Detection of mixed *Mycobacterium tuberculosis* infections in South African TB patients

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Dedicated with love and appreciation to my wonderfully supportive family

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DECLARATION

I, Michael Craig Stead, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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List of Abbreviations

°C	Degrees Celsius
λ	Lamda
3’	Three prime end
5’	Five prime end
ahpC	Alkylhydroperoxide reductase (gene)
BCG	Bacille Calmette-Guérin
bp	Base Pair(s)
BSL3	Biosafety Level 3
CAS	Central Asian Strain
CTAB	Hexadecyl trimethylammonium bromide
ddNTPs	Dideoxinucleotide triphosphates
DNA	Deoxiribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DOTS	Directly observed therapy short-course
DR	Direct repeat
DST	Drug susceptibility testing
EAI	East Asian Indian
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamin tetra-acetic acid
EtBr	Ethidium bromide
ETH	Ethambutol
g	Gram(s)
GSH	Groote Schuur Hospital
gyrA/B	DNA gyrases A/B (genes)
H	Haarlem
HIV	Human immunodeficiency virus

HGDI	Hunter Gaston Discriminatory Index
INH	Isoniazid
inhA	Enoyl-acyl carrier protein (gene)
InhA	Enoyl-acyl carrier protein (protein)
INH^R	Isoniazid mono-resistance
katG	Catylase-peroxidase enzyme (gene)
KatG	Catylase-peroxidase enzyme (protein)
LAT	Latin American
LJ	Lowenstein-Jenson
LSP(s)	Large sequence polymorphism(s)
M	Molar
M. bovis	*Mycobacterium bovis*
M. tuberculosis	*Mycobacterium tuberculosis*
MAS	Multiplex allele-specific
MDR	Multi-drug resistant
$MgCl_2$	Magnesium chloride
MGIT	Mycobacterial growth indicator tube
min	Minutes
MIRU	Mycobacterial interspersed repetitive units
ml	Millilitres
mM	Millimolar
mol	Moles
MWM	Molecular weight marker
NaCl	Sodium chloride
NALC	N-acetyl-L-cystein
NaOH	Sodium hydroxide
ndh	Nicotinamide adenine dinucleotide dehydrogenase (gene)
ng	Nanograms

oxyR	Oxidative stress regulator
PAS	p-aminosalicyclic
PCR	Polymerase chain reaction
pH	Power of hydrogen
pmol	Picomoles
pncA	Pyrazinamidase A
PS	Primer Set
PZN	Pyrizinamide
RIF	Rifampicin
RIF^R	Rifampicin mono-resistance
RFLP	Restriction fragment length polymorphism
rpm	Revolutions per minute
rpoB	Beta-subunit of DNA-dependant RNA polymerase (gene)
rpsL	Ribosomal protein S12 (gene)
RRDR	Rifampicin resistance determining region
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
rrs	16S rRNA (gene)
sec	Seconds
SDS	Sodium dodecyl sulphate
SNP	Small nucleotide polymorphism
STR	Streptomycin
T_a	Annealing temperature
TAE	Tris-acetate EDTA
Taq	*Thermus aquaticus*
TB	Tuberculosis
U	Enzyme Unit
µl	Microlitre(s)

µg	Microgram(s)
µM	Micromolar
UV	Ultraviolet
VNTR	Variable number of tandem repeats
v/v	Volume per volume
W	Week(s)
WT	Wild type
w/v	Weight per volume
XDR	Extremely drug resistant

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Single Letter Nucleotide Code

A	Adenine
T	Thymine
C	Cytosine
G	Guanine
N	Any nucleotide

Single Letter Amino Acid Code

G
Glycine (Gly)

P
Proline (Pro)

A
Alanine (Ala)

V
Valine (Val)

L
Leucine (Leu)

I
Isoleucine (Ile)

M
Methionine (Met)

C
Cysteine (Cys)

F
Phenylalanine (Phe)

Y
Tyrosine (Tyr)

W
Tryptophan (Trp)

H
Histidine (His)

K
Lysine (Lys)

R
Arginine (Arg)

Q
Glutamine (Gln)

N
Asparagine (Asn)

E
Glutamic Acid (Glu)

D
Aspartic Acid (Asp)

S
Serine (Ser)

T
Threonine (Thr)

Abstract

Recently, the widely accepted theory that TB disease resulted from one infecting *M. tuberculosis* strain leading to heightened immune protection against subsequent infections, has been revised. Epidemiological studies and the advances in molecular genotyping techniques have highlighted the rapidly frequent isolation of several different *M. tuberculosis* strain lineages in single disease episodes, often with differing drug susceptibilities. This has important implications on drug susceptibility testing and the treatment of patients. It has also highlighted the relative contributions of exogenous reinfection and endogenous reactivation in TB disease progression. However, our understanding of the nature and frequency of mixed infections is lacking. This study investigated the frequency and detection of mixed TB infections in the Delft region of the Western Cape, as part of a larger clinical trial on the effects of multi-nutrient supplementation and standard treatment on the TB bacteriological response.

Newly diagnosed, adult TB patients (n=154) produced a single weekly sputum sample over an 8-week period. Genomic DNA was extracted from colonies grown from MGIT cultures on LJ slopes. Spoligotyping was used as an initial screen to detect mixed infections as well as to assess the epidemiology of *M. tuberculosis* in these serial isolates (n=686). In addition, clonal relatedness of the isolates was assessed by MIRU-VNTR analysis. Thereafter PCR assays to detect infections of W-Beijing and non-W-Beijing isolates, as well as to differentiate mixed non-W-Beijing isolates were carried out. Phenotypic and genotypic drug susceptibility was carried out.

Spoligotyping indicated that W-Beijing isolates constituted a large proportion (47.8%) of circulating *M. tuberculosis* in this region, with other strains detected including LAM (17.1%), T (14.7%), X (6.4%), H (7.9%), S (4.3%), and F33 (2.1%) strains. Using both spoligotyping and PCR assays, mixed infections were detected 21 (16.3%) of 129 patients screened. Phenotypic and genotypic DST confirmed that all isolates identified in patients as harbouring mixed strains by spoligotyping were fully susceptible to both RIF and INH. MIRU-VNTR

analysis for genetic relatedness identified 1 clonal cluster in the mixed samples identified by spoligotyping, consisting of the T1, T4, W-Beijing and W-Beijing + X3 isolates.

The frequency of mixed infections, particularly in high disease burdened areas, is high, and warrants further attention. This finding has great implications with regards to the interpretation of epidemiological and DST data, and the subsequent treatment of patients.

Chapter 1

Literature Review

Contents

1.1. Introduction

1.1.1. Historical Context

Mycobacterium tuberculosis (*M. tuberculosis*), the causative agent of human tuberculosis (TB) (Koch, 1882), is mankind's oldest and most successful pathogens (Gutierrez *et al*, 2005). So great is its antiquity that *M. tuberculosis* DNA has been found in mummified remains in the Andes Mountains, dating between 140 – 1200 A.D. (Konomi *et al*, 2002); in pre-Columbian Peru, dating between 1000 – 1300 A.D. (Salo *et al*, 1994); and in Egypt, dating between 2050 – 1650 B.C. (Zink *et al*, 2003). Other references date findings in Egypt as early as 3000 and 5000 B.C. (Zimmerman *et al*, 1979; Morse *et al*, 1964 respectively). These findings suggest that TB has been infecting man, certainly in Egypt, since the pre-Dynastic period (Zimmerman *et al*, 1979).

The study of the evolutionary history of *M. tuberculosis* has been difficult and often controversial, due to the relatively static nature of the *M. tuberculosis* complex genomes; *M. tuberculosis* complex bacterial nucleotide sequences are 99.9% identical (Vultos *et al*, 2008). It is believed that the members of the *M. tuberculosis* complex are the clonal progeny of a successful ancestor resulting from a bottleneck event 20000 – 35000 years ago (Gutierrez *et al*, 2005). For decades, scientists have hypothesised that *Mycobacterium bovis* (*M. bovis*) is that ancestor (Brosch, 2002), and started infecting humans around the time when humans began domesticating cattle around the same time as the bottleneck (Smith, 2003). However, recent molecular advances have allowed further analyses into this field and have shown that *Mycobacterium canettii* (*M. canettii*), a tubercule bacillus of rare occurrence and a smooth colony morphology (Gutierrez *et al*, 2005), could represent modern progenies of an ancestral species termed *Mycobacterium prototuberculosis* (*M. prototuberculosis*), from which *M. tuberculosis* may have evolved (Ernst *et al*, 2007)

TB was described clinically in 400 B.C. by Hippocrates in *Of the Epidemics*, detailing patients with bloody sputum and a "wasting disease", with associated chest pain and chronic cough, suggesting that TB was already then well established (Mathema, 2006; Smith, 2003). TB continued to spread across the globe, due mostly to immigration, while scientists debated

what the cause of this disease was (Smith, 2003). Some scientists doubted that it was a single disease, as there were so many varied symptoms among patients, while others denied the infectivity of the disease (Daniel *et al*, 1994). Some of TB's former titles, such as lupis vulgaris, consumption, King's Evil and White Plague (Mathema *et al*, 2006), have been pandemic in Europe and North America for the last 500 years (Iseman, 1994). During the 17th and 18th centuries, at the height of the White Plague, 20% of all adults died of tuberculosis, while an estimated 1 billion people died of tuberculosis between 1850 and 1950 (Iseman, 1994). It wasn't until Robert Koch, in 1882, published his landmark paper, "*Die Atiologie der Tuberkulose*", that the causative agent was identified. He described the TB bacillus and named the pathogen *Mycobacterium tuberculosis* by carrying out staining techniques, which Koch developed to better see microorganisms for characterisation (Koch, 1882).

Following on from Edward Jenner's studies on vaccination, many scientists placed their hope in *M. bovis*, which causes disease in cattle, as a possible vaccine candidate; however, *M. bovis* was equally as contagious in humans (Palomino, 2007). Albert Calmette and Camille Guérin, from 1908 – 1919 attenuated a strain of *M. bovis* by serial passage 230 times, resulting in an avirulent strain termed BCG (Bacille Calmette-Guérin). BCG was later administered into the first human subjects in 1921 and has remained, ever since, the most widely distributed vaccine in the world (Palomino, 2007).

In 1945, Selman Waksman, a soil biologist, discovered the antibiotic streptomycin (STR) and showed that it had a high activity against *M. tuberculosis* (Iseman, 1994). This landmark discovery launched the TB chemotherapeutic era. However, shortly after this discovery, reports of resistance to streptomycin emerged. It was found that streptomycin alone was not sufficient to effectively eradicate the pathogen from patients, but rather selected out, from the numerous numbers of bacilli, those that had a natural resistance to streptomycin (Iseman, 1994). Fortunately, not long after the discovery by Waksman, other antibiotics were discovered or manufactured. The drugs *p*-aminosalicylic (PAS) and isonicotinic acid hydrazide (isoniazid; INH) were found to have a higher efficacy than streptomycin, and when used in combination, it was found that no resistance emerged (Gillespie, 2002). Combination therapy has since then been the backbone of the treatment of TB. Since the establishment of

combination chemotherapy, a host of antibiotics have been included in what is today's standard treatment regime, including INH, ethambutol (ETH), rifampicin (RIF), pyrizinamide (PZN) and STR (Plorde, 1994).

1.1.2. Current Burden of Disease

Today, TB remains one of the most important unresolved diseases around the world, particularly in developing countries such as India, Brazil and South Africa (Iseman, 1994; Iseman and Heifets, 2006). In 2006, 9.2 million new cases of TB were reported as well as 1.7 million deaths (WHO, 2008). It is estimated that 2 billion people, roughly a third of the global population, is infected with *M. tuberculosis*, of which 10% will develop active TB in their lifetime, with HIV sufferers being most vulnerable (WHO, 2007). Africa has the highest per capita rates of TB infections with 28% of the world's TB cases being found in Africa (WHO, 2007). It was estimated in 2006 that there were 500 000 cases of MDR-TB in the world (WHO, 2008). TB is also a disease of poverty, with the majority of cases occurring in developing nations (WHO, 2007).

1.1.3. Antibiotic Resistance in *M. tuberculosis*

Despite an effective therapy regime for TB that has been in place for many years, today we see TB prevalences and incidences that have exceeded those since before the discovery of streptomycin (Gillespie, 2002; Zager and McNerney, 2008). Patient non-compliance as well as inadequate dosing by health practitioners has been blamed for the emergence of multi-drug resistant (MDR) *M. tuberculosis* (MDR-TB) and extensively-drug resistant (XDR) *M. tuberculosis* (XDR-TB), which makes the current treatment programs for TB highly ineffective (Drobniewski and Yates, 1997; Zager and McNerney, 2008). MDR-TB is defined as being resistant to the two first-line drugs INH and RIF (WHO, 2000), while XDR is MDR-TB that is also resistant to a second-line injectable drug, such as capreomycin, and a fluoroquinolone (Zager and McNerney, 2008). Resistance to antibiotics is achieved through various mechanisms such as a mutation in the drug target, which decreases the binding of the drug; reduced uptake of the drug; active efflux of the drug, once inside the cell; the presence of an enzyme that deactivates the drug; over-production of the drug target, resulting in titration of

the antimicrobial agent; or the presence of an alternative enzyme to compensate for the loss of another enzyme inhibited by the drug (Fluit *et al*, 2001). Molecularly, resistance to antibiotics in *M. tuberculosis* develops due to spontaneous chromosomal mutations (Ramaswamy and Musser, 1998). Mathematical models estimate that the rate of spontaneous mutations occur at a frequency of (in mutations per bacterium per cell division) 3.32×10^{-9} for RIF, 2.56×10^{-8} for INH, 2.29×10^{-8} for STR and 1.0×10^{-7} for ETH (Gillespie, 2002).

There are many molecular tools available to scientists today that allow them to detect chromosomally encoded determinants of resistance, such as polymerase chain reaction (PCR), and hybridisation studies (Fluit *et al*, 2001).

1.1.4. *M. tuberculosis* Strain Diversity

For many centuries *M. tuberculosis* has been infecting man and thus has been coevolving with man. As man has a diverse migratory history, *M. tuberculosis* has had to face many different geographical pressures placed on it and has in response to this, evolved clonally (Brudey *et al*, 2006). The various clonal lineages of *M. tuberculosis* have distinct phylogeographical characteristics that separate them from one another (Brudey *et al*, 2006). The hypothesis is that the *M. tuberculosis* complex originated in East-Africa and expanded to the rest of the world with human migration out of and back into Africa (Brudey *et al*, 2006, Gagneux *et al*, 2006). Currently, the six major *M. tuberculosis* lineages recognised circulating around the world include Indo-Oceanic, East-Asian (including the Beijing family), East-African-Indian, Euro-American and West-African lineages 1 and 2 (Gagneux *et al*, 2006). Each have adapted to the various human populations that inhabit the regions of origin of the strain lineages; for example, a person of Chinese decent living in San Francisco is more at risk of acquiring an East-Asian *M. tuberculosis* infection (OR 19.8, 95% CI: 4.6 – 84.2; $P<0.001$) than their American counterparts (Gagneux *et al*, 2006). In South Africa, particularly in the Western Cape, the Beijing and Haarlem strain families are overrepresented among children (Marais *et al*, 2006), while the Beijing and LAM3/F22 families predominate in adults (Nicol *et al*, 2005). It is of importance to scientists, particularly in South Africa where the prevalence of TB is so high, to genotype *M. tuberculosis* strains, as it gives an indication of the circulating lineages, as well as to discern whether or not there is an association between genotype and drug

resistance (Marais *et al*, 2006), or the ability of that lineage to cause extrapulmonary disease (Nicol *et al*, 2005).

1.2. Antibiotic Resistance in *M. tuberculosis*

1.2.1. Anti-tuberculosis Chemotherapy

The current standard treatment regime for TB is known as directly observed therapy short (DOTS) course treatment, and involves the supervised administration of multiple drugs for a minimum of 6 months (Warner and Mizrahi, 2006). There are currently two courses of antibiotics in the treatment of TB, namely the first line and second line antibiotics. Traditionally the first line antibiotics include INH, RIF, PZA, ETH, and STR while the second line of treatment includes the aminoglycosides kanamycin and amikacin, the polypeptide capreomycin, PAS, cycloserine, the thioamides ethionamide and prothionamide and several fluoroquinolones such as moxifloxacin, levofloxacin and gatifloxacin (Palomino, 2007). DOTS treatment follows a two phase plan with the first phase using three or more drugs, normally INH, RIF and PZA, for 2 months to allow the fast killing of dividing bacilli. The second phase is 4 months of 2 drugs, usually INH and RIF, to kill off any remaining bacilli and prevent reoccurrence (Palomino, 2007). Resistance to any of the first line drugs requires the introduction of second line drugs into the regiment (Palomino, 2007).

As it can be seen from the above treatment strategies, INH and RIF are the two most important antibiotics. INH, upon entering the cell through diffusion, is metabolically activated and binds to InhA, an enoyl-acyl carrier protein, involved in mycolic acid elongation (Marrakchi *et al*, 2000). Bacilli death is the result of an accumulation of mycolic acid intermediates in the cytoplasm of *M. tuberculosis* (Vilcheze and Jacobs, 2007). RIF, upon entering the bacillus, binds to the DNA-dependant RNA polymerase and prevents transcription in *M. tuberculosis* (Prescott *et al*, 2005). Resistance to these drugs results in MDR-TB (WHO, 2000).

1.2.2. Emergence of Resistance

In *M. tuberculosis*, resistance to any of the antibiotics is primarily achieved via spontaneous chromosomal mutations in genes responsible for the metabolism of the antibiotic or in one or more of the drug's targets (Ramaswamy and Musser, 1998). A number of different genes are known to be associated with drug resistance in *M. tuberculosis* (Table 1.1).

Table 1.1. Summary of the molecular determinants of antituberculosis drug resistance

Antibiotic	Associated mutated gene or mutation
Rifampicin	*rpoB*
Isoniazid	*katG*
	inhA
	ndh
	oxyR-ahpC
Streptomycin	*rrs*
	rpsL
Pyrazinamide	*pncA*
	IS*6110* Insertion
Ethambutol	*embB*
Fluoroquinolones	*gyrA*
	gyrB

(Adapted from Gillespie *et al,* 2002)

With RIF resistance, 95% of the mutations that confer resistance occur in an 81-bp region in the *rpoB* gene, encoding the β subunit of the DNA-dependant RNA polymerase, known as the RIF resistance-determining region (RRDR), with the most common mutations occurring at codons 516, 526 and 531 (Huitric *et al*, 2006).

In INH resistance, many genes have been associated with conferring resistance, making describing INH resistance complex (Mokrousov *et al*, 2002 (2)). However, the *katG* gene, encoding a bi-functional catalase-peroxidase enzyme, and the *inhA* gene, encoding an enoyl-acyl carrier protein, are the most frequently associated genes in conferring INH resistance (Ramaswamy *et al*, 2003). In *katG* the most common mutation (95%) associated with INH resistance is the Serine-Threonine transition at codon 315 (S315T) (Hazbon, *et al*, 2006), while in *inhA*, the most common mutation (8-20%) is a C-T single nucleotide polymorphism (SNP) at position 15 upstream of the *mabA-inhA* operon in the promoter (C-15T)

(Ramaswamy *et al*, 2003; Hazbon *et al*, 2006). Other genes associated with INH resistance include *ndh*, encoding an NADH dehydrogenase (Cardoso *et al*, 2007), and the *oxyR-ahpC* intergenic region, involved in oxidative stress (Ramaswamy *et al*, 2003). In a study by Ramaswamy and colleagues, 20 different genes associated with INH resistance were studied and some 10% of INH resistant *M. tuberculosis* isolates in their study showed no mutation in any of the genes studied, concluding that there are unknown mechanisms and determinants of INH resistance still to be discovered (Ramaswamy *et al*, 2003).

In STR resistance the two most frequently reported genes involved in resistance are *rrs*, which encodes 16S rRNA, and *rpsL*, which encodes ribosomal protein S12. Both are involved in protein synthesis (Sekiguchi *et al*, 2007).The most frequently reported *rpsL* mutation is K43R, while in the *rrs* gene, mutations are clustered in the highly conserved 530 loop (van Rie *et al*, 2001).

PZA resistance is associated with the gene *pncA*, which encodes pyrazinamidase, an enzyme necessary for the activation of PZA (Sekiguchi *et al*, 2007). Many mutations have been shown to be associated with PZA resistance, with the mutations dispersed throughout the gene; including A3E, D53N, P54L, C72W and M175V (Sekiguchi *et al*, 2007).

ETH resistance is achieved through mutations emerging in the *embABC* gene cluster, with the most common site of mutation occurring within the *embB* gene (Shen *et al*, 2007). The most frequent mutation seen is at codon 306 in the *embB* gene, with estimates of 60% of ETH resistant isolates containing a mutation in this region (Plinke *et al*, 2006). However, recently there has been some controversy over the codon 306 region as a predictor of ETH resistance, with Hazbon and colleagues suggesting that this codon region is associated with broad drug resistance rather than specifically ETH resistance (Hazbon *et al*, 2005). Several authors disagree and maintain the significance of the *embB* codon 306 mutations in the emergence of ETH resistance (Plinke, *et al*, 2006; Shen *et al*, 2007; van Rie *et al*, 2001).

Resistance to the second line fluoroquinolones is achieved through mutations arising in the DNA gyrases A and B encoded by *gyrA* and *gyrB*, respectively (Aubry *et al*, 2006). The gyrases affect the supercoiling of the DNA and are the targets of the fluoroquinolones (Fluit *et al*, 2001). The most common mutations reported occur at codons 90, 91 and 94 in the A subunit, and at codon 510 in the B subunit (Matrat *et al*, 2006; Aubry *et al*, 2006).

The need for so many antibiotics in the effective treatment of TB and the resultant magnitude of molecular determinants of resistance has necessitated the need for accurate methods of determining drug susceptibility. Additionally, given that rapid diagnosis of drug resistance in patients has positive outcomes within the communities, in that the spread of TB from infected contacts is reduced, the need for molecular assays that allow for the rapid and accurate diagnosis of drug resistance is of paramount importance. Several molecular tests are available today to aid clinicians in detecting drug resistance and to determine DST of the many antibiotics used in the treatment of TB (Fluit *et al*, 2001).

1.2.3. Drug susceptibility testing of *M. tuberculosis*

The outcome of a patient suffering from TB is related to the efficiency and turnaround time of the DST performed (Angeby *et al*, 2002). The more rapid an accurate diagnosis of drug resistance is made, the more time a patient has on an adequate regime of medication resulting in less time in the communities shedding bacilli and remaining infectious (Angeby *et al*, 2002). Thus rapid and inexpensive molecular tests are needed for the effective management of TB.

Traditionally, DST has been performed directly and indirectly on solid-based media such as Lowenstein-Jensen (LJ) slopes or Middlebrook 7H10/7H11 plates (Palomino *et al*, 2007). The three routine DST methods that use these media are the proportion method, the resistance ratio method and the absolute concentration method. All three methods require a decontaminated sample (direct) or a culture (indirect) that is inoculated onto the solid media with or without antibiotics (Palomino *et al*, 2007). However, due to the slow nature of *M. tuberculosis* growth, the turnaround time for this DST is anywhere from 4 to often more than 6 weeks (Goloubeva *et al*, 2001). To solve the problem of long turnaround times, several

automated methods using liquid broth have been developed, to greatly reduce the time taken to achieve results (Palomino *et al*, 2007).

The non-radiometric automated BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 DST system (Becton Dickinson Microbiology) has been favoured over the radiometric BACTEC 460 as the standard automated DST system (Rusch-Gerdes *et al*, 2006). The MGIT 960 is a system that allows for the detection and DST of *M. tuberculosis* from an isolated culture and has been validated to provide DST results for the antibiotics STR, INH, RIF, ETH (SIRE) as well as PZA (Piersimoni *et al*, 2006). The MGITs use a modified Middlebrook 7H9 liquid broth supplemented with antibiotic, as well as a silicon disk containing a fluorescent compound (Becton Co, 2005). The fluorescence is quenched by the available oxygen, but as the *M. tuberculosis* isolate grows and uses up the free oxygen, the fluorescence is released and measured by the automated BACTEC system (Piersimoni *et al*, 2006). Results are obtained in 4 to 19 days for positive results and tubes not registering a result after 42 days are considered negative (Becton Co, 2005). Several authors have evaluated the BACTEC MGIT 960 system for DST and *M. tuberculosis* detection (Kruuner *et al*, 2006; Rusch-Gerdes *et al*, 2006; Somoskovi *et al*, 2003), as well as to determine critical concentrations for the second-line antibiotics (Kruuner *et al*, 2006; Rusch-Gerdes *et al*, 2006). The BACTEC MGIT 960 system has shown to have sensitivities and specificities of 1.00 and 1.00 for STR, 0.98 and 1.00 for INH, 1.00 and 1.00 for RIF, and 0.96 and 1.00 for ETH, respectively (Ardito *et al*, 2001). In another study the sensitivities and specificities were 0.97 and 1.00 for STR, 0.95 and 1.00 for INH, 1.00 and 0.97 for RIF, 0.99 and 1.00 for ETH, and 0.96 and 0.87 for PZA, respectively (Scarparo *et al*, 2004). This shows that the MGIT 960 automated system is an excellent method for reliable and relatively fast DST for *M. tuberculosis*. However, this system has a few drawbacks such as the need for heavy and very expensive machinery as well as highly trained personnel and thus is not suited to a poor resource setting (Shiferaw *et al*, 2007).

Another DST tool that has been developed to facilitate a more rapid turnaround time and is inexpensive is known as the microscopic observation drug susceptibility (MODS) assay. This method is based on the characteristic formation of *M. tuberculosis* in liquid media, the so

called “serpentine cord formation” as viewed through an inverted light microscope (Palomino *et al*, 2007). *M. tuberculosis* sputum isolates are inoculated into a standard 24-well plate containing Middlebrook 7H9 liquid broth, with each row containing a standard concentration of test antibiotic, as well as a broth-only control row. Each column represents a sample and 4 to 5 samples can be tested per plate. A non-inoculated and an H37Rv control column are also included in the experiment (Iseman and Heifets, 2006). In a study evaluating the MODS assay, Shiferaw and colleagues reported that of the 262 smear-positive isolates, MODS had a significantly higher detection rate (96.9%) than the culture on LJ (94.3%) (*P*=0.016), had a sensitivity, specificity and accuracy of 92.0, 99.5 and 98.8%, respectively, using the proportion method as a reference (concordance: 98.8%), and their median turnaround time for the MODS assay was 9 days with 95.7% of all isolates being positive in 14 days (Shiferaw *et al*, 2007). This shows that the MODS assay is highly suited to resource poor settings. Draw backs of the assay is that an expensive inverted light microscope is needed as well as training of personnel is required to familiarize oneself with the different growth formations as viewed through the microscope (Palomino *et al*, 2007).

Other DST assays that are currently being used, albeit less frequently, include the Etest (AB BIODISK), which showed a high level of agreement with the proportion method on LJ medium with all first-line antibiotics tested (Hazbon *et al*, 2000); the nitrate reductase assay, also known as the Griess method, an inexpensive and rapid DST assay, based on the ability of *M. tuberculosis* to reduce nitrate to nitrite (Angeby *et al*, 2002); and a rapid high-throughput absolute concentration method, an automated version of the MODS assay (Palomino *et al*, 2007).

1.2.4. Detection of Resistance Determinants

Molecular assays that utilize the fact that resistance to an antibiotic is achieved through spontaneous chromosomal mutations (Ramaswamy and Musser, 1998), and that in most of the cases the mutation and the gene wherein the mutation lies is more often than not known (Ramaswamy *et al*, 2003), are far more likely to be more rapid and sensitive than the phenotypical and biochemical assays used for DST (Hillemann *et al*, 2007). The most common and widely used methods are PCR and hybridization (Fluit *et al*, 2001).

PCR is used to amplify *in vitro* a specific region of DNA (Atlas and Bej *et al*, 1994). The reaction consists of repetitive three stage cycles; one cycle consisting of a high temperature DNA denaturation followed by cooling to allow oligonucleotide primers to anneal to the defined target region (Atlas and Bej *et al*, 1994). Finally the temperature is raised to allow a thermal stable DNA polymerase (often *Taq* polymerase from *Thermus aquaticus*) to extend the newly synthesised DNA strand by the incorporation of free deoxynucleoside triphosphates (dNTPs) (Atlas and Bej *et al*, 1994). Multiplex Allele-Specific (MAS) PCR utilises different pairs of primers that allows simultaneous amplification of several DNA fragments at the same time (Atlas and Bej *et al*, 1994, Mokrousov *et al*, 2002 (1)). The different annealing temperatures (T_a) of the primers result in differential amplification of one or more of the fragments and often no visible detection of some fragments can occur (Atlas and Bej *et al*, 1994). Large differences in the fragment lengths will also favour the amplification of the smaller fragment instead of the larger fragment (Atlas and Bej *et al*, 1994). MAS PCR can thus be used to target commonly reported mutations in genes known to confer resistance to certain antibiotics, and allow one to distinguish between wild-type (WT) sequences and sequences harbouring a mutation (Mokrousov *et al*, 2002 (1)). This is aided by the sequencing of the PCR products and comparing it to published WT sequences, such as H37Rv (Kiepiela *et al*, 2000).

In the sequencing reaction dideoxinucleotide triphosphates (ddNTPs) are incorporated into the growing 3' end of newly synthesised DNA, chain elongation terminates due to the lack of the 3'-hydroxyl group usually present in dNTPs but absent in ddNTPs (Sanger *et al*, 1977; Zimmermann *et al*, 1988). The chance incorporation of ddNTPs instead of dNTPs terminates elongation at all possible sites along the template, in oligonucleotides of varying length, all with the same 5' end but each with a 3' end corresponding to one of four ddNTPs in the template (Sanger *et al*, 1977; Zimmermann *et al*, 1988). The four ddNTPs are fluorescently labelled with a different colour, and upon gel electrophoresis the products migrate towards the positive electrode and each product passes through a laser that reads the different fluorescent labels and computer software converts that signal into a DNA sequence that can later be analysed (Zimmermann *et al*, 1988).

Mokrousov and colleagues developed the MAS PCR assay to originally detect *embB* codon 306 mutations and ETH resistance (Mokrousov *et al*, 2002 (1)) and later modified the assay to detect *katG* codon 315 variations and INH resistance (Mokrousov *et al*, 2002 (3)). Thus the assay can be modified to detect all other resistance determinants provided the sequence of the gene and mutation is known. As PCR and electrophoresis can be completed in a day, this assay could potentially be used as an alternative DST method that provides fast and reliable results as compared to the others mentioned previously.

Hybridization is one of the world's oldest and well used molecular techniques (Fluit *et al*, 2001). Hybridization is the process whereby a segment of single-stranded DNA, known as a probe, covalently binds to another homologous single–stranded segment of DNA, usually immobilized on a support matrix, such as a nitrocellulose membrane or a magnet bead, with a relative stringency (Fluit *et al*, 2001). Probes can be labeled with many enzymatic compounds that allow for the biochemical detection of a hybridization event (Fluit *et al*, 2001).

A commercially available reverse-hybridization assay, known as the GenoType MTBDR*plus* assay (Hain Lifescience GmbH, Nehren, Germany), was recently evaluated by Hillemann and colleagues (Hillemann *et al*, 2007). This assay tests for INH and RIF resistance by a combination of multiplex PCR and reverse-line hybridization onto strips containing oligonucleotides of the common resistance determinants in the *katG*, *rpoB* and *inhA* genes (Palomino *et al*, 2007). Either the omission of a wild-type band or the presence of one or more bands corresponding to the common mutations indicates a resistant strain (Hillemann *et al*, 2007). In the evaluation 125 clinical isolates and 72 smear-positive sputum samples, made up of 106 RIF^R/INH^R, 10 RIF^S/INH^R, and 80 RIF^S/INH^S, were tested with the MTBDRplus assay for its ability to detect RIF and INH resistance (Hillemann *et al*, 2007). The results showed that 71/72 sputum samples and all 125 clinical samples produced discernable results. In addition with the MTBDR assay, which does not detect the *inhA* mutations, both assays, compared to conventional DST methods, were able to identify RIF resistance in 74/75 isolates (98.7%) and 30/31 sputum samples (96.8%), as well as INH resistance in 69/75 isolates (92.0%) and 37/41 sputum samples (90.2%) (Hillemann *et al*, 2007). They concluded that this improved assay is

a reliable method for detecting INH and RIF resistance directly from sputa (Hillemann *et al*, 2007).

Studies have shown a correlation between drug resistance profiles and specific *M. tuberculosis* strain lineages (Gagneux *et al*, 2006 (2); Hillemann *et al*, 2006). Additionally, correlations between strain lineage and relative fitness and virulence have also been postulated (Gagneux *et al*, 2006 (1)). As such, understanding and knowing as much about the evolutionary heritage of the various strains circulating around the world is of great importance.

1.3. *M. tuberculosis* Strain Diversity

1.3.1. Worldwide Prevalence of *M. tuberculosis* Lineages

As mentioned previously, through the co-evolution of mankind and *M. tuberculosis*, several strain lineages have emerged, specific to certain geographical regions around the globe (Gagneux *et al*, 2006 (2)).

In Sweden, from a study by Brudey and colleagues, 23.2% of isolates belonged to the Haarlem family, followed by the W-Beijing (9.8%), Latin American and Mediterranean (LAM; 8%), and the East-Asian-Indian (EAI; 6.2%) families (Brudey *et al*, 2004). However, overall in Scandinavian countries, the ill-defined T family represents 33%, followed by the EAI (22%), Haarlem (20%), LAM (11%), Central Asian (CAS; 5%), X (5%) and W-Beijing (4%) families (Brudey *et al*, 2004).

Since the discovery of the W-Beijing family, this group of strains have predominated in the Asian countries (van Soolingen *et al*, 1995), with Gagneux reporting a prevalence of 33.8% in Far-East Asia, 24.3% in the Middle East and Central Asia, and 22.9% in the Oceana regions (Gagneux *et al*, 2006 (2)).

Recently, in Rio de Janeiro, Brazil, a novel lineage, related to the LAM family of strains, was described and found to be a major cause of TB in that region (Lazzarini *et al*, 2007). The strain was designated RD^{RIO} due to a novel large sequence polymorphism (LSP) that characterizes this strain (Lazzarini *et al*, 2007).

In Pakistan it was reported that the prevalent strain families were the CAS1 (39%) and the W-Beijing (6%) lineages (Hasan *et al*, 2006), while in the Archangel Oblast region of Russia the W-Beijing family represented 44.5% of the strains collected, of which 43.4% were MDR and 92.5% clustered (Toungoussova *et al*, 2002).

Of the 103 MDR isolates collected in Germany (Hillemann *et al*, 2005), 60.2% were of the W-Beijing lineage, and interestingly displayed different resistance determinants than the non-W-Beijing MDR isolates (Hillemann *et al*, 2005).

