can be detected on film (41).

MIRU-VNTR typing

MIRU-VNTR typing is high-throughput PCR analysis of *M. tuberculosis* genomic loci that contain variable-number tandem repeat (VNTR) sequences. *M. tuberculosis* strains can be typed by a numerical code corresponding to the numbers of VNTRs in 12 different loci that contain novel genetic elements named mycobacterial interspersed repetitive units (MIRUs) (78,79). These loci have formed the basis of a PCR-based typing method that has discrimination similar to that of high IS6110 copy number strains and better for low copy number strains (77).

SNP analysis

A SNP (single nucleotide polymorphism) is a variation occurring when a single nucleotide (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual). Almost all common SNPs have only two alleles. SNPs may fall within coding sequences of genes, non-coding regions of genes, or in the intergenic regions between genes. SNPs within a coding sequence will not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code. A SNP in which both forms lead to the same polypeptide sequence is termed *synonymous* (sometimes called a silent mutation) (sSNP) - if a different polypeptide sequence is produced they are *non-synonymous* (nsSNP) (50). SNPs that are not in protein-coding regions may still have consequences for gene splicing, transcription factor binding, or the sequence of non-coding RNA. SNPs can be detected by DNA sequencing.

Genomic deletion analysis

This method is based on large-sequence polymorphisms (LSP) which have been identified by comparative genomic analysis of H37Rv and CDC1551 (23). LSPs mainly occur as a result of genomic deletions (13). Analysis can be performed by a PCR-based method or by automated GeneChip techniques, using deleted fragments (86).

These techniques have detected genotypic variations among *M. tuberculosis* strains and can be used to obtain fingerprints for different isolates of *M. tuberculosis* (41,56). RFLP studies are used to discriminate between individuals by highlighting minor chromosomal differences/changes that are not always related to a variation in phenotype (63).

Mouse models have indicated that there is a difference in pathogenicity in different clinical *M. tuberculosis* strains, but the mouse or any other animal model is unable to measure transmission (4,61). In the absence of a mouse model to directly measure transmission, investigators have used competition assays on culture medium and cell lines to measure virulence and strain fitness (48). Some of the reports indicate that drug resistant strains of *M. tuberculosis* spread less readily than drug-susceptible strains (27), others show no difference in disease transmission (80), and we have shown larger drug resistant clusters than susceptible clusters within the same strain family (75).

2.5 DOWNSTREAM METHODOLOGIES TO FURTHER STUDY VIRULENCE.

The molecular/downstream methods that can be used to further investigate virulence factors that might have been identified, during for example animal studies, are discussed below.

2.5.1 Whole genome sequencing

Whole genome sequencing is one of the most advanced technologies today, and can be used to get a much better understanding of *M. tuberculosis* virulence (19). Comparative genomic analysis can be used to get insight into differential transmission events of *M. tuberculosis* and genetic loci that might be involved. A small number of genomes have been fully sequenced yet. These include the laboratory strain H37Rv (19), the clinical strain CDC1551 (23), *M. tuberculosis* strain C, and Haarlem (1). The first whole genome sequence of an MDR strain originates from our strain collection and the sequence was released recently by SAMJ (91). With Whole genome sequencing, it is now possible to compare the genomes of more or less fit *M. tuberculosis* strains, as defined by molecular epidemiological methods (2,5,19,21).

2.5.2 Microarray analysis

Microarray analysis is a high throughput technique that can be used to analyse every gene in the genome simultaneously (97). Microarrays are made up of DNA sequences (genomic DNA – coding,

intergenic, or non-coding regions; complementary DNA; and oligonucleotides that cover all of the open reading frames in the genome or only specific ones from specific genes of interest) that serve as probes that attached to a solid surface such as glass slides, membranes, or silicon chips (69). The sample of interest is fluorescently labelled and hybridized to the array. A confocal microarray scanner such as Affymetrix 428 dual-laser will detect the fluorescence signal and will generate a gene expression profile (35). The data obtained from the array can then be linked to a gene identity grid which specifies which genes were immobilised on the microarray spots. The data are further analysed using Genespring software which will reveal the identity of the genes, the location of the genes, and whether they are up or down regulated, etc. (93). A list of candidate genes can be compiled which can be used in gene manipulation (knock out) studies to identify the molecular mechanisms associated with a specific phenotype. For example, microarray analysis can be done on two strains, one considered more virulent than the other. From the results of the analysis, a list of candidate genes that differ between the two strains and that might play a role in virulence/transmission can be compiled and used for further investigations (44).

2.5.3 Proteomics

Proteomics is the large-scale study of proteins, particularly their functions and structures (3,9). Proteins are important parts of living organisms, as they are the main components of the physiological metabolic pathways of cells. Proteomics is often considered the next step in the study of biological systems, after genomics. It is much more complicated than genomics, mostly because while an organism's genome is rather constant, a proteome differs from cell to cell and constantly changes through its biochemical interactions with the genome and the environment. One organism has very different protein expression in different parts of its body, different stages of its life cycle and different environmental conditions. Proteins are also very complex relative to nucleic acids. E.g., in a human there are about 25 000 identified genes but an estimated >500 000 proteins that are

derived from these genes. This increased complexity derives from mechanisms such as alternative splicing, protein modification (glycosylation, phosphorylation) and protein degradation which lead to transcripts giving rise to more than one protein. Many proteins also form complexes with other proteins or RNA molecules, and only function in the presence of these other molecules (59).

Proteomics have played an important role in the discovery of biomarkers, such as markers that indicate a particular disease (68). Specific protein biomarkers identified by proteomics, can be used to diagnose disease. It is also very useful for characterising cells and tissues; because there are so much more proteins in the proteome than protein-coding genes, protein diversity cannot be fully characterized by gene expression analysis (38). With proteomics we can also identify which proteins interact, which can give important clues about the functions of newly discovered proteins. One of the most important outcomes from the study of genes and proteins has been the identification of potential new drugs for the treatment of disease. Also, understanding the structure and function of protein-protein interactions is important for the development of effective diagnostic techniques and disease treatments. Proteomics can be used to identify proteins produced during a particular disease, which can be used to diagnose the disease quickly (76).

Various technologies are used for proteomics which include, one- and two-dimensional gel electrophoresis (used to identify the isoelectric point of a protein as well as its relative mass) (9); mass spectrometry, example MALDI-TOF (is used to identify proteins by peptide mass fingerprinting) (37); Affinity chromatography, yeast two hybrid techniques (used to identify protein-protein and protein-DNA binding reactions) (6); software based image analysis (used to automate the quantification and detection of spots within and among gels samples) (74).

2.5.4 QT-PCR analysis

Quantitative reverse transcriptase polymerase chain reaction (QT-PCR) can be used to measure gene expression of candidate genes identified by whole genome sequencing and microarray analysis. The level of differential expression between two different *M. tuberculosis* strains can be measured (33).

2.6 SUMMARY

The models described in this review were very important to better our understanding of the very successful bacterium, *Mycobacterium tuberculosis*, which is responsible for so many deaths each year. Each one of the models has certain advantages as well as limitations, but all of them contributed in their own unique way to further our knowledge of TB and the causative agent, but as we have seen, one specific model does not give the answer to all the questions, therefore a combination of the different types of models is needed. Some of the tissue culture models contain human cells. Therefore these models might give a better representation of the immune and other physiological responses encountered in the human when infected with the bacterium, than what is encountered in an animal model. However, some of these tissue culture models are monolayered, and *in vitro* tissue culture models are artificial and do not represent the complex interactions that occur in humans or animals. Therefore the knowledge gained from tissue culture research have to be used in conjunction with that gained from animal studies to give a better understanding of the host-pathogen interactions. Transmission, which can be used as an indicator of strain virulence or fitness, is difficult to study in tissue culture models or animal models; therefore molecular epidemiological techniques are used to study transmissible clinical *M. tuberculosis* isolates. There are many different molecular techniques available today, which leads to new information about *M. tuberculosis*, and its host, as well as other factors that might play a role in the pathogen's success that are contributing to our knowledge and understanding of the TB disease. There are also very exciting new models that are being investigated. These include *Dictyostelium discoideum* (64), which is used as a surrogate macrophage, *Caenorhabditis elegans* and *Drosophila melanogaster* are being investigated as TB hosts to study conserved innate immune mechanisms (64), and the zebrafish is used to study both innate and adaptive immunity (64,82). These models were developed as model hosts to study aspects of TB that cannot be studied in the mouse model.

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Table 2.1. Methods currently used to study the molecular epidemiology of TB.

METHOD	ADVANTAGES	DISADVANTAGES
RFLP (87)	<p>Gold standard Widely used - much data available for comparison Marker stability very adequate for the study of transmission</p> <p>Extensive diversity in patterns for isolates with more than 6 <i>IS6110</i> insertions</p> <p>Mixed infections can readily be detected Can also be used for evolutionary and phylogenetic studies Laboratory error/cross-contamination can be detected</p>	<p>Labour intensive Requires sub-culturing and DNA isolation Time consuming</p> <p>Cannot be used to reliably type isolates with less than 6 <i>IS6110</i> insertions Cannot be used to type strains with no <i>IS6110</i> insertions Interlaboratory comparative analysis can be difficult</p>
Spoligotyping (41)	<p>Very simple and easy technique</p> <p>No DNA purification required, can perform directly on cell lysate</p> <p>Can be performed on non-viable bacteria Standardized analysis for 43 spacers Hybridization membranes for simultaneous analysis of 40-45 samples commercially available Data can be presented in binary format, allowing inter- and intralaboratory comparisons Two large databases available for comparative analysis</p>	<p>Less discriminatory then <i>IS6110</i> RFLP genotyping</p>
MIRU-VNTR (58)	<p>Rapid, high-throughput technique</p> <p>Can be performed directly on cell lysate, no DNA purification required Digitized results Suited for large-scale genotyping Manual as well as automated analysis is possible</p>	<p>Less discriminatory then <i>IS6110</i> RFLP genotyping Similar patterns may be found in distinct lineages</p>
SNP (43)	<p>Most precise information on strains based on sequencing of polymorphic loci High resolution Some selected SNP can be highly informative Can be automated for large-scale genotyping Other applications include phylogenetic analysis, drug-resistant studies, research on host-pathogen interactions</p>	<p>Requires extensive genome sequencing of multiple chromosome targets</p>
Genomic deletion analysis (86)	<p>High throughput unit microarray analysis Reserve line probe with hybridization membrane possible Results can be digitalized Multiplex PCR for 43 loci available Other applications include phylogenetic analysis, host-pathogen interactions based on specific genomic deletions, facilitation of genome structure-function studies</p>	<p>Not yet standardized Representative target deletions need to be determined Technique has yet to be evaluated in different settings</p>

CHAPTER 3

**SPREAD OF A LOW FITNESS DRUG-RESISTANT *MYCOBACTERIUM*
TUBERCULOSIS STRAIN IN A SETTING OF HIGH HIV PREVALENCE.**

My contribution to this project:

Study design

Drug resistance genotyping

Spoligotyping

Interpretation of data

Writing of manuscript

The results from this study were written up in the form of a note and were recently submitted (October 2007) to the Journal of Clinical Microbiology.

3.1 BACKGROUND

The fitness of *M. tuberculosis* strains circulating in a community is thought to be the driving force perpetuating the TB epidemic. This is particularly true for the spread of drug resistance, as dogma has suggested that the evolution of drug resistance has a fitness cost resulting in the overall attenuation of the pathogen (1). This phenomenon has been demonstrated during the spontaneous evolution of rifampicin (RIF) resistance, where a direct correlation was observed between the frequency at which a specific non-synonymous single nucleotide polymorphism (nsSNP) occurred and the fitness of the mutant clone (4). That study suggested that rarely observed nsSNPs had a high fitness cost, while frequently observed nsSNPs had a low fitness cost. These results correlated well with the frequency of nsSNPs observed in RIF-resistant clinical isolates (4). However, it was not clear whether the genetic background of the clinical isolates with high fitness cost nsSNPs had influenced their ability to spread and cause disease (4).

Molecular studies have suggested that certain *M. tuberculosis* strains are epidemiological more successful than others (3). This has been demonstrated by the spread of strains from the Beijing strain family in most settings world-wide (5). Accordingly, it has been hypothesized that this is a high fitness genotype, possibly as a result of the evolution of unique properties, including the ability to evade the protective effect of BCG vaccination (15), the ability to spread more readily (2) and the ability to acquire drug-resistance more frequently (11).

Analysis of the NTF region of the genome has demonstrated that strains with the Beijing genotype can be broadly grouped as typical or atypical according the presence or absence of an IS6110 insertion in this region, respectively (9). Phylogenetic analysis has provided evidence that these two genotypes are derived from a common progenitor (6). However, they demonstrate vastly different epidemiological characteristics as strains with the atypical Beijing genotype are only rarely

observed (9,13). This has prompted speculation that atypical Beijing strains are of a lower fitness as compared to typical Beijing strains. Thus we hypothesize that high fitness cost RIF resistance causing nsSNPs would only be rarely observed in *M. tuberculosis* strains with an atypical Beijing genotype unless epidemiological factors favouring their spread were present.

3.2 MATERIALS AND METHODS

STUDY DESIGN (The different techniques used in this study are described in detail in Chapter 5.)

To test this hypothesis, sputum specimens were collected from re-treatment cases attending health-care clinics or TB referral hospitals in two regions in SA. Each specimen was subjected to routine culture-based drug-susceptibility testing for isoniazid and rifampicin. To identify the nsSNPs conferring RIF resistance, the RRDR of the *rpoB* gene (12) of the first isolate from each patient was subjected to DNA sequencing. In order to determine whether a relationship existed between strain genotype and the fitness cost of the respective nsSNPs, the isolates from this study were classified as either Beijing or non-Beijing by spoligotyping (8). IS6110 DNA fingerprinting (14) was also done to determine whether the Beijing isolates associated with specific nsSNPs were transmitted. Determination of the regions of difference of the *M. tuberculosis* isolates was done using a PCR-based method.