In South Africa it was found that the F11 strain, which is part of the LAM family, represented 21.4% of isolates collected and was as successful as the W-Beijing family (16.5%) in causing TB in the Western Cape (Victor *et al*, 2004). The Haarlem family is also overrepresented amongst children with drug-resistant TB in the Western Cape (Marais *et al*, 2006).

Still in South Africa, a very recent study carried out was the first to describe the frequency and distribution of *M. tuberculosis* genotypes across most of the provinces (Stavrum *et al*, 2009). Of the 252 collected isolates from eight of the nine provinces, spoligotyping and MIRU-VNTR analysis detected that the ill-defined T lineage was the most prevalent strain family (25.8%) followed by the W-Beijing lineage (10.3%) (Stavrum *et al*, 2009).

1.3.2. The *M. tuberculosis* W-Beijing Family

As seen from the worldwide prevalence above, the W-Beijing family constitutes a major proportion of strains circulating the globe, and is responsible for much morbidity and mortality

(Bifani *et al*, 2002) in part, due to its association with MDR. The W-Beijing family originates from the Beijing province of Eastern Asia and was first described in 1995 (van Soolingen *et al*, 1995). The mechanisms for how this group of strains has managed to spread globally and maintain a high virulence despite its association with MDR, is still not entirely known (Bifani *et al*, 2002).

However, what is known is that the W-Beijing family belongs to the East-Asian lineage (Gagneux *et al*, 2006 (2)) and is a member of the phylogentically termed Principle Genetic Group 1 set of strains (Palomino *et al*, 2007). These strains are characterized by the presence of an inverted IS*6110* copy within the DR region, an IS*6110* element within the origin of replication and one or two IS*6110* copies in a DNA region called NTF. A characteristic W-Beijing lineage-defining small nucleotide polymorphism (SNP) is the G81A in *Rv3815c* as well as LSPs RD105, RD142, RD150 and RD181 (Palomino *et al*, 2007). The standard spoligotyping pattern was described in 1995, which shows that all W-Beijing strains have 9 spacer regions; namely 35 to 43 (van Soolingen *et al*, 1995). W-Beijing strains are significantly associated with drug resistance; OR 1.8 (95% CI: 1.2 – 2.7) for any drug, 1.7 (95% CI: 0.95 – 2.9) for INH, 4.0 (95% CI: 1.4 – 11.9) for RIF, 2.3 (95% CI: 1.4 – 3.7) for STR, 3.0 (95% CI: 0.38 – 23.2) for ETH, and 4.2 (95% CI: 1.2 – 14.7) for MDR (Glynn *et al*, 2006), and as such, represent a group of strains that necessitate a large amount of attention and research.

It has been hypothesized that the widespread nature of the W-Beijing family could be due to the resistance of the lineage to the BCG vaccination, where in areas such as South Africa, is routinely and widely used (van Soolingen *et al*, 1995). The vaccine primes the immune system to mount a specific and rapid immune response towards an infecting strain of *M. tuberculosis* (Kremer *et al*, 2009). Studies showing an association between BCG vaccination (determined by the presence of a BCG scar) and an increased risk of acquiring TB from a W-Beijing strain (van Soolingen *et al*, 1995) have been reported. This implies that although BCG may offer protection from other strains of *M. tuberculosis*, protection against W-Beijing strains is not offered, and that W-Beijing strains represent 'escape variants' of *M. bovis* BCG (Kremer *et al*, 2009). W-Beijing strains are also associated with extrapulmonary TB disease as well as treatment failure and relapse (Reed *et al*, 2007). Recently it was discovered that certain

groups within the W-Beijing family produce a unique phenolic glycolipid, PGL-tb, which is hypervirulent in mouse models, as well as having the dormancy regulon, controlled by the DosR transcription factor, constitutively expressed (Reed *et al*, 2007). The expression of this regulon in times of oxygen deprivation and nitric oxide production, as encountered within the granuloma during *M. tuberculosis* latency, may contribute significantly to the virulence of the W-Beijing family (Reed *et al*, 2007).

1.3.3. Genotyping *M. tuberculosis*

The molecular tools available to researchers today, that allow them to differentiate strain families from clinical samples, are based on differences between strains, such as IS*6110* copy number, spacer sequences present in the direct repeat (DR) or the variable number of tandem repeats (VNTRs) in mycobacterial interspersed repetitive units (MIRUs) (Mathema *et al*, 2006). Each tool has its advantages and disadvantages and most importantly each offer a different level of discriminatory power.

The gold standard in genotyping *M. tuberculosis* has long been IS*6110* restriction fragment length polymorphism (RFLP) typing (Mathema *et al*, 2006). This method is based on the restriction of genomic DNA by *Pvu*II, which cuts IS*6110* once, followed by Southern Blot hybridization and probing for the element (van Embden *et al*, 1993). The resultant banding pattern is descriptive of a specific strain, and can be compared to other strains manually, either with the naked eye, or using computer software (Mathema *et al*, 2006) to determine whether strains are related or not. This typing method has a high level of discrimination for strains with higher copy numbers of IS*6110*, but is not suitable for typing strains with few copies of the element, or in rare circumstances, where a strain contains no element at all (Mathema *et al*, 2006). Another limitation is that this technique is very labour intensive, requiring subculturing and DNA isolation of the strains tested and has a slow turnaround time, of approximately 30 to 40 days (Mathema *et al*, 2006).

Spoligotyping is a PCR-hybridisation technique that simultaneously detects and types *M. tuberculosis* complex bacteria. These organisms contain a unique locus known as the DR

region which consists of directly repeated sequences interspersed with spacer regions of between 35 to 41bp (Kamerbeek *et al*, 1997). The order of the spacer regions remains the same when comparing many different strains, however between the strains, deletions or insertions of spacers often occur. Thus it is possible to compare the presence or absence of the spacer regions to determine whether strains are related (Kamerbeek *et al*, 1997). Spoligotyping involves PCR amplification of the DR region using primers that anneal to the DRs, resulting in the amplification of the spacer regions. The reverse primer is biotin-labelled and therefore all PCR products are biotin-labelled (Kamerbeek *et al*, 1997). The PCR products are then hybridized to a membrane containing parallel lines of covalently linked oligonucleotides from known spacers. The membrane is then incubated in streptavidine-peroxidase and detected using the enhanced chemiluminescence (ECL) Direct Nucleic Acid Labeling and Detection System (Amersham Biosciences) on a light sensitive film. The biotin-labeled probes anneal to regions of homology on the membrane and streptavidine anneals to biotin. A signal is produced when the streptavidine-peroxidase, bound to the biotin-labeled primers, catalyses a light reaction using the ECL detection reagents as a substrate, which is then captured on light sensitive film. The film, after exposure to the membrane, is then developed and fixed and the resulting profile is compared to a database and the strains are genotyped accordingly (Kamerbeek *et al*, 1997).

Mycobacterial interspersed repetitive unit variable number tandem repeat (MIRU-VNTR) typing, is an automated amplification and electrophoresis technique that differentiates strains on the basis of the number of MIRUs in 12 VNTR loci (Supply *et al*, 2000; 2001). The results are reported as a 12-number numerical code representing the number of MIRUs at each locus (Supply *et al*, 2000; 2001). The standard 12 loci studied include locus 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, and 40 (Ali *et al*, 2007). The discriminatory power of MIRU-VNTR analysis is dependent on the number of loci evaluated, but generally when 12 loci are evaluated and combined with spoligotyping, the discriminatory power is greater than or equal to IS*6110*-RFLP (Mathema *et al*, 2006). The advantages of this technique is that it is a rapid, high-throughput technique and has a better resolution than spoligotyping, and along with the digitized output, means than intra-laboratory comparisons are made simpler (Mathema *et al*, 2006). The introduction of fluorescently-labeled primers allows for the automated analysis

when used in conjunction with a capillary sequencer (Mathema *et al*, 2006). The disadvantage is that it is less discriminatory than IS*6110*-RFLP typing (Supply *et al*, 2000; 2001).

In a study by Savine and colleagues, the stability of the VNTRs of MIRUs from 12 loci was assessed over time (Savine *et al*, 2002). They assessed the temporal stability of 123 serial isolates from 56 smear-positive patients. These isolates were separated by up to 6 years and belonged to a variety of distinct IS*6110*-RFLP patterns (Savine *et al*, 2002). They showed that the MIRU-VNTRs are stable over time and are therefore very reliable in the follow-up of patients chronically infected with TB, and that this technique is a highly powerful tool for genotyping *M. tuberculosis* (Savine *et al*, 2002).

In another study, Filliol and colleagues assessed MIRU-VNTR analysis against spoligotyping, IS*6110*-RFLP and double-repetitive-element (DRE) PCR (Filliol *et al*, 2000). The results showed that in 6/12 cases (50%), VNTR-defined clusters were further subdivided by spoligotyping; in comparison to 7/18 (39%) cases where spoligotyping defined clusters were further subdivided by VNTR. When used alone, the least discriminant technique was VNTR analysis, but significantly improved when in association with spoligotyping, however spoligotyping and DRE-PCR had the highest discriminatory power (Filliol *et al*, 2000). They concluded that although the discriminatory power of VNTR was lower than the other tests, this technique may offer additional phylogenetic information that may be helpful to trace the molecular evolution of *M. tuberculosis* (Filliol *et al*, 2000).

Using this typing technique, the CAS1 strain family of *M. tuberculosis*, which is the most prevalent strain in Pakistan, was characterized (Ali *et al*, 2007). A total of 178 CAS1 and 189 'unique' *M. tuberculosis* strains were analyzed and the discriminatory index was calculated using the Hunter Gaston Discriminatory Index (HGDI). The 349 MIRU patterns obtained from the 367 strains tested indicated that MIRU loci 10, 16, 26, 27, 31, 39 and 40 were 'most discriminatory' (DI: ≥0.6), with loci 26 and 31 being 'highly discriminatory'. Loci 4, 20, 23 and 24 were found to be 'moderately discriminatory' (DI: 0.3 – 0.59), and loci 2 was found to be 'poorly discriminatory' (DI<0.3) for typing CAS1 strains specifically (Ali *et al*, 2007). The authors concluded that MIRU typing is an excellent way to estimate the phylogenetic

relatedness amongst CAS1 strains (Ali *et al*, 2007). Interestingly, the association between CAS1 strains and MDR was not significant ($P = 0.21$).

1.4. Mixed *M. tuberculosis* Infections

In the past, TB was thought to have occurred due to a single infection with a single strain of *M. tuberculosis*, resulting in immunity to further infections (Shamputa *et al*, 2004). However, recent studies, with the advent of improved molecular genotyping tools, have highlighted the relevant proportion of TB cases caused by multiple infections of two or more different strains of *M. tuberculosis* (de Viedma *et al*, 2004; Shamputa *et al*, 2004; Shamputa *et al*, 2006; Palomino *et al*, 2007). Despite this knowledge, the study of mixed infections is poorly understood (Shamputa *et al*, 2004, 2006). A mixed infection, or clonal heterogeneity, is the result of a superinfection in a previous TB case by a new *M. tuberculosis* strain, leading to the simultaneous or sequential presence of two or more different strains in the same patient (de Viedma *et al*, 2004), often referred to as exogenous reinfection. This is highly prevalent in high-incidence settings such as South Africa (Shamputa *et al*, 2006). Another class of mixed infections is the presence of multiple populations of *M. tuberculosis* derived from a single ancestral strain, each displaying genetic drift (Palomino *et al*, 2007). Due to the very lobus nature of the lungs, compartmentalization can occur, whereby "pockets" of different clones of the infecting *M. tuberculosis* strain may occur (de Viedma *et al*, 2004), making interpretation of DST and genotyping data difficult and problematic (Shamputa *et al*, 2004).

An epidemiological study carried out in Cape Town revealed that 19% of patients harboured simultaneous infections with both Beijing and non-Beijing lineages of *M. tuberculosis* (Warren *et al*, 2004). Furthermore, it was noted that 57% of all patients infected with a Beijing strain were additionally infected with a non-Beijing strain (Warren *et al*, 2004). The researchers also noted that the occurrence of mixed infections was more frequently detected in retreatment cases (23%) compared to new cases (17%) (Warren *et al*, 2004). This was done using a novel PCR that detected strains in sputum belonging to Beijing and non-Beijing lineages. Utilising four primer sets, designed to anneal to regions of the chromosome specific to the Beijing and non-Beijing strains, Warren and colleagues concluded that the PCR improved the estimate of

mixed infections in the Western Cape (19%) compared to the 4.8% that spoligotyping alone detected, and that this relatively high rate of mixed infections has great implications in the interpretation of DST and other molecular data (Warren *et al*, 2004).

Very recently, an inter-provincial genotyping study was carried out in South Africa, using spoligotyping and MIRU-VNTR typing (Stavrum *et al*, 2009). It showed that 54% of the collected T strains analysed by MIRU-VNTR typing were of mixed *M. tuberculosis* subpopulations (Stavrum *et al*, 2009). MIRU-VNTR can aid in detecting mixed infections; a sample containing different strains will display several alleles for one locus. However, this is based on the assumption that those strains differ in alleles at that specific locus, but even different strains could have the same MIRU-VNTR profile and would not be detected as being mixed (Stavrum *et al*, 2009).

In one study, 10 colonies from each primary *M. tuberculosis* isolate of 97 HIV negative TB patients were screened for heterogeneity and detectable mixed infections by spoligotyping, IS*6110*-RFLP and MIRU-VNTR (Shamputa *et al*, 2004). Infections from heterogeneous bacterial subpopulations were detected in samples from 8 patients (8.2%), with the frequency of detectable mixed infections in the study population being 2.1% (Shamputa *et al*, 2004). Although the researchers allude to the possibility of introducing a bias in the selection criteria of the study population, they conclude that the findings have implications on the interpretation of molecular epidemiological results for patient follow-ups as well as transmission studies (Shamputa *et al*, 2004).

In another study, several pre-treatment isolates from 199 smear-positive male adult inmates were screened using IS*6110*-RFLP as well as MIRU-VNTR analysis (Shamputa *et al*, 2006). Mixed infections were found in 26 cases (13.1%). Using either IS*6110*-RFLP or MIRU-VNTR alone would have missed mixed infections in 26/26 (100%) or 14/26 (54%) cases, respectively (Shamputa *et al*, 2006). They conclude that the current methods to genotype *M. tuberculosis* greatly underestimate the actual heterogeneity of the bacilliary population in TB patients, particularly in high-incidence settings (Shamputa *et al*, 2006).

In a study screening for heterogeneity using IS*6110*-RFLP and spoligotyping, 30 colonies from each primary *M. tuberculosis* isolate from 12 children with TB were collected (de Viedma *et al*, 2004). All cultures from 11 of the 12 children were homogenous, while in 1 child (8.3%), a 2-year old in whom microevolution events were unlikely and who had no risk of overexposure, clonal heterogeneity was found (de Viedma *et al*, 2004). This led to the conclusion that clonal heterogeneity should be expected in primary cases (de Viedma *et al*, 2004).

1.5. Conclusion

Despite its antiquity and vast history, *M. tuberculosis* is described as an emerging pathogen (Hopewell, 1996) and remains the leading cause of death due to an infectious disease worldwide (Plorde, 1994). *M. tuberculosis* is considered an emerging pathogen, not because the bacillus has recently emerged but because scientists, health practitioners and professionals, along with governments have only recently begun working together to formulate treatment regimes in order to stop the spread of this devastating disease (Hopewell, 1996). There is an urgent need for newer and more effective chemotherapies as well as standardized methods of detection, DST as well as strain typing. The fact that TB infection can be the result of several infecting *M. tuberculosis* strains simultaneously requires scientists and clinicians to revaluate the way we analyse and interpret epidemiological and DST data. Furthermore, the fact that the presence of several different strains of *M. tuberculosis* has great consequences on the way that patients are treated, it is imperative that more reliable and sensitive techniques are developed that will differentiate out the mixed strains. In order to reach the 2015 STOP TB Partnership goals of halting and reversing international incidences of TB, and halving prevalence and deaths, in comparison to 1999 (WHO, 2007), we all need to be working together in order to curb TB. By doing this we may see the 2050 goal of eliminating TB as a public health problem (WHO, 2007).

1.6. Project Aims and Objectives

This project aims:

1. To determine the strain lineage of all isolates collected and included in this study by spoligotyping, as a means of assessing the epidemiology of the circulating strains, as well as to screen for mixed infections;

2. To test for Rifampicin and Isoniazid resistance in those isolates determined to be mixed, using the GenoType MTBDR*plus* assay, to identify if there is a corresponding change in drug susceptibility to change in spoligotyping pattern;

3. To assess the genetic relatedness of the mixed isolates by MIRU-VNTR analysis;

4. To screen all isolates, including those identified by spoligotyping to be mixed, for (i) additional mixed isolates, and (ii) to confirm the results obtained by spoligotyping, by carrying out PCR assays that detect mixed isolates belonging to the W-Beijing and non-W-Beijing lineages;

5. To develop a PCR-based assay to differentiate between infecting non-W-Beijing strains, and to screen all isolates, including those identified by spoligotyping to be mixed, for (i) additional mixed isolates, and (ii) to confirm the results obtained by spoligotyping.

Chapter 2

Materials and Methods

Contents

2.1. Bacterial isolates, drug susceptibility testing, and growth conditions

In a larger study conducted by the University of the Western Cape (UWC) in South Africa, the affects of vitamin A and zinc supplementation on time to sputum conversion in newly diagnosed smear-positive tuberculosis (TB) patients, living in the Delft region of the Western Cape, was addressed (M. Visser; personal communication). This trial, a double-blinded, randomized, placebo-controlled trial, investigated whether or not micronutrient supplementation with vitamin A versus placebo, and zinc versus placebo, in addition to anti-TB medication, would reduce the time to sputum conversion over an 8 week period. Adults, aged between 18 and 65 years of age, with newly diagnosed pulmonary TB and living in the Delft community, were enrolled into the trial. Early-morning baseline sputum samples were collected from 154 patients, at the community clinic in Delft, under the supervision of the research nurse. The subsequent weekly samples were brought into the clinic by the patients themselves, unsupervised, over the 8 week study period, from 2005 to 2008. Patients with negative sputum samples were excluded, as were samples contaminated during storage. Contamination from fungi was most common. In addition several samples from patients were misplaced during the study and were thus lost for analysis. Hence, some patients, at the end of the study period, had less than 8 samples for analysis. From the initial 154 patients enrolled in the study a total of 686 isolates were obtained and included in this study.

Collected sputum samples were decontaminated by the NALC/NaOH decontamination/digestion method in the Medical Microbiology Diagnostic laboratory at Groote Schuur Hospital (GSH), Cape Town. These were then inoculated into Mycobacteria Growth Indicator Tubes (MGITs) containing OADC-enriched Middlebrook liquid media supplemented with MGIT PANTA antibiotics and placed into an automated BACTEC MGIT 960 system (BD) for the monitoring of growth. Each tube has imbedded an oxygen-sensitive fluorescent disk that aids in the detection of growth. Oxygen present in the tube quenches the fluorescence and the BACTEC 960 will detect no growth. Active growth uses up the available oxygen in the tubes and the fluorescence is released and captured by the BACTEC 960. The samples were incubated until growth was observed or until the end of the testing protocol (56 days). After the results were recorded, the MGITs were stored in boxes at room temperature in the Biosafety Level III (BSL3) facility in the Division of Medical Microbiology, University of Cape Town.

The strains were inoculated from the MGITs onto Lowenstein-Jenson (LJ) slopes (Bio-Rad) and left to grow at 37°C for 4 – 6 weeks in the BSL3 facility, until growth was observed. Colonies were picked from the slopes and inoculated into 1.25ml ADC-supplemented Middlebrook 7H9 broth (Appendix A) in 2ml screw-cap tubes. These cultures were incubated at 37°C for a week, after which 500µl of 50% glycerol was added to each tube, mixed thoroughly by inverting the tubes several times, and then stored at -80°C.

Phenotypic drug susceptibility DST for rifampicin (RIF) and isoniazid (INH) was performed using the automated BACTEC MGIT 960 system as part of routine diagnostics at the Medical Microbiology diagnostic laboratory at GSH. Genotypic DST was also carried out using the Genotype MTBDR*plus* assay (Hain Lifescience, Germany), on the samples suspected of harboring mixed *M. tuberculosis* isolates. This PCR and reverse hybridization assay detects the most frequently observed chromosomal determinants associated with multi-drug resistance (MDR) *M. tuberculosis*, including resistance to both RIF and INH (WHO, 2000). The membrane used is a strip containing covalently bound DNA probes corresponding to the various resistance determinants. High-level resistance to isoniazid is tested by detecting the S315T mutation of *katG*, while low-level resistance to isoniazid is tested by detecting the C-15T promoter mutation of *inhA*. Resistance to rifampicin is tested by detecting the common rifampicin resistance determining region (RRDR) mutations in *rpoB*, namely the D516V, H526Y, H526D and S531L mutations (Hain Lifesciences). The 50µl amplification mix contained the following components: 35µl of the primer/nucleotide mix (PNM) in the kit, 1X HotStartTaq polymerase buffer, 0.5mM $MgCl_2$, 1X Q-solution and 1U of HotStartTaq DNA polymerase. To each reaction, 5µl of DNA extracted from MGIT cultures (2.2.2) was added. Amplification proceeded with an initial denaturation and polymerase activation at 95°C for 15min followed by 10 cycles of 95°C for 30sec and 58°C for 2min, and a further 20 cycles of 95°C for 25sec, 53°C for 40sec and 70°C for 40sec. Lastly, an elongation at 70°C for 8min completed the amplification.

Hybridization of the amplicons was performed using a TwinCubator®, an automated incubator and shaker specifically designed for the demand of the molecular diagnostics of Hain Lifesciences. The TwinCubator® contains wells for 12 oligonucleotide strips, and is fully

programmable for the timed stages of the protocol. Briefly, the TwinCubator® was pre-warmed to 45°C along with solutions HYB (green) and STR (red) to 45°C. The other reagents, except CON-C (orange) and SUB-C (yellow), were warmed to room temperature. CON-C and SUB-C remained at 4°C (according to manufacturer's instructions). CON-C and SUB-C were diluted 1:100 with CON-D and SUB-D, respectively. In the corner of each well, 20µl of DEN (blue) was applied to which 20µl of the amplified product was added, and mixed by brief aspiration followed by 5min incubation at room temperature. Each DNA strip was marked with a pencil beneath the coloured marker at the end of the strip, for ease of identification. Carefully, avoiding contamination in neighboring wells, 1ml of HYB solution was added to each well and gently homogenized to ensure adequate mixing. A DNA strip was then placed in each well, with the coated surface facing upwards. The tray was then placed onto the TwinCubator® and incubated with shaking at 45°C for 30min. Buffer HYB was then completely aspirated and 1ml of STR buffer was added to each well and incubated at 45°C for 15min. Buffer STR was then carefully poured off and the tray gently tapped to remove any residual buffer in the wells. The strips were then washed once, with 1ml of RIN buffer for 1min in the TwinCubator®. The rinse buffer was then poured off as before and 1ml diluted CON-C was added to each well and incubated on the TwinCubator® at 45°C for 30min. CON-C was then discarded and the strips washed twice in RIN for 1min each, and then once with distilled water, on the TwinCubator® platform. To each strip, 1ml of diluted SUB-C was then added and incubated in the dark, without shaking for 3-20min. The reaction was stopped by rinsing twice with distilled water. The strips were removed with forceps and gently blot dried between two sheets of absorbent paper.

Hybridization signals must be present for all three of the controls, namely the conjugate control, which controls for hybridization-specific anomalies; the amplification control, which ensures that the PCR conditions were optimal; and the *M. tuberculosis* control, which ensures that the sample being tested is in fact from the *M. tuberculosis* complex (Figure 2.1). Hybridization signals for any of the wild-type (WT) probes indicate that no mutation in that region was detected, and that the isolate be considered susceptible to either RIF or INH. The absence of a signal in the WT probes and the presence of a specific mutation probe, indicates that a particular resistance determinant is present in that strain, and that the isolate be

considered resistant to either RIF or INH. A diagrammatic representation of a strip, depicting the bound probes, is featured in Figure 2.1.

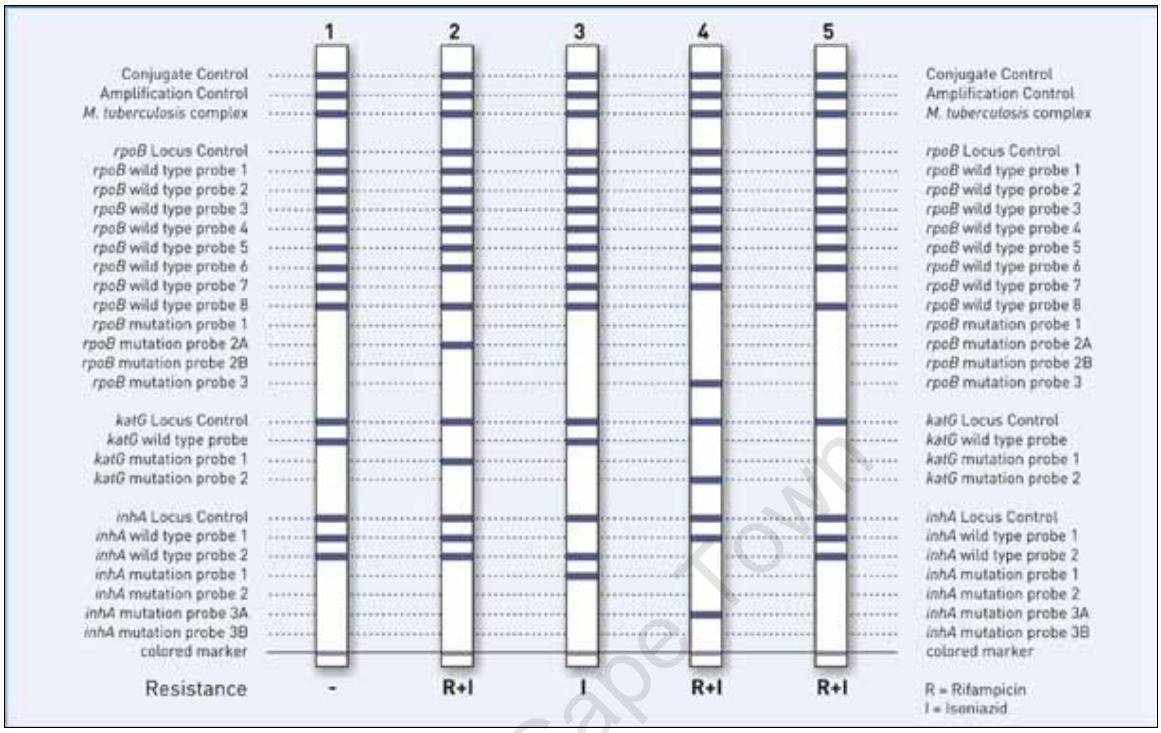


Figure 2.1. Diagrammatic representation of hybridized GenoType MTBDR*plus* assay strips. All probes appear labelled on both the left and right of the strips. Hybridization signals are represented by dark horizontal bands. Strip 1: Signals for all wild-type (WT) probes and the absence of any signals for the mutation probes indicates that this isolate is fully susceptible to both RIF and INH. Strip 2: Signals for both the *rpoB* mutation probe 2A and the *katG* mutation probe 1 indicate that this isolate is resistant to both RIF and INH. Strip 3: The absence of a signal for any *rpoB* mutation probes indicate that this isolate is susceptible to RIF while the presence of a signal for the *inhA* mutation probe 1 indicate that this isolate has a low level of resistance to INH. Strip 4: Signals for the *rpoB* mutation probe 3, *katG* mutation probe 2 and *inhA* mutation probe 3A indicate that this isolate is resistant to both RIF and highly resistant to INH. Strip 5: The absence of a WT probe as well as a mutation probe indicates that a mutation is present but one that isn't covered by the mutation probes, and is considered resistant. Here both the absence of *rpoB* WT probe7 and the *katG* WT probe, as well as the absence of any signals in the mutation probes, indicate resistance to both RIF and INH. Adapted from the GenoType MTDR*plus* Assay online brochure (http://www.hain-lifescience.de/uploadfiles/file/produkte/mikrobiologie/mykobakterien/MTBDR-plus-sl_eng.pdf).

2.2. Extraction of genomic DNA

2.2.1. Extraction of genomic DNA from colonies grown on LJ Slopes

M. tuberculosis colonies were picked from the LJ slopes and inoculated into 500µl of distilled water in 2ml screw-cap tubes and heat killed in a water bath (Memmert) at 80°C for one hour in the BSL3 facility. After one hour of heat killing, 70µl of 10% SDS (BDH, Ltd) and 50µl (10mg/ml) proteinase K (Novagen) was added to each sample and placed in an Eppendorf

thermomixer at 60°C set at 400 rpm for an hour. After shaking, a 100µl aliquot of pre-heated (60°C) 5M NaCl (Saarchem, Merck) and 100µl pre-heated (60°C) 10% CTAB (Sigma) (Appendix A) was added to each sample and mixed thoroughly by inverting several times by hand. Shaking was resumed at 400rpm at 60°C for 15 minutes. The samples were then placed in the -70°C freezer for 15min, thawed and then reincubated in the 60°C Eppendorf thermomixer at 400rpm for 15min. The samples were removed and cooled at room temperature. After cooling, 700µl of chloroform/isoamyl alcohol (24:1) (Saarchem, Merck) (Appendix A) was added to each tube and inverted by hand for 20 – 25 times ensuring that the two phases formed a homogenous suspension. The tubes were centrifuged (Eppendorf 5417C) for 10min at 13000rpm. The aqueous upper phase was then transferred to a fresh 1.5ml Eppendorf tube with 1 volume of cold isopropanol (Saarchem, Merck) and mixed by inverting the tube by hand several times until a DNA precipitate was seen. The samples were incubated at -20°C for 30min, followed by centrifugation for 10min at 13000rpm. The supernatant was removed and the pellet washed with 80% ethanol and centrifuged for a further 10min at 13000rpm. The supernatant was removed and the pellet was air-dried before resuspending in 55µl of distilled water. The DNA was then quantified by electrophoresing 5µl of the purified DNA on a 1% agarose (Whitehead Scientific) (Appendix A) gel and comparing the intensity of the bands with Hyperladder I (Appendix B) and λ DNA. The extracted DNA was stored at 4°C to prevent the shearing effect caused by repetitive freeze-thawing.

2.2.2. Extraction of genomic DNA from MGIT cultures

Genomic DNA was extracted from MGIT cultures for use in PCR assays and for spoligotyping. From the MGIT cultures, 1.8ml was transferred to 2ml screw-cap tubes and centrifuged for 10min at 13000rpm. The supernatant was removed and the cells were resuspended in 500µl of distilled water. The cells were then heat killed in a water bath at 80°C for an hour. After this, the samples were microwaved (LG Multiwave) on high (900 watts) for 1 – 2 minutes to release the DNA from the cells. An aliquot (4µl) was used as template in PCR assays and spoligotyping.

2.3. Agarose gel electrophoresis and DNA visualisation

Gel electrophoresis is a useful technique to separate nucleic acids on the basis of size. When agarose polymerises it forms pores, the size of which is dependent on the percentage of agarose; a higher percentage equates to more agarose being polymerised and thus smaller pores. Usually the larger the nucleic acids in a sample the lower the agarose percentage must be in order for the pore sizes to be large enough to allow the nucleic acids to migrate through the gel. Electrophoresis is performed in a gel tank containing 1X Tris-acetate EDTA (TAE) buffer (Appendix A). A current is passed through the buffer from the negative electrode to the positive electrode, which causes the negatively charged nucleic acids to migrate through the gel towards the positive electrode. The concentrations used ranged from 1% to 2% weight per volume (w/v) agarose dissolved in 1X TAE by heating in a microwave on high for 2 – 3min. Ethidium bromide (EtBr) is then added to a final concentration of 10ng/µl to visualise the migrated nucleic acid band(s). This is achieved because EtBr intercalates between DNA and fluoresces in the presence of UV light. The *GoTaq* reaction buffer contains a loading dye that allows one to monitor the migration of the nucleic acids as well as keeping the sample heavy in the wells. Depending on the size of the DNA fragments either HyperLadder I or HyperLadder IV (Bioline) (Appendix B), for larger to smaller fragments, respectively, was loaded alongside to determine the size and concentrations of DNA fragments. After electrophoresis the gel was placed onto a UV box (Fotodyne Inc.) for visualisation and the image captured using a digital camera (Kodak EDAS 290).

2.4. DNA purification

In order for PCR amplified DNA products to be sequenced they must be of a high quality and thus the need for purification. To purify DNA fragments from agarose gels, a MinElute Gel Extraction kit (Qiagen) was used. Gel bands containing DNA fragments of interest were visualised on a UV box wearing the appropriate face guard and excised using a scalpel removing as much surplus agarose as possible. The gel bands were placed into separate Eppendorf tubes and weighed. After the tubes were weighed, 3 gel volumes of buffer QG was added to each tube and incubated at 50°C for 10min until all the agarose had melted. An equal volume of isopropanol was added to each tube to precipitate the DNA. Each sample was then transferred to a MinElute spin column and centrifuged for 1 minute at 13000rpm to

allow the DNA to bind to the column matrix. The flow-through was discarded and 500µl of buffer QG was added to each spin column and centrifuged for a further minute at 13000rpm. Once again the flow-through was discarded and 750µl of buffer PE was added to wash the DNA, and was centrifuged for 1min at 13000rpm. The flow-through was discarded and the tubes were centrifuged for 1min at 13000rpm to remove residual ethanol from the DNA. The spin columns were placed in 1.5ml Eppendorf tubes and 10µl of distilled water was applied to each column. The DNA was then eluted by centrifugation for 1 minute at 13000rpm and stored at 4°C until needed.

2.5. DNA Sequencing and analysis

Sequencing reactions were carried out according to the protocol by Zimmerman and colleagues (Zimmermann *et al*, 1988). Essentially, when ddNTPs are incorporated into the growing 3' end of newly synthesised DNA, chain elongation terminates due to the lack of the 3'-hydroxyl group usually present in dNTPs but absent in ddNTPs. The chance incorporation of ddNTPs instead of dNTPs terminates elongation at all possible sites along the template, in oligonucleotides of varying length, all with the same 5' end but each with a 3' end corresponding to one of four ddNTPs in the template. The four ddNTPs are fluorescently labelled with a different colour, and upon gel electrophoresis the products migrate towards the positive electrode and each product passes through a laser that reads the different fluorescent labels and computer software converts that signal into a DNA sequence that can later be analysed. One microliter of purified DNA was quantified on a 2% agarose gel (Appendix A) alongside 5µl of the DNA marker HyperLadder IV (Bioline) (Appendix B). The intensity of the purified DNA band was compared to that of the HyperLadder bands to determine the concentration of DNA in ng/µl. Based on the size of the DNA template approximately 5 – 10ng of DNA was used if the template size was 400 – 450bp, or 50 – 100ng of DNA was used if the template size was 2000 – 3000bp. In addition to the DNA template, the 20µl sequencing reaction volume contained 1X reaction buffer, 4µl of Sequencing Mix (Applied Biosystems) and 3.2pmol/µl of primer. The cycle conditions were as follows: an initial denaturation at 96°C for 30 seconds followed by 25 cycles of 96°C for 30sec, 50°C for 15sec and 60°C for 4min. The reactions were sent to the DNA Sequencing facility at the University of Stellenbosch where an ABI Prism 3100 Genetic Analyser (Applied Biosystems) was used to determine the

sequence of the samples. The chromatogram was analysed using the ChromasPro Version 1.34 (Technelysium Pty Ltd) software package. Alignments and sequence modifications were done using the DNAMAN Version 4.0 (Lynnon BioSoft) software package.

2.6. Spoligotyping

Genetic relatedness between the different strains was determined using spoligotyping. This PCR-hybridisation technique simultaneously detects and types *M. tuberculosis* complex bacteria. These organisms contain a unique locus known as the Direct Repeat (DR) region which consists of directly repeated sequences interspersed with spacer regions of between 35 to 41bp. The order of the spacer regions remains the same when comparing many different strains, however between the strains, deletions or insertions of spacers often occur. Thus it is possible to compare the presence or absence of the spacer regions to determine whether strains are related.

Spoligotyping involves PCR amplification of the DR region using primers that anneal to the DRs, resulting in the amplification of the spacer regions. The reverse primer is biotin-labelled (Isogen) and therefore all PCR products are biotin-labelled (Figure 2.2).

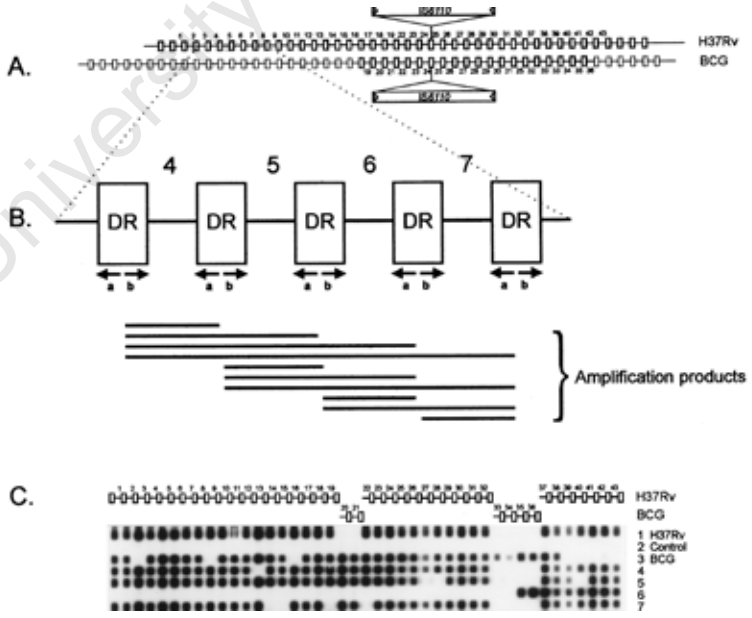


Figure 2.2. Schematic representation of the spoligotyping technique. (A) A depiction of the DR locus containing IS*6110*. (B) PCR primers anneal to the DR region and amplify the intervening spacer regions. The reverse primer DRa is biotin-labelled. (C) After developing the film following incubation with the ECL detection reagents, black square signals represent the spacer sequences that are present in each strain. (Adapted from Kamerbeek *et al*, 1997)

The PCR products are then hybridised to a membrane (Isogen) containing parallel lines of covalently linked oligonucleotides from known spacers. The membrane is then incubated in streptavidine-peroxidase (Sigma) and detected using the enhanced chemiluminescence (ECL) Direct Nucleic Acid Labelling and Detection System (Amersham Biosciences) on a light sensitive film. The biotin-labelled probes anneal to regions of homology on the membrane and streptavidine anneals to biotin. A signal is produced when the streptavidine-peroxidase, bound to the biotin-labelled primers, catalyses a light reaction using the ECL detection reagents as a substrate, which is then caught on the light sensitive film. The film, after exposure to the membrane, is then developed and fixed and the resulting profile is compared to a database and typed accordingly.

2.6.1. Amplification of the DR spacer regions

The spoligotyping PCR was performed using reagents from Isogen – Lifescience. The 25µl reaction contains 1X Reaction Buffer, 1mM $MgCl_2$, 200µl of each dNTP, 1X Reagent Q, 10µM each of biotin-labelled primer DRa, and primer DRb, 1U *GoTaq* (Promega). If genomic DNA is being used then 100 – 200ng is added as template otherwise a 4µl aliquot from a MGIT preparation is used as template. Included in the kit from Isogen were the *M. tuberculosis* H37Rv and the *M. bovis* BCG P3 controls which, along with the negative water control, were included in the reaction using 1µl each. H37Rv contains spacer sequences 1 – 19, 22 – 32, and 37 – 43, while BCG contains spacer sequences 20 – 21 and 33 – 36. Therefore by including both strains as positive controls, ensures that all spacer sequences are amplified equally from all strains (Kamerbeek *et al*, 1997). The negative water control is included to ensure that none of the reagents used are contaminated. The cycling conditions in the thermalcycler were an initial denaturation at 96°C for 15min, followed by 25 cycles of 96°C for 1min, 55°C for 1min and 72°C for 30sec. A final elongation at 72°C for 5min completed the reaction. Amplified products were then stored at 4°C until needed.

2.6.2. Hybridisation

The reagents used for hybridisation were pre-warmed as follows: 2X SSPE/0.1% SDS at 60°C, 2X SSPE/0.5% SDS at 60°C, 2X SSPE/0.5% SDS at 42°C and 2X SSPE at room

temperature (Appendix A). The amplified DR spacer PCR products were added to 150µl of 2X SSPE/0.1% SDS and heat denatured in the thermalcycler for 10 minutes at 99°C and immediately cooled on ice. The membrane (Biodyne C negatively charged nylon) was washed in 250ml of 2X SSPE/0.1% SDS in a 60°C oven with gentle shaking for 5min. The membrane was then placed inside the miniblotter apparatus (Isogen) (Figure 2.3) on a support cushion in such a way as to ensure the wells are perpendicular to the oligonucleotide probes.

Figure 2.3. Miniblotter apparatus supplied with the Spoligotyping kit (Isogen) (www.blossombio.com.tw)

The blotter has 45 wells that connect to parallel grooves running the length of the apparatus. When the membrane is placed inside the blotter these grooves run perpendicular to the oligonucleotides on the membrane, and thus after hybridisation the spacers can be visualised on the film as black squares. The fasteners were then tightened and residual fluid was aspirated out of the wells. The diluted PCR products were then transferred to the wells ensuring that no air bubbles were introduced and that no cross-well contamination occurred. The entire apparatus was placed in a 60°C oven and left to hybridise for 1 hour without shaking. After the 1 hour incubation the PCR products were removed from the blotter by aspiration. The membrane was then removed from the blotter with forceps and washed twice in 250ml 2X SSPE/0.5% SDS in a 60°C oven for 10min with gentle shaking. The membrane was then placed inside a rolling bottle to cool down to room temperature before the addition of the 5µl of 5U/ml streptavidine-peroxidase in 10ml of 2X SSPE/0.5% SDS and incubated in the

rolling bottle in a 42°C oven with gentle rolling for 1 hour. The membrane was then washed twice in 250ml 2X SSPE/0.5% SDS for 10min in a 42°C oven with gentle shaking and then rinsed twice in 250ml 2X SSPE at room temperature for 5min.

2.6.3. Detection

The ECL Direct Nucleic Acid Labelling and Detection System (G.E. Healthcare, Amersham) was used to detect the spacer regions. In a dark room, 5ml of detection reagent 1 and 5ml detection reagent 2 was mixed together and poured over the membrane. The membrane was then wrapped in a sheet of Saran-wrap and incubated for 1min. A piece of light sensitive film (Agfa) was then placed over the membrane in a film cassette and tightly closed to prevent light from entering. The film was then exposed to the membrane for 10min and developed. The film was placed in developer for 3min, rinsed in water for 1min and then placed in fixer for 1min before hanging out to dry. The pattern of spacers obtained for each strain, represented by black squares in rows, was then compared to known profiles in order to determine the strain family to which the strain belongs. The membrane can be reused after it has been stripped by incubating the membrane in 1% SDS (Appendix A) at 80°C for 30min twice, then in 20mM EDTA at pH 8 for 15min at room temperature. The membrane is then covered in Saran-wrap, to avoid dehydration, and stored at 4°C till reused.

2.7. Mycobacterial interspersed repetitive units (MIRU) – variable number of tandem repeats (VNTR) typing

MIRU-VNTR utilises regions in the chromosome of *M. tuberculosis* known as mycobacterial interspersed repetitive units (MIRU). There are 41 MIRU loci, spaced throughout the *M. tuberculosis* complex genome, 12 of which display variable number tandem repeats (VNTR) that differ in copy number within the MIRU loci (Supply *et al*, 2000, 2001). These regions are exploited in this assay to further characterize relatedness between strain lineages, by the formation of a numerical code that corresponds to the numbers of VNTRs in the 12 MIRU loci. This is achievable due to the high discriminatory power that these loci have in differentiating related and unrelated strains. The results from this assay, the numerical codes, are fully reproducible and allow for inter-laboratory comparison of strains (Supply *et al*, 2001).

MIRU-VNTR has been enhanced by the use of fluorescently labelled primers, the products of which can be detected using an automated sequencer. MIRU-VNTR was used to determine the relatedness of the sequential isolates collected from each patient indicated as harbouring mixed isolates. The assay consists of a multiplex PCR assay that utilises four groups of three labelled primer sets each, to detect the 12 MIRU loci (Table 2.1)

Table 2.1. MIRU-VNTR primer mixes and the corresponding amplified MIRU loci

Multiplex PCR Mixture	MIRU Locus	[a]Primer Sequence 5' à 3' (Label)
A	4	GCGCGAGCCCGAACTGC (FAM) GCGCAGCAGAAACGTCAGC
	26	TAGGTCTACCGTCGAAATCTGTGAC CATAGGCGACCAGGCGAATAG (HEX)
	40	GGGTTGCTGGATGACAACGTGT (NED) GGGTGATCTCGGCGAAATCAGATA
B	10	GTTCTTGACCAACTGCAGTCGTCC GCCACCTTGGTGATCAGCTACCT (FAM)
	16	TCGGTGATCGGGTCCAGTCCAAGTA CCCGTCGTGCAGCCCTGGTAC (HEX)
	31	ACTGATTGGCTTCATACGGCTTTA GTGCCGACGTGGTCTTGAT (NED)
C	2	TGGACTTGCAGCAATGGACCAACT TACTCGGACGCCGGCTCAAAAT (FAM)
	23	CTGTCGATGGCCGCAACAAAACG (HEX) AGCTCAACGGGTTCGCCCTTTTGTC
	39	CGCATCGACAAACTGGAGCCAAAC CGGAAACGTCTACGCCCCACACAT (NED)
D	20	TCGGAGAGATGCCCTTCGAGTTAG (FAM) GGAGACCGCGACCAGGTACTTGTA
	24	CGACCAAGATGTGCAGGAATACAT GGGCGAGTTGAGCTCACAGAA (HEX)
	27	TCGAAAGCCTCGCGTGCCAGTAA GCGATGTGAGCGTGCCACTCAA (NED)

[a] Forward primer sequences are listed above the reverse primer sequence in each case, with the fluorescent dye indicated in each mixture.

The 50µl reaction volume was made up in 96-well reaction plates and consisted of the following components: 1U HotStartTaq DNA polymerase (Qiagen, Germany), 1X HotStartTaq buffer (Qiagen, Germany), 1X Q-solution (Qiagen, Germany), 0.2mM each of dNTPs (Fermentas, Inqaba Biotech), 0.4µM of each of the primers, and 3.0, 2.0, 2.5 and 1.5mM $MgCl_2$ (Qiagen, Germany) for mixtures A to D, respectively. To this 3µl of DNA extracted from MGIT cultures (2.2.2) was added to each mixture. All four mixes had the same amplification protocol, performed in an ABI Thermocycler, which consisted of an initial denaturation at 95°C

for 15min, followed by 40 cycles of 1min 30sec at 94°C, 1min 30sec at 58°C and 2min at 72°C. A final elongation at 72°C for 10min completed amplification. After amplification the products were diluted 1:20 using distilled water into a fresh 96-well reaction plate, in order to reduce the signal from primer-dimers. Into a third reaction plate, 2µl of the diluted amplicons were added to 8µl of HiDi formamide (Applied Biosystems) and 0.2µl of MapMarker. MapMarker is used by the sequencing software to standardize the size of the signal peaks during analysis. The samples were then run on an automated sequencer (ABI 3100 Analyser; Applied Biosystems) in the Division of Human Genetics, University of Cape Town. The fluorescently labelled products are detected and an image of the peaks based on the size of the products generated. The product size corresponds to the number of VNTRs in that MIRU locus (Appendix D). The number of VNTRs in each MIRU locus forms a 12-digit numerical code, which is then used to compare the relatedness of each strain. Using the MIRU-VNTR*plus* algorithm (www.miru-vntrplus.org) with a maximum allele difference between clusters set at 2, the 12-digit numerical codes are compared and analysed. Thus isolates with differences in two or less alleles are considered related and form part of a clonal complex, while isolates with more than two allele differences are considered unrelated. Isolates with identical alleles are considered the same strain.

2.8. Detection of mixed *Mycobacterium tuberculosis* infections by PCR assay based on the direct repeat region polymorphism

The Polymerase Chain Reaction (PCR) is used to amplify *in vitro* a specific region of DNA. The reaction consists of repetitive three stage cycles; one cycle consisting of a high temperature DNA denaturation followed by cooling to allow oligonucleotide primers to anneal to the defined target region. Finally the temperature is raised to allow a thermal stable DNA polymerase, often *Taq* polymerase from *Thermus aquaticus*, to extend the newly synthesised DNA strand by the incorporation of free deoxynucleoside triphosphates (Atlas and Bej, 2004) (Figure 2.4).

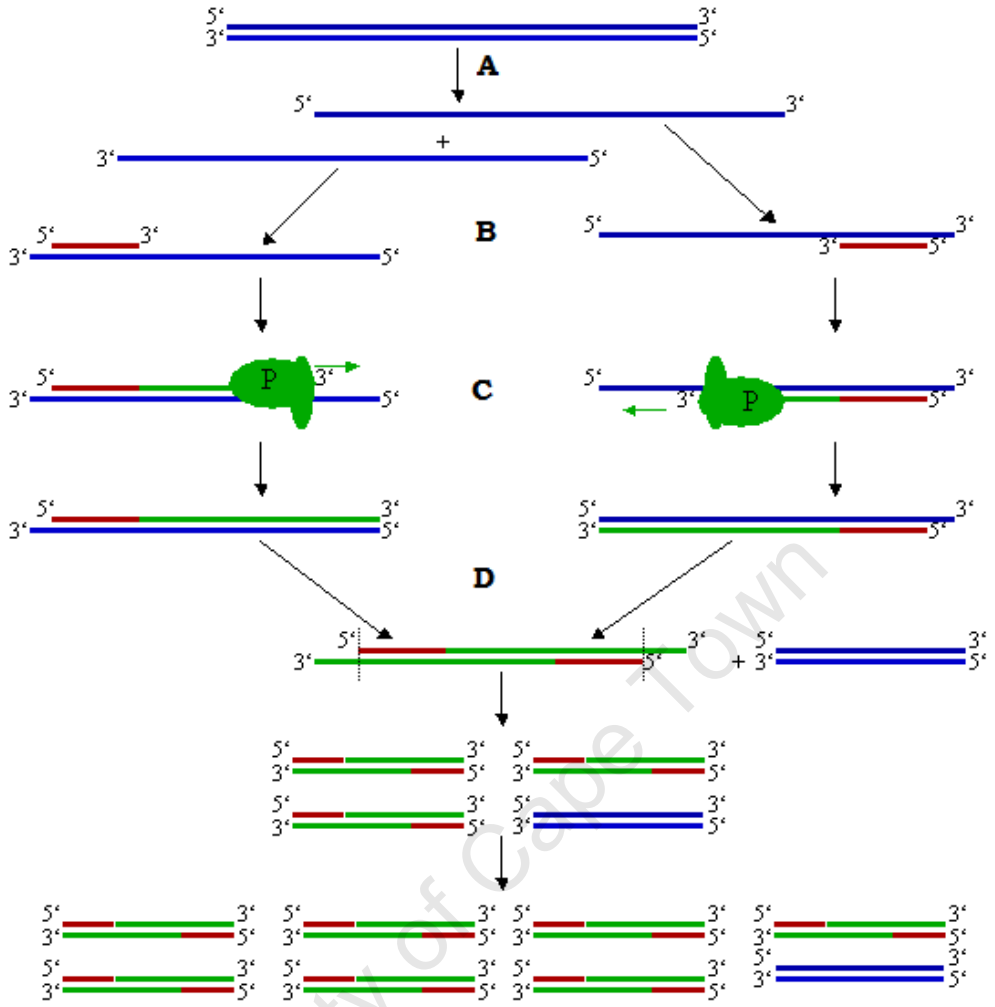


Figure 2.4. Schematic representation of the Polymerase Chain Reaction (PCR). A: Denaturing. B: Annealing. C: Elongation (P=Polymerase). D. The first cycle is complete. The two resulting DNA strands make up the template DNA for the next cycle, thus doubling the amount of DNA duplicated for each new cycle. (Taken from www.biologydaily.com/biology/PCR).

A PCR assay to detect a mixed infection from W-Beijing and non-W-Beijing *M. tuberculosis* isolates in sputum (Warren *et al*, 2004), was used in this study. The assay differentiates isolates based on the presence or absence of chromosomal markers unique to the defined evolutionary lineages of *M. tuberculosis* (Warren *et al*, 2004). A useful region in the chromosome of *M. tuberculosis* that is often used in genotyping studies, such as spoligotyping, is the direct repeat region (DR), which contains highly conserved direct repeats interspersed with highly variable spacer sequences (Kamberbeek *et al*, 1997). The polymorphism present in this region that makes it suited for delineating strains is that the

spacer sequences across the lineages may be deleted. Thus it is possible to genotype strains of *M. tuberculosis* based on the presence or absence of the spacer regions, as is done in spoligotyping (2.8.). Unique to the Beijing lineages is the absence of the majority of the direct repeats and spacer sequences, except for spacer sequences 35 to 43, as well as the presence of the insertion sequence (IS) IS*6110* in region *Rv2820* of the *M. tuberculosis* chromosome.

This PCR assay (Warren *et al*, 2004) utilizes four separate sets of overlapping primers; primer sets 1 and 2 detect Beijing isolates, while primer sets 3 and 4 detect non-Beijing isolates (Figure 2.5).

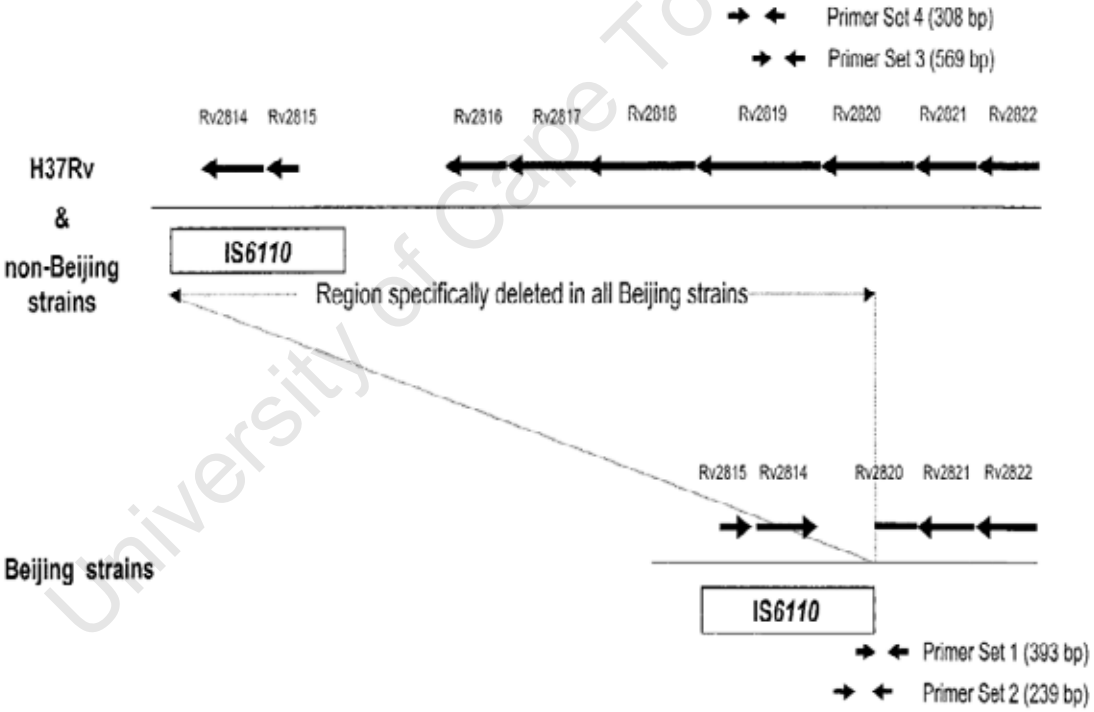


Figure 2.5. Schematic depicting the relative positions of primer sets 1 – 4 in the DR in all non-W-Beijing and W-Beijing strains. Large arrows: direction of open reading frames. Square boxes: insertion of IS*6110* in the DR (in Beijing isolates IS*6110* is inverted). Dotted lines: region deleted in all Beijing strains. Small arrows: position of primer sets 1 – 4. (Adapted from Warren *et al*, 2004).

Primer sets 1 and 2 (Table 2.1) are complimentary to a portion of IS*6110* and Rv2820 (Figure 2.5) and following PCR amplification results in an amplicon of 393bp and 239bp, respectively (Figure 2.6). Primer sets 3 and 4 (Table 2.2) are complimentary to Rv2819, which is deleted in

all W-Beijing strains, and upon amplification and electrophoresis, results in an amplicon of 569bp and 308bp, respectively (Figure 2.6). Correctly amplified products visualized from all for primer sets indicates a mixed infection consisting of a W-Beijing and a non-W-Beijing isolate.

Table 2.2. Primer sets and their sequences used in the PCR assay to detect mixed infections of *M. tuberculosis*.

Primer Name	Primer Sequence (5' à 3')[a]	Region of Amplification
PS1F	TTC AAC CAT CGC CGC CTC TAC	5' portion of IS*6110*
PS1R	CAC CCT CTA CTC TGC GCT TTG	Rv2820
PS2F	ACC GAG CTG ATC AAA CCC G	5' portion of IS*6110*
PS2R	ATG GCA CGG CCG ACC TGA ATG AAC C	Rv2820
PS3F	GAT CGC TTG TTC TCA GTG CAG	Rv2819
PS3R	CGA AGG AGT ACC ACG TGG AG	Rv2819
PS4F	GGT GCG AGA TTG AGG TTC CC	Rv2819
PS4R	TCT ACC TGC AGT CGC TTG TGC	Rv2819

[a] Sequences as described by Warren *et al*, 2004. PS: Primer Set; F: Forward primer; R: Reverse primer.

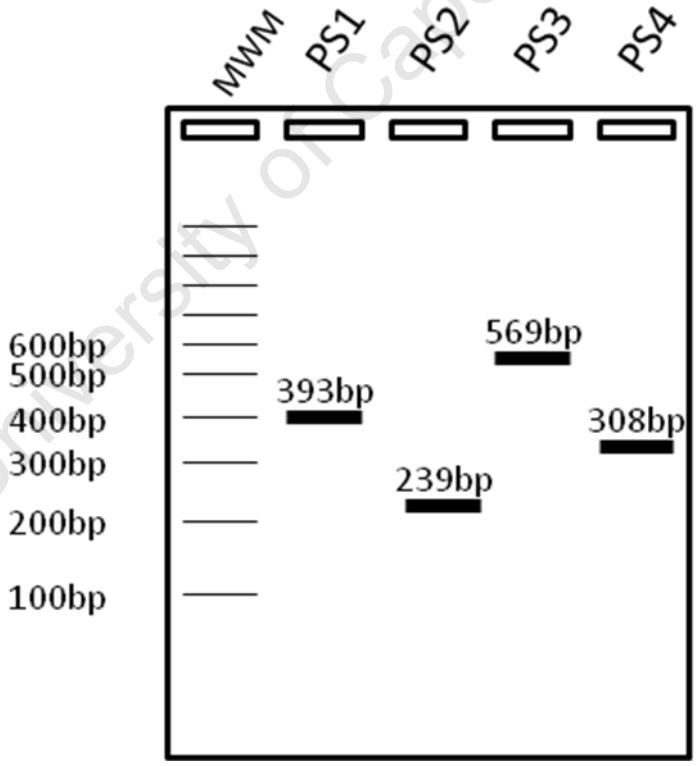


Figure 2.6. Schematic representation of the expected products following amplification from assays PS1, PS2, PS3 and PS4. The molecular weight marker (MWM) is represented as thin parallel lines, while amplicons are represented as thick horizontal lines. The appropriate MWM is labelled above each specific amplicon.

The PCR assay consisted of four separate reactions made up to a final volume of 25µl with 1X HotStarTaq enzyme buffer (Qiagen), 3.5mM $MgCl_2$ (Qiagen), 4mM dNTPs (Fermentas), 25pmol of each primer, 0.5U of HotStarTaq DNA Polymerase (Qiagen) and 1µl of MGIT DNA template (2.2.2). Amplification was performed in ABI2720 Thermocycler (Applied Biosystems) under the following conditions: initial denaturation and enzyme activation at 95°C for 15min followed by 45 cycles of 94°C for 1min, 62°C for 1min and 72°C for 1min. Finally, an elongation at 72°C for 10min completed the amplification. The amplified products were electrophoresed with 5µl loading buffer on a 1.5% agarose gel at 100 volts for 1 hour and visualized (2.3).

2.9. Differentiation of non-W-Beijing *Mycobacterium tuberculosis* infections by a 3-stage PCR algorithm

The PCR assay (Warren *et al*, 2004), as described previously (2.4), allows for a modest approximation of mixed infections of *M. tuberculosis* in a high-burdened setting. However, as stated, a mixed population of infecting non-W-Beijing strains would go undetected if the assay was used in isolation. Thus there exists a need for an additional assay to separate out the various prevalent non-W-Beijing strains circulating in the Western Cape.

According to several South African researchers (Nicol *et al*, 2005; Marais *et al*, 2006), there are four circulating non-W-Beijing strain lineages that are prevalent in the Western Cape (Table 2.3.).

Table 2.3. Prevalence of major circulating *M. tuberculosis* lineages in the Western Cape

Strain Lineage	Prevalence (% of total study population)	
	Study 1†	Study 2‡
All LAM	33	28.6
LAM3	30	NR
X	10.5	6.3
T	7	NR
Haarlem	4	3

†Nicol *et al*, 2005
‡Marais *et al*, 2006. NR: Not Reported

The DR was used to differentiate each lineage based on the presence or absence of spacer sequences of each prevalent lineage (Figure 2.7.)

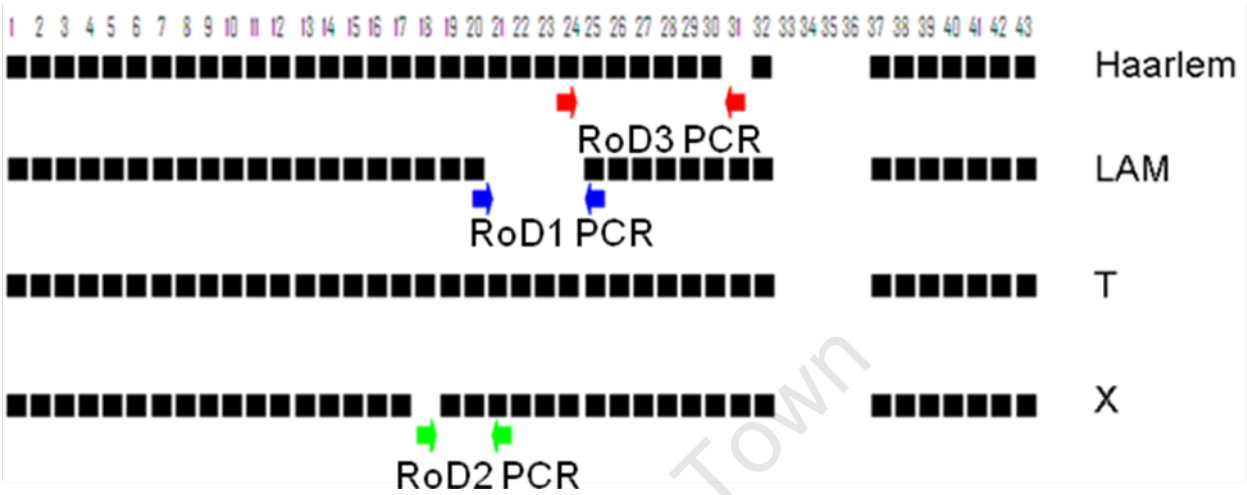


Figure 2.7. Schematic depicting the consensus spacer sequences of each prevalent non-W-Beijing lineage. The lineages are labeled on the right. Each spacer is numbered at the top from 1 through to 43 and is depicted as a black square. Spacer sequences that are absent in that lineage appear as a blank space. Arrows depict the forward and reverse primers of each PCR in the algorithm. Blue: RoD1 PCR; Green: RoD2 PCR; Red: RoD3 PCR. (Adapted from the SpolD4 database; Brudey *et al*, 2006)

As it can be seen in Figure 2.7, each lineage is characterized by a different arrangement of spacer sequences. Based on these regions of difference (RoD), three separate PCR assays (RoD1 – 3) were designed in an algorithm format; starting from RoD1 one would work down toward RoD3 based on the results obtained from the previous PCR (Figure 2.8).

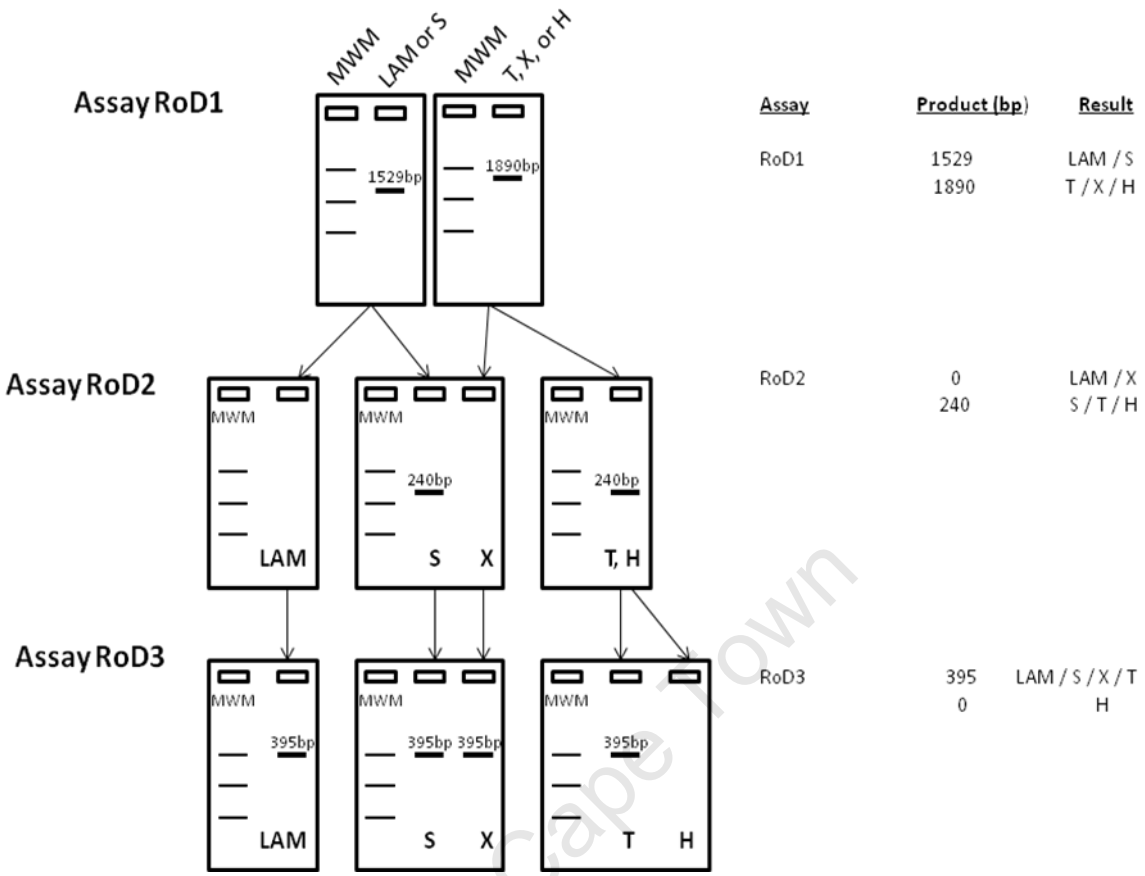


Figure 2.8. A 3-Step RoD PCR algorithm for the detection and differentiation of mixed non-Beijing *M. tuberculosis* isolates. Gels depicted as large rectangles. Molecular weight marker (MWM) depicted as 3 parallel thin lines. Amplicons represented as bold horizontal lines. Molecular weights of amplicons labeled above each band. An amplicon of 1529bp after RoD1 indicates a LAM or S strain while the larger 1890bp amplicon indicates either T, X or H strains. An amplicon of 240bp after RoD2 indicates either a T or S strain (or an S strain, if in RoD1 the same sample yielded a 1529bp product) while the absence of a product indicates an X strain (or a LAM strain, but this would have been determined at RoD1). An amplicon of 395bp after RoD3 indicates a T strain (or LAM, X, or S strains but these would have been determined after RoD1 and RoD2), while the absence of an amplicon is indicative of an H strain.

2.9.1. Differentiation using RoD1 PCR assay

The first step in the PCR algorithm, RoD1 differentiates LAM strains from non-LAM strains based on the size of the amplicon. LAM strains are characterized by the absence of spacer sequences 21 – 24, whereas they are present in H, T and X lineages. RoD1F is designed to anneal to spacer sequence 20 while RoD1R is designed to anneal to spacer sequence 25 (Table 2.4; Figure 2.7). In non-LAM strains a larger product of 1890bp is amplified while in LAM strains a smaller product of 1529bp is amplified (Figure 2.8), due to the absence of the

intervening spacer sequences. The insertion of *IS*6110 in spacer 25 accounts for the apparent large size of both products.

Table 2.4. Primers and primer sequences used in RoD1 to differentiate non-Beijing *M. tuberculosis* isolates.

Primer Name	Primer Sequence (5' à 3')	Amplified Region
RoD1F	ATT GCG CTA ACT GGC TTG	Spacer sequences 21 - 24
RoD1R	TGC GGT GGT CGC TGA TC	Spacer sequences 21 - 24

F: Forward primer; R: Reverse primer.