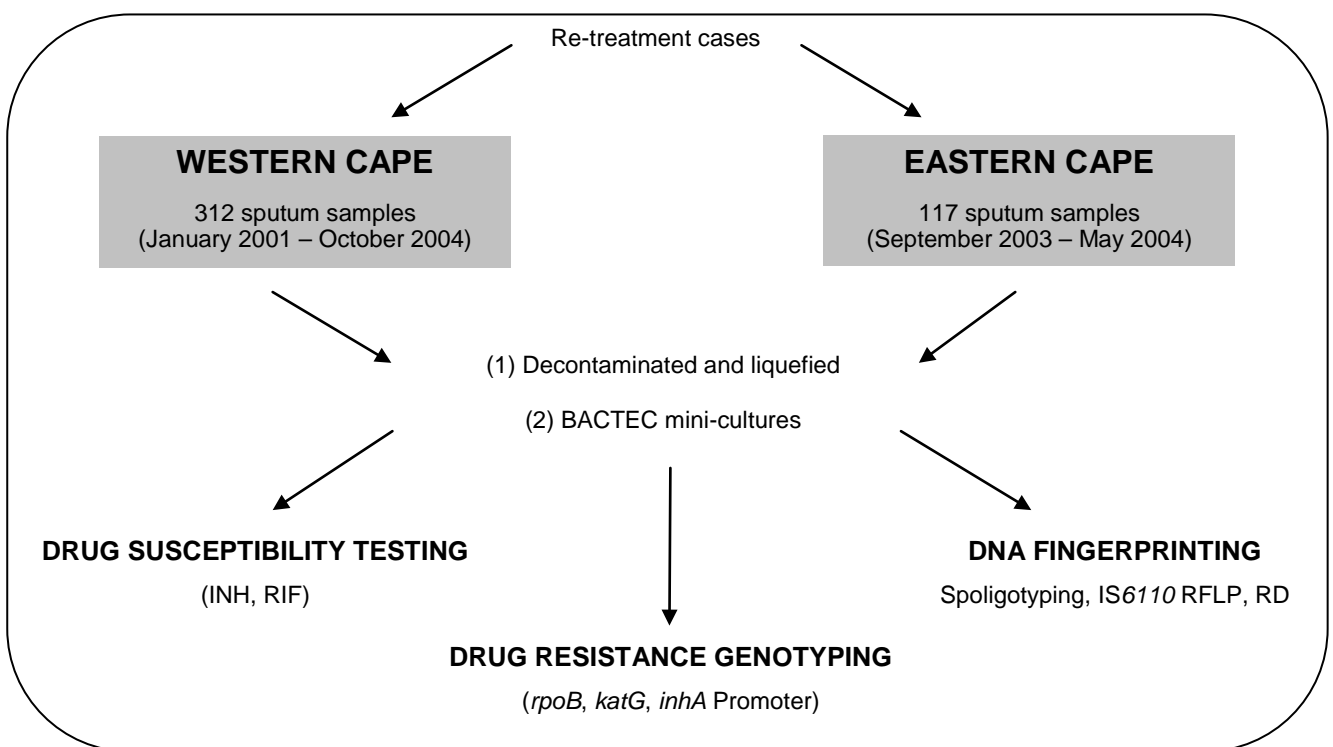


Figure 3.1. Experimental design of this study. INH=isoniazid; RIF=rifampicin; RFLP=restriction fragment length polymorphism; RD=region of difference.

3.3 RESULTS AND DISCUSSION

Sequence analysis showed that >90% of RIF-resistant isolates had a nsSNP in the RRDR region of the *rpoB* gene. Of the 30 nsSNPs identified, 25 nsSNPs appeared at a frequency consistent with frequencies reported in *in vitro* generated rifampicin-resistant mutants (4,7,10) (Table 3.1). However, the frequency of appearance of the remaining 5 nsSNPs was discordant when compared to the *in vitro* generated rifampicin-resistant mutants (Table 3.1). The nsSNPs at codons 516 (GAC→GTC) and (GAC→TAC) and 533 (CTG→CCG) were significantly over-represented in the rifampicin-resistant clinical isolates, while nsSNPs at codon 522 (CTG→CCG) and codon 526 (CTG→CCG) were significantly under-represented (Table 3.1). This suggests that the two nsSNPs at codon 516 had a lower high fitness cost in clinical TB as compared to *in vitro* generated rifampicin-resistant mutants. Conversely, the nsSNPs at codons 522 (CTG→CCG) and 526 (CTG→CCG) appear to have a high fitness cost in clinical TB.

In order to determine whether a relationship existed between strain genotype and the nsSNPs conferring rifampicin-resistance, the isolates from this study were classified as either Beijing or non-Beijing by spoligotyping (8). The results showed that 116 (37%) of the rifampicin-resistant cases from the WC region and 59 (50%) of the rifampicin-resistant cases from the EC region were infected with a Beijing genotype strain, respectively (Table 3.1). Sub-classification of the Beijing isolates as either typical or atypical (6) showed that the population structure of rifampicin-resistant Beijing strains was significantly different in the two study settings [Fisher's exact test OR = 21.6; CI95% 9.6 to 48.6, $p < 0.0001$] (Table 3.1). The nsSNP at codon 516 (GAC→GTC) was associated with the atypical Beijing genotype from the EC [Fisher's exact test OR = 45; CI95% 3.8 to 525, $p = 0.0008$], while the nsSNP at codon 533 was mostly found in isolates with the typical Beijing genotype from the WC (Table 3.1).

IS6110 DNA fingerprinting (14) was done to determine whether the Beijing isolates with nsSNPs at codons 516 (GAC→GTC) and 533 (CTG→CCG) were transmitted. Analysis of these DNA fingerprints showed that isolates from the WC region with the typical Beijing genotype and either an nsSNP at codon 516 or codon 533 were not clustered, thereby suggesting that these nsSNPs had evolved independently and that the resulting clones were not transmitted. In contrast, the isolates from the EC region with the atypical Beijing genotype and an nsSNP at codon 516 (GAC→GTC) were clustered and also shared the rare -17 *inhA* promoter mutation (GAC→TAC) (data not shown), suggesting ongoing transmission. The above isolates were also clustered with the atypical Beijing strains from the WC which had an nsSNP at codon 516 (GAC→GTC) and the -17 *inhA* promoter mutation, suggesting inter-provincial spread. This finding is contrary to previous reports which have suggested that atypical Beijing strains are attenuated in their ability to transmit (9,13), while the mutation at codon 516 (GAC→GTC) would have been expected to further compromise the ability of these strains to transmit unless compensatory mutations were present or the epidemiological context allowed transmission to occur. Analysis of the host population in the EC region showed HIV co-infection to be a risk factor for the spread of the atypical Beijing strains [z-test for the hypothesis that proportion of HIV + cases = 0.42, p=0.029]. In contrast, the frequency of atypical Beijing strains was low in the WC region which in turn has a low incidence of HIV/TB co-infection (6). This raises concern for the spread of all drug-resistant strains in vulnerable populations.

3.4 CONCLUSION

The fitness cost associated with the evolution of rifampicin-resistance in *Mycobacterium tuberculosis* may be different in clinical tuberculosis as compared to *in vitro* generated mutants. The results from this study are very alarming. An atypical Beijing strain (attenuated phenotype) demonstrated the ability to spread despite acquiring rifampicin-resistance. This atypical Beijing strain has an *rpoB* mutation at codon 516 that are not frequently seen in *in vitro* generated *rpoB* mutants and is not dominant in clinical isolates, and are therefore thought to be a high fitness cost mutation. HIV co-infection could be linked to this specific strain [$p=0.029$] and might be the reason for its spread; therefore, greater vigilance is required to contain the drug-resistant TB epidemic in high HIV prevalence settings. This can be achieved by the development and implementation of rapid diagnostics, provision of appropriate therapy, ensuring treatment adherence and intensified screening of contacts. However, in order for diagnosis and treatment to be effective it is essential that communities are educated to improve health seeking behaviour.

3.5

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Table 3.1. Distribution of mutations conferring RIF resistance in clinical isolates from the Western and Eastern Cape regions of South Africa.

<i>rpoB</i> codon	In vitro RIF ^R mutants ^a (n=304)	Fitness cost ^b	Western Cape region			Eastern Cape region		
			All RIF ^R cases (n=312)	Beijing RIF ^R cases (n=116)		All RIF ^R cases (n=117)	Beijing RIF ^R cases (n=59)	
				Typical (n=101)	Atypical (n=15)		Typical (n=14)	Atypical (n=45)
490		high	1					
511		high	3	2	1	3	3	
512		high	3					
513	8	high	5		1			
516	3	high	32	7	4	35	2	30
519	1	high						
522	37	low						
526	110	low	39	8	1	8	2	2
529	2	high						
531	123	low	181	61	8	60	5	11
533	1	high	17	12				
Multiple nsSNPs	2	high	1					
Insertions	1	high	1					
Deletions	15 ^c		2					
nsSNPs absent from RRDR	1	high	27	11		11	2	2

Legend to Table 3.1:

^a Combined data from (4,7,10)

^b Assigned according to the definition described in (4)

^c Eleven different deletion events (7,10)

RIF^R = rifampicin-resistant, RRDR = rifampicin resistance determining region.

CHAPTER 4

PROTEIN EXPRESSION PROFILES OF LARGE, SMALL, AND ATYPICAL DRUG-RESISTANT CLUSTERS OF THE BEIJING STRAIN FAMILY.

My contribution to this project:

Planning of project

Strain culture and growth

Protein isolation

2-D gel electrophoresis

Data analysis

4.1 BACKGROUND

Previous studies have shown that the drug-resistant TB epidemic in SA is driven predominantly by transmission of *M. tuberculosis* strains (5,7,10,11,13-15). Evident from these studies was that certain family specific drug-resistant strains from within the same strain family have a higher propensity to transmit, despite the maintenance of a constant control program and a stable community (Figure 4.1). The definition that was assigned to a strain family was that the strains have to have $\geq 65\%$ IS6110 RFLP banding pattern similarity. Those strains could be grouped into clusters according to DNA fingerprints (with identical or similar IS6110 RFLP fingerprints, and similar or identical spoligotype patterns) and drug resistance conferring mutations in different genes associated with drug resistance (e.g. *rpoB* (RIF resistance) (6), *katG* (INH resistance) (4), *inhA* promoter gene (INH resistance) (4)). The Beijing strain family was one of the largest strain families identified in the longitudinal reference database representing 31% of all the drug-resistant strains, and could be divided into large (>10 isolates with identical or near identical genotypic characteristics) and small clusters (2-5 isolates with identical or near identical genotypic characteristics). The Beijing strain family can broadly be grouped into typical and atypical according to the presence or absence of an IS6110 insertion in the NTF region (8). It has been speculated that the typical Beijing strains are hyper mutable and therefore have an increased ability to develop drug resistance, and are able to spread more readily (2,9). Defects in DNA repair genes are unique to the typical Beijing strains and it has been proposed that this may partly explain the high prevalence of typical Beijing drug-resistant strains (9). In contrast, the atypical Beijing strains are not frequently associated with drug resistance, are rarely observed, and are thought to be attenuated in their ability to transmit (8,12). In this study, the typical Beijing strain is Beijing cluster 220, which is a dominant cluster representing 42% of the Beijing drug-resistant isolates in a region in the Western Cape province of South Africa called the Boland-Overberg-Karoo-Southern Cape (BOKS) region, and was responsible for an outbreak in a high school in Cape Town in 2005 (7). All cluster 220 strains identified in this region have a -15

inhA (C→T) promoter mutation, and no drug-susceptible Beijing Cluster 220 isolate has been identified yet (Johnson R *et al.* - manuscript in preparation). Our observations showed that this strain is highly transmissible and therefore very fit, but we also observed other drug-resistant strain clusters, within the Beijing strain family, that consist of only one or two isolates per cluster. This is puzzling, since they also belong to this highly successful Beijing strain family, but are less transmitted and therefore implying that they are less fit.

In this study we aim to understand on a proteomic level, why specific drug-resistant *M. tuberculosis* strains like Beijing Cluster 220, transmit better than other drug-resistant strains from the same strain family, under a constant TB control programme and a stable community. We also aim to understand what the proteomic differences are between the typical Beijing strain (regarded as more fit) and the atypical Beijing strain (Chapter 3), which is regarded as less fit.

4.2 Problem statement

We have previously found that certain family specific drug-resistant *M. tuberculosis* strains within the same strain family have a higher propensity to transmit, despite the maintenance of a constant TB control program and a stable community (Figure 4.1). This suggests that the strain dynamics within a strain family are not the same.

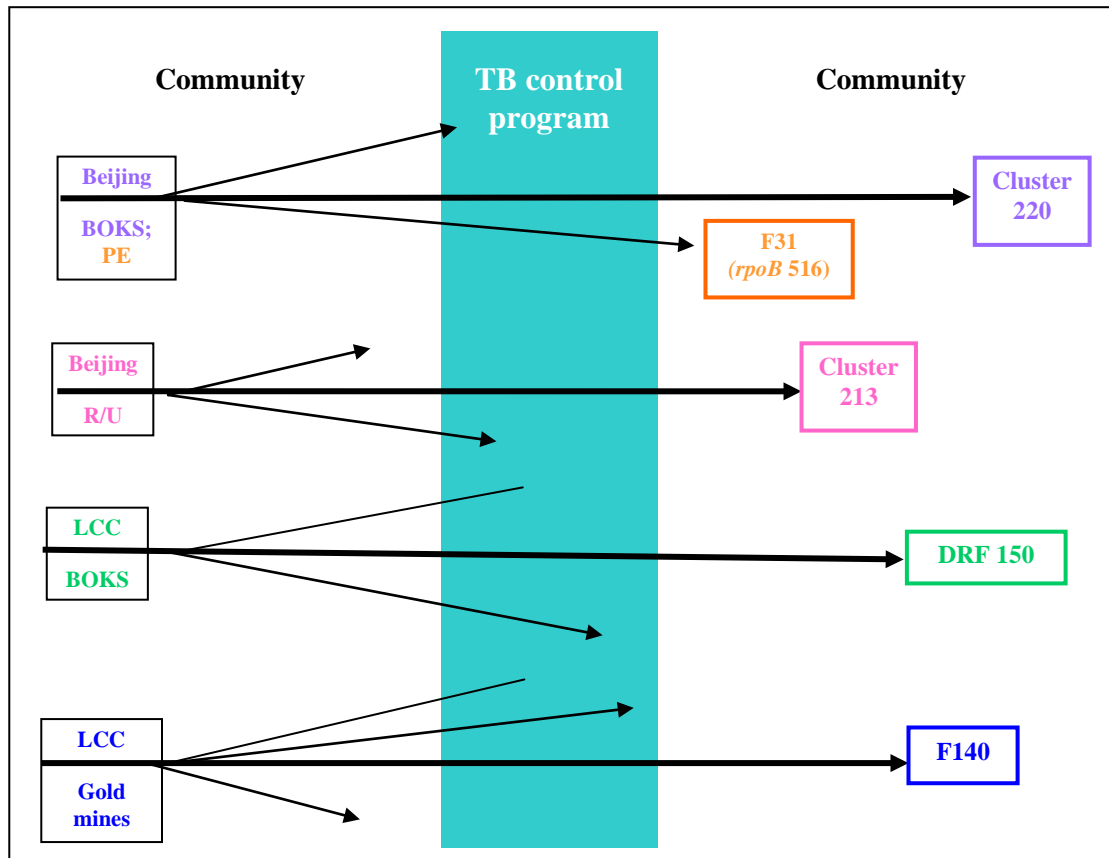


Figure 4.1. The figure illustrates the TB phenomenon that is seen in different settings in SA. The arrows illustrate that certain clusters (specifically the smaller clusters) can be stopped by the TB control programme, whereas some of the large clusters (indicated by darker arrows) cannot be controlled. Examples of large clusters that were responsible for drug-resistant TB outbreaks in different regions in SA include Beijing Cluster 220 (7), LCC (Low Copy Clade) DRF150 (Drug-resistant Family 150) (15), LCC F140 (Family 140) (Manuscript in preparation), and Atypical Beijing F31 (Family 31) (this thesis, Chapter 3)

Hypothesis

Differential expression of proteins in Beijing drug-resistant strains will aid in the identification of proteins that allow certain strains to transmit more than others.