The master PCR mix, with a final volume of 50µl, contains 1X GoTaq Flexi enzyme buffer, 3.5mM $MgCl_2$, 4mM dNTPs, 25pmol of each primer, 1U GoTaq DNA polymerase and 2µl of MGIT DNA template (2.2.2). Amplification took place in a thermocycler under the following conditions: an initial denaturation at 95°C for 5min, followed by 35 cycles of 95°C for 1min, 56°C for 1min and 72°C for 2min. Finally an elongation at 72°C for 5min completed the amplification. The products were electrophoresed on a 1% agarose gel and visualized (2.3).

2.9.2. Differentiation using RoD2 PCR assay

Further differentiation is needed after RoD1 if a product of 1831bp is amplified, which is indicative of either a T, X or H lineage. RoD2 separates strains of the X lineage from the T and H lineage based on the presence or absence of a 240bp amplicon. Strains from the X lineage are characterized by the absence of spacer sequence 18 (Figure 2.7). RoD2F (Table 2.5.) is designed to anneal to spacer sequence 18 and along with RoD2R (Table 2.5), which anneals to spacer sequence 21, will amplify a product of 240bp in strains H and T, in whom spacer 21 is present. An absence of a product will indicate and X strain, but will also indicate strains of LAM lineage due to the absence of spacer 21 in these strains. However, all LAM strains will be separated out in RoD1 and thus RoD2 should serve as a confirmation (Figure 2.8).

Table 2.5. Primers and primer sequences used in RoD2 to differentiate non-Beijing *M. tuberculosis* isolates

Primer Name	Primer Sequence (5' à 3')	Amplified Region
RoD2F	AGC TGC AGA TGG TCC GGG A	Spacer sequences 18 – 21
RoD2R	ATT GGG ACA TCG ACA TCG AC	Spacer sequences 18 - 21

F: Forward primer; R: Reverse primer

The master PCR mix, with a final volume of 50µl, contains 1X GoTaq Flexi enzyme buffer, 3.5mM $MgCl_2$, 4mM dNTPs, 25pmol of each primer, 1U GoTaq DNA polymerase and 2µl of MGIT DNA template (2.2.2). Amplification took place in a thermocycler under the following conditions: an initial denaturation at 95°C for 5min, followed by 35 cycles of 95°C for 1min, 62°C for 1min and 72°C for 30sec. Finally an elongation at 72°C for 5min completed the amplification. The products were electrophoresed on a 1.5% agarose gel and visualized (2.3).

2.9.3. Differentiation using RoD3 PCR assay

The last differentiation step in the algorithm, RoD3 will separate out the H and T strains based on the presence or absence of a 395bp amplicon (Figure 2.8). H strains are characterized by the absence of spacer sequence 31, which is present in the other strains (Figure 2.7). RoD3F (Table 2.6) is designed to anneal to spacer 26 while RoD3R is designed to anneal to spacer 31, which, in T strains, will result in the amplification of a 395bp product. It could also indicate X and LAM strains, due to the presence of both spacer sequences 26 and 31, but as these would have been detected in the previous PCRs, this serves as a confirmation of those results. Thus the absence of this product will indicate an H strain, due to the absence of spacer 26 (Figure 2.8).

Table 2.6. Primers and primer sequences used in RoD3 to differentiate non-Beijing *M. tuberculosis* isolates

Primer Name	Primer Sequence (5' à 3')	Amplified Region
RoD3F	TTC AGC ACC ACC ATC ATC C	Spacer sequences 26 – 31
RoD3R	TGA TCG ACG CGA ACC TGT C	Spacer sequences 26 – 31

F: Forward primer; R: Reverse primer

The master PCR mix, with a final volume of 50µl, contains 1X GoTaq Flexi enzyme buffer, 3.5mM $MgCl_2$, 4mM dNTPs, 25pmol of each primer, 1U GoTaq DNA polymerase and 2µl of MGIT DNA template (2.2.2). Amplification took place in a thermocycler under the following conditions: an initial denaturation at 95°C for 5min, followed by 35 cycles of 95°C for 1min, 58°C for 1min and 72°C for 1min. Finally an elongation at 72°C for 5min completed the amplification. The products were electrophoresed on a 1% agarose gel and visualized (2.3).

Chapter 3

Results

Contents

3.1. *Mycobacterium tuberculosis* isolates included in this study

By the end of the study period, 154 adult patients with newly diagnosed TB were enrolled and included in this study. Of the 154 patients, 8 were excluded on the basis that no positive sputum samples were obtained during the 8 week study. An additional 6 patients were excluded due to samples being lost during the course of the study. An initial sputum sample was collected prior to the start of treatment and is referred to as a baseline sample. Follow up samples were taken weekly for 8 weeks thereafter resulting in a collection of 9 samples from each patient. However, a number of samples had to be excluded due to the contamination of the samples, mainly due to fungal spores that may have been transferred into the MGIT cultures during subculturing from the original sputum sample. As a result, a total of 686 samples from 140 patients were included in this study (Appendix D).

Genomic DNA was extracted from *M. tuberculosis* colonies grown on LJ slopes at 37°C (2.2.1) and electrophoresed along with a Lamda (λ) DNA titration and Hyperladder I (Appendix A) in order to quantify the DNA (Figure 3.1). A selection of strains comprising LAM, X, H and T strains were included as controls.

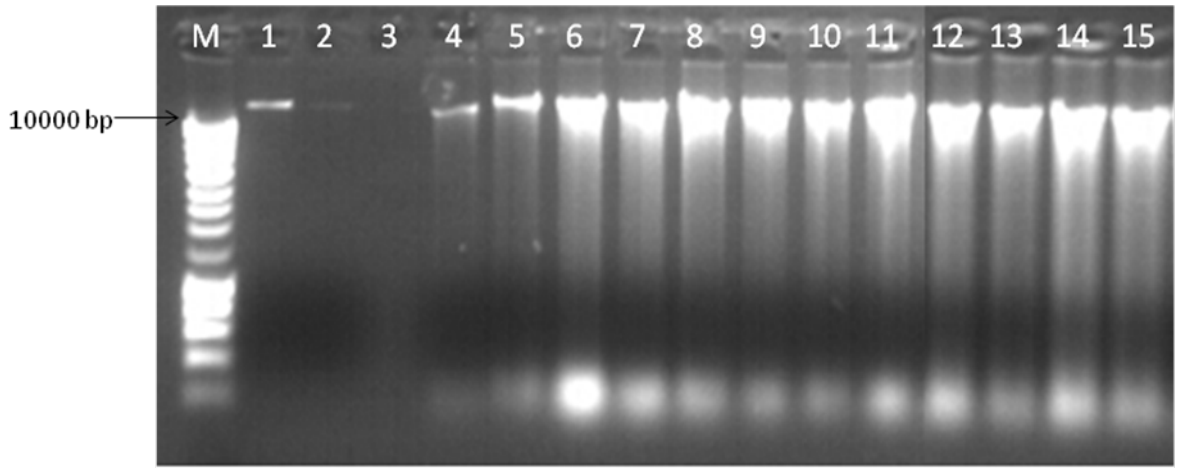
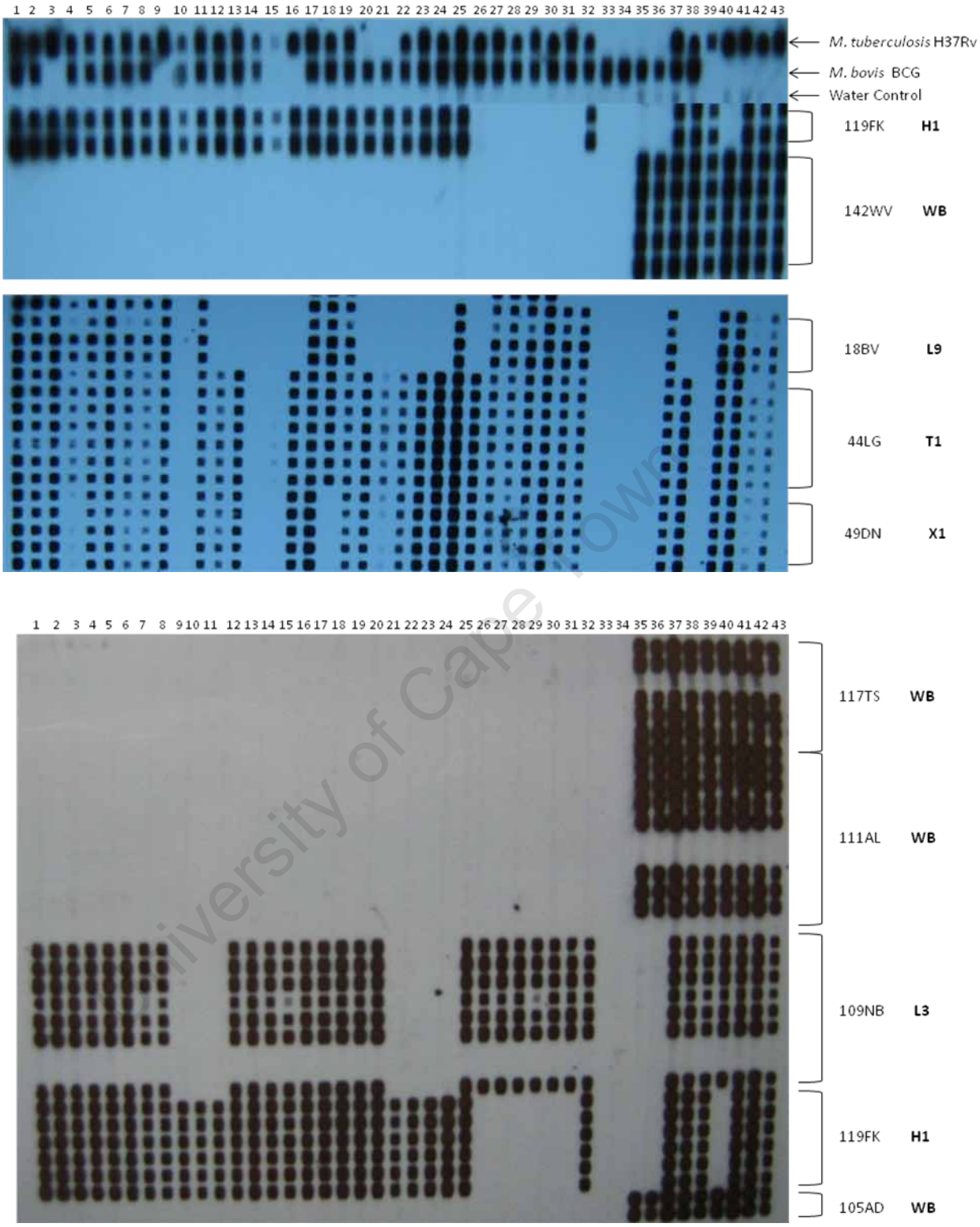


Fig 3.1. Agarose electrophoresis of genomic DNA from *M. tuberculosis* strains used as controls. M: Hyperladder I; Lane1: λ DNA (35ng); Lane 2: λ DNA (3.5ng); Lane 3: Void; Lane 4: LAM 1; Lane 5: LAM 2; Lane 6: LAM 3; Lane 7: T 1; Lane 8: T 2; Lane 9: T 3; Lane 10: X 1; Lane 11: X 2; Lane 12: X 3; Lane 13: H 1; Lane 14: H 2; Lane 15: H 3

Based on the intensity of the genomic DNA bands relative to the λ DNA (Figure 3.1) the concentration of DNA following extraction (2.2.1) is 30ng/µl for LAM 1 is (Lane 4; Figure 3.1), 50ng/µl for LAM 2 (Figure 3.1; Lane 5) and 100ng/µl for the remaining control strains (Figure 3.1; Lanes 6 – 15). In subsequent PCR assays, 2µl of 1:10 dilution of all strains other than LAM 1 and 2, equating to 20ng of DNA, was added as template. LAM 1 was diluted 1:3 and LAM 2 was diluted 1:5; 2µl of each, equating to 20ng of DNA, was added as template.

3.2. Spoligotyping of isolates collected from patients

To investigate the epidemiology of the isolates obtained from the patients enrolled in this study as well as to screen for potential mixed samples, spoligotyping (2.8) was performed on DNA prepared from MGIT cultures (2.2.2). Following amplification of the spacer regions in the DR locus, the products were hybridised in a miniblotter to a membrane containing the known spacer oligonucleotides and later visualised using the ECL method on a light-sensitive film. The profiles were compared to published lineages (Brudey *et al*, 2006) to determine the strain family to which the isolates belonged (Figure 3.2).



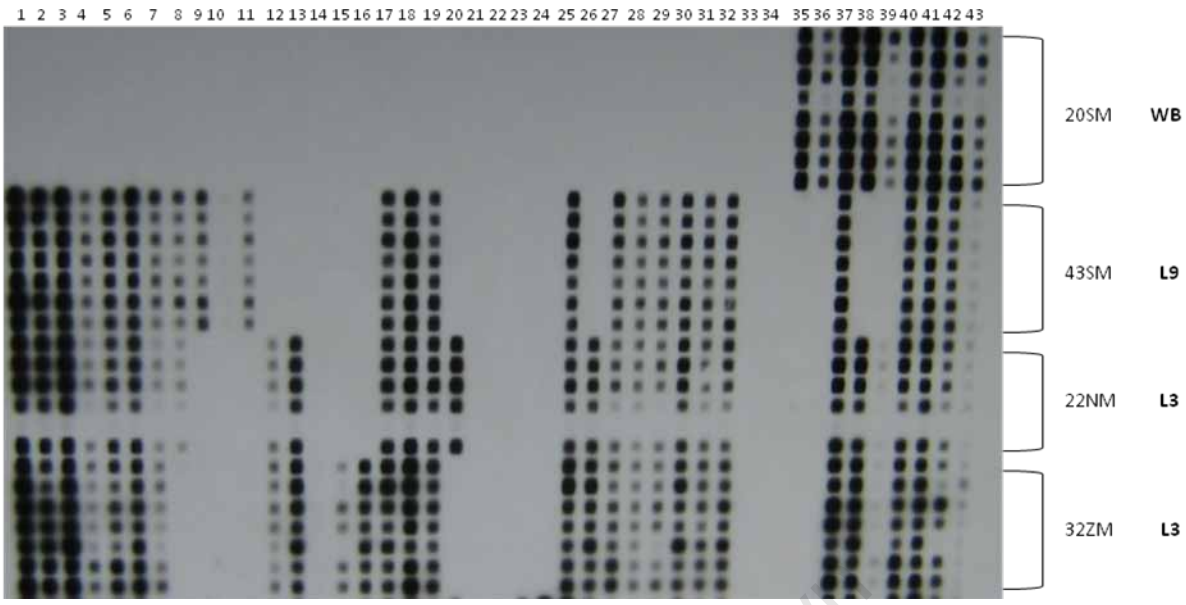


Figure 3.2. Autoradiographs of spoligotyping, of a selection of *M. tuberculosis* isolates from patients included in the study. Black squares represent spacer sequences present in the DR of each *M. tuberculosis* isolate, and are indicated above each autoradiograph. H37Rv and BCG controls are included to represent all spacer sequences. The negative water control is to rule out the possibility of contamination. Patient names are indicated to the right of each autoradiograph with isolates from each patient grouped together by a square bracket. Profiles representing all genotype families detected in the study are indicated in bold font after each patient name. H: Haarlem; WB: W-Beijing; L: LAM; T: T Family; X: Low-copy Clade X.

The positive controls H37Rv and *M. bovis* BCG are included to ensure that the PCR amplifies all spacer sequences in the DR region. Strain H37Rv contains spacer sequences 1 – 19, 22 – 32, and 37 – 43 (Figure 3.2). Spacer sequences 20, 21, 33 – 36 are present in *M. bovis* BCG (Figure 3.2). The negative water control is included to ensure no contamination is present in the assay.

Analysis of the spoligotyping profiles obtained from isolates from 123 patients (88%) indicated that these patients were infected with a single genotype (Figure 3.2; Table 3.1). Only 1 viable culture was obtained from 11 patients; these could not be analysed with regards to mixed infections.

Table 3.1. Spoligotypes of sequential isolates obtained from 140 patients included in the study

Patient Number	Patient Name	Number of Samples Genotyped	†Genotype (n)
2	FA	1	WB
4	UO	3	L3
5	GS	1	WB
6	RB	1	L3
7	XT	2	WB
8	RJ	2	WB
9	OA	4	WB
10	MO	2	L3
11	DP	6	WB
12	TM	3	WB
13	BB	6	WB
14	MM	4	WB
15	JS	5	WB
16	DM	5	WB
17	MM	1	T1
18	WJ	6	WB
19	BB	5	L3
20	TM	7	WB
21	CJ	4	WB
22	JE	9	L5
23	FM	5	WB
24	NC	7	*T4 (6); S (1)
25	ZD	3	X3
26	YK	2	L3
28	JJ	2	WB
29	FJ	6	L3
30	AV	2	WB
32	WC	2	S
33	WH	4	X3
34	CI	3	WB
35	NN	3	WB
36	BQ	2	L3
37	KM	1	WB
38	TP	3	X2
39	JD	4	T1
40	SD	6	WB
41	SM	2	X1
42	MM	4	T1
43	XM	6	WB
44	DL	3	T1
45	LG	7	T1
46	JP	6	*T1 (4); S(2)
47	ZM	6	L3
48	ML	8	H1

49	DN	5	X1
50	SD	8	WB
51	SM	5	L3
52	SM	9	L9
53	TN	3	L3
54	ER	5	WB
55	BM	7	H1
56	TZ	2	L3
57	SM	8	WB
58	NM	8	L3
59	AM	8	WB
60	JS	6	F33
61	WM	8	WB
62	CG	1	WB
63	MC	4	T1
64	BV	4	L9
65	RR	5	L3
66	RP	7	X1
67	CE	5	T1
68	NM	5	S
69	BD	3	WB
70	JB	2	T4
71	NV	5	F33
72	VF	2	WB
73	NC	10	*WB (9); T4 (1)
74	SE	4	WB
75	CK	3	F33
76	BD	2	T1
78	NM	4	WB
79	RC	7	WB
80	GI	7	WB
81	SM	3	*WB (2); WB + X3 (1)
85	SA	4	WB
86	AF	8	WB
87	GK	9	WB
88	NG	8	T1
89	ZB	6	WB
90	CD	3	L3
91	IF	6	H1
92	DS	1	T1
93	SB	8	S
94	CJ	7	H3
95	AM	9	WB
96	PJ	6	T1
98	HV	4	S
99	TN	6	X1
100	CW	4	S

101	GP	8	WB
103	RP	7	L3
104	MM	7	WB
105	AD	9	WB
107	MD	6	*WB (4); WB + X3 (2)
108	NM	1	WB
109	NB	8	L3
111	AL	7	WB
112	AP	7	WB
113	DM	1	L3
114	VJ	2	WB
115	JM	4	H1
116	NS	4	L3
117	TS	8	WB
118	AG	3	*L3 (2); T4 (1)
119	FK	9	H1
120	IB	6	WB
121	VS	3	WB
122	HC	3	T1
123	EJ	5	WB
124	TJ	3	T1
126	NS	4	WB
127	ZC	2	WB
128	SK	6	WB
129	MN	5	WB
130	MJ	4	H1
131	DD	5	H1
133	SM	5	WB
134	DG	3	T1
135	SS	2	L3
136	JI	5	WB
137	BJ	4	X3
138	PD	6	WB
139	DG	5	T1
140	PW	5	WB
141	NE	7	T1
142	WV	5	H1
143	JB	1	WB
144	EJ	8	WB
145	NN	8	WB
146	GM	8	T1
147	SD	7	S
148	DJ	8	WB
149	WM	7	H1
150	ME	7	X3
151	BH	1	L3
152	CB	7	WB

153	WI	6	WB
154	MM	9	H1

†WB: W-Beijing; X: Low Copy Number Clade X; T: Strain Family T; H: Haarlem; F33: Family 33; S: Strain Family S; L: LAM. (*Where 2 strain lineages are indicated sequentially in the same patient, that patient is considered to harbour a mixed infection).

The remaining 6 patients (4.3%) displayed colonization with mixed isolates, as indicated by the heterogeneous patterns observed following spoligotyping (Figure 3.3).

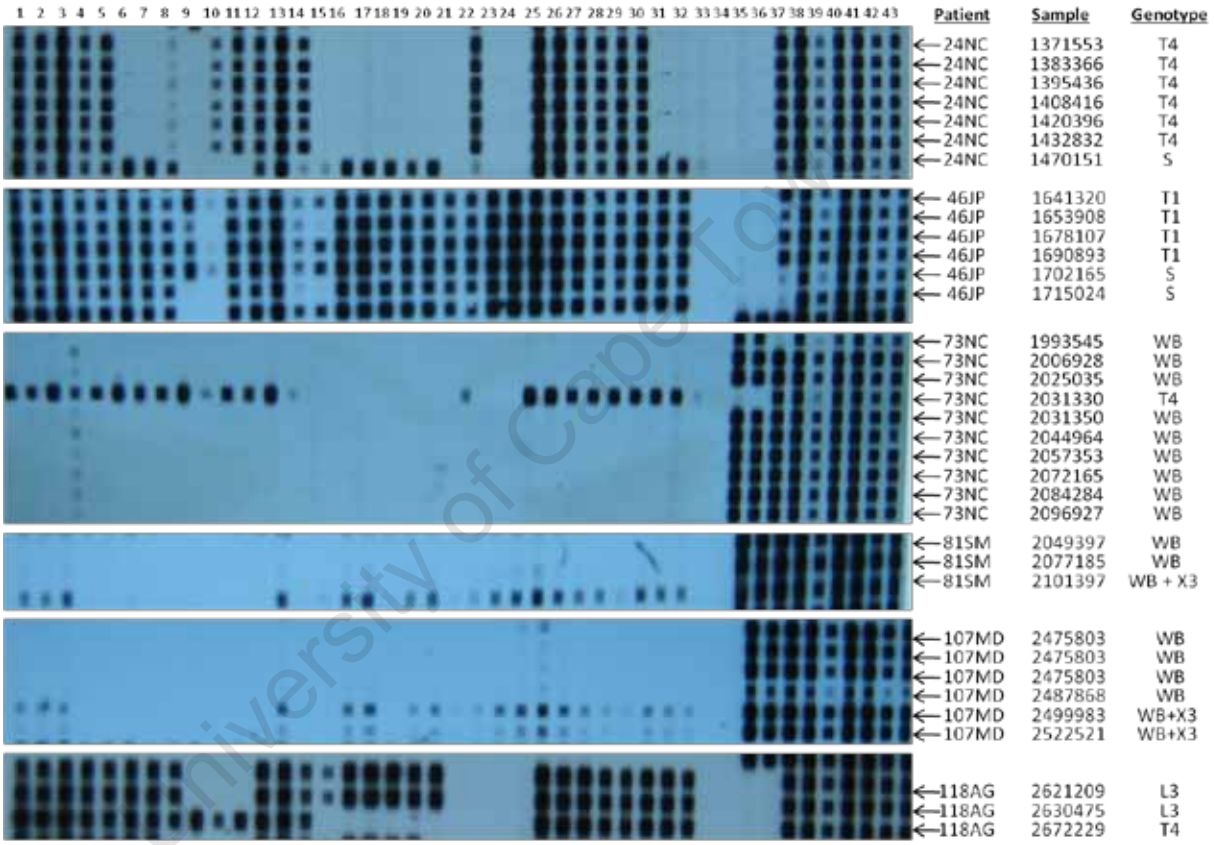


Figure 3.3. Mixed samples as detected by spoligotyping. Spacer sequences are represented by black squares and are arranged in order from 1 through to 43, as labelled at the top of the figure. Patient names as well as sample numbers and genotypes for each sample are indicated to the right of the figure. T: T Family strains; S: S Family strains; WB: W-Beijing; X: Low-copy clade X; L: LAM.

Therefore, of the 129 patients with more than 2 viable cultures that were typed, 6 (4.7%) harboured isolates of different genotypes; 4 of these patients (24NC, 46JP, 73NC, and 118AG) showed evidence of reinfection with a different strain, while 2 patients (81SM, and 107MD) harboured mixed isolates (Figure 3.3; Table 3.1).

Of the 140 patients included, 67 (47.8%) were colonised with W-Beijing isolates, 24 (17.1%) with isolates from the LAM family, 20 (14.3%) with isolates from the T family, 9 (6.4%) with isolates from the low-copy number clade X, 11 (7.9%) with isolates from the Haarlem family, 6 (4.3%) with isolates from the S family and 3 (2.1%) patients were colonised with isolates from Family 33 (Figure 3.4).

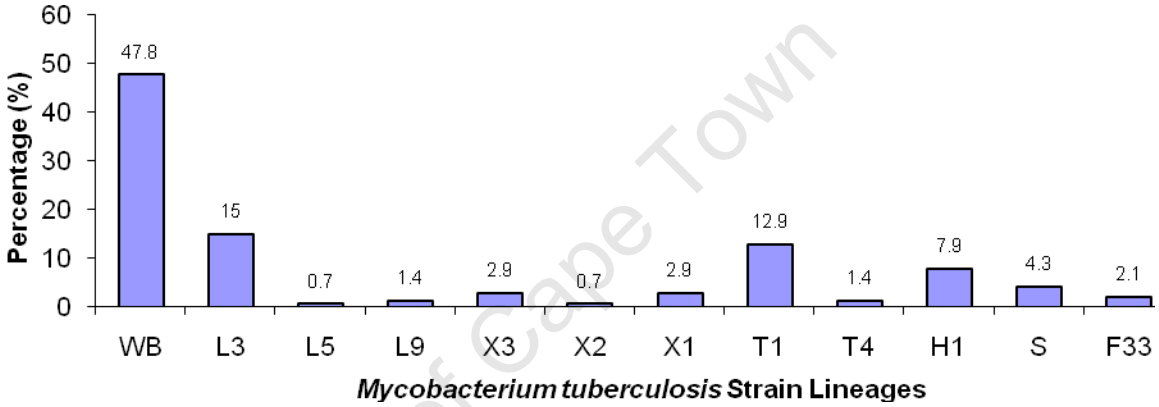


Figure 3.4. Distribution of circulating *Mycobacterium tuberculosis* strains in the Delft region of the Western Cape. The percentages of the total of 140 patients is indicated above each bar.

Of the patients initially colonized with either T4 (24NC) or T1 (46JP) strains, both were found to harbour an additional S strain at the end of the study period (Figure 3.3). The first 3 isolates obtained from patient 73NC were of the W-Beijing lineage (Figure 3.3). The 4th isolate emerged as a T4 strain, thereafter the remaining 5 isolates were of the W-Beijing lineage. Two patients (SM81 and MD107) harbouring W-Beijing had a dual infection with X3 strains. In both patients, the X3 strain emerged after the initial isolation of the W-Beijing strains. Two LAM3 strains were initially isolated from patient AG118, with a T4 isolated 4 weeks later. Due to missing or contaminated samples, it is unclear as to what the bacillary population encompasses during those 4 weeks, but would be of great interest.

3.3. Mycobacterial interspersed repetitive units (MIRU) – variable number of tandem repeats (VNTR) typing of sequential isolates of patients with mixed isolates

To ascertain the genetic relatedness of the sequential isolates obtained from each of the patients colonized with mixed isolates, and to determine the relatedness of the infecting isolates between patients, 12-locus MIRU-VNTR was carried out. Following amplification of the MIRU loci using the grouped multiplex primers, the labelled products were detected in an ABI 3100 Analyser (Applied Biosystems) in the Division of Human Genetics, University of Cape Town. The sizes of the products were compared to published alleles (Supply *et al*, 2000) rendering the number of VNTRs in each isolate to a numerical code (Appendix D), and the genetic relatedness of the isolates was analysed using the MIRU-VNTR*plus* algorithm (http://www.miru-vntrplus.org/miru). Isolates differing from one another at 2 alleles or less were defined as being part of the same clonal complex, whilst those that differed at 3 or more loci were considered outliers.

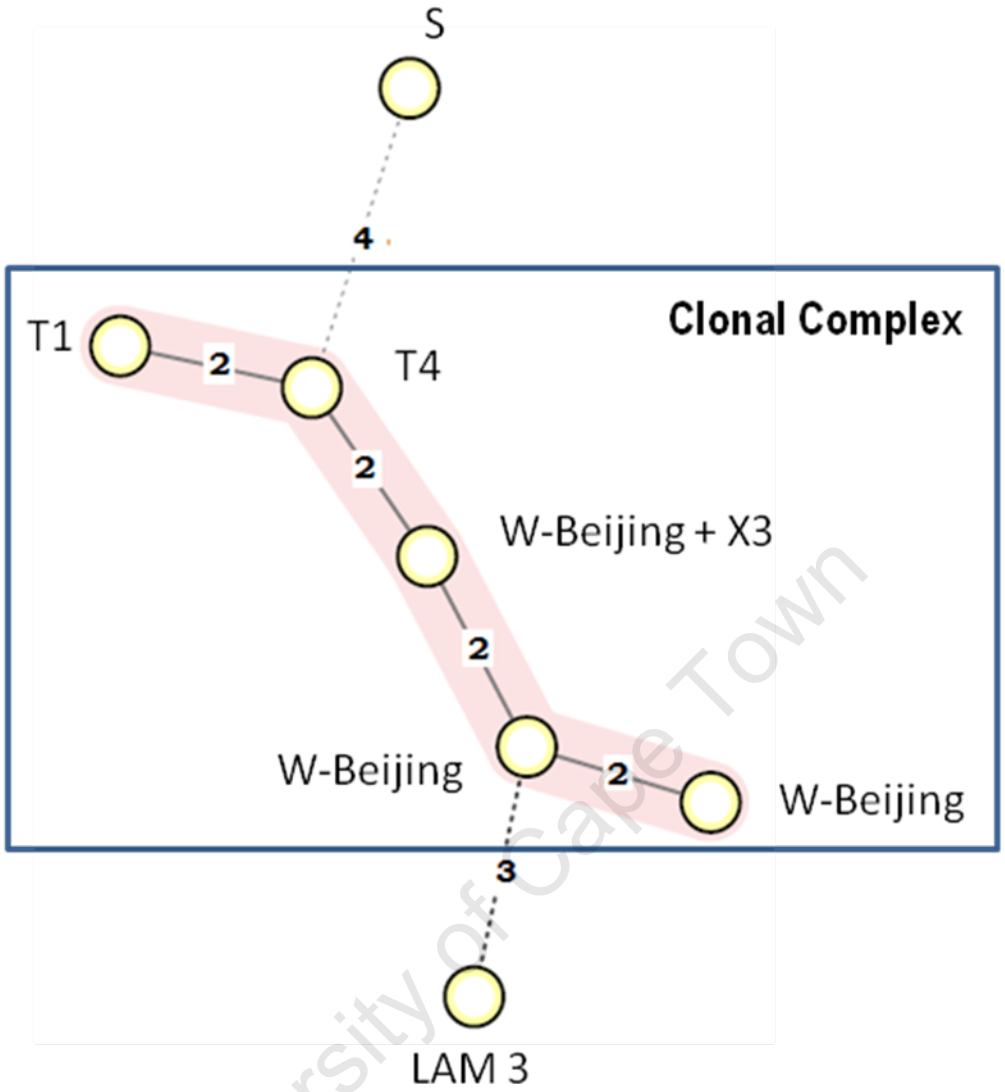


Figure 3.5. Minimum spanning tree indicating the genetic relatedness of *M. tuberculosis* isolates obtained from patients with mixed infections. Genotypes are represented by circles and are labelled accordingly. Solid lines indicate a difference of 2 alleles between genotypes, indicating a clonal complex. Dotted lines indicate differences of 3 or more alleles between genotpyes and represent outliers. Numbers above the joining lines indicate the allelic difference between joining genotypes. Isolates from the T1, T4, W-Beijing and X3 lineages formed a clonal complex, as demarcated by the square. WB: W-Beijing.

Analysis of the MIRU loci revealed sequential isolates obtained from each individual patient are clonal and display the same number of VNTR alleles per locus; that is all T4 isolates (patients 24NC, 73NC and 118AC) were identical as were the T1 isolates (patient 46JP), S isolates (patients 24NC and 46JP), W-Beijing + X3 isolates (patients 81SM and 107MD) and LAM 3 isolates (patient 118AG) (Figure 3.5). Although the W-Beijing isolates from patients 73NC and 107MD were identical, they differed from the W-Beijing isolates from patient 81SM

by 2 alleles (Figure 3.5). The T4 isolates (patients 24NC, 73NC and 118AG), the T1 isolates (patient 46JP), and the W-Beijing isolates (patients 73NC, SM81, and 107MD) differed from one another by 2 or less alleles and form part of the same clonal complex (Figure 3.5). The S isolates (patients 24NC and 46JP) and the LAM 3 isolates (patient 118AG) had more than 2 alleles different from the clonal complex genotypes and are therefore represented as outliers (Figure 3.5), and are thus considered unrelated.

Interpretation of the electrophorerogram data of the W-Beijing + X3 mixed sample was difficult, as the expected separate peaks; one for each isolate, at each locus, was not present. Instead, one signal was obtained at each locus, and represented an amalgamation of alleles for each isolate, as the alleles neither represent a W-Beijing isolate, nor an X3 isolate (Appendix D). However the profile was related to the W-Beijing profiles of patients 73NC and 107MD, as there was only a difference of 2 alleles between them (Figure 3.5). Interestingly, the W-Beijing + X3 mixed samples of patient 81SM and 107MD were identical (Appendix D; Figure 3.5).

3.4. Drug Susceptibility Testing of mixed isolates using the GenoType MTBDR*plus* assay

Drug susceptibility testing (DST) was performed routinely in the diagnostic microbiology laboratory in GSH using the BACTEC MGIT 960 system (2.1) on the baseline and final samples collected from each patient enrolled in the study. Additional DST was performed on all samples from patients with mixed isolates using the GenoType MTBDR*plus* assay (2.2), to observe determine whether the DST profiles changes with corresponding changes in genotype profiles. Following PCR amplification of the regions on the chromosome known to contain determinants associated with resistance to RIF and INH, amplicons were hybridized to a membrane strip containing the corresponding oligonucleotides. No signal was detected for any of the probes corresponding to either RIF or INH resistance, indicating that all strains assayed are susceptible to both RIF and INH (Table 3.2). These results correspond with the DST results obtained using the BACTEC MGIT 960 system (Table 3.2).