Aim

To make use of 2-D gel electrophoresis in order to determine whether the transcriptome between a large and small Beijing cluster, as well as a typical and atypical Beijing cluster, is differentially expressed.

4.3 MATERIALS AND METHODS

The different methods as well as the buffers and solutions used in this study are described in detail in Chapter 6.

STUDY DESIGN

Strain selection

A laboratory H37Rv strain and a representative drug-resistant *M. tuberculosis* clinical isolate from a large (Cluster 220), small (Cluster 6018), and atypical Beijing cluster were selected from a longitudinal reference database maintained at the University of Stellenbosch. The criteria used for therefore strain selection was as follows: i) the two strains from the large and small Beijing clusters had to be drug-resistant (at least MDR); ii) the strains had to be collected at the same time period, so that it could be determined whether both strains had equal opportunity to transmit; iii) additional confounding factors, such as patient compliance were also taken into account in the selection of these strains.; iv) an atypical strain, which did not necessarily fit above criteria, was also selected to compare its protein expression profile to that of a typical Beijing strain.

M. tuberculosis culture conditions

The strains were grown on LJ slants with continuous aeration for approximately three weeks. After three weeks, colonies were scraped from the LJ slants and inoculated into 20ml 7H9 Middlebrook medium and incubated at 37°C without shaking, until an OD₆₀₀ of 0.6-0.8 was reached. Whole cell lysate (WCL) proteins were extracted and the concentrations of the proteins were determined using the Bradford assay (3). The proteins were purified using the ReadyPrep 2-D CleanUp Kit and subsequent protein concentrations were determined using the RC DC Protein assay.

2-D Gel Electrophoresis

The WCL proteins were prepared for isoelectric focusing (IEF) where proteins were separated according to pH, after which 2-D gel electrophoresis was done as described (1), with minor changes described in detail in Chapter 5. In this study, the 2-D gels were stained with silver stain compatible with mass spectrometry. The gels were then scanned and recorded using a GS-800 Calibrated Densitometer (Bio-Rad Laboratories). Detection of differentially expressed protein spots was done using Quantityone and PDQuest (Bio-Rad) software, together with careful visual inspection. After the 2-D gels (two duplicate gels for each strain) were scanned into the computer, PDQuest created a master gel, which is a combination of all 4 gels; all protein spots are therefore visible on the master gel. Using this master gel, the computer then measured the intensity of each spot in the 2-D gels of one strain and compared it to the spots in the 2-D gels of the other strain to see which proteins are present or absent in the two strains or which proteins are up or down regulated according to spot intensities (larger spots mean proteins are up regulated and if the spot is smaller it means the protein is down regulated).

Differentially expressed proteins were excised and stored in milliQH₂O for future mass spectrometric analyses. Due to time constraints mass spectrometric analyses could not be done for this study. Figure 2 gives a diagrammatic illustration of the study design.

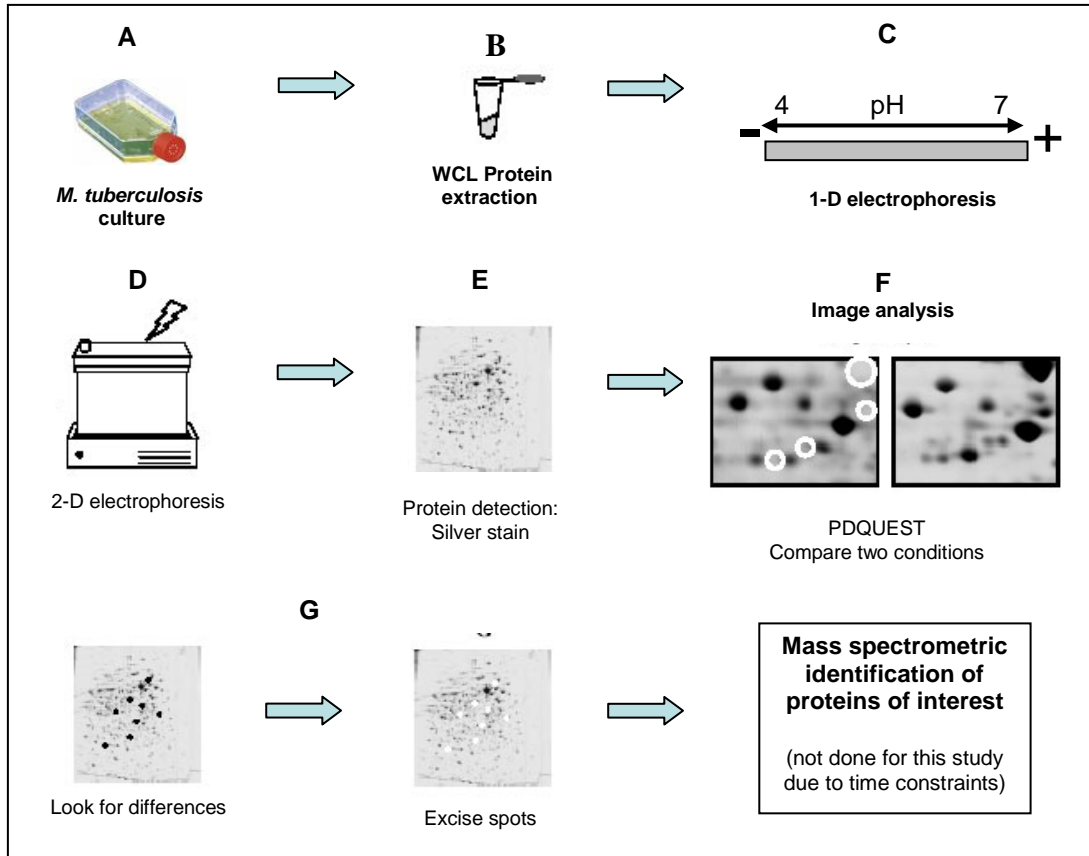


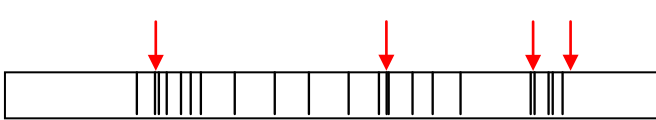
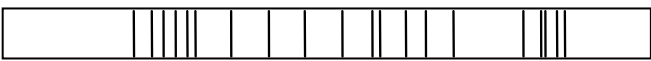
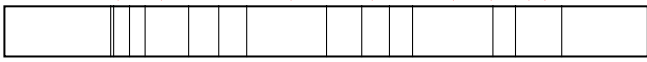
Figure 4.2. A diagrammatic illustration of the study design.

Legend to Figure 4.2: WCL=whole cell lysate; 1-D=1 dimensional; 2-D=2 dimensional.

4.4 RESULTS

Table 4.1 shows the characteristics of the large, small, and atypical clusters from the Beijing strain family that were investigated in this study. To make a comparison between the protein expression profiles of the large and small Beijing clusters, both strains fit the criteria mentioned under study design. Both strains were at least resistant to INH and RIF, both strains were collected from the same time period (between February 2001 and February 2002), and both the patients from which the strains were collected originated from the same town and clinic in the Southern Cape region, which is in the WC province of SA. The red arrows in Table 4.1 indicate the differences between the IS6110 RFLP fingerprints of the large and small Beijing clusters. The IS6110 RFLP fingerprint of the atypical Beijing strain is also different from both the other two typical Beijing strains (differences indicated by the red arrows in Table 4.1).

Table 4.1. Characteristics of the small, large and atypical Beijing clusters.

Strain	Drug resistance phenotype	IS6110 RFLP pattern
Beijing Cluster 220 (large cluster) n=148/362 – all drug-resistant Beijing isolates from BOKS	H,R,E,S,Et,Thia	
Beijing Cluster 6018 (small cluster) n=2/362 - all drug-resistant Beijing isolates from BOKS	H,R,Z,S	
Atypical Beijing n=45/59 all drug-resistant atypical Beijing isolates from the EC sample collection	H,R	

Legend to Table 4.1:

RFLP=restriction fragment length polymorphism; H=isoniazid; R=rifampicin; E=ethambutol; S=streptomycin; Et=ethionamide; Thia=thiacetazone; Z=pyrizinamide; BOKS=Boland-Overberg-Karoo-Southern Cape; EC=Eastern Cape province.

In figure 4.3, each differentially expressed spot is reviewed independently. Figure 4.3A, represents the master gel. Figures 4.3B and 4.3C represents two duplicate gels of the large Beijing cluster. Figures 4.3D and 4.3E represents two duplicate gels of the small Beijing cluster. Figure 4.3F, represents one spot (SSP2002) that is present in the small Beijing cluster (black squares) but absent in the large Beijing cluster (black circles). The green bars represent the two duplicate gels from the small Beijing cluster indicating that spot 2002 is present and no bars are shown for the large Beijing cluster indicating that spot 2002 is absent.

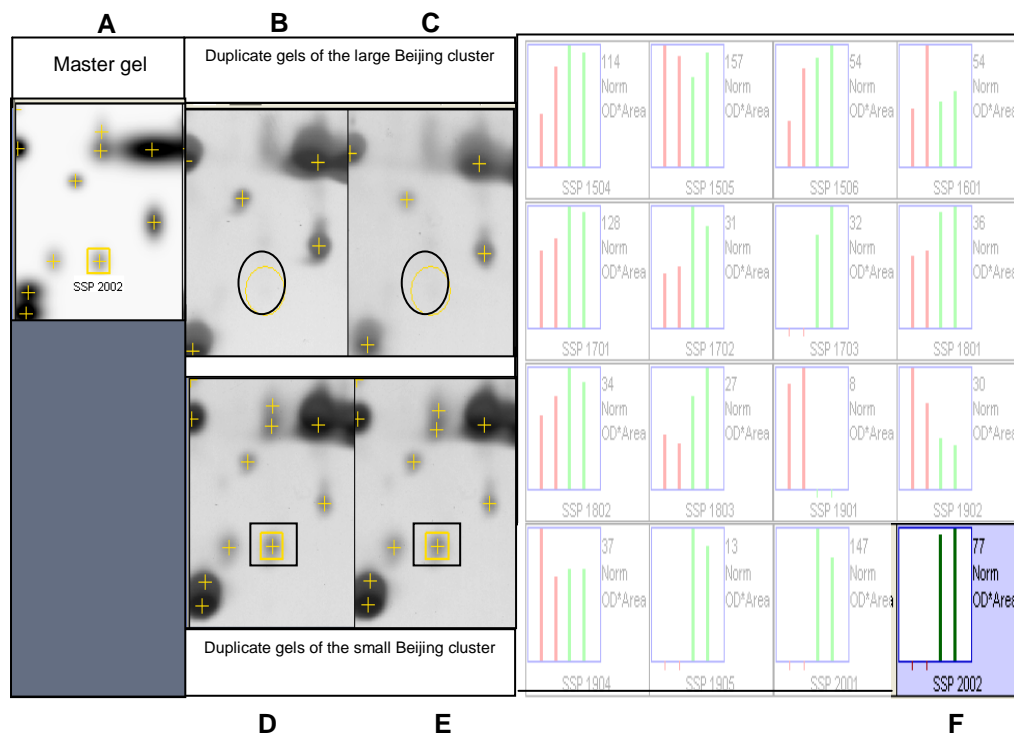


Figure 4.3. Reviewing of differentially expressed protein spots using the Spot Review Tool. Figure 4.3A, represents the master gel which is a combination of all the gels, all protein spots are therefore visible on the master gel, figures 4.3B and 4.3C represents two duplicate gels of the large Beijing cluster. Figures 4.3D and 4.3E represents two duplicate gels of the small Beijing cluster. Figure 4.3F, represents one spot (SSP2002) that is present in the small Beijing cluster (black squares) but absent in the large Beijing cluster (black circles). The green bars represent the two duplicate gels from the small Beijing cluster indicating that spot 2002 is present and no bars are shown for the large Beijing cluster indicating that spot 2002 is absent.

Figures 4.4, 4.6, and 4.8, show the electrophoretic fractionation of WCL proteins in 2-D silver-stained gels (pI range of pH 4-7, with a molecular weight of 10-100 kDa). Each differentially expressed protein has a significant p value of < 0.05 . The p value was determined using a PDQuest integrated statistical analysis tool, T-test, with confidence interval of 95%. Comparison of protein expression between the large and small clusters by PDQuest analysis showed approximately 120 distinct protein spots (Figure 4.4), of which 64 were significantly differentially expressed ($p=0.05$) between the two strains (Table 2). Figure 4.5, shows a representation of a few differentially expressed proteins between the two different clusters. Twelve (12) proteins were absent in the large Beijing cluster and present in the small Beijing cluster, and 11 proteins were absent in the small Beijing cluster but present in the large Beijing cluster. Furthermore, 24 proteins were down regulated in the large Beijing cluster, but were up regulated in the small Beijing cluster, and 17 proteins were up regulated in the large Beijing cluster but down regulated in the small Beijing cluster.

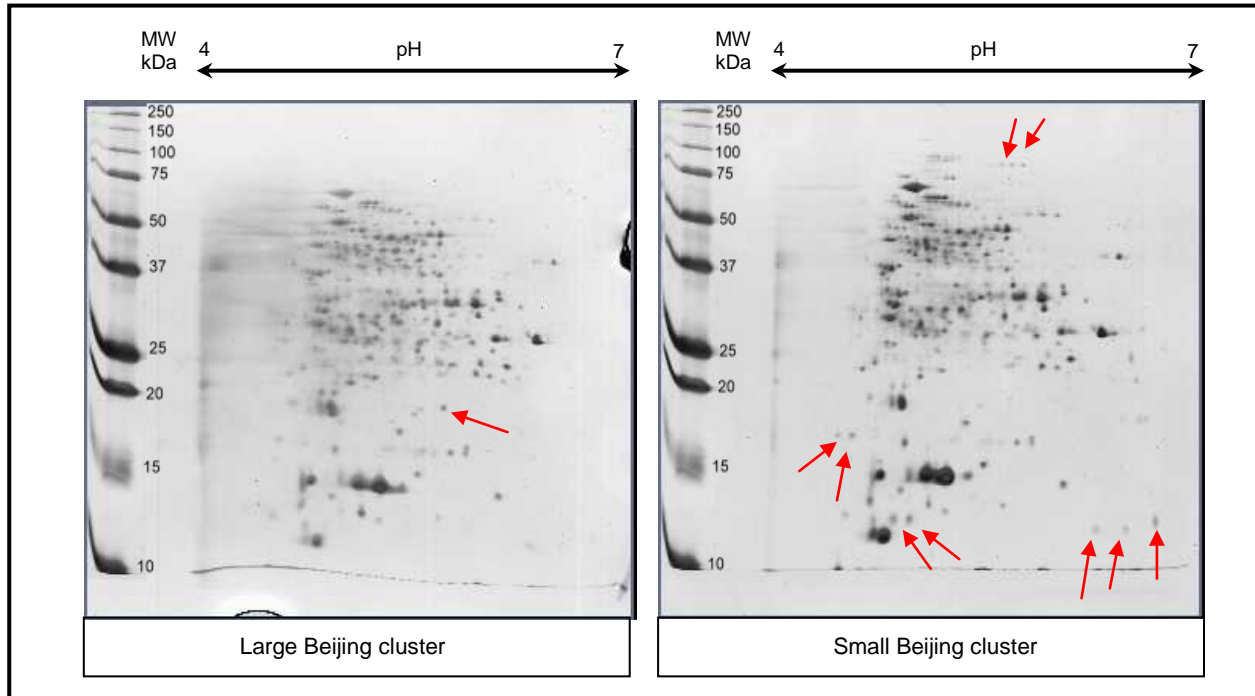


Figure 4.4. Examples of the 2-D protein gels showing protein spots of the large and small Beijing clusters used in this study. A=small Beijing cluster; B=large Beijing cluster. The red arrows indicate some of the protein spots that were different between the two clusters.

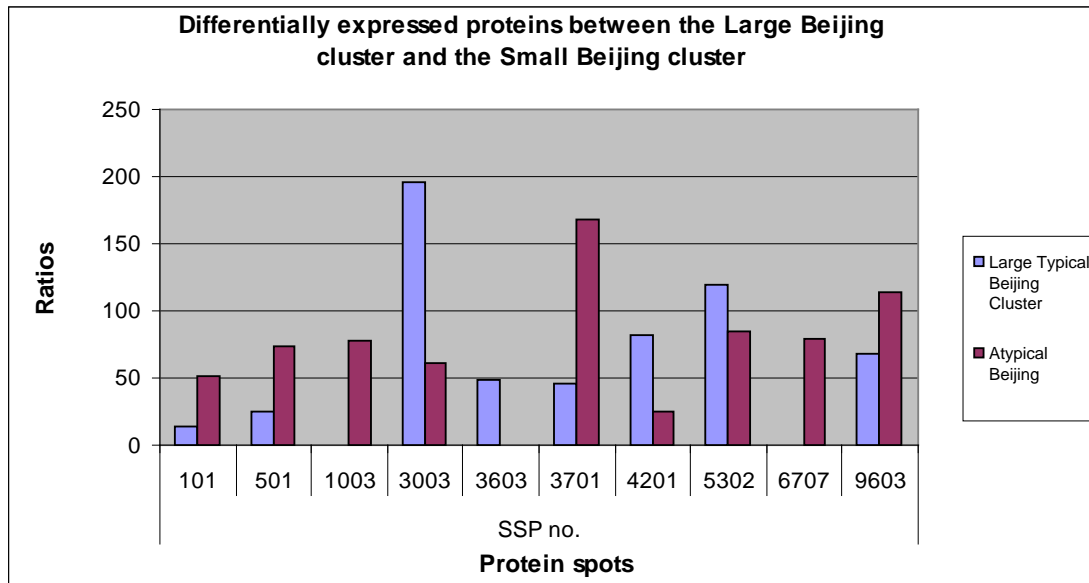


Figure 4.5. A representation of a few differentially expressed proteins (including proteins that are present or absent), between the large and small Beijing clusters.

Table 4.2. Differentially expressed protein spots between Beijing cluster 220 (large cluster) and Beijing cluster 6018 (small cluster).

Protein spots	Large cluster	Small cluster	Ratio
SSP number			
10	-	11.1	18.56
101	13.6	50.9	3.37
104	-	28	46.75
402	-	23.8	39.63
501	25.4	73.6	2.9
503	-	47.5	79.25
1003	-	77.2	128.85
1201	23	36.6	1.59
1701	87.7	125.1	1.43
1801	22.5	35.8	1.6
1905	-	123	20.56
2001	-	131.5	219.47
2002	-	75.4	125.72
2102	-	18.3	30.58
2602	49.9	66.6	1.33
2701	23.7	34.6	1.46
2801	14	20	1.43
2804	29.7	66.6	2.24
3003	195.3	60.8	0.31
3203	11.4	-	0.13
3602	50.7	89.9	1.77
3603	48.9	-	1.77
3604	40.5	105.8	2.61
3701	45.5	167.9	3.69
3901	15.1	50.7	3.35
4101	96.6	43.6	0.45
4201	81.6	24.4	0.3
4302	48	35	0.73
4501	22.2	-	0.07
4603	123.7	101.8	0.82
4605	-	39	65.04
4802	11	-	0.14
4903	22.2	50.9	2.29
5302	119.3	84.2	0.71
5403	79.3	49.3	0.62
5404	112.5	20.6	0.18
5601	34.2	19.6	0.57
5605	13.4	43.7	3.27
5701	251.2	158	0.63
5702	65.1	95	1.46
5703	-	19.5	32.57
5802	32	68.1	2.13
5803	19.2	-	0.08
5804	16.6	42.3	2.54
6001	60.1	96.1	1.6
6203	27.4	-	0.06
6501	41.5	28.9	0.7
6502	29.8	-	0.05

6602	86.1	61.3	0.71
6605	14.7	-	0.1
6704	76.2	60.9	0.8
6707	-	78.5	131.02
6802	18.5	7.3	0.4
7101	60.5	-	0.02
7103	59.4	-	0.03
7301	19.4	-	0.08
7304	33.9	15.4	0.45
7502	82.5	44.8	0.54
7703	76.9	62.2	0.81
8001	64.8	32.4	0.5
8101	80.5	12.5	0.16
8302	26.7	21	0.79
8801	12.8	28.7	2.25
9603	68.6	113.8	1.66

Legend to Table 4.2:

MW = molecular weight; pI = pH range; SSP number = the number that the computer assign to the protein spots; - = protein absent.

Comparison of protein expression between the large typical Beijing cluster and the atypical Beijing strain showed 260 distinct protein spots, of which 59 were significantly differentially expressed between the two strains ($p=0.05$) (Table 3). Figure 4.7, shows a representation of a few differentially expressed proteins between the two different clusters. Ten (10) proteins were absent in the large typical Beijing cluster and present in the atypical Beijing cluster, and 6 proteins were absent in the atypical Beijing cluster but present in the large typical Beijing cluster. Furthermore, 26 proteins were down regulated in the large typical Beijing cluster, but were up regulated in the atypical Beijing cluster, and 17 proteins were up regulated in the large typical Beijing cluster but down regulated in the atypical Beijing cluster.

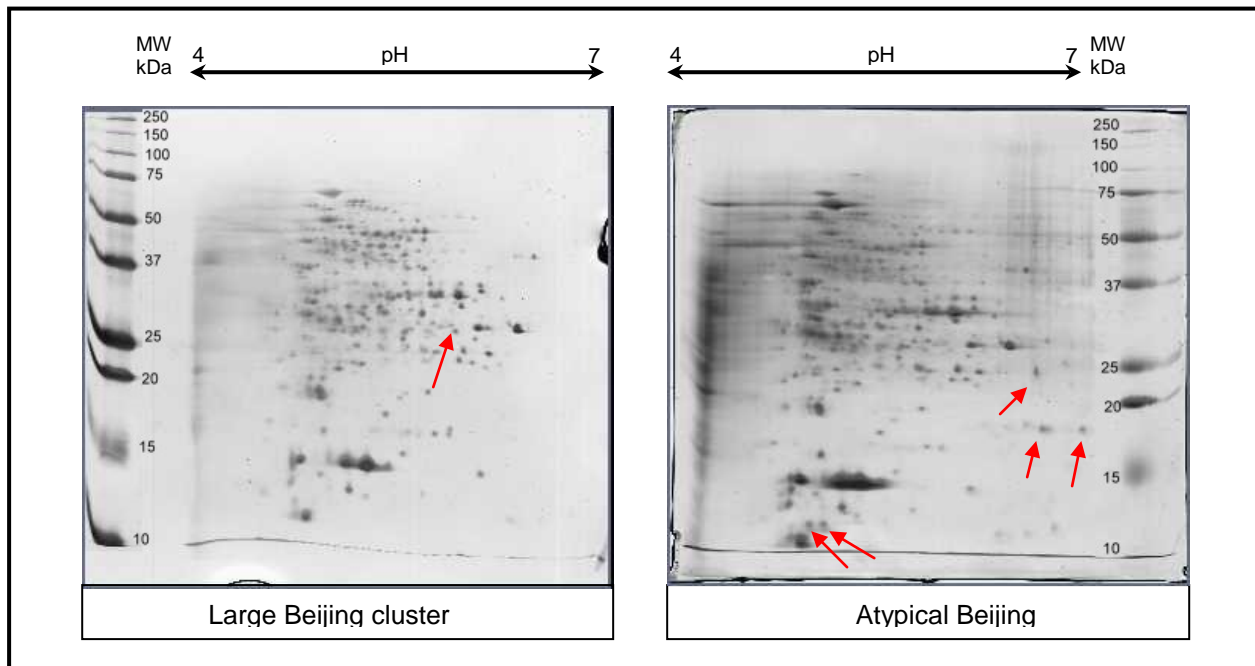


Figure 4.6. Comparison between 2D protein gels from a typical Beijing strain (large cluster) and the atypical Beijing strain. The red arrows indicate some of the differential protein spots between the two strains.

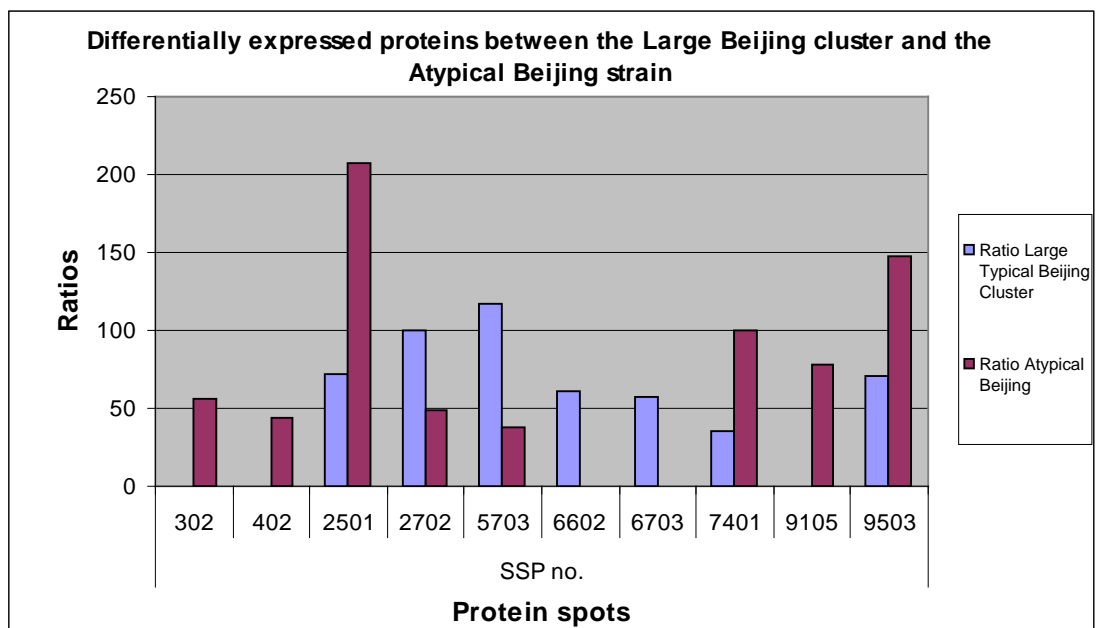


Figure 4.7. A representation of a few differentially expressed proteins (including proteins that are present or absent), between the large and atypical Beijing clusters.

Table 4.3. Differentially expressed protein spots between Beijing cluster 220 (large cluster) and the atypical Beijing strain.

Protein spots	Large cluster	Atypical cluster	Ratio
SSP number			
1	59.5	116.8	0.51
101	25.3	46.2	0.32
302	-	56.6	0.01
303	-	23.7	0.02
306	30.9	-	37.3
402	-	44.5	0.01
501	16.6	48.7	0.34
603	35.6	150	0.24
701	-	28.2	0.01
801	19.2	59.6	0.32
1301	133.3	647.8	0.21
1303	-	39.3	0.01
1401	119.6	160.8	0.74
1601	78	112.8	0.69
1701	64.8	144.2	0.45
1801	14.1	35.3	0.4
1906	21	48.2	0.44
2002	-	69.9	0.01
2103	48.4	97.8	0.49
2201	-	38.6	0.01
2501	71.4	206.9	0.35
2602	31.8	46.6	0.68
2702	100.5	48.7	2.06
2802	26.3	17.4	1.51
3002	113.1	116.6	0.97
3705	36.8	-	44.33
3706	32.5	9.2	3.54
3801	62.9	22.1	2.84
3802	23	12.3	1.86
4302	68.9	-	83.12
4802	78.5	51.6	1.52
4902	31.9	34.2	0.93
5301	29.8	52.2	0.57
5403	91.8	30.9	2.97
5501	39.3	19.4	2.03
5703	117.2	38.3	3.06
5902	15.2	-	18.37
6303	-	17.4	0.02
6602	61.3	-	73.89
6703	57.2	-	68.98
7001	38.1	10.3	3.69
7303	12.1	26.8	0.45
7401	34.8	100.4	0.35
7501	25.9	39.3	0.66
7503	31.4	36.2	0.87
7601	50.1	33.2	1.51
7605	16.5	10.5	1.57

8201	45.1	102.9	0.44
8303	21.2	31.4	0.67
8701	49.8	32.3	1.54
9001	52.2	29	1.8
9101	57	34.5	1.65
9103	95.9	77	1.25
9104	-	46.7	0.01
9105	-	78	0.05
9303	24.2	49.3	0.49
9402	62.4	13.7	4.54
9501	32.9	68.2	0.48
9503	70.7	147.2	0.48

Legend to Table 4.3:

MW = molecular weight; pI = pH range; SSP number = the number that the computer assign to the protein spots; - = protein absent.