Table 3.2. Resistance to RIF and INH of the *M. tuberculosis* isolates from patients harbouring mixed infections

Patient Number	Patient Name	Sample Number	Week	Genotype	MGIT		MTBDRplus	
					RIF	INH	RIF	INH
24	NC	1371553	0	T4	s	s	s	s
		1383366	1	T4	nd	nd	s	s
		1395436	2	T4	nd	nd	s	s
		1408416	3	T4	nd	nd	s	s
		1420396	4	T4	nd	nd	s	s
		1432832	5	T4	nd	nd	s	s
		1470151	8	S	s	s	s	s
46	JP	1641320	0	T1	s	s	s	s
		1653908	1	T1	nd	nd	s	s
		1678107	2	T1	nd	nd	s	s
		1690893	3	T1	nd	nd	s	s
		1702165	4	S	nd	nd	s	s
		1715024	5	S	s	s	s	s
73	NC	1993545	0	WB	s	s	s	s
		2006928	1	WB	nd	nd	s	s
		2025035	2	WB	nd	nd	s	s
		2031330	3	WB	nd	nd	s	s
		2031350	4	T4	nd	nd	s	s
		2044964	5	WB	nd	nd	s	s
		2057353	6	WB	nd	nd	s	s
		2072165	7	WB	nd	nd	s	s
		2084284	8	WB	nd	nd	s	s
		2096927	9	WB	s	s	s	s
81	SM	2049397	0	WB	s	s	s	s
		2077185	1	WB	nd	nd	s	s
		2101397	2	WB + X3	s	s	s	s
107	MD	2420713	0	WB	s	s	s	s
		2450525	1	WB	nd	nd	s	s
		2463471	2	WB	nd	nd	s	s
		2475803	3	WB	nd	nd	s	s
		2499983	4	WB + X3	nd	nd	s	s
		2522521	5	WB + X3	s	s	s	s
118	AG	2621209	0	L3	s	s	s	s
		2630475	1	L3	nd	nd	s	s
		2672229	2	T4	s	s	s	s

RIF: Rifampicin; INH: Isoniazid; s: susceptible; nd: not determined, 0: Baseline isolate.

All isolates tested were indicated as being fully susceptible to both RIF and INH (Table 3.2).

3.5. Detection of mixed infections harbouring W-Beijing and non-W-Beijing *M. tuberculosis* isolates

A PCR assay was designed to screen for the presence of W-Beijing isolates and non-W-Beijing isolates in samples obtained from patients (Warren *et al*, 2004). The assay, comprising four reactions, PS1, PS2, PS3 and PS4, is designed to differentiate W-Beijing isolates from non-W-Beijing isolates. PS1 and PS2 contain primers that specifically amplify unique W-Beijing associated sequences and will therefore detect isolates belonging to the W-Beijing lineage. Similarly, PS3 and PS4 specifically amplify unique non-W-Beijing sequences and therefore will detect isolates belonging to non-W-Beijing lineages. Following amplification using PS1 and PS2, a product of 393bp and 239bp, respectively, is expected. Products of 570bp and 309bp are obtained following amplification using PS3 and PS4, respectively. Positive amplification of all expected products for all four of the primer sets would indicate a mixed infection consisting of W-Beijing and non-W-Beijing isolates (Warren *et al*, 2004).

Isolates, known to be W-Beijing strains following spoligotyping and MIRU-VNTR (Evans *et al*, 2009) termed W-Beijing 1 and W-Beijing 2, were used as W-Beijing control strains. The laboratory reference strain H37Rv was used as a non-W-Beijing control. Genomic DNA was extracted from these control strains (2.2.1) and used as template for optimization of the PCR assays.

The assay conditions were as described (Warren *et al*, 2004) with the exception that *GoTaq* DNA polymerase (Promega) was used instead of HotStartTaq DNA polymerase and Q-Solution (Qiagen). Following amplification, the PCR products were separated by agarose gel electrophoresis (Figure 3.5).

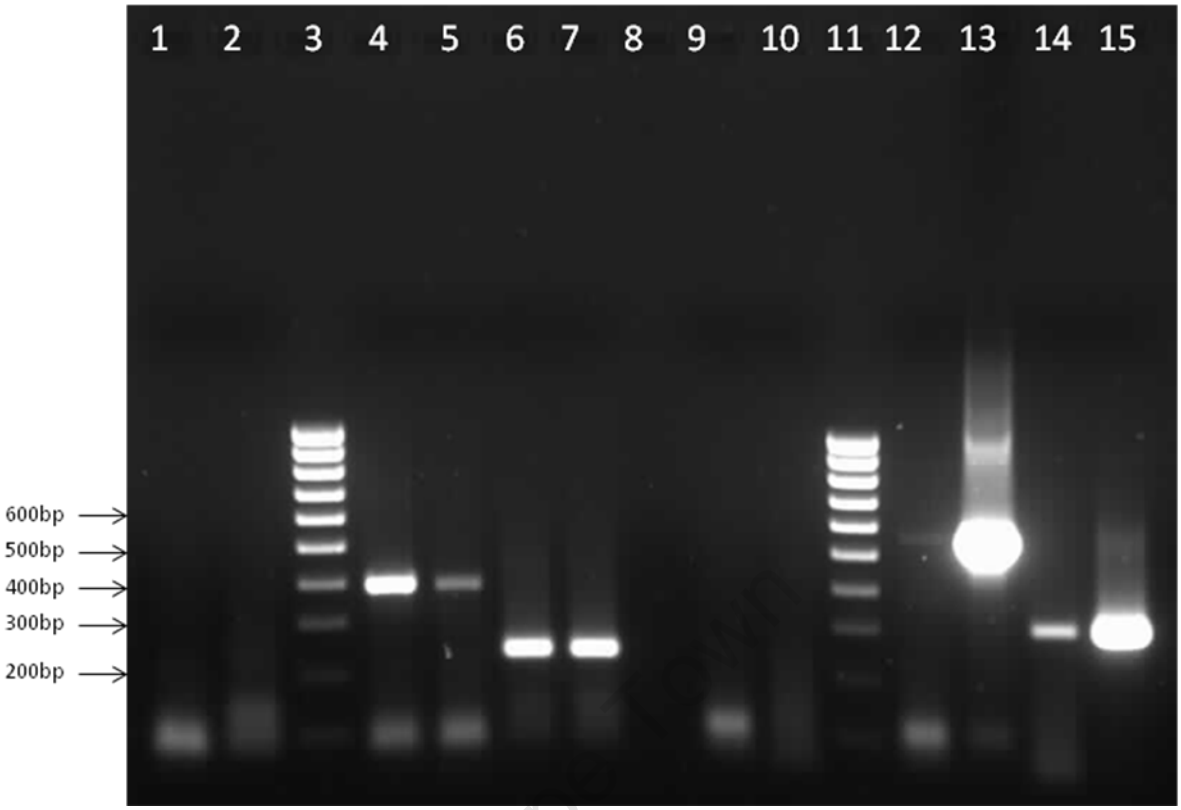


Figure 3.6. Agarose gel electrophoresis of PCR products obtained using PS1, PS2, PS3 and PS4 for the detection of mixed W-Beijing and non-W-Beijing infections. Lane 1: PS1 negative water control; Lane 2: PS2 negative water control; Lane 3: Hyperladder IV; Lane 4: PS1 W-Beijing control; Lane 5: PS1 non-W-Beijing control; Lane 6: PS2 W-Beijing control; Lane 7: PS2 non-W-Beijing; Lane 8: Void; Lane 9: PS3 negative water control; Lane 10: PS4 negative water control; Lane 11: Hyperladder IV; Lane 12: PS3 W-Beijing control; Lane 13: PS3 non-W-Beijing control; Lane 14: PS4 W-Beijing control; Lane 15: PS4 non-W-Beijing control.

No signal was detected in the negative water controls ensuring no contamination of the reagents used (Figure 3.6; Lanes 1, 2, 9, 10). Products of expected size of 393bp and 239bp were obtained using PS1 and PS2, respectively (Figure 3.6; Lanes 4 and 5, and 6 and 7). Though PS1 and PS2 are designed to amplify W-Beijing only, products were obtained from the non-W-Beijing control in both PS1 and PS2. PCR products of 570bp and 309bp were observed using PS3 and PS4, respectively, when using non-W-Beijing as template. Additionally, amplification occurred in the W-Beijing control using PS3 and PS4, respectively (Figure 3.6; Lanes 12 and 14). The detection of non-W-Beijing using W-Beijing-specific primers, and vice versa, indicated that the PCR assay conditions were not fully optimized.

As products of equal intensity were obtained from both the W-Beijing and non-W-Beijing controls using PS2, optimizations were carried out on the non-W-Beijing control so as to detect conditions that would prevent non specific amplification from H37Rv. Optimizations included primer, $MgCl_2$ and dNTP titrations, as well as adjusting the annealing temperature.

The concentration of primers is a crucial parameter that must be carefully controlled in PCR (Atlas and Bej, 1994). If the primers are prone to dimerisation, too high a concentration will lead to the formation of more primer-dimers. Additionally, if the primer concentration is too high, and the primers are not prone to dimerisation, then the risk of non-specific amplification of spurious products, resulting from mispriming, increases (Atlas and Bej, 1994). A primer titration was performed on H37Rv to determine the optimum primer concentration. Following amplification, the products were separated by agarose gel electrophoresis (Figure 3.7).

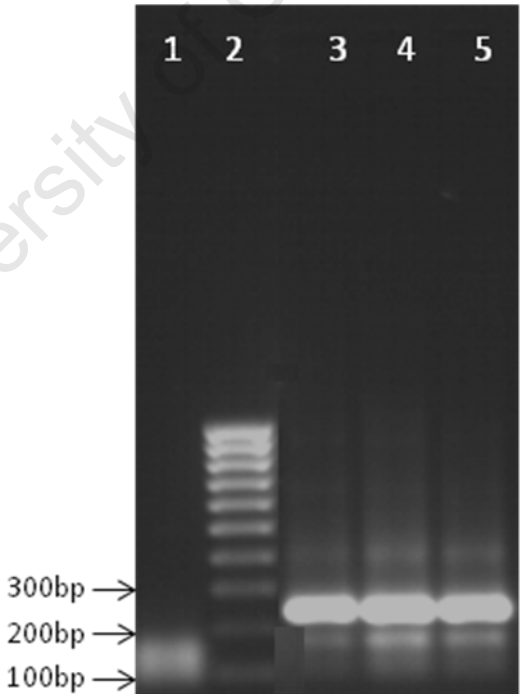


Figure 3.7. Agarose gel electrophoresis of PS2 primer titration. Lane 1: PS2 negative water control; Lane 2: Hyperladder IV; Lane 3: PS2 15pmol; Lane 4: PS2 20pmol; Lane 5: PS2 25pmol.

No signal was observed in the negative water controls, indicating that the reagents used were not contaminated (Figure 3.7; Lane 2). A product, corresponding to 239bp, was obtained from H37Rv genomic DNA using 15pmol, 20pmol and 25pmol of each primer in assay PS2 (Figure 3.7; Lanes 3 – 5)

Since dNTPs, the DNA polymerase and primers all require Mg for optimum binding, $MgCl_2$ is essential for DNA:DNA interactions and binding (Atlas and Bej, 1994). The dNTPs compete for magnesium more than any of the other components, and as such, requires an $MgCl_2$ concentration higher than that of the dNTPs (Atlas and Bej, 1994). However, higher concentrations of $MgCl_2$ can increase the presence of non-specific products, as mismatching of the primers to the template is tolerated better at higher concentrations of $MgCl_2$. Too little Mg^{2+} and little to no products are formed (Atlas and Bej 1994). The concentration of dNTPs is also a crucial parameter to control. The higher the concentration of dNTPs the more Mg^{2+} is sequestered in the reaction resulting in less free Mg^{2+} resulting in poor polymerase activity (Atlas and Bej, 1994). Thus the interrelationship between dNTPs and $MgCl_2$ is very important in optimizing PCR conditions. A $MgCl_2$ titration as well as a dNTP titration were carried out for assay PS2. Following amplification, the products were separated using agarose gel electrophoresis (Figure 3.8).

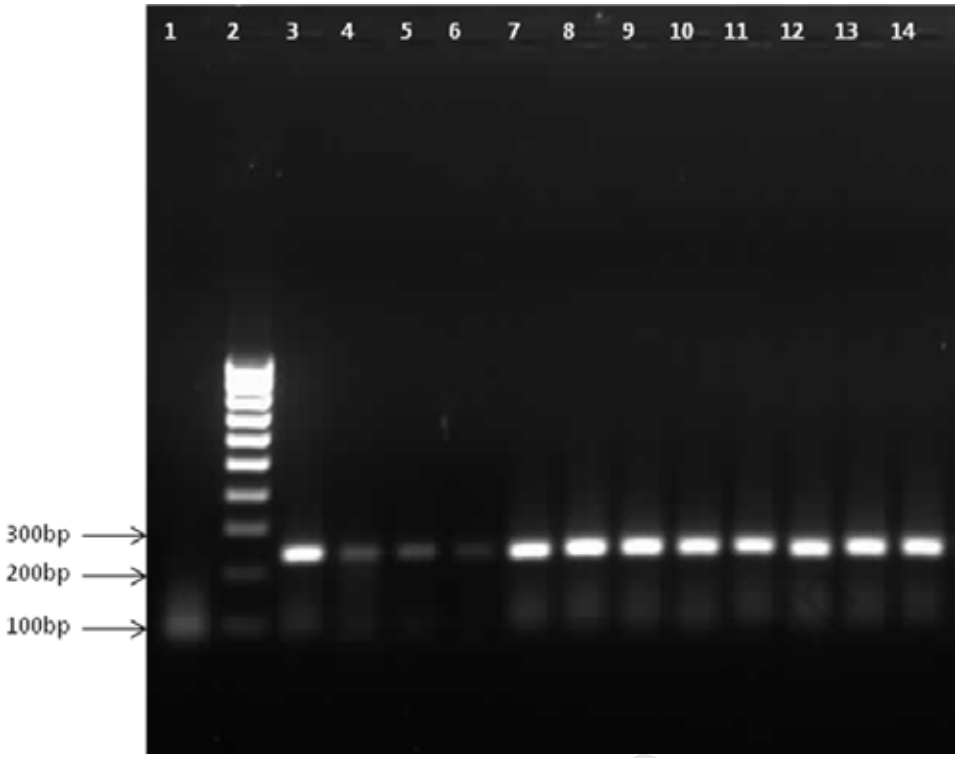


Figure 3.8. Agarose gel electrophoresis of assay PS2 following $MgCl_2$ and dNTP titrations. H37Rv genomic DNA was used as template. Lane 1: negative water control; Lane 2: Hyperladder IV; Lane 3 1mM $MgCl_2$ and 0.1mM dNTP; Lane 4: 1mM $MgCl_2$ and 0.2mM dNTP; Lane 5: 1mM $MgCl_2$ and 0.3mM dNTP; Lane 6: 1mM $MgCl_2$ and 0.4mM dNTP; Lane 7: 2mM $MgCl_2$ and 0.1mM dNTP; Lane 8: 2mM $MgCl_2$ and 0.2mM dNTP; Lane 9: 2mM $MgCl_2$ and 0.3mM dNTP; Lane 10: 2mM $MgCl_2$ and 0.4mM dNTP; Lane 11: 3mM $MgCl_2$ and 0.1mM dNTP; Lane 12: 3mM $MgCl_2$ and 0.2mM dNTP; Lane 13: 3mM $MgCl_2$ and 0.3mM dNTP; Lane 14: 3mM $MgCl_2$ and 0.4mM dNTP.

No signal was observed in the negative water control indicating the reagents were not contaminated (Figure 3.8). Products corresponding to 239bp were obtained in all reactions using H37Rv genomic DNA. Reaction conditions using $MgCl_2$ at 1mM and dNTPs at 0.4mM yielded product with the lowest intensity (Figure 3.8; Lane 6). Subsequent optimizations of dNTPs using 1mM $MgCl_2$, were carried out using H37Rv genomic DNA and DNA from W-Beijing 1 and W-Beijing 2 control strains. Following amplification, the products were separated by agarose gel electrophoresis (Figure 3.9).

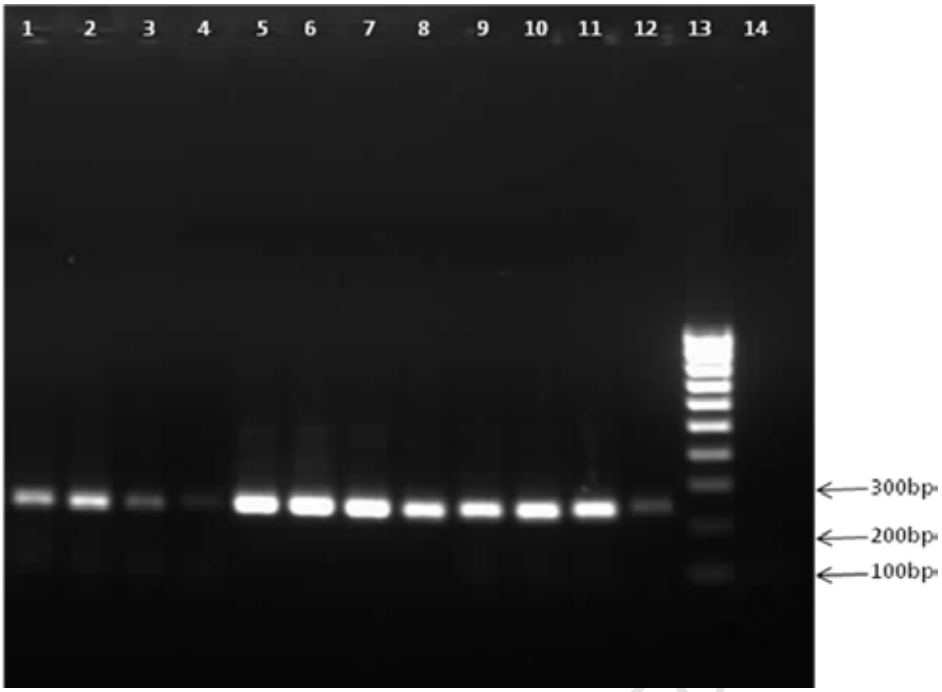


Figure 3.9. Agarose gel electrophoresis of assay PS2 dNTP titration using 1mM $MgCl_2$ on H37Rv and W-Beijing templates. Lane 1: H37Rv 0.1mM, Lane 2: H37Rv 0.2mM ; Lane 3: H37Rv 0.3mM, Lane 4: H37Rv 0.4mM; Lane 5: W-Beijing (1) 0.1mM, Lane 6: W-Beijing (1) 0.2mM ; Lane 7: W-Beijing (1) 0.3mM, Lane 8: W-Beijing (1) 0.4mM; Lane 9: W-Beijing (2) 0.1mM, Lane 10: W-Beijing (2) 0.2mM ; Lane 11: W-Beijing (2) 0.3mM, Lane 12: W-Beijing (2) 0.4mM; Lane 13: Hyperladder IV; Lane 14: negative water control.

No signal was observed in the negative water control, indicating that the reagents used were not contaminated (Figure 3.9). Products corresponding to 239bp were amplified in both H37Rv and W-Beijing 1 and W-Beijing 2 control templates (Figure 3.9; Lanes 1 – 12). Reaction conditions including $MgCl_2$ at 1mM and 0.4mM dNTPs yielded product with the lowest intensity (Figure 3.9; Lane 4) yet retains the amplicons from the W-Beijing controls (Figure 3.9; Lanes 8 and 12). The dNTP concentration was further increased to 0.5mM and 0.6mM in order to reduce the non-specific amplification from the H37Rv template. Following amplification, the products were separated by agarose gel electrophoresis (Figure 3.10).

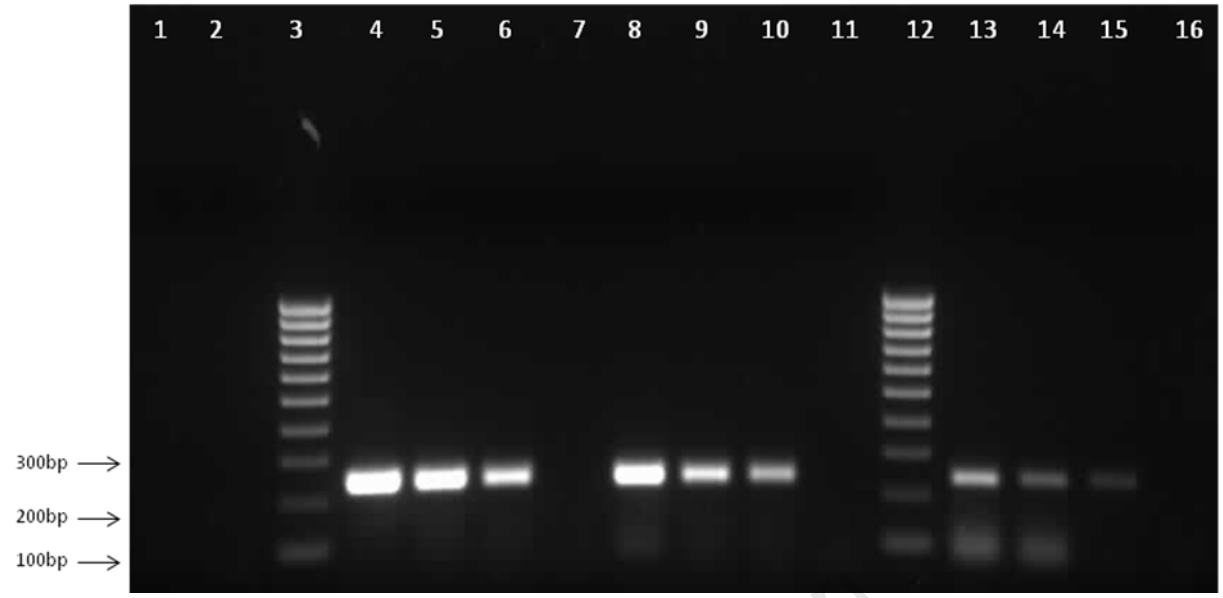


Figure 3.10. Agarose gel electrophoresis of products from assay PS2 with an increase in dNTP concentration using H37Rv and W-Beijing control templates. Lane 1: Negative water control (0.5mM dNTPs); Lane 2: Negative water control (0.6 mM dNTPs); Lane 3: Hyperladder IV; Lane 4: WB (1) – 0.3mM dNTPs; Lane 5: WB (1) – 0.4mM dNTPs; Lane 6: WB (1) – 0.5mM dNTPs; Lane 7: WB (1) – 0.6mM dNTPs; Lane 8: WB (2) – 0.3mM dNTPs; Lane 9: WB (2) – 0.4mM dNTPs; Lane 10: WB (2) – 0.5mM dNTPs; Lane 11: WB (2) – 0.6mM dNTPs; Lane 12: Hyperladder IV; Lane 13: H37Rv – 0.3mM dNTPs; Lane 14: H37Rv – 0.4mM dNTPs; Lane 15: H37Rv – 0.5mM dNTPs; Lane 16: H37Rv – 0.6mM dNTPs.

No signal was observed in either of the negative controls, ruling out the possibility of contamination (Figure 3.10). The expected products of 239bp were observed for both W-Beijing controls (1 and 2) (Figure 3.10; Lanes 4 – 11). The same size product was also observed from the non-W-Beijing control (Figure 3.10; Lanes 13 – 16). An increase in dNTP concentration to 0.6mM results in the absence of signal from all templates (Figure 3.10; Lanes 7, 11 and 16). This is most likely due to the increase in demand of free $MgCl_2$ from the increase in dNTPs resulting in inadequate amounts of $MgCl_2$ for the polymerase to function. Thus it appears that 0.5mM dNTPs results in a decrease of the signal from H37Rv while still maintaining the W-Beijing-specific signals.

Another critical parameter in the PCR is the annealing temperature. This is the temperature at which the primers anneal to the template. Generally the optimum annealing temperature is 5°C lower than the melting temperature of the primers (Atlas and Bej, 1994). Annealing temperatures that are too low can cause mis-priming of the primers and can result in the non-specific amplification of undesirable products (Atlas and Bej, 1994). Temperatures too high will

prevent the primers from binding to the template and can result in no amplification of the target sequence (Atlas and Bej, 1994). The annealing temperature for the PS2 primers was increased from 62°C to 64°C in order to decrease the non-specific products. Additionally, the concentration of dNTPs was assessed at 0.4mM and 0.5mM at 64°C. The $MgCl_2$ concentration was maintained at 1mM. Following amplification, the products were separated using agarose gel electrophoresis (Figure 3.11).

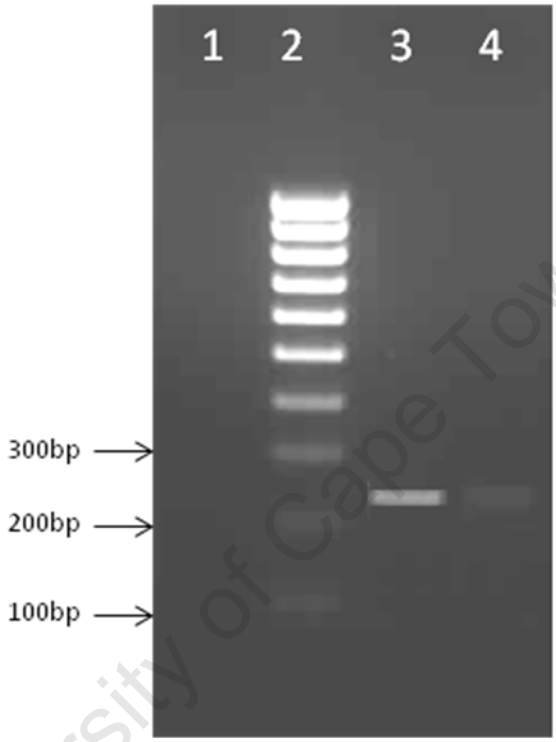


Figure 3.11. Agarose gel electrophoresis of products from PS2 with increased dNTPs an annealing temperature using H37Rv control template. Lane 1: Negative water control; Lane 2: Hyperladder IV; Lane 3: 0.4mM dNTPs and 64°C; Lane 4: 0.5mM dNTPs and 64°C.

No signal was observed in the negative water control indicating that the reagents used were not contaminated (Figure 3.11). Products of 239bp were amplified using the H37Rv control DNA (Figure 3.11; Lanes 3 – 4). Amplification conditions using 1mM $MgCl_2$ and 0.5mM dNTPs at 64°C decreased the intensity of the observed product (Figure 3.11; Lane 4).

An additonal increase in $MgCl_2$ and dNTP concentration at both 62°C and 64°C and whether or not these conditions could improve the amplification, was assessed. Following amplification the products were separated by agarose gel electrophoresis (Figure 3.12).

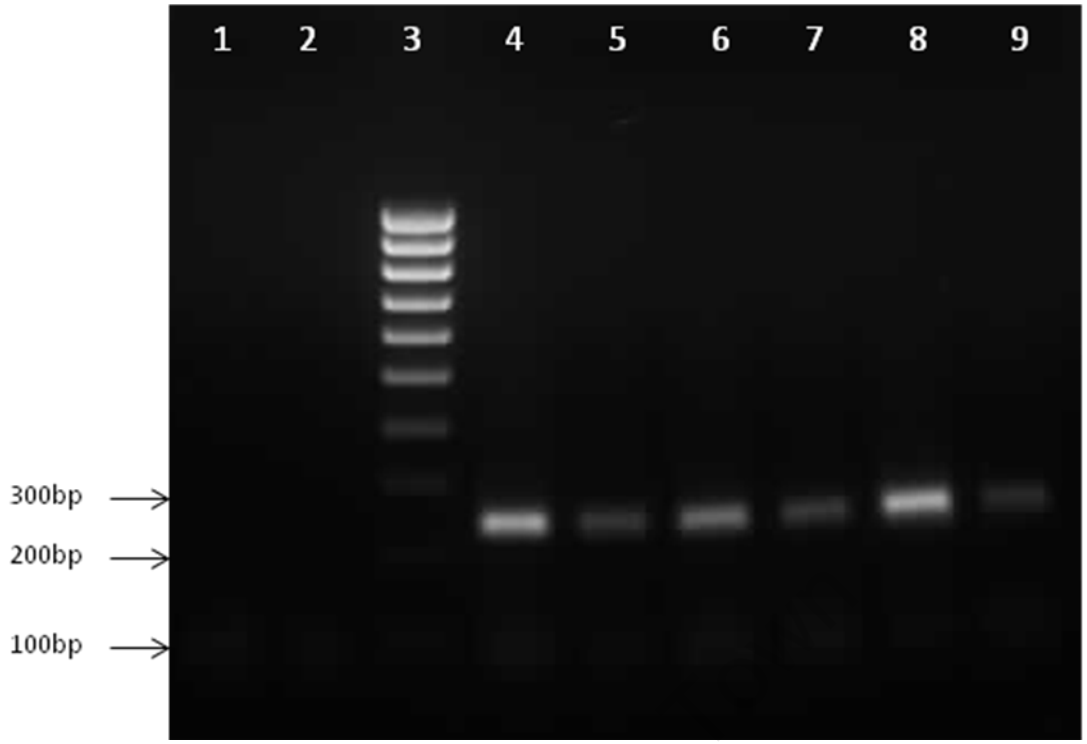


Figure 3.12. Agarose gel electrophoresis of products from PS2 $MgCl_2$, dNTP and annealing temperature optimizations. Lane 1: Negative water control (1.5mM $MgCl_2$, 0.6mM dNTPs and 62°C); Lane 2: Negative water control (1.5mM $MgCl_2$, 0.6mM dNTPs and 64°C); Lane 3: Hyperladder IV; Lane 4: 1.5mM $MgCl_2$, 0.4mM dNTPs and 62°C; Lane 5: 1.5mM $MgCl_2$, 0.4mM dNTPs and 64°C; Lane 6: 1.5mM $MgCl_2$, 0.5mM dNTPs and 62°C; Lane 7: 1.5mM $MgCl_2$, 0.5mM dNTPs and 64°C; Lane 8: 1.5mM $MgCl_2$, 0.6mM dNTPs and 62°C; Lane 9: 1.5mM $MgCl_2$, 0.6mM dNTPs and 64°C.

No signal was observed in either of the negative water controls, indicating the reagents used were not contaminated (Figure 3.12). Products corresponding to 239bp were observed for the non-W-Beijing H37Rv control (Figure 3.12; Lanes 4 – 9). Amplification conditions with 1.5mM $MgCl_2$ at 64°C, regardless of the dNTP, concentration yielded products of lesser intensity (Figure 3.12; Lane 5; 7 and 9). The intensity, however, is still less at 1mM $MgCl_2$ and 0.5mM dNTPs at 64°C (Figure 3.11). However, additional increases in dNTPs cannot occur without additional $MgCl_2$, as the signal observed from the W-Beijing control will also decrease. Therefore, final amplification conditions of 1mM $MgCl_2$ and 0.5mM dNTPs at 64°C reduced the intensity of the 239bp from the H37Rv non-W-Beijing control significantly. However further optimizations are still clearly needed to ensure no amplification of these products, and could include the use of alternative DNA polymerase systems and corresponding buffers, using enhancers, such as those found in the HotStartTaq system (Solution Q; Qiagen, Germany), or to redesign the primers for amplification in a similar region.

Several optimizations were similarly carried out for PS1, PS3 and PS4 using the H37Rv and W-Beijing (1) control strains as template (data not shown). Firstly, both the forward and reverse primers were titrated by adding 1pmol, 5pmol, 10pmol, 15pmol, 20pmol or 25pmol of each. PCR amplification of the expected products was optimal at 15pmol. $MgCl_2$ was titrated from 1mM to 1.5mM, 2.0mM, 2.5mM, 3.0mM and to the recommended 3.5mM; amplification at 1.5mM yielded the more intense product. Using 15pmol of each primer and 1mM of $MgCl_2$, dNTP titrations were carried out. The recommended conditions use 4mM dNTPs, which is unusually high for a PCR assay. Following titration, 0.4mM of dNTPs yielded the most intense product. Finally, the number of amplification cycles was reduced from 45 cycles to 35 cycles, in order to reduce the intensity of the non-specific amplification without compromising the intensity of the positive controls. Amplification using 30 cycles reduced the intensity of the non-specific products. PCR amplification for PS1, PS3 and PS4 were carried out using a reaction mixture consisting of 15pmol of each primer, 1mM $MgCl_2$, 0.4mM dNTPs and 1U of *GoTaq* in the buffer supplied. Cycling conditions were as follows: an initial denaturation of 15min at 95°C, followed by 35 cycles of 94°C for 1min, 62°C for 1min and 72°C for 1min. Finally an additional elongation of 10min at 72°C completed the amplification.

These conditions were carried out using W-Beijing and non-W-Beijing controls. Following amplification, products were separated by agarose gel electrophoresis (Figure 3.13).

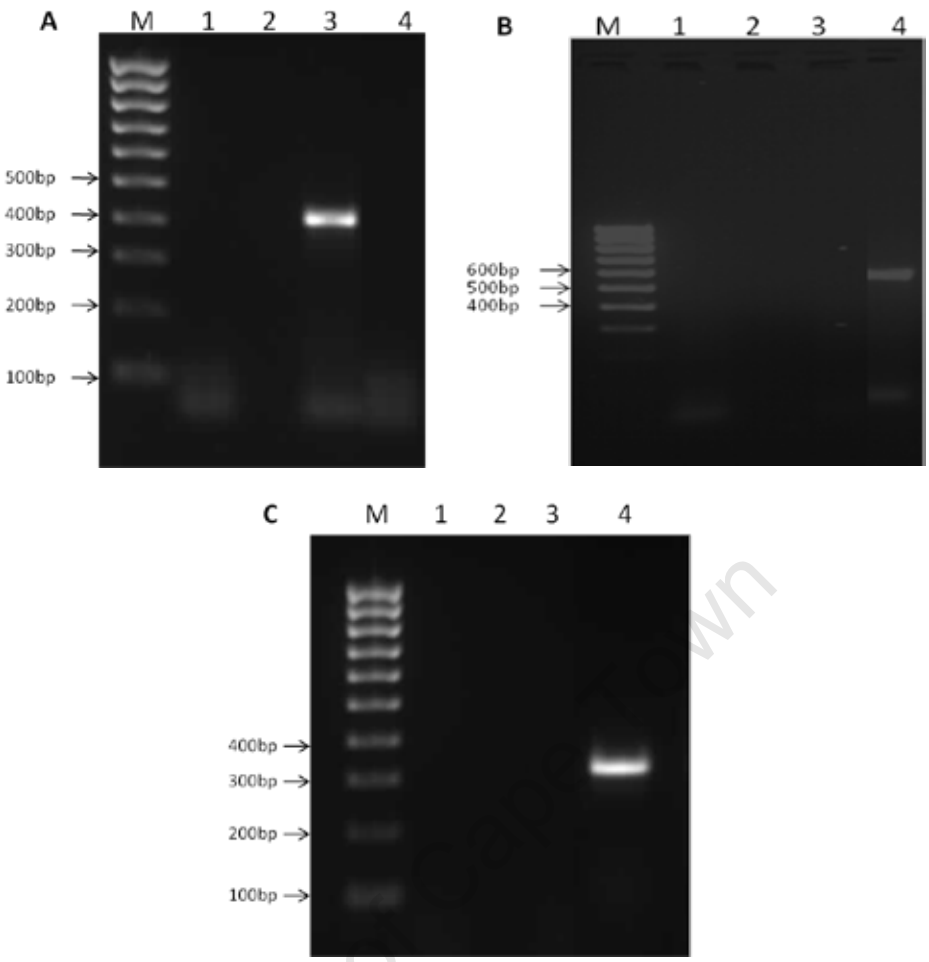


Figure 3.13. Agarose gel electrophoresis of A: PS1, B: PS3 and C: PS4, using W-Beijing 1 and non-W-Beijing control strains. A – M: Hyperladder IV; Lane 1: negative water control; Lane 2: Void; Lane 3:W-Beijing control; Lane 4: non-W-Beijing control. B – M: Hyperladder IV; Lane 1: negative water control; Lane 2: Void; Lane 3: W-Beijing control; Lane 4: non-W-Beijing control. C – M: Hyperladder IV; Lane 1: negative water control; Lane 2: Void; Lane 3: W-Beijing control; Lane 4: non-W-Beijing control.