Comparison of protein expression between the small Beijing cluster and the atypical Beijing strain showed approximately 293 distinct protein spots, of which 132 were differentially expressed ($p=0.05$) between the two strains (Table 4). Figure 4.9, shows a representation of a few differentially expressed proteins between the two different clusters. Eighteen (18) proteins were absent in the small typical Beijing cluster and present in the atypical Beijing cluster, and 11 proteins were absent in the atypical Beijing cluster but present in the small typical Beijing cluster. Furthermore, 50 proteins were down regulated in the small typical Beijing cluster, but were up regulated in the atypical Beijing cluster, and 53 proteins were up regulated in the small typical Beijing cluster but down regulated in the atypical Beijing cluster.

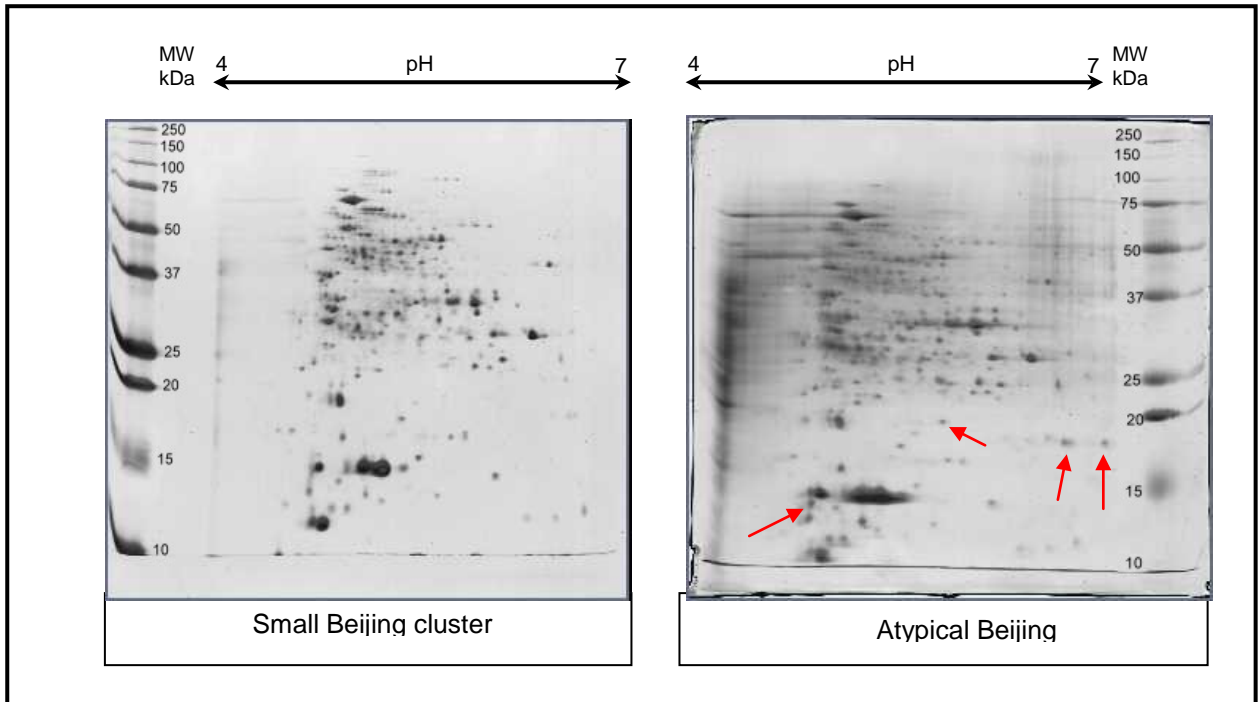


Figure 4.8. Comparison between 2D protein gels from a typical Beijing strain (small cluster) and the atypical Beijing strain. The red arrows indicate some of the differential protein spots between the two strains.

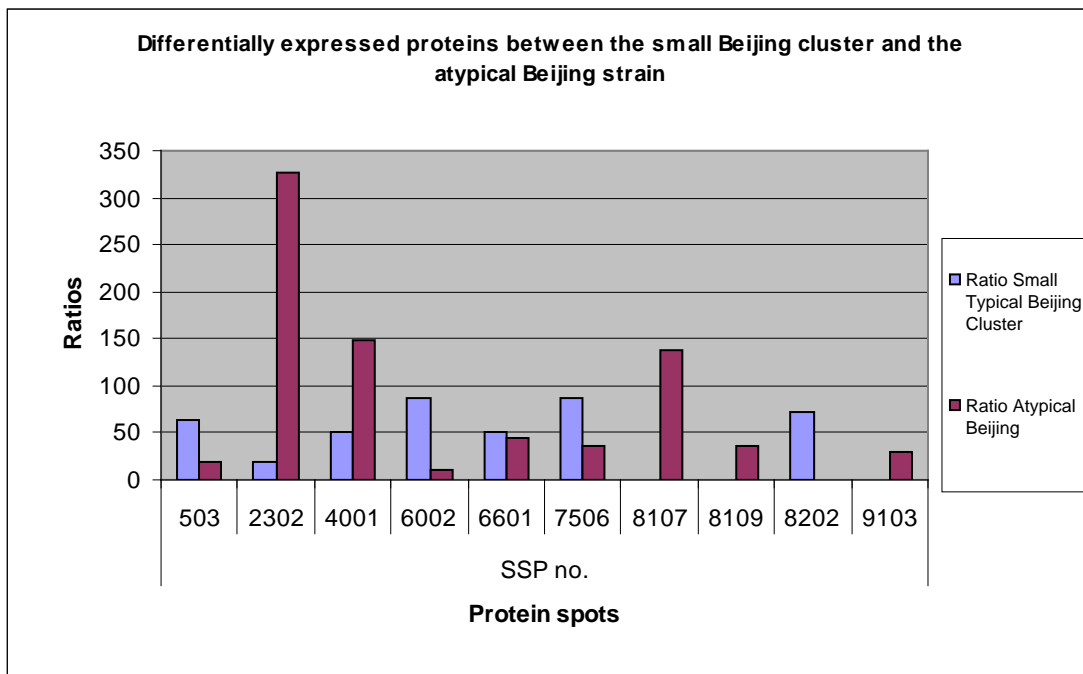


Figure 4.9. A representation of a few differentially expressed proteins (including proteins that are present or absent), between the small Beijing cluster and atypical Beijing strain.

Table 4.4. Differentially expressed protein spots between Beijing cluster 6018 (small cluster) and the atypical Beijing strain.

Protein spots	Small cluster	Atypical cluster	Ratio
SSP number			
1	97.2	128.9	1.33
2	475.5	-	0
102	27.9	19.1	0.68
103	70.3	25.5	0.63
104	17.5	48.3	2.77
105	28.8	56.1	1.95
301	26.1	73	2.8
401	21.6	119.3	5.51
503	63.5	19.1	0.3
504	108	153.9	1.43
1001	67	104.4	1.56
1101	21.9	-	0.07
1201	33.2	25.3	0.76
1203	29.9	21.2	0.71
1401	57.7	218.2	3.78
1406	119.1	203.6	1.71
1407	142.4	60	0.42
1502	177.2	93.8	0.53
1503	41.2	23.5	0.57
1601	116.2	35	0.3
1802	22.1	47.1	2.13
2001	46.3	28.9	0.62
2003	65.2	77.2	1.18
2005	-	49.6	110.7
2101	496.2	437.4	0.88
2103	100.4	123.9	1.23
2302	18.5	326.8	17.62
2401	66.9	190.5	2.85
2701	16.5	-	0.09
2702	29.9	8.1	0.27
2802	43.1	30.2	0.7
2803	201	3.1	0.02
2805	55.7	10.8	0.19
3001	1053.4	1344.4	1.28
3101	48.4	52.2	1.08
3201	48.3	81.1	1.68
3501	10.9	21.5	1.97
3602	68	57.1	0.84
3606	137.4	71.4	0.52
3703	64.7	18.7	0.29
3801	69.7	-	0.02
4001	50.1	148.5	2.96
4004	-	16.6	36.94
4202	41.7	96.1	2.3
4204	68.5	112	1.63
4304	-	94.5	210.86
4501	80.2	40.1	0.5
4601	97.7	-	0.02
4603	67.6	9.4	0.14

4701	111.8	24	0.21
4805	37	15.8	0.43
5101	-	11.8	26.37
5301	113.2	41.7	0.37
5302	29.1	66.3	2.28
5304	130.1	33.2	0.26
5401	66.1	50.5	0.76
5403	37	70.3	1.9
5501	30.8	46	1.49
5503	44	20.3	0.46
5601	30.2	134.3	4.45
5602	123.9	-	0.01
5604	107.3	47.1	0.44
5607	134.2	44	0.33
5701	94	58.5	0.62
5703	153	46.3	0.3
5803	52	-	0.03
6002	86.3	10	0.12
6101	45.6	36.5	0.8
6102	-	81.9	182.72
6201	21.3	23.3	1.1
6202	57.5	30.1	0.52
6302	-	18.8	41.85
6403	56.9	101	1.77
6405	89.6	83.9	0.94
6501	34.3	62.6	1.82
6502	13	29.7	1.82
6601	50.6	45.4	0.9
6602	50.7	-	0.03
6604	51.1	38.1	0.74
6703	93.7	36.8	0.39
6705	50.7	73.9	1.46
6706	68.4	-	0.02
6801	34.3	-	0.04
6802	5.1	21	4.12
6803	5.1	7.4	1.45
7102	-	50	111.56
7203	-	74	165.11
7204	-	31.3	69.82
7302	101.1	85.9	0.85
7402	17.1	170.5	9.98
7501	23.9	45.6	1.91
7502	24.2	17.4	0.72
7503	6.7	18.9	2.82
7504	36	62.8	1.75
7505	6.3	7.4	1.18
7506	87.4	36.8	0.42
7601	14	10.5	0.75
7701	34.8	49.5	1.42
7702	136.8	82.9	0.61
7704	51.6	36.8	0.71
7801	10.3	17.2	1.68
7802	-	17.1	38.2
8001	85.2	32.7	0.38
8101	34.4	16.1	0.47
8102	9.5	45.2	4.74

8104	88.1	39.6	0.45
8105	29.2	33.6	1.15
8106	140.9	97.4	0.69
8107	-	138.2	308.15
8108	-	130.6	291.37
8109	-	35.1	78.26
8202	72.1	-	0.02
8203	16.6	14.4	0.87
8204	177.6	191.2	1.08
8205	106.8	46.3	0.43
8206	-	23.8	53.1
8301	12.2	35.9	2.95
8302	147.5	96.4	0.65
8403	203.7	383.7	1.88
8404	83.8	66.2	0.79
8405	18.2	31.6	1.74
8501	49.4	87.9	1.78
8502	44.4	75.6	1.7
8803	16.6	15.5	0.93
9101	27.1	73.2	2.71
9102	-	46.2	103.01
9103	-	30.3	67.51
9104	-	7.8	17.35
9201	15.9	36.6	2.3
9205	62.4	71	1.14
9501	-	8	17.84
9602	104	70.6	0.68

Legend to Table 4.4:

MW = molecular weight; pI = pH range; SSP number = the number that the computer assign to the protein spots; - = protein absent.

Figure 4.10 is another way of presenting the data. It shows two scatter plots which shows the relatedness of the small Beijing cluster duplicate protein gels (Figure 4.10A), as well the relatedness between the protein spots of the small Beijing cluster and the atypical Beijing strain (Figure 4.10B). The intensity of each spot from the one strain is plotted on a log scale against the intensity of the same spot from the other strain. If a spot falls above or below the centre line (black line in both graphs, Figure 4.10) the spot's intensity for the two different strains are not same and if the spot falls on the centre line the spot's intensity is the same for both strains. The green line in Figure 4.10A and 4.10B is the regression line that is generated from the plot, and the red and blue parallel lines in the graphs will appear when a fold-factor range (e.g. 2-fold, 3-fold, or 4-fold) are chosen. All the spots

that lie between these two lines in the graphs (Figure 4.10A and 4.10B) have intensities that fall within the selected fold-factor range. The correlation coefficient is represented by r in the graphs. A correlation coefficient of 1.00 indicates that the spots for the two different strains are the same and a low correlation coefficient (e.g. 0.3) indicates that the spots are different in the two strains. The correlation coefficient for the small Beijing cluster duplicates is almost 1.00 (Figure 4.10A), which is what would be expected, and the correlation coefficient for the small Beijing cluster and the atypical Beijing cluster is low (Figure 4.10B), which is an indication of the un-relatedness of the two strains.

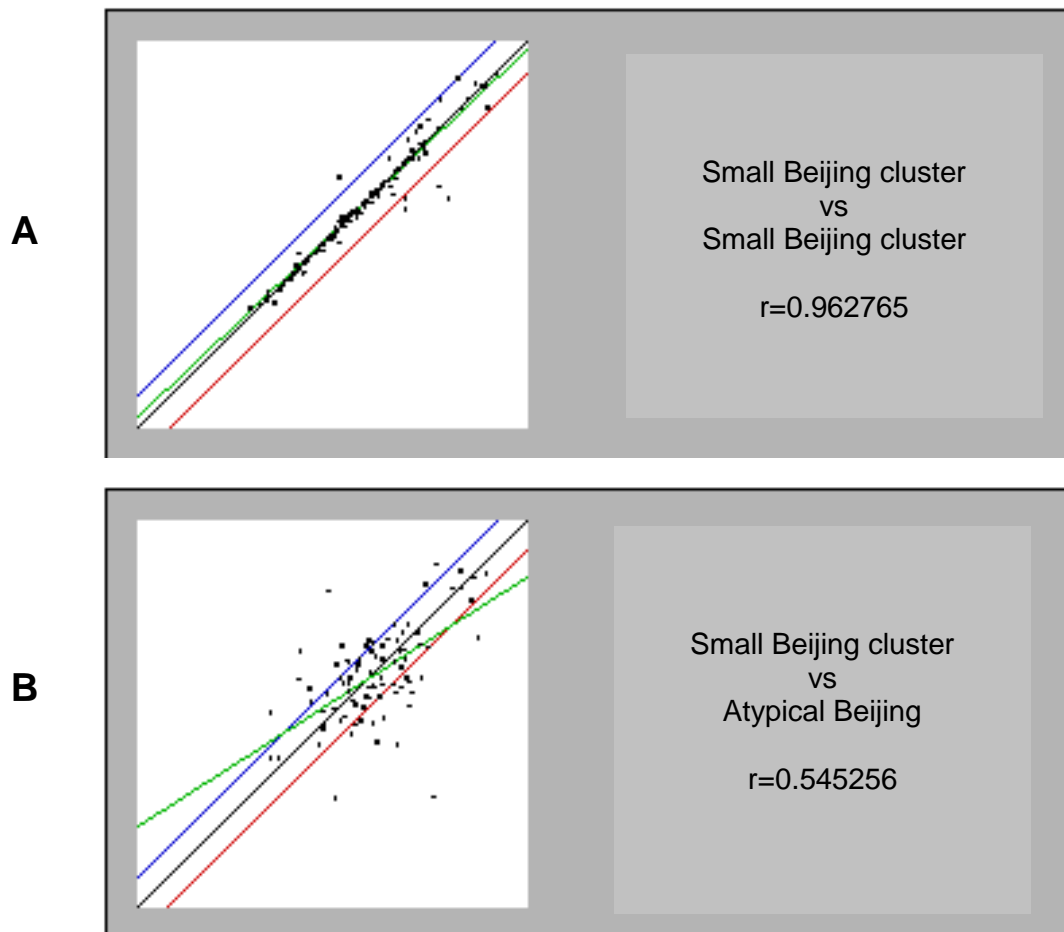


Figure 4.10. Scatter Plot of the Small Beijing cluster and Atypical Beijing strain.

4.5 DISCUSSION AND CONCLUSION

There could be many reasons why Beijing cluster 220 (large Beijing cluster) is more successful in transmitting to a secondary host than Beijing cluster 6018 (small Beijing cluster). One of the reasons could be that cluster 6018 did not have the same opportunities to transmit as cluster 220, but if we consider the time period when the two strains were collected (Table 1), it suggests that cluster 6018 had the same opportunity to transmit than cluster 220. The protein expression profiles for the two strains are very different indicating there are differences between the two strains and some of the differentially expressed proteins might give insight into why the one strain is more successful than the other, which is from the same strain family. The protein expression comparisons between the large and small typical Beijing clusters show 120 protein spots of which 64 were differentially expressed. This is over half and far higher than would be expected. Even though the large and small Beijing clusters belong to the same strain family, the two clusters seem to be very different in terms of their ability to transmit. Both have the typical Beijing characteristics and the Beijing strain family is one of the most dominant *M. tuberculosis* strain families, so why is the small cluster not as successful? Therefore one would expect that the expression profiles of the two clusters be different. It would be very interesting to find out what proteins are different between the two clusters that cause the one to be more successful than the other. Other factors (host and environmental) might also play a role, but for this study we focused on the proteins that might play a role. One should also bear in mind that the 64 differentially expressed spots are mostly the same proteins in both strains but the protein expression is different, its only a few proteins that are present in the one strain but absent in the other. The computer program used to analyse the 2-D gels is also very sensitive so minute differences will be detected.

The protein expression profiles of the typical and atypical Beijing strains are also different. Fifty-nine (59) differentially expressed proteins were found when the protein expression profiles of the

large typical Beijing cluster and atypical Beijing cluster were compared. This is less than the large typical Beijing vs. the small typical Beijing cluster, yet these strains are more phenotypically and genetically divergent. An explanation might be that the atypical Beijing cluster is also seen as a “large cluster”/high transmitter, like the large typical Beijing cluster, it is also successful in its ability to transmit. Similar bacterial-related factors might play a role in the success of transmission of these clusters (large typical and atypical Beijing) that might result in a more similar protein expression profile than large typical Beijing cluster vs. small cluster. Overall there is a large divergence between total protein spots observed (ranges from 293 (high) to 120 (low)). This is a very large difference for such similar organisms. One would think that the protein expression profiles of the high and low transmitted strains would be very different. The large cluster, which transmits more, is therefore more virulent than the small cluster. Therefore more genes might be upregulated or more proteins that play a role in transmission/virulence are expressed in the large cluster. One might wonder whether this suggest inaccuracies in the 2D gel electrophoresis technique? The protein extractions from the three different Beijing clusters (large typical, small typical, and atypical) were done in duplicate (biological duplicates) and the 2D gels containing the protein spots from the different clusters were also done in duplicate. The duplicate gels were exactly the same, which is an indication that the laboratory error was minimal (I would not say that there was absolutely no laboratory error). One limitation of the computer program that is used to analyse the 2D gels is that it is very sensitive. Any background that might be on the 2D gels will be scored as protein spots therefore manual analysis of the gels together with the 2D gel program is needed. And the reason for the large difference between the protein expression profiles of the three clusters might be that the program recognises very minute changes.

Unfortunately due to time constraints differentially expressed proteins between the three clusters could not be identified yet and therefore there was also no time to investigate other comparisons for example the protein expression profiles between two different clinical isolates from the same cluster as well as secreted proteins. The next step now would be to sequence the differentially expressed

proteins using MALDI TOF analysis, to identify the proteins which might play a role in the success of one strain and the failure of the other.

4.6

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CHAPTER 5

MATERIALS AND METHODS

In this chapter the methods will be described, followed by a list of buffers and solutions at the end of this chapter.

5.1 DRUG RESISTANCE GENOTYPING

Mini-cultures for the preparation of DNA templates for PCR reactions

The sputum samples were decontaminated by standard procedures (3) as follows. An aliquot of 500ml of a NaOH/NaCl solution was added to 500ml of each sputum sample and mixed for 20 minutes. The samples containing the NaOH/NaCl solution were centrifuged for 10 minutes at 1200g. The supernatant from each sample was removed and the remaining pellet was re-suspended in 1.5ml 1XTE-buffer and centrifuged for 10 minutes at 12000g. The supernatant was removed and the resulting pellet was re-suspended in 1ml of 1XTE-buffer. In preparation for the mini-cultures, the decontaminated and liquefied sputum samples (1ml) were centrifuged for 20 minutes at 14000xg, after which the supernatant was removed, and 500 μ l BACTEC medium, containing 100 μ l Panta Plus, were added to each sample. The mini-cultures were then incubated at 37°C. After 7 days of incubation, the mini-cultures were centrifuged for 20 minutes at 14000xg, and most of the supernatant was from each sample was discarded, leaving approximately 100 μ l of supernatant behind. The bacterial cell pellet was dissolved in the remaining 100 μ l supernatant and boiled for 20 minutes at 100°C. These crude DNA templates were used for PCR reactions.

PCR amplification of various genes

DNA amplifications were executed in 100µl reaction volumes. The PCR master-mix consisted of 5µl 10Xreaction buffer, 1µl MgCl₂, 4µl deoxyribonucleotide triphosphates (dNTPs) (0.2mM of each dNTP), 0.25µl Forward primer (10pM), 0.25µl Reverse primer (10pM), 0.15µl/sample Hotstar Taq polymerase, ~ 5µl of the crude DNA template, and dH₂O to make up a final volume of 50µl. Reaction mixtures were heated in a thermal cycler (GeneAmp PCR System 2400, Applied Biosystems, Foster City, CA 94404, USA) as follows: an initial activation step of 15 minutes at 95°C, followed by 40 cycles of a denaturing step at 94°C for 1 minute, an annealing step at T_m for 1 minute, and an extension step at 72°C for 1 minute. A final extension was done at 72°C for 10 minutes. The primers used for the PCR reactions are tabulated in Table 1. The PCR products were loaded onto a 1.5% agarose gel (SeaKem[®] LE Agarose, Cambrex BIO Science Rockland, USA) prepared by adding 1.5g agarose to 100ml 1XTBE buffer. The gel was then electrophoresed for 30 minutes to 1 hour at 80V and visualized under ultra violet light using the Kodak Digital Science Electrophoresis Documentation and Analysis System 120 (Vilber Lourmat, France).

TABLE 5.1. Primers used for PCR reactions.

Target gene	Primer		T _m (°C)	Product Size (bp)
<i>rpoB</i>	rpoB For	5' TGGTCCGCTTGCACGAGGGTCAGA 3'	78	437
	rpoB Rev	5' CTCAGGGGTTTCGATCGGGCACAT 3'	76	
<i>katG</i>	RTB 59	5' TGGCCGCGGCGGTCGACATT 3'	66	804
	RTB 36	5' TCGGGGTCGTTGACCTCCCA 3'		
<i>inhA</i> promoter	P5	5' CGCAGCCAGGGCCTCGCTG 3'	60	246
	P3	5' CTCCGGTAACCAGGACTGA 3'		
<i>IS6110</i>	IS6110 XhoI	5' TTCAACCATCGCCGCTCTAC 3'	62	270
	954.19	5' AATCGTTGATGCTGGCGCTATGAACC 3'		
	Esat-6 For	5' GAGCAGCAGTGGAATTTTCGC 3'		
	Esat-6 Rev	5' GAAGGCAACGTCCTGGG 3'		

References for primers: *IS6110* primers=(1), *rpoB*, *katG*, *inhA* primers=(6)

5.2 DNA SEQUENCING

PCR-products were purified using the ExoSAP-IT Clean-up enzyme (AEC-Amersham, UK). Two microliters (2µl) of enzyme (ExoSAP-IT) were added to 5µl of PCR-product after which the mixture was incubated at 37°C for 15 minutes. The enzyme was then inactivated, by heating the samples at 80°C for 15 minutes. Direct sequencing of the purified, diluted PCR products was done with an ABI PRISM DNA Sequencer (Model 3100 PERKEN ELMER, AME Bioscience, Norway) using the forward primer.

5.3 DNA FINGERPRINTING

5.3.1 Spoligotyping

Spoligotyping is the PCR amplification of a unique highly polymorphic locus (DR locus) in the genome of *M. tuberculosis* (2). The DR locus consists of directly repeated sequences, DRs, of 36 bp each, which are interspersed by non-repetitive variable repeat sequences, each 35 to 41 bp in length, which in combination is termed DVRs (2). This method detects the presence or absence of 43 unique DVRs by line-blot hybridisation. It has been shown previously that the DR locus is highly polymorphic in different *M. tuberculosis* strains (5), which makes this a good method for genotypic classification and strain differentiation.

Spoligotyping was done using the international standardised spoligotype method (2). PCR amplifications were done in 50µl reaction volumes and the PCR mix consisted of 5µl 10xreaction buffer (without MgCl₂), 5µl MgCl₂, 4µl dNTPs (0.2mM of each dNTP), 4µl forward primer (DRa-biotinilated), 4µl reverse primer (DRb), 0.2µl Taq polymerase (5U/µl) (Promega), DNA template (20ng), and dH₂O to male up a final volume of 50µl. The primers used are tabulated in Table. DRa: 5'-GGT TTT GGG TCT GAC GAC-3', biotinilated at the 5'-end, and DRb: 5'-CCG AGA GGG

GAC GGA AAC-3'. The reaction mixtures were heated in a thermal cycler as follows: An initial denaturing step of 3 minutes at 96°C, followed by 20 cycles of a denaturing step at 96°C for 1 minute, an annealing step at T_m of 55°C for 1 minute, and an extension step at 72°C for 30 seconds. A final extension was done at 72°C for 5 minutes.

After completion of the amplification of the DNA samples spoligotyping was done using the following protocol. A spoligotype membrane (Isogen, Life Science, Lagedijk Noord, 3401 VA IJsselstein) containing denatured sequence specific oligonucleotide probes covalently linked to it was incubated at room temperature for 5 minutes in 2xSSPE/0.1%SDS. A 20µl aliquot of each PCR product was added to 150µl 2xSSPE/0.1%SDS and denatured for 10 minutes at 100°C and immediately put on ice after denaturation was finished. After incubation, the membrane was placed on a support cushion in a mini-blotter (Immunetics, Cambridge, MA 02139) by aspiration. Each of the slots was filled with the diluted PCR products and allowed to hybridise for 1 hour at 57°C on a horizontal surface without shaking. After hybridization the samples were removed from the mini-blotter by aspiration and the membrane was removed from the mini-blotter and washed twice in 2xSSPE/0.5%SDS at 60°C for 10 minutes. The membrane was then incubated in 30µl 2xSSPE/0.5%SDS containing 7.5µl strepavidin-peroxidase conjugate (Roche Diagnostics, Mannheim, Germany) for 45-60 minutes at 42°C. To clear the membrane of all unbound strepavidin-peroxidase conjugate, the membrane was washed twice in 2xSSPE/0.5%SDS at room temperature after which the membrane was incubated for 60-90 seconds in 20ml of ECL detection fluid (AEC-Amersham, SA) and covered with a transparent plastic sheet with X-ray film. The developing ranged from 5-20 minutes. If necessary, the membrane can directly be used again to expose another film for an optimum period. After use, the membrane is stripped, by incubating it in 1% SDS for 1 hour at 80°C, after which it is stored in 20mM EDTA at 4°C for further use.

5.3.2 IS6110 RFLP ANALYSIS

IS6110 RFLP analysis of the *M. tuberculosis* strains was done according to the international standardised method.