No signal was observed in the negative water controls, indicating that the reagents used were not contaminated (Figure 3.13). Products corresponding to 393bp was observed for the W-Beijing control (Figure 3.13; A: Lane 3) and was absent in the non-W-Beijing control (Figure 3.13; A: Lane 4), as expected. Products of 570bp was observed in the non-W-Beijing control (Figure 3.12; B: Lane 4) and was absent in the W-Beijing control (Figure 3.13; B: Lane 3), as expected. Similarly, products corresponding to 309bp was observed in the non-W-Beijing control (Figure 3.13; C: Lane 4), while it was absent in the W-Beijing control (Figure 3.13; C: Lane 3), as expected.

3.5.1. Screening of isolates from patients confirmed by spoligotyping as harbouring mixed infections

Since conditions for PS1 and PS3 were optimized (3.5), these assays, which detect W-Beijing and non-W-Beijing, respectively, were carried out on all isolates from the 6 patients indicated as carrying mixed infections following spoligotyping. Genomic DNA from isolates obtained from each patient (2.2.1) was used as template in the PCR assays. Following amplification, products were separated by agarose gel electrophoresis for PS1 (Figure 3.14), and PS3 (Figure 3.15).

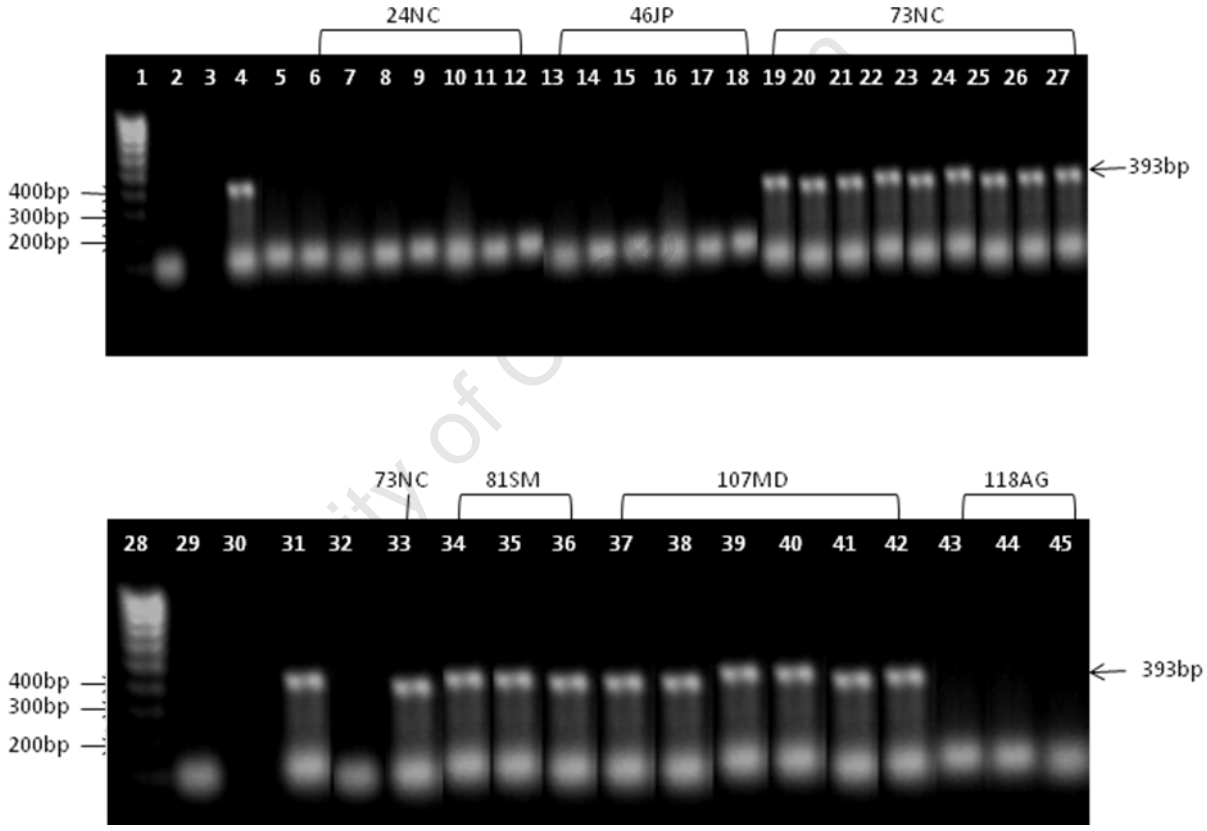


Figure 3.14. Agarose gel electrophoresis of products of assay PS1 from mixed samples. Lane 1: Hyperladder IV; Lane 2: negative water control; Lane 3: VOID; Lane 4: W-Beijing control; Lane 5: non-W-Beijing control; Lane 6: 24NC week (W) 0; Lane 7: 24NC W1; Lane 8: 24NC W2; Lane 9: 24NC W4; Lane 10: 24NC W4; Lane 11: 24NC W5; Lane 12: 24NC W6; Lane 13: 46JP W0; Lane 14: 46JP W1; Lane 15: 46JP W2; Lane 16: 46JP W3; Lane 17: 46JP W4; Lane 18: 46JP W5; Lane 19: 73NC W0; Lane 20: 73NC W1; Lane 21: 73NC W2; Lane 22: 73NC W3; Lane 23: 73NC W4; Lane 24: 73NC W5; Lane 25: 73NC W6; Lane 26: 73NC W7; Lane 27: 73NC W8; Lane 28: Hyperladder IV; Lane 29: negative water control; Lane 30: VOID; Lane 31: W-Beijing control; Lane 32: non-W-Beijing control; Lane 33: 73NC W9; Lane 34: 81SM W0; Lane 35: 81SM W1; Lane 36: 81SM W2; Lane 37: 107MD W0; Lane 38: 107MD W1; Lane 39: 107MD W2; Lane 40: 107MD W3; Lane 41: 107MD W4; Lane 42: 107MD W5; Lane 43: 118AG W0; Lane 44: W1; Lane 45: W2.

No signal was observed in the negative water control, indicating that the reagents used were not contaminated (Figure 3.14). Products corresponding to 393bp were observed for the W-Beijing control (Figure 3.14; Lanes 4 and 31). These products were not observed in the non-W-Beijing control (Figure 3.14; Lanes 5 and 32). Isolates from week (W) 0 – 5 from patient 24NC were indicated as being from the T4 lineage following spoligotyping, with the isolate from W6 being indicated as belonging to the S lineage. The absence of products corresponding to 393bp confirms the results that all isolates from this patient belong to the non-W-Beijing group of strains (Figure 3.14; Lanes 6 – 12). Similarly, isolates from W0 – 3 from patient 46JP were indicated as being from the T1 lineage following spoligotyping, with isolates from W4 – 5 belonging to the S lineage. The absence of products corresponding to 393bp confirms the result that all isolates from this patient belong to the non-W-Beijing group of strains (Figure 3.14; Lanes 13 – 18).

Isolates from W0 – 2 from patient 73NC were indicated as being from the W-Beijing lineage, with the isolate from W3 belonging to the T4 lineage, and isolates from W4 – 9 belonging to the W-Beijing lineage. Products corresponding to 393bp in all isolates confirm the result that these isolates belong to the W-Beijing lineage, and that the W-Beijing isolate is still present at W2 (Figure 3.14; Lanes 19 – 27, and 33). Isolates from W0 – 3 from patient 81SM were identified as belonging to the W-Beijing lineage by spoligotyping, with the emergence of an additional X3 isolate in W3. Products for 393bp for all isolates confirm the result that these isolates belong to the W-Beijing lineage (Figure 3.14; Lanes 34 – 36). Similarly isolates from W0 – 5 from patient 107MD, were identified as belonging to the W-Beijing lineage by spoligotyping, with the emergence of an additional X3 isolate in W4 – 5. Products of 393bp in all isolates confirm the result that these isolates belong to the W-Beijing lineage (Figure 3.14; Lanes 37 – 42). Isolates from W0 – 1 from patient 118AG were identified as belonging to the LAM3 lineage by spoligotyping, and from the T4 lineage in W2. The absence of products corresponding to 393bp in all isolates confirms the result that these isolates belong to the non-W-Beijing group of strains (Figure 3.14; Lanes 43 – 45).

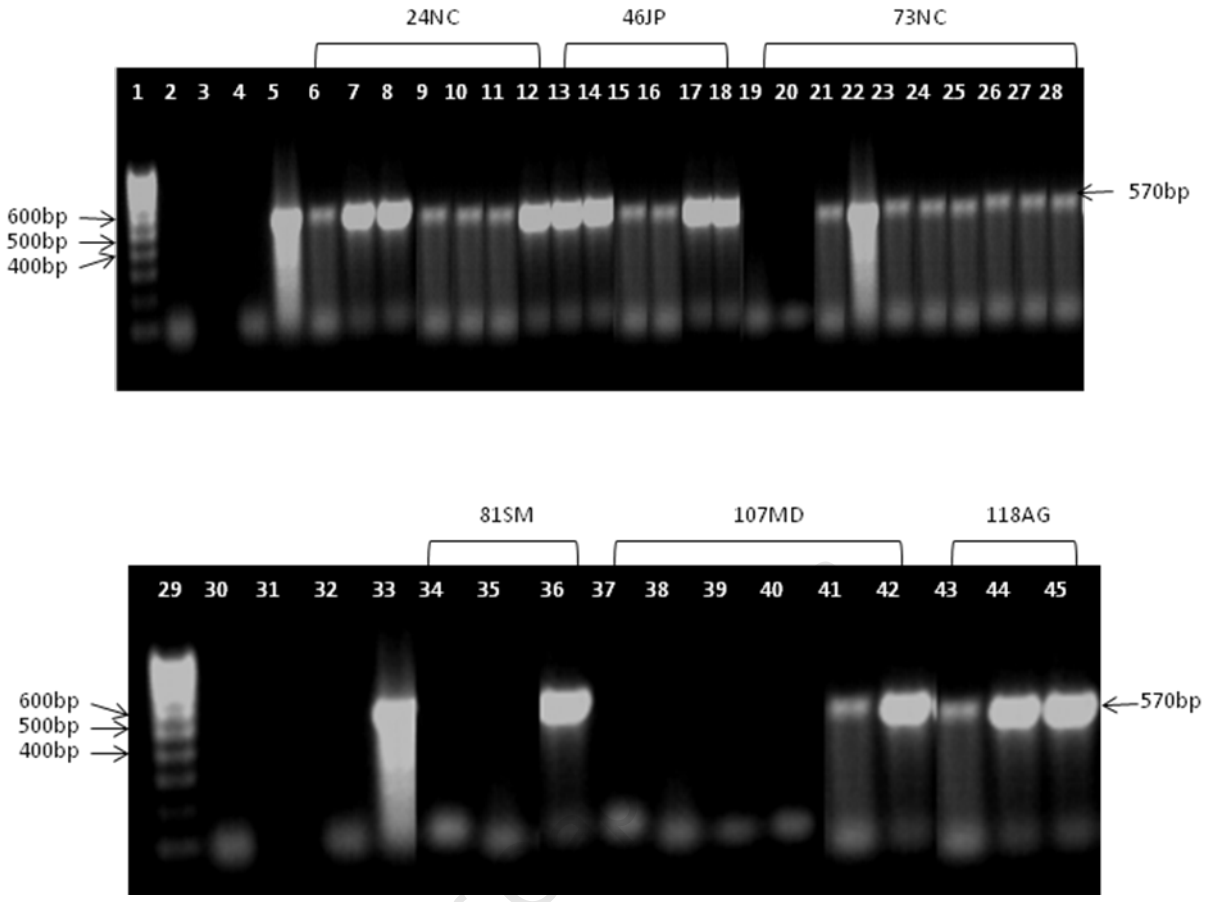


Figure 3.15. Agarose gel electrophoresis of products of assay PS3 from mixed samples. Lane 1: Hyperladder IV; Lane 2: negative water control; Lane 3: VOID; Lane 4: W-Beijing control; Lane 5: non-W-Beijing control; Lane 6: 24NC week (W) 0; Lane 7: 24NC W1; Lane 8: 24NC W2; Lane 9: 24NC W4; Lane 10: 24NC W4; Lane 11: 24NC W5; Lane 12: 24NC W6; Lane 13: 46JP W0; Lane 14: 46JP W1; Lane 15: 46JP W2; Lane 16: 46JP W3; Lane 17: 46JP W4; Lane 18: 46JP W5; Lane 19: 73NC W0; Lane 20: 73NC W1; Lane 21: 73NC W2; Lane 22: 73NC W3; Lane 23: 73NC W4; Lane 24: 73NC W5; Lane 25: 73NC W6; Lane 26: 73NC W7; Lane 27: 73NC W8; Lane 28: 73NC W9; Lane 29: Hyperladder IV; Lane 30: negative water control; Lane 31: VOID; Lane 32: W-Beijing control; Lane 33: non-W-Beijing control; Lane 34: 81SM W0; Lane 35: 81SM W1; Lane 36: 81SM W2; Lane 37: 107MD W0; Lane 38: 107MD W1; Lane 39: 107MD W2; Lane 40: 107MD W3; Lane 41: 107MD W4; Lane 42: 107MD W5; Lane 43: 118AG W0; Lane 44: W1; Lane 45: W2.

No signal was observed in the negative water controls indicating that the reagents used were not contaminated (Figure 3.15). Products corresponding to 570bp were observed in the non-W-Beijing control (Figure 3.15; Lanes 5 and 33), while were absent in the W-Beijing control (Figure 3.15; Lanes 4 and 32). Isolates from week (W) 0 – 5 from patient 24NC were indicated as being from the T4 lineage following spoligotyping, with the isolate from W6 being indicated as belonging to the S lineage. The presence of products corresponding to 570bp confirms the results that all isolates from this patient belong to the non-W-Beijing group of strains (Figure 3.15; Lanes 6 – 12). Similarly, isolates from W0 – 3 from patient 46JP were indicated as being

from the T1 lineage following spoligotyping, with isolates from W4 – 5 belonging to the S lineage. The presence of products corresponding to 570bp confirms the result that all isolates from this patient belong to the non-W-Beijing group of strains (Figure 3.15; Lanes 13 – 18). Isolates from W0 – 2 from patient 73NC were indicated as being from the W-Beijing lineage, with the isolate from W3 belonging to the T4 lineage, and isolates from W4 – 9 belonging to the W-Beijing lineage. Products corresponding to 570bp in isolates from W2 – W9 confirm the result that these isolates harbour the T4 lineage, with the products in W3 being the most intense. The absence of products corresponding to 570bp in isolates from W0 – 1 indicates that the emergence of the T4 isolate did not occur until W2 (Figure 3.15; Lanes 19 – 28).

Isolates from W0 – 3 from patient 81SM were identified as belonging to the W-Beijing lineage by spoligotyping, with the emergence of an additional X3 isolate in W3. Products corresponding to 570bp the isolate on W2 confirm the result that that isolate belongs to the non-W-Beijing lineage. The absence of the product in W0 – 1, indicates that the emergence of the X3 did not occur until W2 (Figure 3.15; Lanes 34 – 36). Similarly isolates from W0 – 5 from patient 107MD, were identified as belonging to the W-Beijing lineage by spoligotyping, with the emergence of an additional X3 isolate in W4 – 5. Products of 570bp in isolates from W4 - 5 confirms the result that these isolates belong to the non-W-Beijing lineage. The absence of products in W0 – 3 indicates that the emergence of the X3 isolate did not occur until W4 (Figure 3.15; Lanes 37 – 42). Isolates from W0 – 1 from patient 118AG were identified as belonging to the LAM3 lineage by spoligotyping, and from the T4 lineage in W2. The presence of products corresponding to 570bp in all isolates confirms the result that these isolates belong to the non-W-Beijing group of strains (Figure 3.15; Lanes 43 – 45). Therefore both assays PS1 and PS3 confirms the results from spoligotyping.

3.5.2. Screening for additional mixed isolates in the study population

To determine whether any additional patients harbour mixed isolates, the first and last isolate from each of the 129 patients included in this study, were screened with PS1 and PS3. The first and last isolates were screened as was previously noticed, the majority of mixed isolates

occurred in the final samples. Following amplification products were separated by agarose gel electrophoresis for PS1 (Figure 3.16) and PS3 (Figure 3.17).

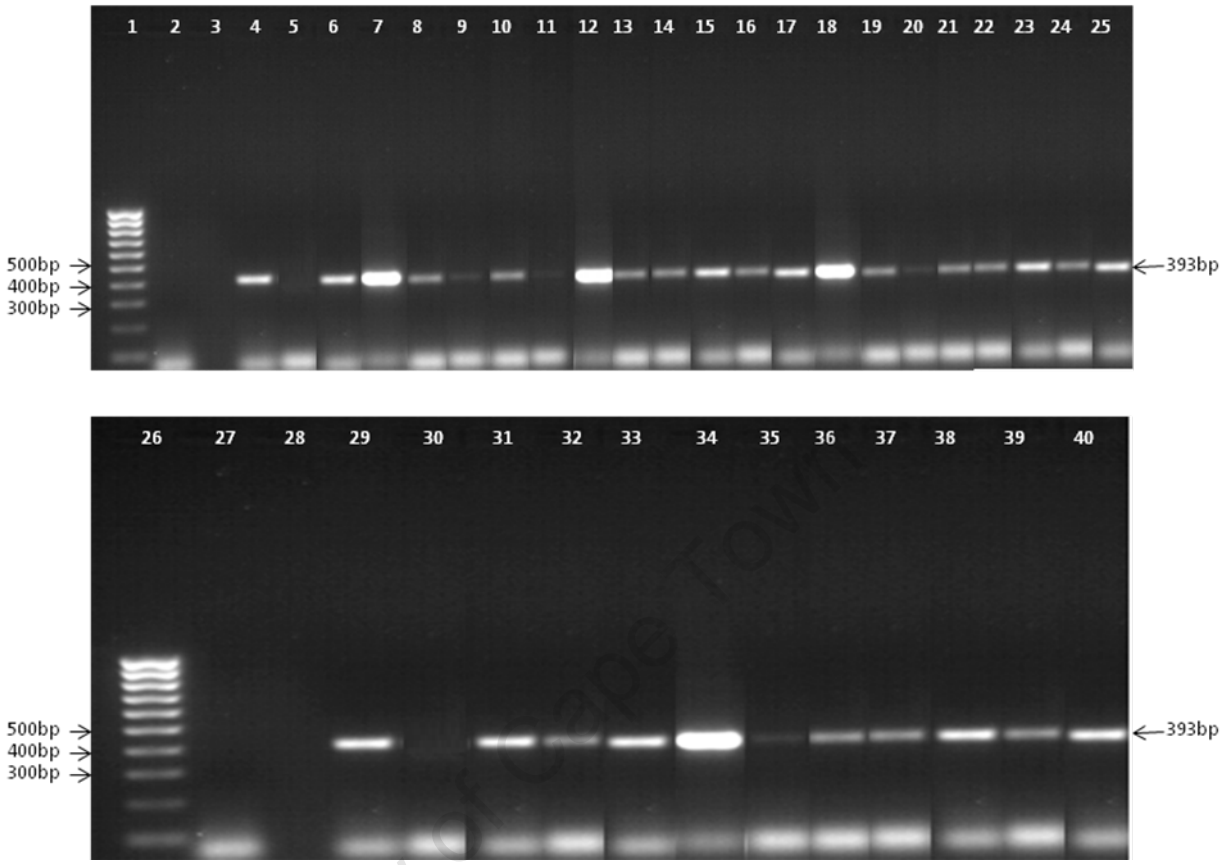


Figure 3.16. Agarose gel electrophoresis of products from PS1 on first and last isolate of 129 patients included in the study. Lane 1: Hyperladder IV; Lane 2: negative water control; Lane 3: VOID; Lane 4: W-Beijing control; Lane 5: non-W-Beijing control; Lane 6: 20TM W0; Lane 7: 20TM W6; Lane 8: 54ER W0; Lane 9: 54 W4; Lane 10: 61WM W0; Lane 11: 61WM W7; Lane 12: 79RC W0; Lane 13: 79RC W6; Lane 14: 80GI W0; Lane 15: 80GI W6; Lane 16: 85SA W0; Lane 17: 85SA W3; Lane 18: 86AF W0; Lane 19: 86AF W7; Lane 20: 87GK W0; Lane 21: 87GK W8; Lane 22: 95AM W0; Lane 23: 95AM W8; Lane 24: 101GP W0; Lane 25: 101GP W7; Lane 26: Hyperladder IV; Lane 27: negative water control; Lane 28: VOID; Lane 29: W-Beijing control; Lane 30: non-W-Beijing control; Lane 31: 104MM W0; Lane 32: 104MM W6; Lane 33: 105AD W0; Lane 34: 105AD W8; Lane 35: 114VJ W0; Lane 36: 114VJ W1; Lane 37: 117TS W0; Lane 38: 117TS W7; Lane 39: 121VS W0; Lane 40: 121VS W2.

No signal was observed in the negative water control indicating that the reagents used were not contaminated (Figure 3.16). Products corresponding to 393bp were observed for the W-Beijing control (Figure 3.16; Lanes 4 and 29), and were absent in the non-W-Beijing control (Figure 3.16; Lanes 5 and 30). All additional patients screened were identified as harbouring W-Beijing isolates as determined by spoligotyping. Products corresponding to 393bp were observed for both the first and last isolate for each patient, confirming the result that these isolates belong to the W-Beijing lineage (Figure 3.16; Lanes 6 – 25, and 31 – 40).

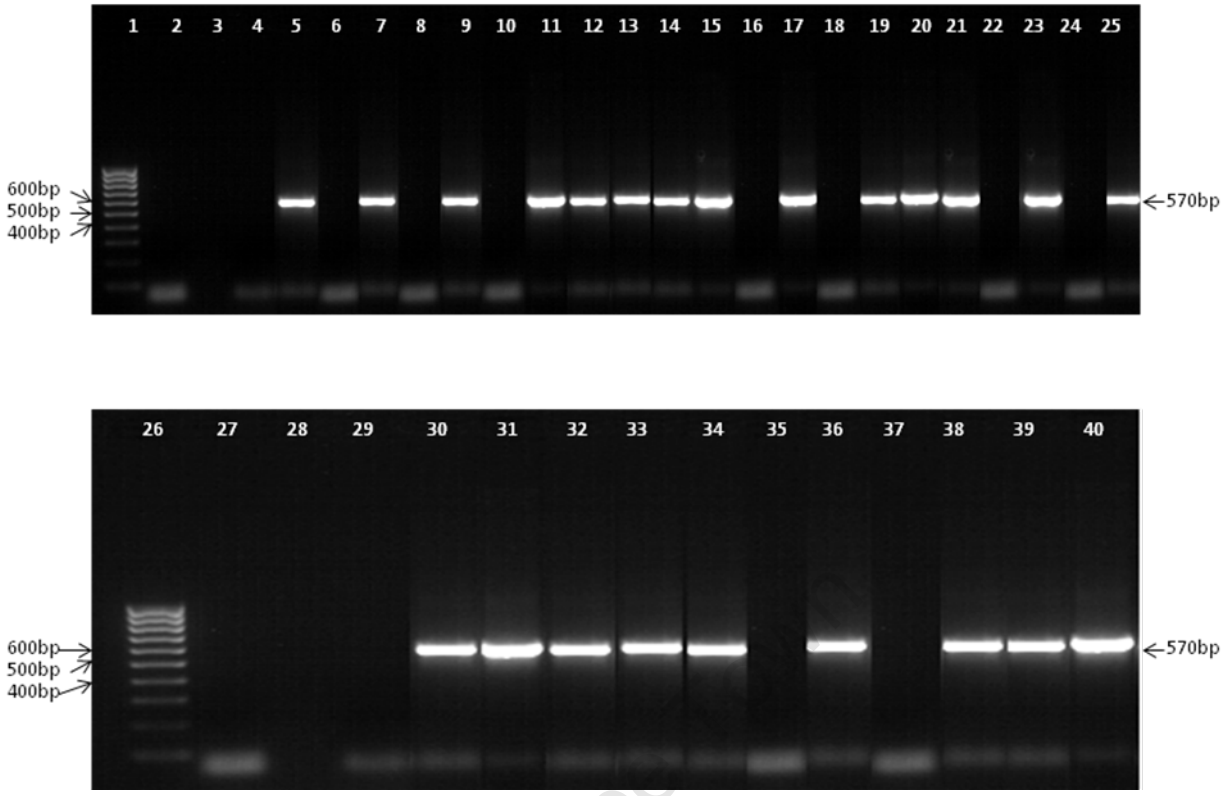


Figure 3.17. Agarose gel electrophoresis of products from PS3 on first and last isolate of 129 patients included in the study. Lane 1: Hyperladder IV; Lane 2: negative water control; Lane 3: VOID; Lane 4: W-Beijing control; Lane 5: non-W-Beijing control; Lane 6: 20TM W0; Lane 7: 20TM W6; Lane 8: 54ER W0; Lane 9: 54 W4; Lane 10: 61WM W0; Lane 11: 61WM W7; Lane 12: 79RC W0; Lane 13: 79RC W6; Lane 14: 80GI W0; Lane 15: 80GI W6; Lane 16: 85SA W0; Lane 17: 85SA W3; Lane 18: 86AF W0; Lane 19: 86AF W7; Lane 20: 87GK W0; Lane 21: 87GK W8; Lane 22: 95AM W0; Lane 23: 95AM W8; Lane 24: 101GP W0; Lane 25: 101GP W7; Lane 26: Hyperladder IV; Lane 27: negative water control; Lane 28: VOID; Lane 29: W-Beijing control; Lane 30: non-W-Beijing control; Lane 31: 104MM W0; Lane 32: 104MM W6; Lane 33: 105AD W0; Lane 34: 105AD W8; Lane 35: 114VJ W0; Lane 36: 114VJ W1; Lane 37: 117TS W0; Lane 38: 117TS W7; Lane 39: 121VS W0; Lane 40: 121VS W2.

No signal was observed in the negative water control indicating that the reagents used were not contaminated (Figure 3.17). Products corresponding to 570bp were observed in the non-W-Beijing control (Figure 3.17; Lanes 5 and 30), while were absent in the W-Beijing control (Figure 3.17; Lanes 4 and 29). Products of 570bp were observed in all of the last isolates in each patient screened, indicating the emergence of a non-W-Beijing isolate (Figure 3.17; Lanes 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 31, 33, 36, 38, and 40). Products of 570bp were additionally observed in the W0 isolate in patients 79RC, 80GI, 87GK, 104MM, 105AD, and 121VS, indicating that the presence of the non-W-Beijing isolate was part of the initial infection at the start of the study period (Figure 3.17; Lanes 12, 14, 20, 31, 33, and 39).

A total of 15 patients (20TM, 54GR, 61WM, 79RC, 80GI, 85SA, 86AF, 87GK, 95AM, 101GP, 104MM, 105AD, 114VJ, and 121VS) were indicated as carrying mixed isolates from the screening with PS1 and PS3. This increases the number of mixed infections detected to 21 patients (16.3%).

3.6. Differentiation of mixed non-W-Beijing *M. tuberculosis* isolates identified from clinical samples by spoligotyping.

A PCR algorithm, consisting of 3 sequential PCRs, was developed to delineate the major non-W-Beijing isolates circulating in the Western Cape, by the amplification or the lack of amplification of regions of difference (RoD) associated with each of the LAM, X, T and H strains (2.9). Sequentially, the algorithm will delineate the sample to each of the major circulating strain families. Genomic DNA, extracted from colonies grown from the suspected mixed MGIT cultures on LJ slopes (2.2.1), was used as template in the subsequent PCR assays.

The control isolates of the LAM (LAM 1, LAM 2, and LAM 3), T (T 1, T 2 and T 3), X (X 1, X 2 and X 3) and H (H 1, H 2 and H 3) families (kindly provided by J. Evans) as detected by spoligotyping, were used to optimize the RoD1, RoD2 and RoD3 assays (data not shown). Additionally, W-Beijing control 1 was included as a negative control as the regions amplified in RoD1, RoD2 and RoD3, are deleted in all W-Beijing isolates.

For the optimization of RoD1, a $MgCl_2$ titration was performed by adding 1mM, 1.5mM, 2.0mM, 2.5mM, 3.0mM, 3.5mM, 4.0mM, and 4.5mM $MgCl_2$ to the reaction. Amplification at a $MgCl_2$ concentration of 3.5mM yielded products of the greatest intensity. Both the forward and reverse primers of RoD1 were titrated, both individually and together, by adding 25pmol, 20pmol, 15pmol, 10pmol, 5pmol or 1pmol of each to the reaction mixture. Amplification at 10pmol for the forward primer and 5pmol for the reverse primer yielded expected products and decreased the intensity of the non-specific amplicons. Further experiments were carried out and the annealing temperature was increased from 56°C to 58°C, in order to decrease the

intensity of the non-specific amplification further. Amplification of the expected product size occurred at an annealing temperature of 58°C, while decreasing the non-specific amplification. Lastly, the number of amplification cycles was reduced from 35 cycles to 30 cycles, to reduce amplification of non-specific products. The RoD1 PCR assay was carried out using a reaction mixture containing 3.5mM $MgCl_2$, 10pmol forward primer, 5pmol of the reverse primer and 1U of *GoTaq* DNA polymerase. The cycling conditions were 5min at 95°C, followed by 30 cycles of 95°C for 1min, 58°C for 1min and 72°C for 2min. Finally, an elongation at 72°C for 5min completed the amplification. Following amplification, the products were separated by agarose gel electrophoresis (Figure 3.18).

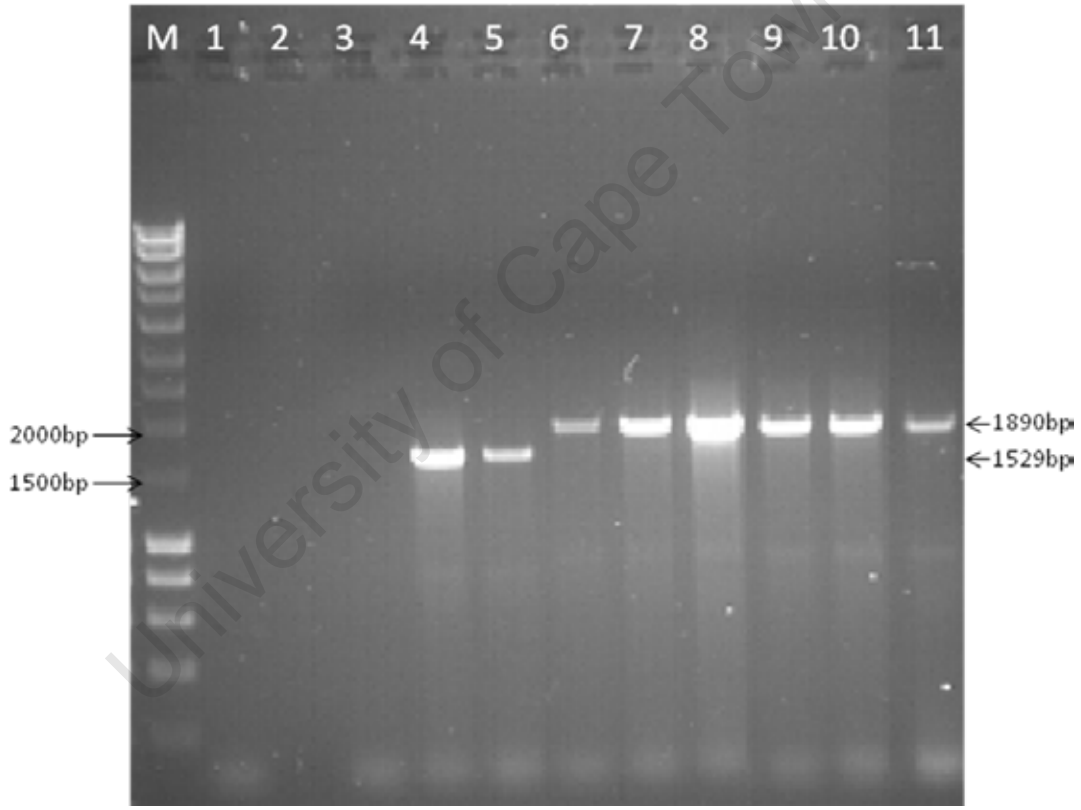


Figure 3.18. Agarose gel electrophoresis of assay RoD1 using control strains as template. HI: Hyperladder I; Lane 1: Negative water control; Lane 2: VOID; Lane 3: W-beijing control 1; Lane 4: LAM 1; Lane 5: LAM 2; Lane 6: T 1; Lane 7: T 2; Lane 8: X 1; Lane 9: X 2; Lane 10: H 1; Lane 11: H 2.

No signal was observed in the negative water control, indicating that the reagents used were not contaminated (Figure 3.18). Products corresponding to 1529bp were observed, as expected, for the LAM control strains (Figure 3.18; Lanes 4 – 5), while the larger 1890bp

product was observed, as expected, for the T, X and H control strains (Figure 3.18; Lanes 6 – 11). No product was observed for the W-Beijing isolate (Lane 3), as the target sequence is deleted in all W-Beijing isolates.

Following RoD1 PCR, the RoD2 assay was carried out to further delineate non-W-Beijing isolates into T, X or H strains. Following amplification, the products were separated by agarose gel electrophoresis (Figure 3.19).

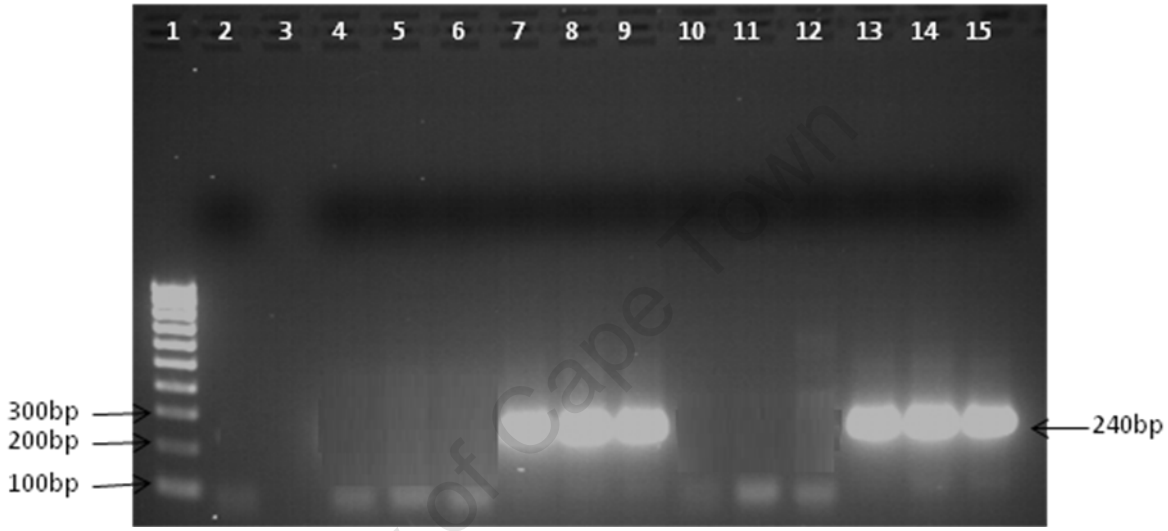


Figure 3.19. Agarose gel electrophoresis of assay RoD2 using control strains as template. Lane 1: Hyperladder IV; Lane 2: Negative water control; Lane 3: VOID; Lane 4: LAM 1; Lane 5: LAM 2; Lane 6: LAM 3; Lane 7: T 1; Lane 8: T 2; Lane 9: T 3; Lane 10: X 1; Lane 11: X 2; Lane 12: X 3; Lane 13: H 1; Lane 14: H 2; Lane 15: H 3.