Extraction of *M. tuberculosis* Genomic DNA:

LJ slants containing *M. tuberculosis* were heated at 80°C for 1 hour, to kill the bacteria. Fifty millilitre (50ml) polypropylene tubes were filled to approximately 2ml (conical section) with glass balls (20x5mm). After incubation, the samples were allowed to cool down for 5-10 minutes in a biosafety level 2 flow hood, where the rest of the DNA extraction procedure took place. To each LJ slant, 3ml of extraction buffer (pH 7.4,) were added and using a sterile 10µl plastic loop, all the bacteria were gently scraped loose from the LJ slants and transferred to the tubes containing the glass balls. To each of the scraped slants, another 3ml of extraction buffer were added to get the remains of the cells left in the bottles. The tubes were then vortexed vigorously for approximately 2 minutes. To the vortexed tube containing the bacterial cell suspension, 500µl of lysozyme (Roche, Germany) with a concentration of 50mg/ml and also 2.5µl of RNaseA (Roche, Germany) with a concentration of 10mg/ml, were add. The tubes were gently inverted (not shaken) to mix and were then incubated at 37°C for 2 hours in a preheated oven. Volumes of 600µl of 10xProteinase K buffer and 150µl Proteinase K with a concentration of 10mg/ml were added to the bacterial cell suspension. The tubes were mixed gently by inversion and were incubated at 45°C over night. Five millilitres (5ml) of phenol/chloroform/isoamylalcohol (PCI) solution in a ratio of 25:24:1 were added to the cell suspension and mixed gently every 30 minutes for 2 hours at room temperature. The tubes were then centrifuged at 3000rpm for 20 minutes at room temperature. This step was included to ensure complete phase separation. Five millilitres (5ml) of chloroform/isoamylalcohol (CI) solution with a ratio of 24:1 were added into new sterile 50ml polypropylene tubes and by using a 5ml pipette, the top phase containing the DNA were carefully collected without taking up

any of the interface and were transferred to the new sterilised polypropylene tubes containing the CI solution. The tubes were mixed gently by inverting them and the centrifugation step was repeated. The tubes were then centrifuged at 3000rpm for 20 minutes at room temp. To new sterile 50ml polypropylene tubes, 600µl of 3M sodium-acetate (NaAc) at pH 5.2 were added and, using a 5ml pipette, the top phase containing the DNA was carefully collected, without taking up any of the interface, and transferred to the new sterilised polypropylene tubes containing the NaAc solution. Seven millilitres (7ml) of ice-cold isopropanol were added and the tubes were inverted gently back and forth until DNA became visible. The precipitated DNA was fished out immediately, using a thin glass rod, and the rods containing the DNA were placed into 15ml tubes containing 1ml of 70% EtOH for 10 minutes. The 1.5ml tubes and glass rods were then incubated at room temperature for ±2.5 hours, to dry the DNA. Once the DNA was dry, it was re-hydrated by adding 300–600µl of TE buffer (pH 8.0), and release from the glass rods by mixing it slowly. The DNA was allowed to re-dissolve by incubating it at 4°C overnight or at 65°C for 2 hours, after which it was and stored at -20°C.

PvuII Restriction Endonuclease Digestion

The concentration of the DNA was determined spectrophotometrically. The PvuII digestion of the DNA was carried out by adding 10µl of 10Xrestriction buffer, 6µg of DNA, 30U of the restriction enzyme PvuII endonuclease to a 1.5ml tube and made up with ddH₂O to a final volume of 100µl. The mixture was vortexed and incubated at 37°C for 3–16 hours after which the PvuII enzyme was inactivated by incubating the tubes at 65°C for 10 minutes.

Gel Electrophoresis of Restricted DNA

Eight microlitres (8µl) of the PvuII digested DNA was run on a 1% agarose gel test gel to determine whether the PvuII digestion was complete, and if the DNA was at the correct concentration. The DNA was separated at 1.45V/cm for 16 hours/overnight, or 4V/cm for 4 hours

and to visualise the digested DNA, the gel was stained in 500ml 1xTBE containing 50µl of a 10mg/ml ethidium bromide and viewed under a 245nm UV light box to visualise the digested DNA bands.

A final gel was then prepared and the remaining PvuII-digested DNA (92µl) was precipitated as follows and run on the gel: Nine microlitres (9µl) of 3M Sodium Acetate (pH5.2) and 300µl ice cold 100% EtOH were added to the digested DNA and the tubes were mixed gently. The tubes were then incubated at -20°C for 16 hours/overnight. The PvuII-digested DNA was centrifuged at 10 000xg for 30 minutes at 4°C to pellet the DNA. A suction device attached to a sterile 10ml plastic pipette was used to slowly and carefully aspirate the supernatant down to ~50µl. The DNA pellet was washed again with 500µl 70% ice-cold ethanol after which it was centrifuge at 10 000xg for 30 minutes at 4°C to pellet the DNA once again. The supernatant was again aspirated down to ~50µl and the DNA pellet was allowed to dry at room temperature for 16 hours/overnight to remove all residual 70% ethanol. The DNA was re-dissolved in 1x loading buffer/Internal Molecular Weight Marker (Marker X) and mixed gently every hour at 4°C for 16hours/overnight or 65°C for 4hours. A 0.8% agarose gel were prepared and the PvuII-digested DNA samples were loaded onto the gel and was separated and visualised as previously described.

Southern Transfer of the Fingerprinting Gel

The gel was placed inverted into a flat plastic container and the DNA in the gel was denatured by incubating in 500ml denaturing buffer at 25°C for 30 minutes with gentle shaking. The denaturing solution was aspirated off and the gel was neutralized with 500ml neutralizing buffer and incubated at 25°C for 30 minutes with gentle shaking. A nylon membrane (Hybond N+) was labelled to allow further recognition by spotting 0.2µl aliquots of orientation marker onto the membrane. The membrane was hydrated in ddH₂O for a few seconds and was immediately transferred to a 20XSSPE solution to allow equilibration. The Southern Blot apparatus was set up by soaking a

large sheet of Whatman 3MM paper (46x57cm) in 20XSSPE and placing it on a blotting tray. The gel was then placed in its inverted orientation, onto the Whatman 3MM paper. By using a surgical blade, the edges of the gel as well as the wells were cut away and all air bubbles were removed from under the gel. Strips of parafilm were placed around the gel ensuring that they are positioned right up against the gel and the Hybond N+ membrane were placed onto the agarose gel with the orientation markers and labelled facing the gel. Air bubbles were removed from between the gel and membrane. One large sheet of Whatman paper (46x57cm) was cut into two identical pieces such that they overlap the gel by 5 to 10mm on each side. Both pieces of paper were wet in 20X SSPE (pH1.4) and place one on top of the other on the nylon membrane. Again air bubbles were removed.

In order to create a capillary flow of buffer through the gel and membrane, folded paper towels were stacked onto the Whatman 3MM papers. The blotting tray was filled with 20XSSPE (pH7.4) and the Southern transfer was allowed to proceed for 16 hours (or overnight). When the transfer was finished, the nylon membrane was removed and washed in 2XSSPE for 10 minutes. The membrane was placed between 2 sheets of Whatman 3MM paper and baked at 80°C for 2 hours. The membrane was then sealed in a plastic sleeve and was stored at 4°C until further use.

Preparing the IS6110 Probe by PCR Amplification

The PCR amplification mixture was prepared by adding, in order, 9.375µl Sterile nuclease free dH₂O to 5µl Q-Solution, 2.5µl 10× PCR Buffer, 2µl MgCl₂ (25mM), 4µl dNTPs (10mM), 1µl of each IS6110 primer (50pmol/µl) and 0.125µl HotStarTaq DNA polymerase to a total volume of 25µl.

The amplification process was started by incubating the samples at 95°C for 15 minutes. Then the samples were incubated at 94°C for 1 minute, for 35-45 cycles, followed by incubation at 62°C for 1 minute, 72°C for 1 minute, and lastly incubation at 72°C for 10 minutes. After the PCR

amplification was complete, 5µl aliquots of the PCR products were run on a 2% agarose gel (containing 1×TBE pH8.3) followed by staining with ethidium bromide. The IS6110 PCR products were purified with a clean up kit (Wizard SV Gel & PCR Clean-up System) according to the manufacturer's specifications. The DNA was eluted in nuclease free H₂O and the concentration of the probe was determined spectrophotometrically by reading the optical density at 260nm.

[OD_{260nm} = 50µg/µl of double stranded DNA]

Labelling the IS6110 & Internal Marker Probes

A total of 200ng of probe DNA were added to a 0.5ml tube and made up to a volume of 15µl with nuclease free H₂O. The tube was then incubated at 100°C for 5 minutes to denature the probe DNA and immediately after was put on ice for 5 minutes to ensure that the denatured DNA stays in single stranded form so that hybridization can take place. The probe was labelled by adding 15µl of Horse Radish Peroxidase (HRP) to it. A total of 15µl of Gluteraldehyde solution were added to the tube containing the probe and HRP and mixed well. The tube was then incubated for 10 minutes at 37°C and then added to the membrane.

Prehybridisation and Hybridisation

The nylon membrane was re-hydrated by incubating it in 500ml dH₂O. The membrane was then put in a plastic sleeve (25x35cm) containing 48ml of ECLTM Gold Hybridization Buffer. All air bubbles were removed from the bag. The plastic sleeve containing the membrane and buffer was then sealed and the ECLTM Gold Hybridization Buffer was spread over the membrane by gently rolling a 10ml pipette over the bag. The plastic sleeve containing the membrane and ECL buffer was then put into a flat plastic container. A second plastic sleeve containing 500ml of H₂O were placed on top of the plastic sleeve containing the membrane and pre-hybridised by incubating the plastic container in a shaking water bath at 42°C for at least 60 minutes at 90rpm. Hybridization was started by removing the sleeve containing the membrane from the water bath, one corner of the sleeve was cut off and

the labelled probe was added directly to the ECL™ Gold Hybridization Buffer. Air bubbles were removed and the membrane was placed back in the water bath and hybridised at 42°C for 16 hours with shaking at 90rpm. After hybridisation was complete, the membrane was removed from the sleeve and washed twice by adding 400ml pre-warmed (to 42°C) Primary wash (Section ... Buffers and Solutions), in a water bath at 42°C for 20 minutes shaking at 90rpm. The membrane was then washed twice with 400ml 2XSSC for 5 minutes at room temperature on a shaker.

Detection of Hybridisation

To confirm whether hybridisation took place, 4ml of each of the two Amersham ECL™ Detection Reagents were mixed together and added to the membrane. The detection fluid was spread over the membrane for 90 seconds. All excess Amersham ECL™ Detection fluid was removed and the membrane was exposed to x-ray for 1 minute to up to 2 hours.

Stripping the membrane

To strip the membrane from all probe and DNA, the membrane was placed in a plastic container containing 400ml of boiling 0.1% SDS. The container was then placed on a shaker at room temperature for 60 minutes and the membrane was put in a clean plastic sleeve stored at 4°C.

5.3.3 DETERMINATION OF THE PRESENCE OR ABSENCE OF REGIONS OF DIFFERENCE

Each Beijing strain from each patient was subjected to PCR amplification in a reaction mixture containing 0.2g DNA template, 5ul Q buffer, 2.5ul 10xbuffer, 2ul 25mM MgCl₂, 4ul 10mM dNTPs, 1ul of each primer (50pmol/ul) (4) (Table 4) and 0.125ul HotStarTaq DNA polymerase (QIAGEN, Germany) and made up to 25ul with dH₂O. Amplification was initiated by incubation at 95°C for 15 minute, followed by 35 to 45 cycles at 94°C for 1 minute, annealing temperature (62°C) for 1 minute, and 72°C for 1 minute. After the last cycle, the samples were incubated at 72°C for 10 minutes. PCR amplification products were electrophoretically fractionated in 3.0% agarose in 1xTris-buffered EDTA, pH8.3, at 6 V/cm for 4 hours and visualized by staining with ethidiumbromide. The existence of a mutational event was determined by the presence or absence and the size of the respective PCR product.

Table 5.2. Primers used to determine regions of difference.

OLIGO NAME	SEQUENCE
RD105F	5'ACA GCG CGG GTC ATA TCA C 3'
RD105INT	5'GCA ACA CCC GCT TGT CTT TG 3'
RD105R	5'AAC CAG CTC CTC GAC GCT ATC 3'
RD181F	5'AAA TCC GCC CAT ACC CGT C 3'
RD181R	5'AGC TTC GAC TGG CCA TAG GC 3'
RD150F	5'AGT GCT GGC AAT AGC GGT TG 3'
RD150INT	5'CAC CGG CAC TTA CCA TCT CG 3'
RD150R	5'CCA GCA CTT GTT GCA ACT TCG 3'
RD142F	5'CCG GTG GTA CGG GTA TTT CC 3'
RD142INT	5'GCT CGA GCA TGA TCA GCA AAG 3'
RD142R	5'TAG CAC CAG TAC CGG ATG TCC 3'

5.4 CULTIVATION OF *M. TUBERCULOSIS* STRAINS

Strains were first cultured on solid media slants. The solid medium used in this study to culture *M. tuberculosis* was Lowenstein-Jensen (LJ) medium which is an egg-based medium. The medium contains inhibitors to keep contaminants from outgrowing *M. tuberculosis*. After 2-3 weeks, small buff coloured colonies that had a breadcrumb (Figure 5.1) appearance became visible.



Figure 5.1. *M. tuberculosis* colonies on an LJ slant.

From the positive LJ, a few colonies were picked and inoculated into liquid medium known as Middlebrook 7H9 medium (Becton, Dickinson and Company, Sparks, USA), which is an agar-based medium (ref) (supplemented with 10% albumin-dextrose-catalase (ADC), 0.2% (v/v) glycerol (Merck Laboratories, Saarchem, Gauteng, SA) and 0.1% Tween80 (Merck Laboratories)). The bacteria were cultured in filtered-cap tissue culture flasks (Greiner Bio-one, Maybach Street, Germany) without shaking at 37 °C. An initial culture of 5ml was prepared and incubated for ~ 2 weeks until the culture had a milky appearance and reached an optical density (OD_{600}) of mid-logarithmic phase (0.6-0.8). This starting culture was used to make 1:100 dilution sub-cultures until the required volume of bacteria was reached (Figure 5.2).

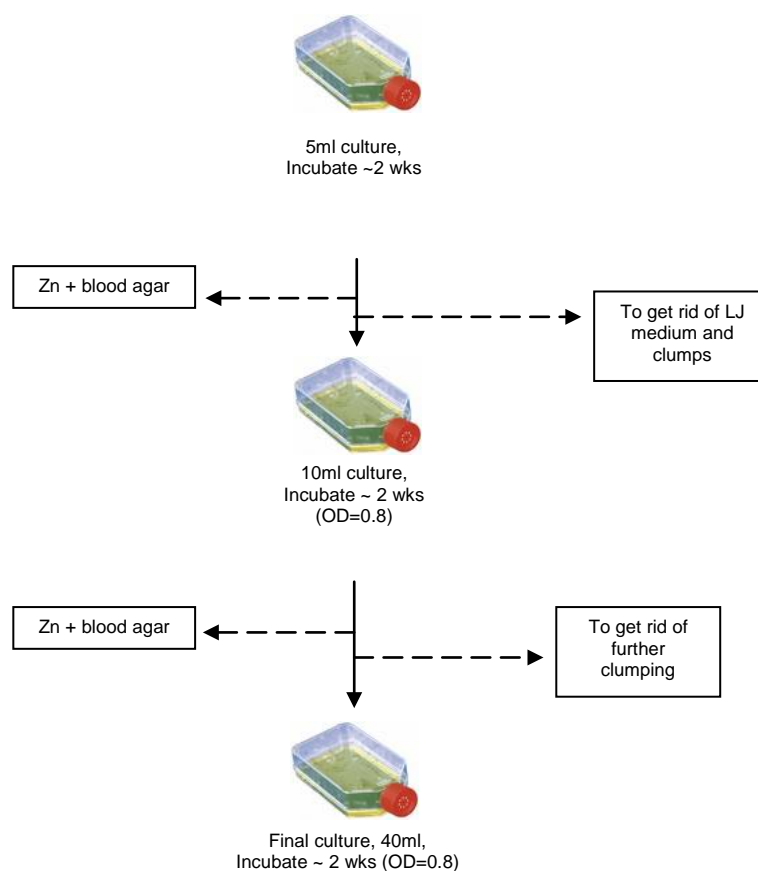


Figure 5.2. Diagrammatic illustration of how *M. tuberculosis* was cultured for protein extraction.

The cultures were tested continuously for contaminants by doing a Ziehl-Neelsen (Zn) test (to make sure that the organism present is *M. tuberculosis*) as well as plating a drop of the culture onto a blood agar plate (*M. tuberculosis* does not grow on blood agar). *M. tuberculosis* is classified as acid-fast bacteria due to their impermeability by certain dyes and stains. Despite this, once stained, acid-fast bacteria will retain dyes when heated and treated with acidified organic compounds. The acid-fast staining method for *M. tuberculosis* that we used was the Zn stain. When this method is used, the *M. tuberculosis* smear was fixed (heated for 2 hours at 100°C), stained with carbol-fuchsin (a pink dye) (Becton, Dickinson and Company, Maryland, USA), and decolorized with acid alcohol. The smear was then counterstained with methylene blue (Becton, Dickinson and Company, Maryland, USA). Acid-fast bacilli appear pink in a contrasting background (Figure 5.3).

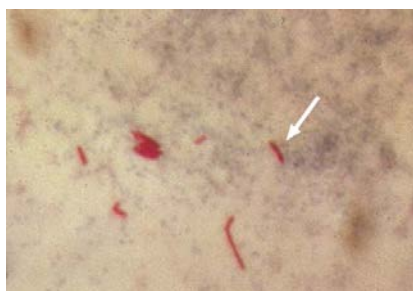


Figure 5.3. The white arrow indicates acid-fast *M. tuberculosis* stained with ZN stain.

5.5 PROTEIN EXTRACTIONS OF *M. TUBERCULOSIS* WHOLE CELL LYSATE

After the *M. tuberculosis* culture reached an OD_{600} of mid log phase (0.6-0.8), it was centrifuged (Eppendorf, Centrifuge 5810R) at 3000rpm for 20 minutes at 20°C. The supernatant was discarded and the pellet re-suspended in 1ml Phosphate buffer saline (PBS), pH 7.4, containing 1% (v/v) Tween. The suspension was then transferred to a 2ml screw cap tube and centrifuged at 6000rpm for a further 5 minutes. The supernatant was discarded and the resulting pellet was washed in 1ml PBS and centrifuged at 6000rpm for 5 minutes. The supernatant was discarded and an equal volume (approximately the same size as the pellet) of silica hybrid beads (Bio 101, Vista, USA) and approximately 200-500 μ l of Lysis buffer (Sigma-Aldrich, Missouri, USA) (depending on the size of the pellet) were added to the cell pellet. The tube containing the bacterial pellet, beads and Lysis buffer was then vortexed to mix the beads and Lysis buffer with the cell pellet. The mixture was then incubated at 80°C for 20 minutes after which it was cooled to room temperature. The bacterial cells were ribolysed using a FastPrep FP120 ribolyzer (Bio101 SAVANT, Vista, USA), with highest intensity (6.5 m/s) for 2-4 \times 45 seconds with 1 minute of cooling on ice between the cycles. The cells were then incubated at 100°C for 5 minutes allowed cool to room temperature, and centrifuged for 10 minutes at 13 000 rpm. The supernatant, which contained the whole cell lysate proteins were then transferred to a clean 2ml eppendorf tube and stored at -20°C until needed for further analysis.

5.6 DETERMINATION OF PROTEIN CONCENTRATIONS

The Bradford protein assay in which a differential colour change of a dye occurs in response to various concentrations of protein, were used to determine the concentrations of the extracted whole cell lysate proteins. The linear concentration range is 0.1-1.4 mg/ml of protein, using BSA (bovine serum albumin) as the standard protein. To determine the concentrations of the proteins in this study a total of 8 dilutions ranging from 0.2 to 0.9mg/ml, were prepared (Table 5.3) and used to establish a standard curve from which the unknown protein concentrations could be determined.

Table 5.3. Range of BSA used for standard curve

BSA range	BSA dilutions	
	BSA stock (µl)	H₂O (µl)
Blank	0	20
0.1	0.7	19.3
0.2	1.5	18.5
0.4	2.9	17.1
0.5	3.6	16.4
0.6	4.4	15.6
0.8	5.8	14.2
1	7.24	12.75

One microlitre (1µl) of each sample was added to 19µl of dH₂O. The Bradford reagent (containing BSA) was diluted (1 part reagent to 3 parts milliQH₂O) with milliQH₂O to a volume according to the number of protein samples. A volume of 980µl diluted Bradford reagent was then added to each standard and sample, and vortexed to mix the reagent with the BSA standards and samples. The mixtures were then incubated at room temperature for 5 minutes and the ODs of the samples were taken in duplicate at wavelength 595 with a spectrophotometer.

Protein purification

The proteins were purified using the ReadyPrep 2-D CleanUp Kit (Bio-Rad Laboratories, Hercules, CA 94547) according to the manufacturer's instructions. The concentrations of the proteins were then determined with the RC DC Protein assay (Bio-Rad Laboratories, Hercules, CA 94547) according to the instructions of the manufacturers.

5.7 PROTEIN SEPARATION

5.7.1 Isoelectric focusing

Proteins differ from each other in terms of their mass and charge. The first dimension (1-D) polyacrylamide gels used in IEF were pH4-7 IPG (immobilised pH gradients) strips (Bio-Rad, Laboratories, Hercules, CA 94547), which provide a supporting matrix through which proteins can migrate. This gel has a pH gradient from top to bottom, the top more acidic than the bottom. The proteins migrate through the gel until they reach their isoelectric point (the point at which their charge is the same as the surrounding pH).

The protein samples were added to re-hydration (RH) buffer, which is used to solubilise and run protein mixtures during IEF. The IPG strips were then added to the protein/RH buffer mix and allowed to re-hydrate at room temperature for 20 hours. When re-hydration was finished the IPG strips containing the proteins, were electrophoresed under the following running conditions: 100V for 2 hours, 300V for 3 hours, 1000V for 1 hour, and 3500V for 20 hours, at 17°C.

5.7.2 2-Dimensional gel electrophoresis

For the second step of the experiment, the IPG strips were placed in equilibration buffer containing DTT (used for disruption of protein disulfide bonds) (Sigma-Aldrich, Missouri, USA) for 15 minutes and then in buffer containing Iodoacetamide (an alkylating agent which aids in unfolding the proteins) (Sigma-Adrich, Missouri, USA) for 15 minutes. The 1-D gels were then placed on top of the 2-D gels, which are SDS-PAGE gels, and sealed to the 2-D gels with agarose (ReadyPrep™ Overlay agarose, Bio-Rad Laboratories, Hercules, CA 94547). The proteins were separated by a second property in a direction 90 degrees from the first. Proteins with a higher mass, migrates slower through the gel than proteins with a smaller mass. The proteins were therefore spread out on the surface of the gel. The 2-D gels were electrophoresed for 2-3 hours.

5.8 PROTEIN DETECTION

The proteins can be detected by a variety of methods but the most commonly used are silver nitrate and coomassie brilliant blue staining methods. For this study we made use of the silver stain method.

The proteins were fixed in the 2-D protein gels with Fixing buffer for 1 hour. The protein gels were then soaked in Soaking buffer overnight. After the gels were soaked, they were washed with milliQH₂O 3 times for 10 minutes each and then sensitised twice for 15 minutes with Sensitise buffer. The gels were washed 3 times for 10 minutes each with chilled milliQH₂O and incubated in chilled (4°C) silver nitrate solution for 1.5 hours at 4°C with shaking. The silver nitrate solution was discarded and the gels were rinsed twice for 1 minute with milliQH₂O. Developing buffer was then added to the gels to allow the silver nitrate to develop. As soon as the Developing buffer turned a yellow colour, it was discarded and replaced with fresh buffer. The new Developing buffer was discarded as soon as the protein spots were visible and the gels were washed with Stop buffer to stop the developing. The silver-stained gels were stored in Store buffer at room temperature.

5.9 2-D GEL ANALYSIS

Silver stained gels were scanned and recorded using a GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, CA 94547) and quantitative spot detection and matching was done visually as well as using PDQuest and Quantityone 2-D software (Bio-Rad Laboratories, Hercules, CA 94547). Differentially expressed protein spots were excised and stored for future Mass spectrometric analysis.

5.10

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5.11

LIST OF BUFFERS AND SOLUTIONS

PCR BUFFERS

10XTE Buffer:

10mM TRIS

1mM EDTA

dH₂O**10XTBE Buffer (pH 8.3):**

0.45M Tris

0.44M Boric acid

10mM EDTA

dH₂O to make up 1L

SPOLIGOTYPING SOLUTIONS

10XSSPE:100mM HNa₂PO₄

1.8M NaCl

10mM EDTA

dH₂O to make up 1L**10% SDS:**

50g SDS

500ml dH₂O**0.5M EDTA:**

93g EDTA

500ml H₂O

IS6110 RFLP BUFFERS AND SOLUTIONS

Chloroform/isoamyl alcohol:	24:1 v/v
Denaturing Buffer (5 litres):	1.5M NaCl, 0.5M NaOH
:	438.3g NaCl 100g NaOH
70% Ethanol:	store at -20°C use 'ice-cold'
100% Ethanol:	store at -20°C
Ethidium Bromide:	Final concentration 10mg/ml NB: store in dark container
Extraction Buffer pH 7.4 (1litre):	5% Mono Sodium Glutamic Acid 50mM Tris-HCl (pH 7.4), 25mM EDTA Adjust pH with Hydrochloric Acid
100% Isopropanol:	store at -20°C use 'ice-cold'

23 μ l TE

45 μ l 0.8M NaOH

Phenol/Chloroform/isoamyl alcohol: 25:24:1v/v

Primary wash buffer: 720g urea

8g SDS

25ml 20 x SSC

dH₂O to make up to 2 litres

store at room temperature

Proteinase K (Roche, Germany): 10mg/ml in dH₂O

store in aliquots at -20°C

Proteinase K buffer (pH 7.8, 500ml): 5% sodium dodecyl sulphate

100mM Tris-HCl (pH7.8)

50mM EDTA

Adjust pH with HCl

PvuII restriction endonuclease (10u/ μ l) (New England Biolabs, USA)

Restriction Buffer, NE Buffer 2 (10X) (New England Biolabs, USA)

RNaseA (Roche, Germany):	10mg/ml in 10mM Tris-HCl (pH7.5) 15mM NaCl Heat to 100°C for 15 minute Allow to cool to room temperature Store in aliquots at -20°C
3M Sodium Acetate pH 5.5 (500ml):	204.1g Na-Ac-3H ₂ O adjust pH with Glacial Acetic Acid store at room temperature
Sodium Dodecyl Sulphate (SDS):	10% w/v
SSPE Solution (20X) (pH 7.4) (5 litres):	3M NaCl 0.2 NaH ₂ PO ₄ · H ₂ O 20mM EDTA 876.5g NaCl 138g NaH ₂ PO ₄ · H ₂ O 37g EDTA 40g NaOH pellets adjust pH with ~8g NaOH store at room temperature
5XTBE (pH 8.3):	0.45M Tris 0.44M Boric acid 10mM EDTA dH ₂ O to make up 2L

store at room temperature

NB: DO NOT pH THIS SOLUTION

TE (pH 8.0):

10mM Tris-HCl (pH 8.0)

1mM EDTA

1.211g Tris

0.372g EDTA

adjust by using HCl, autoclave

store at room temperature

CULTIVATION OF *M. TUBERCULOSIS* STRAINS

Middlebrook 7H9 medium:

4.7g 7H9 medium

900ml dH₂O

2ml Glycerol

0.5ml Polysorbate (Tween80)

ADC:

25g BSA

10g Glucose

0.75ml Catalase

dH₂O to make up 500ml

EXTRACTION OF WCL PROTEINS

Lysis buffer:

0.3% (w/v) SDS

200mM DTT (for intracellular proteins)

50mM Tris-HCL (pH 7.0)

1mM PMSF (inhibit serine proteases)

1 Protease inhibitor tablet

dH₂O to make up 25ml

PBS (pH 7.3):

8g NaCl

0.2g KCL

1.15g diNaHPO₄

0.2g KH₂PO₄

1% Tween80

dH₂O to make 1L

2-D GEL ELECTROPHORESIS SOLUTIONS

re-hydration (RH) buffer:

8M Urea

2% Chaps

10mM DTT

2% IPg buffer

Bromophenol blue (trace amount)

Equilibration buffer 1 (containing DTT): 0.375M Tris-HCL (pH 8.8)

6M Urea

30% Glycerol

2% SDS

2% DTT

Equilibration buffer 2 (containing Iodoacetamide):

0.375M Tris-HCL (pH 8.8)

6M Urea

30% Glycerol

2% SDS

1g Iodoacetamide

Electrophoresis buffers:

Cathode buffer:

192M Glycine (pH 8.3)

pH with Tris

add 0.1% SDS

milliQH₂O to make up 1L**Anode buffer** (2x):

0.375M Tris (pH 8.8)

pH with AcOH

milliQH₂O to make up 1.5L**Fixing buffer:**50% MeOH (Merck, Darmstadt,
Germany)5% AcOH (Merck, Darmstadt,
Germany)**Soak buffer:**

50% MeOH

Sensitise buffer:0.02% Na₂S₂O₃ (Sigma-Aldrich,
Missouri, USA)

Silver nitrate solution (Sigma-Aldrich): 0.1%

Developing buffer: 0.04% formalin (Sigma-Aldrich)

2% Na₂CO₃ (Sigma-Aldrich)

Store buffer: 1% AcOH