No signal was observed in the water control, indicating that the reagents used were not contaminated (Figure 3.19). No products were obtained for the X control strains (Figure 3.19; Lanes 10 – 12). Products corresponding to 240bp were observed as expected in the T control strains (Figure 3.19; Lanes 7 – 9) and the H control strains (Figure 3.19; Lanes 13 - 15). No amplification was observed using the LAM control strains (Figure 3.19; Lanes 4 – 6). This served as a confirmation of the LAM result obtained in the RoD1 assay (Figure 3.18).

The presence of the 240bp product following assay RoD2 is indicative of either a T or an H strain. The RoD3 assay was then carried out to differentiate between these 2 strain families.

Amplification was carried out as described (2.9.3) except the number of cycles was decreased from 35 cycles to 30 cycles, to eliminate non-specific amplification. Following amplification, the products were separated by agarose gel electrophoresis (Figure 3.20).

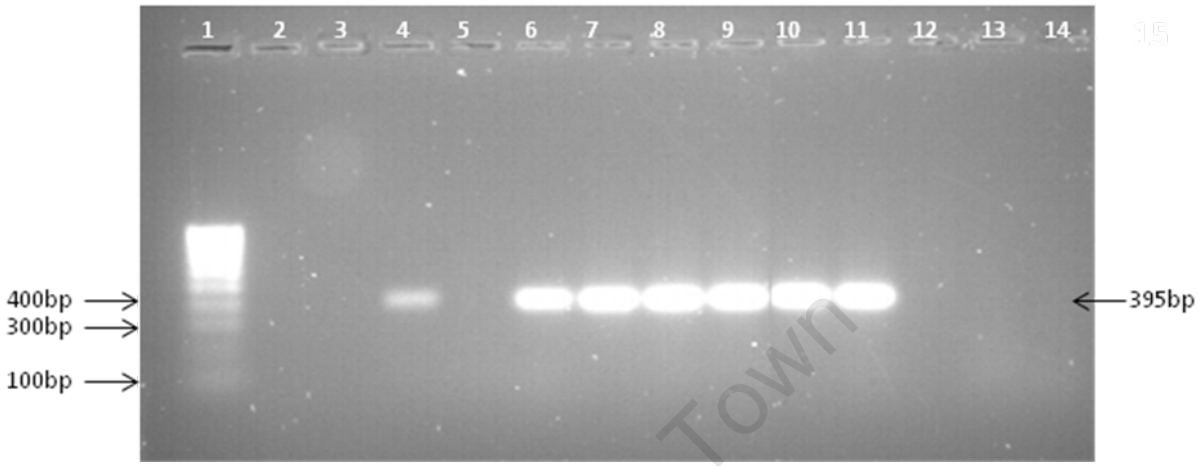


Figure 3.20. Agarose gel electrophoresis of assay RoD3 using control strains as template. Lane 1: Hyperladder IV; Lane 2: Negative water control; Lane 3: VOID; Lane 4: LAM 1; Lane 5: W-Beijing 1; Lane 6: LAM 2; Lane 7: T 1; Lane 8: T 2; Lane 9: T 3; Lane 10: X 1; Lane 11: X 2; Lane 12: H 1; Lane 13: H 2; Lane 14: H 3.

No signal is observed in the water control, indicating that the reagents used were not contaminated (Figure 3.20). Products corresponding to 395bp were observed as expected in the T and X control strains (Figure 3.20; Lanes 7 - 11). As RoD2 detects isolates of the X family RoD3 serves to confirm the RoD2 result. Similarly, a product observed with the LAM control (Figure 3.20; Lanes 4 and 6) confirms the result obtained using assay RoD1 (Figure 3.18). No PCR amplification was detected for the H control strains (Figure 3.20; Lanes 12 – 14) as well as the W-Beijing control (Figure 3.20; Lane 5), indicating that these isolates are neither T nor X strains.

3.6.1. Screening of isolates from patients identified by spoligotyping as harbouring mixed infections

Assay RoD1 was carried out on all isolates for patients indicated as harbouring mixed infections following spoligotyping. Following amplification, the products were separated by agarose gel electrophoresis (Figure 3.21).

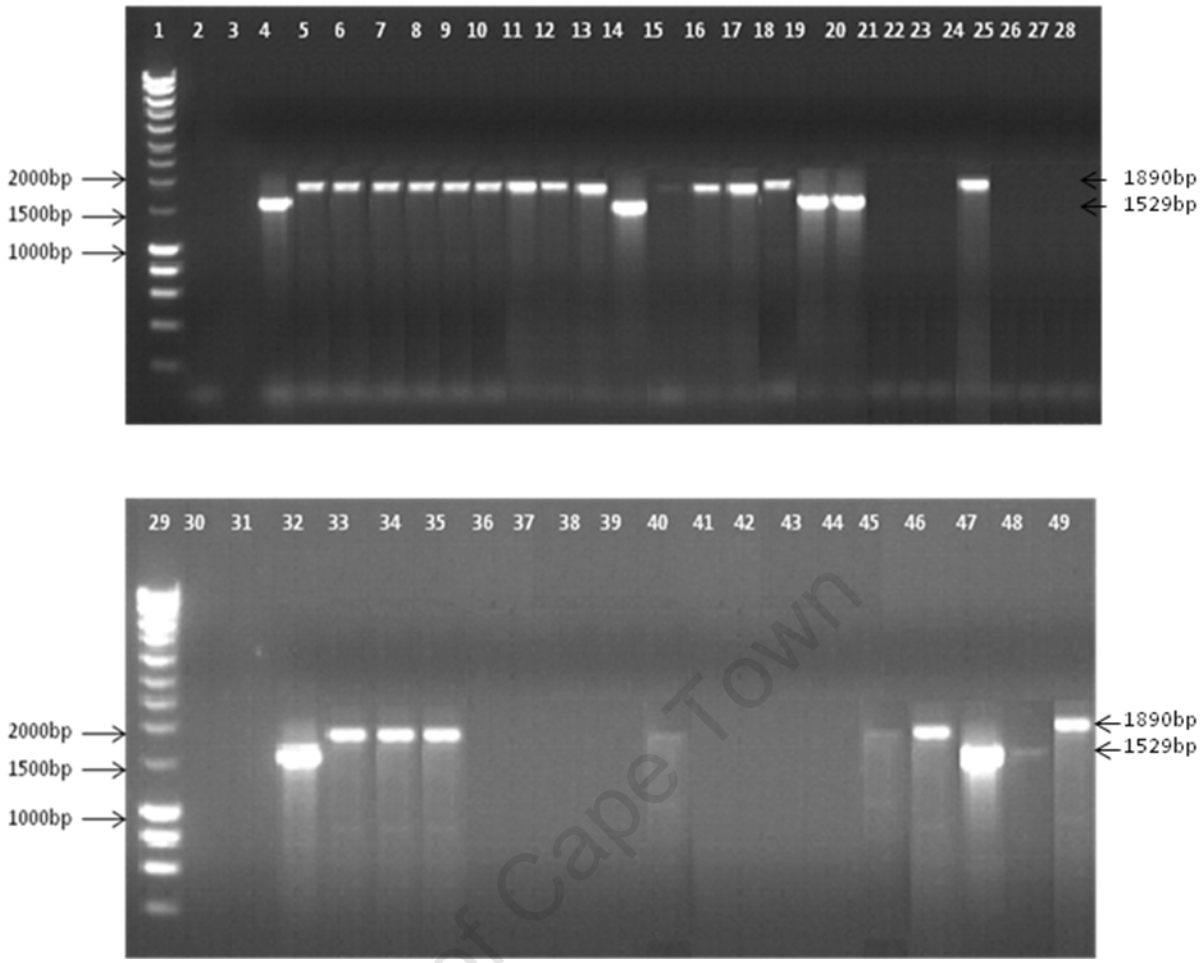


Figure 3.21. Agarose gel electrophoresis of assay RoD1 from mixed patient samples. Lane 1: Hyperladder I; Lane 2: negative water control; Lane 3: VOID; Lane 4: LAM 1; Lane 5: T 1; Lane 6: X 1; Lane 7: H 1; Lane 8: 24NC Week (W) 0; Lane 9: 24NC W 1; Lane 10: 24NC W 2; Lane 11: 24NC W 3; Lane 12: 24NC W 4; Lane 13: 24NC W 5; Lane 14: 24NC W 6; Lane 15: 46JP W 0; Lane 16: 46JP W 1; Lane 17: 46JP W 2; Lane 18: 46JP W 3: Lane 19: 46JP W 4; Lane 20: 46JP W 5; Lane 21: 73NC W 0; Lane 22: 73NC W 1; Lane 23: 73NC W 2; Lane 24: 73NC W 3; Lane 25: 73NC W 4; Lane 26: 73NC W 5; Lane 27: 73NC W 6; Lane 28: 73NC W 7; Lane 29: Hyperladder I; Lane 30: negative water control; Lane 31: VOID; Lane 32: LAM 1; Lane 33: T 1; Lane 34: X 1; Lane 35: H 1; Lane 36: 73NC W 8; Lane 37: 73NC W 9; Lane 38: 81SM W 0; Lane 39: 81SM W 1; Lane 40: 81SM W 2; Lane 41: 107MD W 0; Lane 42: 107MD W 1; Lane 43: 107MD W 2; Lane 44: 107MD W 3; Lane 45: 107MD W 4; Lane 46: 107MD W 5; Lane 47: 118AG W 0; Lane 48: 118AG W 1; Lane 49: 118AG W 2.

No signal is observed in the either of the negative water controls, indicating that the reagents used were not contaminated (Figure 3.21). Products corresponding to 1529bp were observed for the LAM control strains, as expected (Figure 3.21; Lanes 4 and 32), as were products corresponding to 1890bp for the non-LAM control strains, T, X and H (Figure 3.21; Lanes 5 – 7, and 33 – 35, respectively). Patient 24NC, which was identified using spoligotyping, as harbouring T4 strains in the samples isolated in W0 – 5 and an S strain in the sample isolated in W6, displays the expected 1890bp product for the samples isolated in W0 - 5 (Figure 3.21;

Lanes 8 – 13) and a product corresponding to 1529bp for the sample isolated in W6 (Figure 3.21; Lane 14). Products corresponding to 1890bp are observed for the samples isolated in W0 - 3 of patient 46JP (Figure 3.21; Lanes 15 – 18), identified by spoligotyping as harbouring T1 strains in those isolates. Products of 1529bp were observed in the samples isolated in W4 - 5 of patient 46JP, identified by spoligotyping as harbouring S strains in these samples (Figure 3.21; Lanes 19 – 20). No products were observed in the samples isolated in W0 - 2 of patient 73NC, identified by spoligotyping as harbouring W-Beijing isolates (Figure 3.21; Lanes 21 – 23), while a product corresponding to 1890bp was observed, as expected for the emerged T4 strain, in the sample isolated in W3 (Figure 3.21; Lane 24), while the samples isolated in W4 – 9 failed to yield products, due to the W-Beijing strains re-emerging in those samples (Figure 3.21; Lanes 25 – 28, and 36 – 37).

Similarly no products were observed for the samples isolated in W0 - 1 of patient 81SM (Figure 3.21; Lanes 38 – 39), due to the identified W-Beijing isolates, while a low intensity product of 1890bp is observed in the sample isolated in W2 (Figure 3.21; Lane 40). This is most likely due to the low amount of X3 DNA in the mixed sample. Additionally, no products are observed in the samples isolated in W0 - 3 of patient 107MD, identified as harbouring W-Beijing isolates (Figure 3.21; Lanes 41 – 44), and an additional X3 strain in the samples isolated in W4 - 5, where products of 1890bp were observed (Figure 3.21; Lanes 45 – 46). Finally, products of 1529bp are observed in the samples isolated in W0 – 1 of patient 118AG, identified as harbouring LAM3 strains (Figure 3.21; Lanes 47 – 48), while a 1890bp product was observed in the sample isolated in W2, identified as an emerged T4 strain (Figure 3.21; Lane 49). RoD1 confirmed the identification of strains detected by spoligotyping as either LAM or non-LAM.

The RoD2 assay was subsequently used to further delineate non-LAM isolates as either T, X or H strains. RoD2 separates out T and H strains from X strains based on the presence or absence of a product corresponding to 240bp. Positive amplification is indicative of either T or H strains, while the absence of amplification indicates the presence of X or LAM strains. Following RoD2 amplification, products were separated by agarose gel electrophoresis (Figure 3.22).

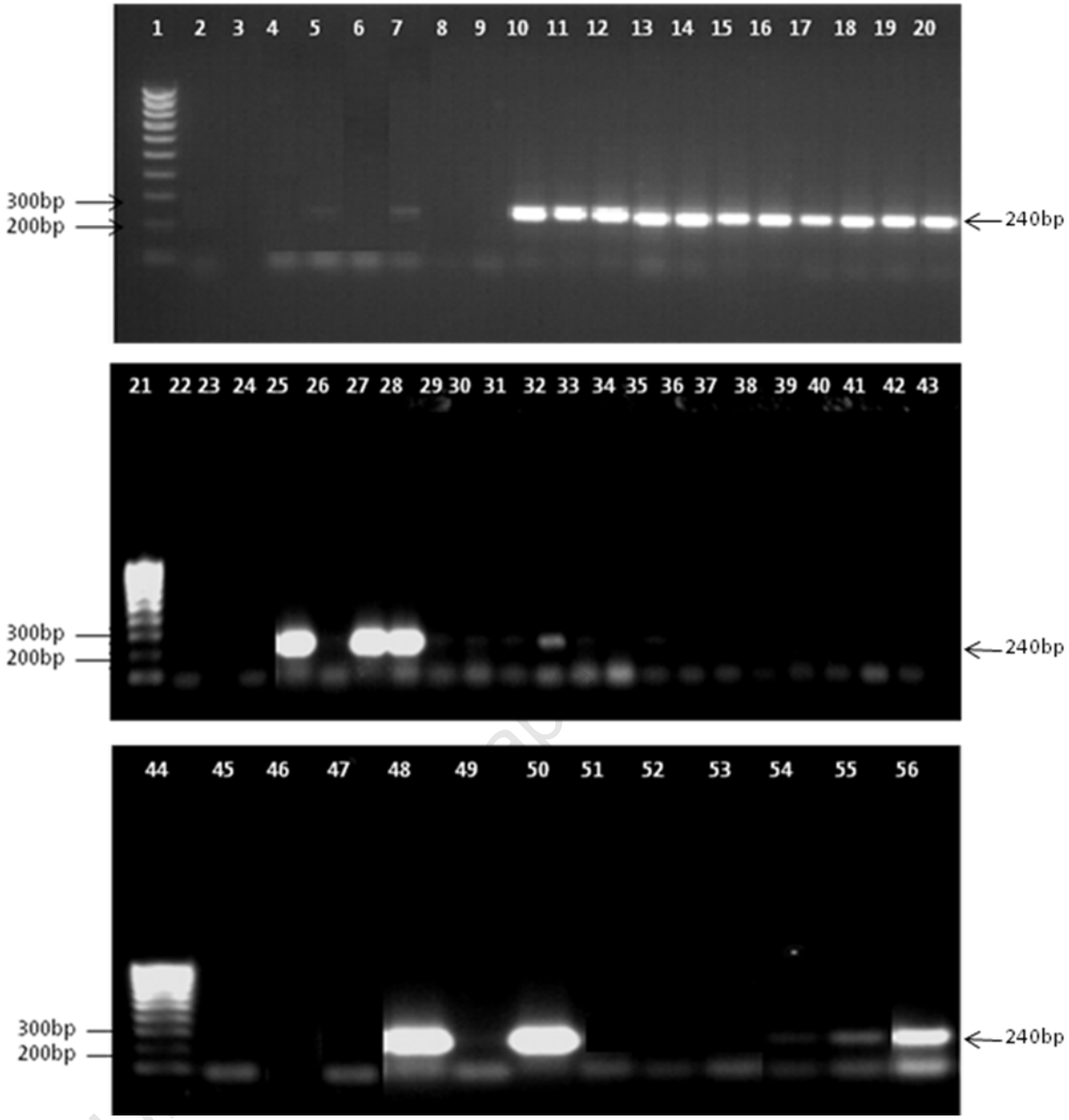


Figure 3.22. Agarose gel electrophoresis of assay RoD2 from mixed patient samples. Lane 1: Hyperladder IV; Lane 2: negative water control; Lane 3: VOID; Lane 4: LAM 1; Lane 5: T 1; Lane 6: X 1; Lane 7: H 1; Lane 8: 24NC W 0; Lane 9: 24NC W 1; Lane 10: 24NC W 2; Lane 11: 24NC W 3; Lane 12: 24NC W 4; Lane 13: 24NC W 5; Lane 14: 24NC W 6; Lane 15: 46JP W 0; Lane 16: 46JP W 1; Lane 17: 46JP W 2; Lane 18: 46JP W 3: Lane 19: 46JP W 4; Lane 20: 46JP W 5; Lane 21: Hyperladder IV; Lane 22: negative water control; Lane 23: VOID; Lane 24 LAM 1; Lane 25: T 1; Lane 26: X 1; Lane 27: H 1; Lane 28: 73NC W 0; Lane 29: 73NC W 1; Lane 30: 73NC W 2; Lane 31: 73NC W 3; Lane 32: 73NC W 4; Lane 33: 73NC W 5; Lane 34: 73NC W 6; Lane 35: 73NC W 7; Lane 36: 73NC W 8; Lane 37: 73NC W 9; Lane 38: 81SM W 0; Lane 39: 81SM W 1; Lane 40: 81SM W 2; Lane 41: 107MD W 0; Lane 42: 107MD W 1; Lane 43: 107MD W 2; Lane 44: Hyperladder IV; Lane 45: negative water control; Lane 46: VOID; Lane 47: LAM 1; Lane 48: T 1; Lane 49: X 1; Lane 50: H 1;Lane 51: 107MD W 3; Lane 52: 107MD W 4; Lane 53: 107MD W 5; Lane 54: 118AG W 0; Lane 55: 118AG W 1; Lane 56: 118AG W 2

No signal was observed in any of the negative water controls indicating that the reagents used were not contaminated (Figure 3.22). Products corresponding to 240bp were observed for the T control strains (Figure 3.22; Lanes 5, 25, and 48) and H control strains (Figure 3.22; Lanes 7, 27, and 50), while no signal was observed in the LAM control strains (Figure 3.22; Lanes 4, 24, and 47) and X control strains (Figure 3.22; Lanes 6, 26, and 49), as expected. Products of 240bp were observed for samples isolated in W0 - 5 from patient 24NC, including the sample isolated in W6. This was expected, having previously been identified by spoligotyping to be of the T4 lineage (W0-5) and the S lineage (W6) (Figure 3.22; Lanes 8 – 14). The lack of product amplification in lanes 8 and 9 is most likely due to template concentration in these samples being too low for detection using this assay. It is not likely that it was an inability of the PCR to amplify T4 strains as the other samples were successfully amplified.

Similarly, products of 240bp were observed, as expected, for samples isolated in W0 - 3 from patient 46JP, including the samples isolated in W4 – 5, having previously been identified as belonging to the T1 lineage (W0 – 3) and the S lineage (W4-5) (Figure 3.22; Lanes 15 – 20). Products of 240bp were observed in the samples isolated in W0 - 5, and again in the sample isolated in W7 (Figure 3.22; Lanes 28 – 33, and 35), of patient 73NC, which is unexpected, as these samples are known to be of the W-Beijing lineage, except for the sample isolated in W3, which belongs to the T4 lineage (Figure 3.22; Lane 32). However, this indicates that the T4 strain was present, albeit in fractional amounts, from the initial infection. However, subsequent samples isolated in W6, and W8 to W9, failed to detect T4 DNA, and no amplification signals were observed, indicative of W-Beijing isolates (Figure 3.22; Lanes 34, and 36 - 37).

No signals were observed in samples isolated in W0 – 2 from patient 81SM, indicative of the W-Beijing and X3 strains, both yielding no amplification (Figure 3.22; Lanes 38 – 40). Similarly, no signals were observed, as expected from the samples isolated in W0 - 5 from patient 107MD, as these isolates were identified to be of the W-Beijing lineage in the samples W0 – 3 (Figure 3.22; Lanes 41 – 43), while an additional X3 isolate emerged in samples W4 and W5 (Figure 3.22; Lanes 51 – 53). Products corresponding to 240bp were observed for samples isolated in W0 - 2 from patient 118AG, indicative of the underlying T4 strain that emerges in sample W2. Signals in samples W0 and W1 are less intense than in sample W2,

due most likely to the low numbers of T4 isolates present in the first 2 samples (Figure 3.22; Lanes 54 – 56). Assay RoD2 correctly identified and differentiated the T isolates from the X isolates and corresponded with the spoligotyping data. Furthermore, the assay was able to detect the underlying strains, present in low quantities, before they were detectable in subsequent samples by spoligotyping, thus indicating that the assay is more sensitive than spoligotyping in detecting mixed isolates earlier.

Assay RoD3 differentiates T strains from H strains based on the presence or absence of a 395bp product. The presence of the product is indicative of T strains present in the sample, as well as LAM or X strains, whiles the absence of amplification suggests the presence of H strains. As RoD1 and RoD2 assays detect LAM and X strains prior to RoD3, the presence of a signal here serves to confirm results obtained in the previous two assays. Following RoD3 amplification, products were separated by agarose gel electrophoresis (Figure 3.23).

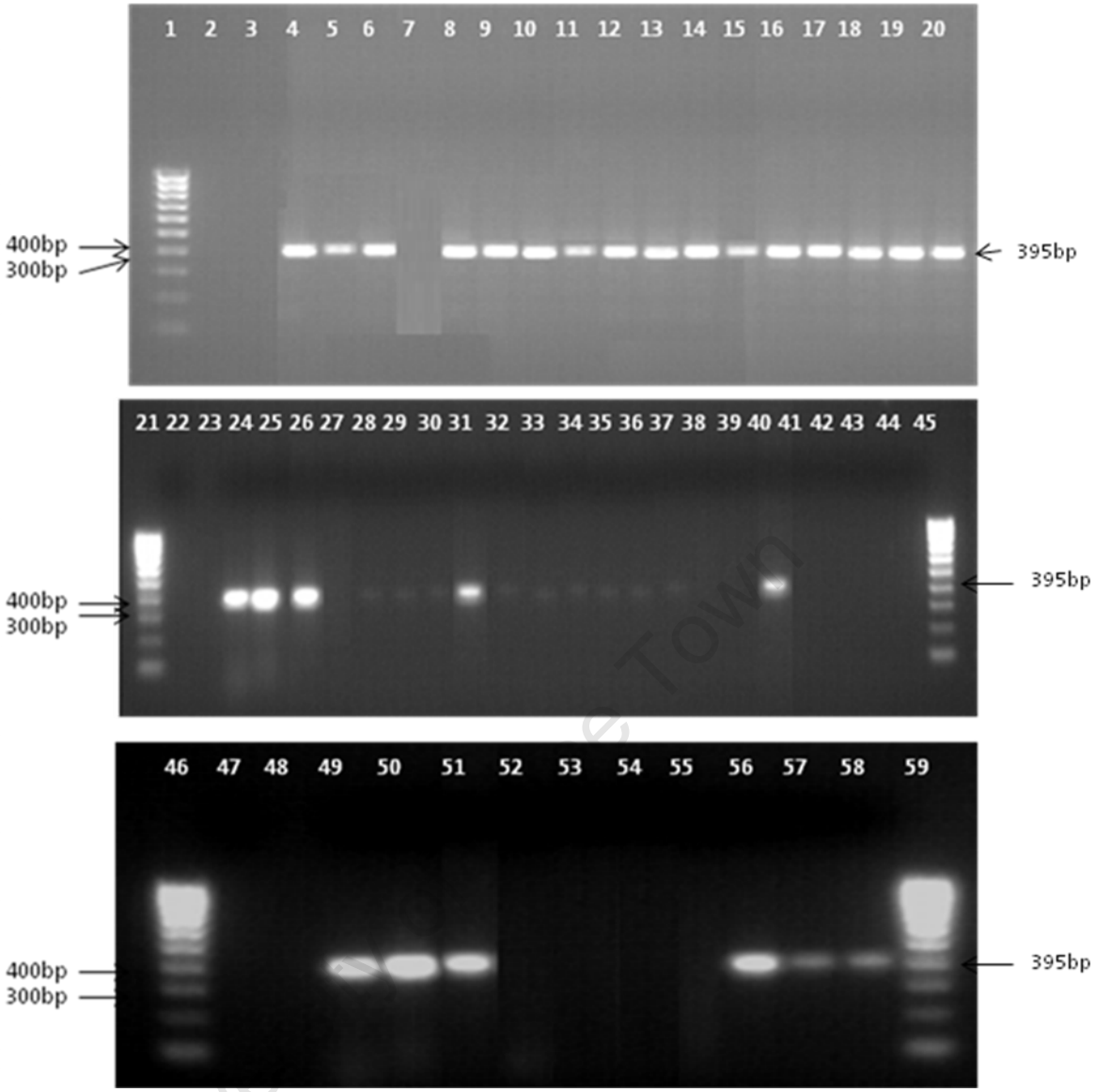


Figure 3.23. Agarose gel electrophoresis of assay RoD3 from mixed patient samples. Lane 1: Hyperladder IV; Lane 2: negative water control; Lane 3: VOID; Lane 4: LAM 1; Lane 5: T 1; Lane 6: X 1; Lane 7: H 1; Lane 8: 24NC W 0; Lane 9: 24NC W 1; Lane 10: 24NC W 2; Lane 11: 24NC W 3; Lane 12: 24NC W 4; Lane 13: 24NC W 5; Lane 14: 24NC W 6; Lane 15: 46JP W 0; Lane 16: 46JP W 1; Lane 17: 46JP W 2; Lane 18: 46JP W 3: Lane 19: 46JP W 4; Lane 20: 46JP W 5; Lane 21: Hyperladder IV; Lane 22: negative water control; Lane 23: VOID; Lane 24 LAM 1; Lane 25: T 1; Lane 26: X 1; Lane 27: H 1 Lane 28: 73NC W 0; Lane 29: 73NC W 1; Lane 30: 73NC W 2; Lane 31: 73NC W 3; Lane 32: 73NC W 4; Lane 33: 73NC W 5; Lane 34: 73NC W 6; Lane 35: 73NC W 7; Lane 36: 73NC W 8; Lane 37: 73NC W 9; Lane 38: 81SM W 0; Lane 39: 81SM W 1; Lane 40: 81SM W 2; Lane 41: 107MD W 0; Lane 42: 107MD W 1; Lane 43: 107MD W 2; Lane 44: VOID; Lane 45: Hyperladder IV; Lane 46: Hyperladder IV; Lane 47: negative water control; Lane 48: VOID; Lane 49: LAM 1; Lane 50: T 1; Lane 51: X 1; Lane 52: H 1; Lane 53: 107MD W 3; Lane 54: 107MD W 4; Lane 55: 107MD W 5; Lane 56: 118AG W 0; Lane 57: 118AG W 1; Lane 58: 118AG W 2; Lane 59: Hyperladder IV.

No signal was observed in any of the negative water controls indicating that the reagents used were not contaminated (Figure 3.23). Products corresponding to 395bp were observed for the LAM control strains (Figure 3.23; Lanes 4, 24 and 49), T control strains (Figure 3.23; Lanes 5, 25, and 50) and X control strains, as expected (Figure 3.23; Lanes 6, 26, and 51) and were absent using the H control strains (Figure 3.23; Lanes 7, 27, and 52). Products corresponding to 395bp were observed for the samples isolated in W0 - 6 of patient 24NC (Figure 3.23; Lanes 8 – 14). The products were also observed in the samples isolated in W0 - 5 for patient 46JP (Figure 3.23; Lanes 15 – 20). This confirms the presence of the T and S strains in each patient.

Products corresponding to 395bp were observed, at a low intensity, for samples isolated in W0 – 2 (Figure 3.23; Lanes 28 – 30) of patient 73NC, and samples isolated in W4 – 9 (Figure 3.23; Lanes 32 – 37), indicative of the underlying T4 strain which emerges in the sample isolated in W3, showing a stronger intensity (Figure 3.23; Lane 31). No products were observed in the samples isolated in W0 – 1 from patient 81SM, corresponding to the W-Beijing strains isolated (Figure 3.23; Lanes 38 – 39). Product corresponding to 395bp was observed in the sample isolated in W2 (Figure 3.23; Lane 40) of patient 81SM. This indicates that X3, which emerged in the sample isolated in W2, was present in small quantities, too low to be detected in the first two samples. Similarly, no amplification was observed in samples isolated in W0 - 5 from patient 107MD (Figure 3.23; Lanes 41 – 43, and 53 – 55), despite the emergence of X3 in samples isolated in W4 – 5 (Figure 3.23; Lanes 54 – 55). Amplification of expected size was observed in samples isolated in W0 - 2 from patient 118AG, confirming the emergence of the T4 strain in sample W2 (Figure 3.23; Lanes 56 – 58). As there were no H strains detected by spoligotyping, assay RoD3, therefore confirmed the results obtained from the other two assays.

3.6.2. Screening for additional mixed infections in the study population.

Assays RoD1, RoD2 and RoD3 were carried out on the first and last isolates of the 129 l patients included in the study including the 15 patients identified as having mixed isolates by PCR. The RoD assays did not identify any additional patients that harboured mixed non-W-

Beijing isolates, and confirmed the spoligotyping results of those patients. The RoD assays were used to screen the first and last isolate of the additional 15 patients. Following amplification with RoD1 products were separated by agarose gel electrophoresis (Figure 3.24).

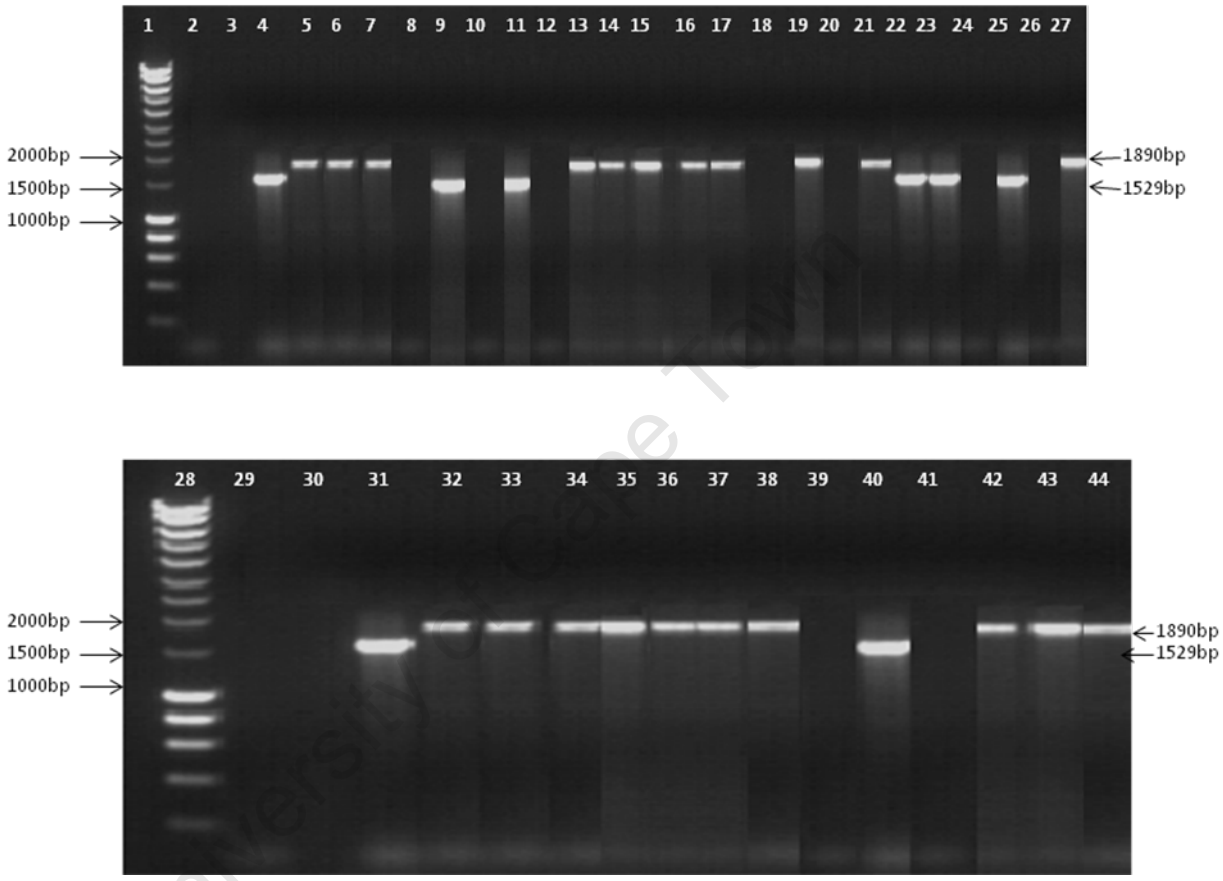


Figure 3.24. Agarose gel electrophoresis of assay RoD1 of additional mixed isolates. Lane 1: Hyperladder I; Lane 2: negative water control; Lane 3: VOID; Lane 4: LAM 1; Lane 5: T 1; Lane 6: X 1; Lane 7: H 1; Lane 8: 20TM W0; Lane 9: 20TM W6; Lane 10: 54ER W0; Lane 11: 54 W4; Lane 12: 61WM W0; Lane 13: 61WM W7; Lane 14: 79RC W0; Lane 15: 79RC W6; Lane 16: 80GI W0; Lane 17: 80GI W6; Lane 18: 85SA W0; Lane 19: 85SA W3; Lane 20: 86AF W0; Lane 21: 86AF W7; Lane 22: 87GK W0; Lane 23: 87GK W8; Lane 24: 95AM W0; Lane 25: 95AM W8; Lane 26: 101GP W0; Lane 27: 101GP W7; Lane 28: Hyperladder I; Lane 29: negative water control; Lane 30: VOID; Lane 31: LAM 1; Lane 32: T 1; Lane 33: X 1; Lane 34: H 1; Lane 35: 104MM W0; Lane 36: 104MM W6; Lane 37: 105AD W0; Lane 38: 105AD W8; Lane 39: 114VJ W0; Lane 40: 114VJ W1; Lane 41: 117TS W0; Lane 42: 117TS W7; Lane 43: 121VS W0; Lane 44: 121VS W2.

No signal was observed in the negative water control indicating that the reagents used were not contaminated (Figure 3.24). Products corresponding to 1529bp were observed for the LAM control strain (Figure 3.24; Lanes 4 and 31) while products of 1890bp were observed for

the T, X and H control strains (Figure 3.24; Lanes 5 – 7, and 32 – 34). No product is observed in the W0 isolate of patient 20TM, indicative of the W-Beijing isolate confirmed present by spoligotyping, while product corresponding to 1529bp in the W6 isolate indicates that this isolate is either a LAM or S strain (Figure 3.24; Lanes 8 – 9). Similarly, no product is observed in the W0 isolate of patient 54GR, while the 1529bp product in the W4 isolate is indicative of either a LAM or S strain (Figure 3.24; Lanes 10 – 11).

No product is observed in the W0 isolate of patient 61WM indicative of the W-Beijing isolate in that sample, while the 1890bp product observed in the W7 isolate, indicative of either a T, X or H strain (Figure 3.24; Lane 12 – 13). Products corresponding to 1890bp were observed in both isolates of patient 79RC indicative of the non-W-Beijing isolate present in both samples, and may correspond to either a T, X, or H strain (Figure 3.24; Lanes 14 – 15). Similarly products of 1890bp were observed in both isolates of patient 80GI, indicative of the non-W-Beijing isolate present in both samples and may belong to either the T, X or H lineages (Figure 3.24; Lanes 16 – 17). No product was observed in the W0 isolate of patient 85SA indicative of the W-Beijing isolate confirmed in that sample, while product corresponding to 1890bp observed in the W3 isolate is indicative of either a T, X or H isolate (Figure 3.24; Lanes 18 – 19). Similarly, no product was observed in the W0 isolate from patient 86AF, indicative of the W-Beijing isolate confirmed in that sample, while product of 1890bp in the W7 isolate corresponds to either a T, X or H strain (Figure 3.24; Lanes 20 – 21).

Product of 1529bp observed in both isolates of patient 87GK is indicative of the non-W-Beijing strains found in both samples and is indicative of either a LAM or S strain (Figure 3.24; Lanes 22 – 23). No product was observed in the W0 isolate of patient 95AM, indicative of the W-Beijing isolate confirmed in that sample, while product corresponding to 1529bp in the W8 isolate indicates the presence of either a LAM or S strain (Figure 3.24; Lanes 24 – 25). No product was observed in the W0 isolate of patient 101GP, confirming the presence of the W-Beijing isolate present in that sample, while product corresponding to 1890bp in the W7 isolate indicates the presence of either a T, X or H strain (Figure 3.24; Lanes 26 – 27). Products corresponding to 1890bp were observed in both isolates of patient 104MM, indicative of the non-W-Beijing strain present in both samples and corresponds to the presence of either a T, X

or H strain (Figure 3.24; Lanes 35 – 36). Similarly products corresponding to 1890bp were observed in both isolates of patient 105AD, indicative of the non-W-Beijing isolate present in both samples and corresponds to the presence of either a T, X or H strain (Figure 3.24; Lanes 37 – 38).

No product was observed in the W0 isolate of patient 114VJ, confirming the presence of the W-Beijing strain present in that sample, while product corresponding to 1529bp in the W1 isolate indicates the presence of either a LAM or S strain (Figure 3,24; Lanes 39 – 40). No product was observed in the W0 isolate of patient 117VS, confirming the presence of the w-Beijing strain present in that sample, while product corresponding to 1890bp was observed in the W7 isolate, which corresponds to the presence of either a T, X or H strain (Figure 3.24; Lanes 41 – 42). Products corresponding to 1890bp were observed in both isolates from patient 121VS, indicative of the non-W-Beijing isolate present on both samples, and corresponds to the presence of either a T, X or H strain (Figure 3.24; Lanes 43 – 44).

The RoD2 assay was carried out subsequent to amplification of RoD1, to further discriminate between the T, X and H strains. Following amplification of RoD2 the products were separated by agarose gel electrophoresis (Figure 3.25).

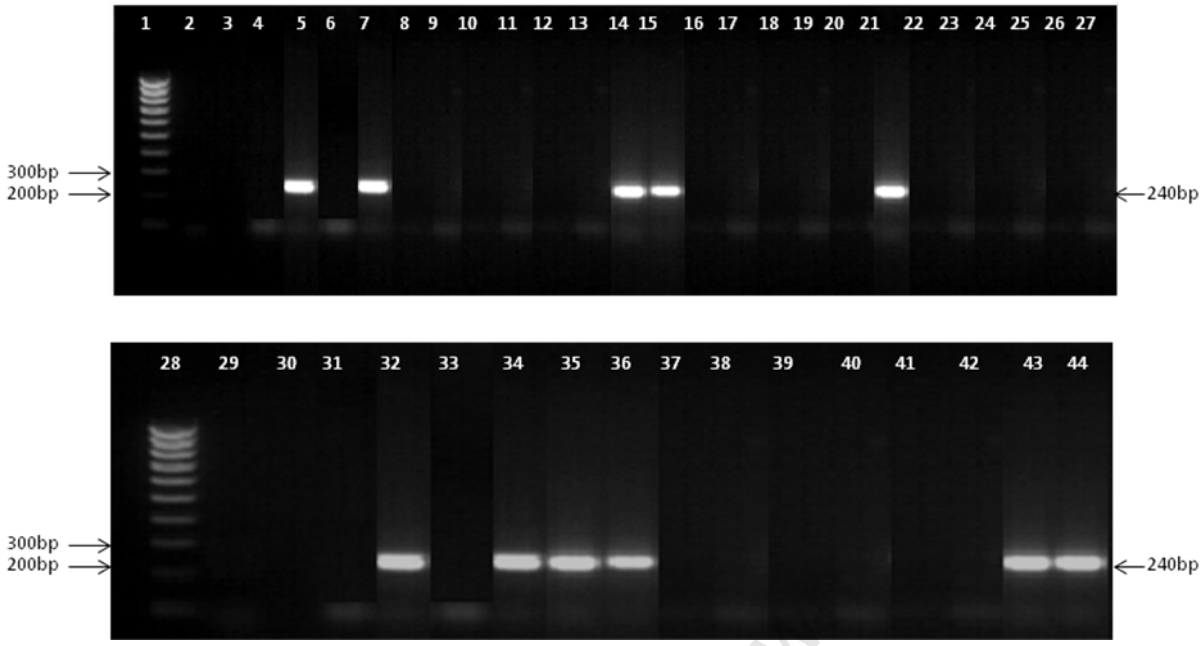


Figure 3.25. Agarose gel electrophoresis of assay RoD2 of additional mixed isolates. Lane 1: Hyperladder I; Lane 2: negative water control; Lane 3: VOID; Lane 4: LAM 1; Lane 5: T 1; Lane 6: X 1; Lane 7: H 1; Lane 8: 20TM W0; Lane 9: 20TM W6; Lane 10: 54ER W0; Lane 11: 54 W4; Lane 12: 61WM W0; Lane 13: 61WM W7; Lane 14: 79RC W0; Lane 15: 79RC W6; Lane 16: 80GI W0; Lane 17: 80GI W6; Lane 18: 85SA W0; Lane 19: 85SA W3; Lane 20: 86AF W0; Lane 21: 86AF W7; Lane 22: 87GK W0; Lane 23: 87GK W8; Lane 24: 95AM W0; Lane 25: 95AM W8; Lane 26: 101GP W0; Lane 27: 101GP W7; Lane 28: Hyperladder I; Lane 29: negative water control; Lane 30: VOID; Lane 31: LAM 1; Lane 32: T 1; Lane 33: X 1; Lane 34: H 1; Lane 35: 104MM W0; Lane 36: 104MM W6; Lane 37: 105AD W0; Lane 38: 105AD W8; Lane 39: 114VJ W0; Lane 40: 114VJ W1; Lane 41: 117TS W0; Lane 42: 117TS W7; Lane 43: 121VS W0; Lane 44: 121VS W2.

No signal was observed in the negative water control indicating that the reagents used were not contaminated (Figure 3.25). Products corresponding to 240bp were observed for the T and H control strains (Figure 3.25; Lanes 5 and 7, 32 and 35) while no products were observed for the LAM and X control strains (Figure 3.25; Lanes 4 and 6, and 31 – 33). No products are observed in both isolates of patient 20TM, indicative of the W-Beijing isolate confirmed present by spoligotyping in the W0 isolate, while the absence of product in the W6 isolate confirms the presence of a LAM strain, rather than an S strain, as these strains yield products in all 3 RoD assays (Figure 3.25; Lanes 8 – 9). Similarly, no products were observed in both isolates of patient 54GR, indicating the W-Beijing strain in the W0 isolate and a LAM strain in the W4 isolate (Figure 3.25; Lanes 10 – 11).

No products are observed in both isolates of patient 61WM indicative of the W-Beijing isolate in the W0 sample, while the absence of product observed in the W7 isolate, confirms the

presence of an X strain due to the larger product being amplified in RoD1 (Figure 3.25; Lane 12 – 13). Products corresponding to 240bp were observed in both isolates of patient 79RC indicative of the non-W-Beijing isolate present in both samples, and may correspond to either a T or an H strain (Figure 3.25; Lanes 14 – 15). No products were observed in either of the isolates from patient 80GI, confirming the presence of an X strain in both samples (Figure 3.25; Lanes 16 – 17). No products were observed in the both isolates of patient 85SA indicative of the W-Beijing isolate confirmed in the W0 sample, while absence of product observed in the W3 isolate is indicative of an X isolate (Figure 3.25; Lanes 18 – 19). No product was observed in the W0 isolate from patient 86AF, indicative of the W-Beijing isolate confirmed in that sample, while product of 240bp in the W7 isolate corresponds to either a T or H strain (Figure 3.25; Lanes 20 – 21).

No products were observed in both isolates of patient 87GK and are indicative of a LAM strain found in both samples (Figure 3.25; Lanes 22 – 23). No product was observed in both isolates of patient 95AM, indicative of the W-Beijing isolate confirmed in the W0 isolate, while the absence of product in the W8 isolate indicates the presence of a LAM strain (Figure 3.25; Lanes 24 – 25). No product was observed in the either isolate of patient 101GP, confirming the presence of the W-Beijing isolate present in the W0 sample, while the absence of product in the W7 isolate indicates the presence of an X strain (Figure 3.25; Lanes 26 – 27). Products corresponding to 240bp were observed in both isolates of patient 104MM, indicative of the non-W-Beijing strain present in both samples and corresponds to the presence of either a T or H strain (Figure 3.25; Lanes 35 – 36).

No products were observed in both isolates of patient 105AD, indicative of the non-W-Beijing isolate present in both samples and corresponds to the presence of an X strain in both samples (Figure 3.25; Lanes 37 – 38). No product was observed in either of the isolates of patient 114VJ, confirming the presence of the W-Beijing strain present in the W0 sample, while the absence of product in the W1 isolate indicates the presence of a LAM strain (Figure 3.25; Lanes 39 – 40). No product was observed in either of the isolates of patient 117VS, confirming the presence of the W-Beijing strain present in the W0 sample, while the absence of product in the W7 isolate corresponds to the presence of an X strain (Figure 3.25; Lanes 41

– 42). Products corresponding to 240bp were observed in both isolates from patient 121VS, indicative of the non-W-Beijing isolate present on both samples, and corresponds to the presence of either a T or H strain in those samples (Figure 3.25; Lanes 43 – 44).

RoD3 was carried out, subsequent to amplification by RoD2, to further discriminate between T and H strains. Following amplification the products were separated by agarose gel electrophoresis (Figure 3.26).

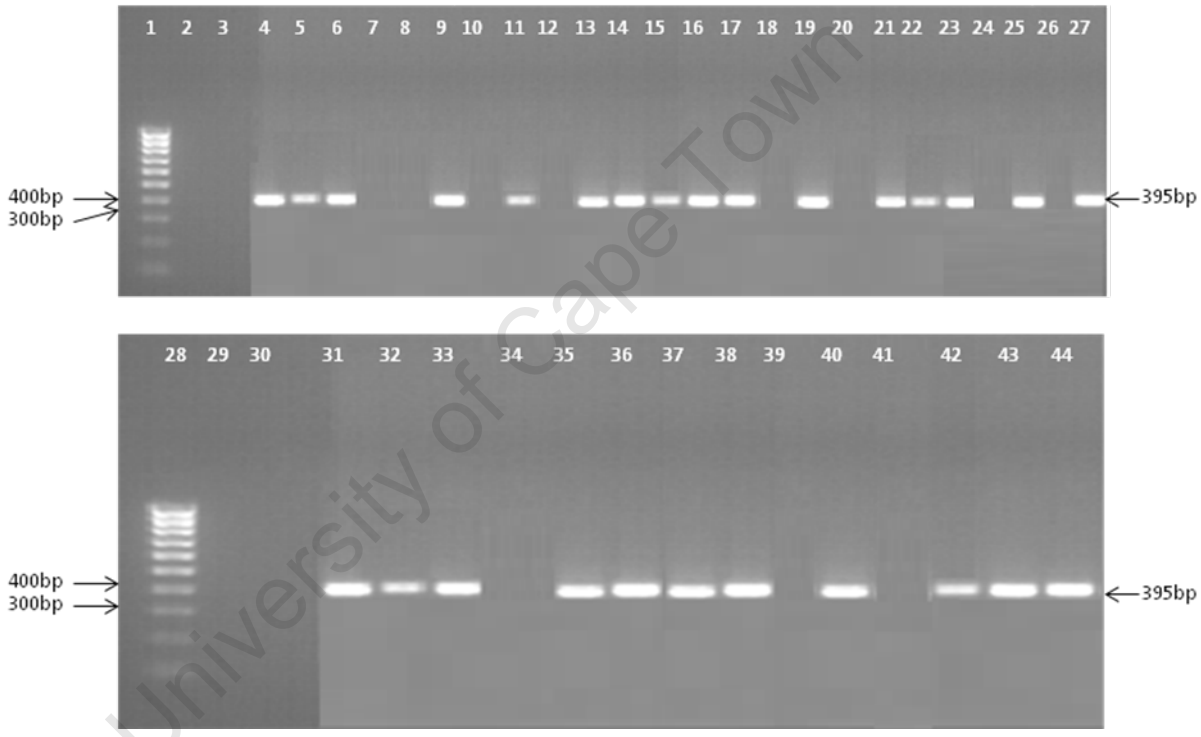


Figure 3.26. Agarose gel electrophoresis of assay RoD3 of additional mixed isolates. Lane 1: Hyperladder I; Lane 2: negative water control; Lane 3: VOID; Lane 4: LAM 1; Lane 5: T 1; Lane 6: X 1; Lane 7: H 1; Lane 8: 20TM W0; Lane 9: 20TM W6; Lane 10: 54ER W0; Lane 11: 54 W4; Lane 12: 61WM W0; Lane 13: 61WM W7; Lane 14: 79RC W0; Lane 15: 79RC W6; Lane 16: 80GI W0; Lane 17: 80GI W6; Lane 18: 85SA W0; Lane 19: 85SA W3; Lane 20: 86AF W0; Lane 21: 86AF W7; Lane 22: 87GK W0; Lane 23: 87GK W8; Lane 24: 95AM W0; Lane 25: 95AM W8; Lane 26: 101GP W0; Lane 27: 101GP W7; Lane 28: Hyperladder I; Lane 29: negative water control; Lane 30: VOID; Lane 31: LAM 1; Lane 32: T 1; Lane 33: X 1; Lane 34: H 1; Lane 35: 104MM W0; Lane 36: 104MM W6; Lane 37: 105AD W0; Lane 38: 105AD W8; Lane 39: 114VJ W0; Lane 40: 114VJ W1; Lane 41: 117TS W0; Lane 42: 117TS W7; Lane 43: 121VS W0; Lane 44: 121VS W2.

No signal was observed in the negative water control indicating that the reagents used were not contaminated (Figure 3.26). Products corresponding to 395bp were observed for the LAM,

T and X control strains (Figure 3.26; Lanes 4 – 6 and 31 – 33) while no products were observed for the H control strain (Figure 3.26; Lanes 7 and 34). No product was observed in the W0 isolate of patient 20TM, indicative of the W-Beijing isolate confirmed present by spoligotyping in that sample, while the presence of product corresponding to 395bp in the W6 isolate confirms the presence of a LAM strain in that sample (Figure 3.26; Lanes 8 – 9). Similarly, no product was observed in the W0 isolates of patient 54GR, indicating the W-Beijing strain, while product corresponding to 395bp in the W4 isolate confirms the presence of a LAM strain in that sample (Figure 3.26; Lanes 10 – 11).

No product was observed in the W0 isolate of patient 61WM indicative of the W-Beijing strain, while the presence of product corresponding to 395bp observed in the W7 isolate, confirms the presence of an X strain in that sample (Figure 3.6 Lane 12 – 13). A product corresponding to 395bp was observed in both isolates of patient 79RC indicative of the non-W-Beijing isolate present in both samples, and thus corresponds to a T strain in both samples (Figure 3.26; Lanes 14 – 15). Similarly products of 395bp were observed in both isolates of patient 80GI, confirming the X strain identified from RoD2 (Figure 3.26; Lanes 16 – 17). No product was observed in the W0 isolate of patient 85SA, confirming the presence of the W-Beijing isolate in that sample, while the presence of product corresponding to 395bp confirms the presence of an X isolate in that sample (Figure 3.26; Lanes 18 – 19).

No product was observed in the W0 isolate from patient 86AF, indicative of the W-Beijing isolate confirmed in that sample, while product of 395bp in the W7 isolate corresponds to a T strain (Figure 3.26; Lanes 20 – 21). Products corresponding to 395bp were observed in both isolates of patient 87GK and are indicative of a LAM strain found in both samples (Figure 3.26; Lanes 22 – 23). No product was observed in the W0 isolate of patient 95AM, indicative of the W-Beijing isolate confirmed in that sample, while the presence of product corresponding to 395bp in the W8 isolate confirms the presence of a LAM strain (Figure 3.26; Lanes 24 – 25). No product was observed in the W0 isolate of patient 101GP, confirming the presence of the W-Beijing isolate present in that sample, while the presence of product in the W7 isolate confirms the presence of an X strain (Figure 3.26; Lanes 26 – 27). Products corresponding to 395bp were observed in both isolates of patient 104MM, indicative of the non-W-Beijing strain

present in both samples and corresponds to the presence of a T strain in both samples (Figure 3.26; Lanes 35 – 36).

Products of 395bp were observed in both isolates of patient 105AD, confirming the presence of an X strain present in both samples (Figure 3.26; Lanes 37 – 38). No product was observed in the W0 isolate of patient 114VJ, confirming the presence of the W-Beijing strain present in that sample, while the presence of product corresponding to 395bp in the W1 isolate confirms the presence of a LAM strain (Figure 3,26; Lanes 39 – 40). No product was observed in the W0 isolate of patient 117VS, confirming the presence of the W-Beijing strain present in that sample, while the presence of product corresponding to 395bp in the W7 isolate confirms to the presence of an X strain (Figure 3.26; Lanes 41 – 42). Products corresponding to 395bp were observed in both isolates from patient 121VS, indicative of the non-W-Beijing isolate present on both samples, and corresponds to the presence of a T strain in those samples (Figure 3.26; Lanes 43 – 44). A summary of the lineages as confirmed by both PCR systems is reported in Table 3.3.

Table 3.3. Genotype of additional isolates from patients identified as harbouring mixed isolates following PS1 and PS3 PCR assays and RoD1, RoD2, and RoD3 PCR assays

Patient Code	Sample†	Lineage
20TM	W0	W-Beijing
	W6	W-Beijing + LAM
54GR	W0	W-Beijing
	W4	W-Beijing + LAM
61WM	W0	W-Beijing
	W7	W-Beijing + X
79RC	W0	W-Beijing + T
	W6	W-Beijing + T
80GI	W0	W-Beijing + X
	W6	W-Beijing + X
85SA	W0	W-Beijing
	W3	W-Beijing + X
86AF	W0	W-Beijing
	W7	W-Beijing + T
87GK	W0	W-Beijing + LAM
	W8	W-Beijing + LAM
95AM	W0	W-Beijing
	W8	W-Beijing + LAM
101GP	W0	W-Beijing
	W7	W-Beijing + X
104MM	W0	W-Beijing + T

	W6	W-Beijing + T
105AD	W0	W-Beijing + X
	W8	W-Beijing + X
114VJ	W0	W-Beijing
	W1	W-Beijing + LAM
117TS	W0	W-Beijing
	W7	W-Beijing + X
121VS	W0	W-Beijing + T
	W2	W-Beijing + T

†W: Week

No H strains were identified in the patient samples as described earlier. The results indicate clearly the need for additional genotyping assays to be performed in conjunction with spoligotyping, as this increases the rate of isolating mixed infections in the study setting.

Chapter 4

Discussion

Previously, it was widely accepted that TB disease is the result of one infecting *M. tuberculosis* strain, and that that strain led to immunity from other infecting strains (Shamputa *et al*, 2004). The widely used *M. bovis* BCG vaccine is based on this theory (Kremer *et al*, 2009). The attenuated strain is intended to prime the host immune system, allowing for the heightened detection and protection against future infecting strains (Kremer *et al*, 2009). Through advances in molecular genotyping, this theory has recently been revised to include that; firstly, disease progression can be due to multiple infecting strains. In addition, little to no immunity is conferred as a result of such infection and infections could result from exogenous reactivation from prior dormant strains or reinfection with a new superinfection (de Viedma *et al*, 2004; Shamputa *et al*, 2004; Shamputa *et al*, 2006; Palomino *et al*, 2007). Thus the efficacy of the protection afforded by BCG in this setting becomes unknown, as BCG alone may not be sufficient for protection later on (Kremer *et al*, 2009). Despite advances in strain genotyping, little is known about the scope of mixed infections, or clonal heterogeneity, which could greatly impact interpretation of epidemiological data and subsequent treatment of patients (Shamputa *et al*, 2004, 2006; Warren *et al*, 2004). This study investigated the detection of mixed infections in weekly sputum samples collected from newly diagnosed TB patients using a number of molecular typing tools. The patients were recruited in the Delft region of the Western Cape as part of a larger study conducted to look at the effects of micronutrient supplementation, in conjunction with standard therapy, on sputum conversion (M. Visser, personal communication).

At the end of the 8-week study period, samples from 154 enrolled adults had been collected. A number of patients and samples had to be excluded from analysis. Samples from 8 patients were excluded due to the lack of positive cultures, most likely due to early sputum conversion as a result of treatment (M. Visser, personal communication). Samples from a further 6 patients were excluded as they were misplaced or discarded erroneously during storage.

Additionally, many individual samples were excluded due to contamination. Of the samples collected 12.8% of the total collection of samples was contaminated, with the majority containing heavy fungal growth. Reports suggest that the general contamination rate for liquid media is between 8 and 10% (Burman and Reves, 2000). Increases in this rate of contamination may be due to laboratory contamination, incorrect handling and storage of samples, inadequate sputum decontamination and technician error (Burman and Reves, 2000), which could account for the slightly elevated contamination rate in this study. As a result of this contamination, many patients had less than the anticipated 9 samples available for analysis. A final total of 686 samples, collected from 140 patients, were included in this study.

To assess the epidemiology of the circulating *M. tuberculosis* strains in Delft, as well as an initial screen for mixed infections, spoligotyping was carried out on all patient isolates collected. Based on the differences in the DR of each isolate, spoligotyping detected 6 different strain families infecting the 140 patients enrolled. A large proportion of patients (48.7%) harboured W-Beijing strains. This is highly elevated compared to previous data obtained in similar settings, but lends credence to the claim that the rate of isolation of W-Beijing strains is increasing in the Western Cape (Hillemann *et al*, 2006). In 2004, 16.5% of isolates were of the W-Beijing lineage (Victor *et al*, 2004), while 25% of strains were W-Beijing in a 2005 study (Nicol *et al*, 2005); this increased to 36% in 2006 (Marais *et al*, 2006).

Other *M. tuberculosis* strain families detected in this study included LAM (17.1%), T (14.3%), X (6.4%), Haarlem (7.9%), S (4.3%), and Family 33 (2.1%). The isolation of LAM strains (17.1%) is much lower than the 30% previously reported (Nicol *et al*, 2005; Marais *et al*, 2006) with the prevalence of T strains (14.3%) twice that indicated earlier (7%) (Nicol *et al*, 2005). These discordant results may reflect the different populations of *M. tuberculosis* circulating in adults versus children as both previous studies were conducted using isolates obtained from children, whereas only adults were included in the Delft study. Furthermore, the differences observed could be uniquely attributed to the community of Delft. However, it may, too, represent a shift in *M. tuberculosis* populations circulating in the Western Cape.

Of the 140 patients examined by spoligotyping, 11 had only 1 viable isolate, and were thus excluded from the analysis for mixed infections. Of the 129 patients with multiple sequential isolates, 123 patients (95.3%) displayed homogenous genotypes for all isolates tested and 6 patients (4.7%) displayed evidence of mixed infections, as determined by spoligotyping. This is concordant with the previous report of 4.8% detection of mixed infection using spoligotyping (Warren *et al*, 2004). Of the 6 patients indicated as containing mixed infections, 4 patients (66.6%) (24NC, 46JP, 73NC, and 118AG) were first infected by one strain which was then replaced by a second strain. The mixed infection in the remaining 2 patients (33.3%) (81SM and 107MD) are a result of a dual infection, suggesting that these two patients harboured two strains each.

Of the 7 isolates obtained from patient 24NC, the first 6 isolates were of the T4 lineage while the 7th isolate was of the S lineage. In patient 46JP the first 4 isolates of the total 6 isolates collected were of the T1 lineage, with the last 2 isolates from the S lineage. Similarly, in patient 118AG, the first 3 strains collected were of the LAM3 lineage while the last isolate was of the T4 lineage. Of the 10 isolates obtained from patient 73NC, however, the first 4 strains were of the W-Beijing lineage, then in week 5 a T4 strain emerged, thereafter from weeks 4 through to 8 W-Beijing strains re-emerged. With patients 24NC and 118AG, the change in genotype occurred in the last isolates, with no additional samples to confirm the change. One can argue therefore that these may not represent true mixed infections, and could point to clerical error, or laboratory cross-contamination, both of which have been reported as confounders in sample genotyping studies (Warren *et al*, 2004). The 2 sequential isolates obtained from patient 46JP in the last 2 weeks suggest that this is a true mixed infection in this patient. Due to the infrequent isolation of S isolates (4.3%) and the fact that 2 sequential isolates both indicate the change, the likelihood of this patient harbouring a mixed infection is strengthened. In patient 73NC, the change in genotype from W-Beijing to T4 occurred mid-way through the study period. The fact that the next isolate was once again a W-Beijing could also indicate possible clerical error, in this case, the mislabelling of a sample tube. Considering the frequency of isolating a strain from the T4 lineage (1.4%) is a rare event however, could aid in confirming the mixed nature of these samples. The isolation of 1 mixed sample, or a sample that displays a change in genotype, out of a collection of several homogeneous sequential isolates, particularly at the end of the study period, places doubt

onto the validity of that patient harbouring mixed infections. One would ideally need to follow up after the change is noticed, in order to confirm the presence of that additional isolate, otherwise one could argue that laboratory cross contamination, or a clerical error, could more likely account for the change, and would not in fact, represent a true mixed infection.

Patients 81SM and 107MD both displayed a mixed spoligotype signal indicating that W-Beijing strains were isolated and that the patients then acquired *M. tuberculosis* of the X3 lineage. Since the change in strain lineage was only detected with the last isolates in both patients, the true mixed nature of the sample could not be confirmed. However, up to 57% of patients infected with W-Beijing strains were repeatedly infected with an additional non-W-Beijing strain (Warren *et al*, 2004). This along with the fact that X lineage strains were identified at a very low frequency, suggests that these patients are dually infected with a W-Beijing strain and an X isolate.

The 4.7% reported here in terms of the frequency of mixed infections is clearly an underestimate, due mainly to the problems associated with spoligotyping to detect mixed non-W-Beijing infections. As W-Beijing isolates display such a distinct spoligotyping pattern, and due to the sensitivity of the PCR assay used (Kamberbeek *et al*, 1997), one can clearly observe mixed infections of W-Beijing and non-W-Beijing samples. Unfortunately, one cannot so easily distinguish between mixed non-W-Beijing isolates as the spoligotyping patterns are so similar in appearance, and any underlying strains will not be detected. Thus a need for differentiating these possible mixed non-W-Beijing isolates is evident.

It has been reported that a change in genotype can be associated with a change in drug susceptibility profiles (Kruuner *et al*, 2002). To this end phenotypic and genotypic DST was performed on a selection of isolates, to ascertain whether the changes in genotype observed in these 6 patients corresponded to a change in drug susceptibility. Phenotypic DST was performed routinely on the baseline and week 8 samples from each patient, at C18, GSH. As the isolation of drug resistant strains is more frequently observed in retreatment cases rather than new cases (Warren *et* al, 2004) one can expect that the frequency of drug resistance was

low. Of the 140 patients analysed in this study, 6 (4.3%) patients had isolates that were resistant to either RIF or INH in week 8. Of these 6 patients, 1 patient (105AD) had an isolate that is resistant to both RIF and INH in week 8, 4 patients (20TM, 48ML, 52SM and 95AM), had RIF mono-resistant strains isolated in week 8, and 1 patient (22JE), had an INH mono-resistant strain isolated in week 8. Of the 5 patients with RIF resistant isolates, 3 patients (60%) were harbouring isolates of the W-Beijing lineage, supporting the association of drug resistance with W-Beijing strains (Bifani *et al*, 2001; Hillemann *et al*, 2006; Evans *et al*, 2009).

Recently, an association between INH mono resistance and X3 strains in the Western Cape was reported (Evans *et al*, 2009). It is therefore interesting to note the association of X3 strains with W-Beijing isolates in patient samples isolated towards the end of the study period. Although these X3 isolates are neither resistant to RIF nor INH, these may represent isolates with a greater potential to becoming resistant to INH. It would therefore be worth following up with these patients to monitor when drug resistant strains emerge and to detect the strain lineages of these isolates. Several hypotheses to why certain strains become drug resistant, or their ability to remain virulent, have been postulated in recent years (Gagneux *et al*, 2006 (1 and 2)). W-Beijing strains are widely known to illicit powerful immune responses in patients and it has been hypothesised that the W-Beijing isolates are hypervirulent, despite their propensity to become drug resistant and MDR (Lopez *et al*, 2003, Gagneux *et al*, 2006 (1)). It has also been reported that the phenolic glycolipids associated with W-Beijing strains allow them to evade the immune response (Reed *et al*, 2007). Also it has been seen in mouse models and hypothesised to occur in humans that the immune protection afforded by BCG is circumvented by W-Beijing isolates, and that W-Beijing strains might represent "escape variants" of *M. bovis* BCG vaccination. This might highlight a possible route to the global circulation of these strains, owing to the wide-use of the vaccine (Kremer *et al*, 2009). Either or a combination of these factors may contribute to the emergence of these X3 clones, with their associated drug resistance, in the Western Cape.

Of the 6 patients with mixed infections, all isolates from each patient were fully susceptible to both RIF and INH by genotypic DST with the GenoType MTBDR*plus* assay. However, this neither confirms nor disproves the mixed nature of these isolates, as this could represent the

isolation of mixed drug susceptible strains, which seems probable due to the fact that these patients have newly diagnosed TB and have therefore not been treated prior to this study.

Further discrimination of all the mixed isolates identified by spoligotyping was carried out by 12-locus MIRU-VNTR analysis (Supply *et al*, 2000, 2001). To determine whether the sequential isolates were clonal in nature or if over the study period several different clones of the same lineage had been isolated. Isolates from each patient showed clonal homogeneity using MIRU-VNTR up until the change in spoligotype, where a corresponding change in the MIRU profile was observed. Interestingly, the MIRU-VNTR profiles of the initial isolates from patients 73NC and 107MD were identical, suggesting that these patients may be have been infected with a clonally related strain. Furthermore, MIRU-VNTR analysis revealed a 2 allele difference between the W-Beijing strains from patients 73NC and 107MD as compared to 81SM. This suggests that even in a close community such as Delft, several different clonal sub-populations of the same lineage may be circulating and causing disease. Using the MIRU-VNTR*plus* algorithm (www.miru-vntrplus.org/miru), employing a maximum difference of 2 alleles between clonal complexes, the T1, T4, W-Beijing and W-Beijing + X3 strains were found to form a clonal complex, which indicates that these isolates are related.

This confirms the need for genotyping assays to be carried out in combination with each other. Although the MIRU data identifies that the clonal complex isolates are related, in terms of the spoligotyping data, they are unrelated to each other, representing discreet lineages. Thus the discriminatory power of just a single genotyping assay is not sufficiently high enough, and requires additional assays to be carried out to increase the total discriminatory power when detecting for mixed infections. Isolates obtained from patients 81SM and 107MD that had different spoligotypes from the initial isolates produced discordant MIRU data. The profiles obtained following the change in spoligotyped pattern from W-Beijing to X3 were of an amalgamation of the W-Beijing strain observed in the initial isolates and that of a different strain, which is suggestive of a mixed infection (Shamputa *et al*, 2006; Stavrum *et al*, 2009). It should be noted that the initial W-Beijing isolate and the W-Beijing isolate in the mixed infection may not be the same strain. These patients may have acquired a subsequent infection with a different W-Beijing strain as well as with an X3 isolate. However, that the

MIRU profiles of the initial W-Beijing isolate and the mixed sample differed at only 2 loci suggests that this is not the case. It is interesting to note that the MIRU-VNTR profiles of all of the W-Beijing + X3 isolates are identical, which suggests that these patients may have become co-infected with the same X3 strain.

To obtain a more accurate estimation of the rate of mixed *M. tuberculosis* infections in patients, it is worth investigating inter-lineage differences associated with specific strains. Using a PCR assay, the prevalence of mixed infections was estimated at 19% in the Western Cape (Warren *et al*, 2004). This PCR assay consists of four separate primer sets for the differentiation of W-Beijing and non-W-Beijing isolates. Amplification products obtained using assays PS1 and PS3 confirmed the spoligotyping results obtained for the 6 patients (24NC, 46JP, 73NC, 81SM, 107MD, and 118AG) indicated as containing mixed infections. It is interesting to note that the majority of the 6 patients harbouring mixed infections were infected with W-Beijing isolates, which formed the basis of the infection. This could suggest that infection with a W-Beijing strain may predispose a patient to subsequent infections with other strains. However it cannot be assumed that infection with W-Beijing isolates occurred first with subsequent infections occurring later; as the methods employed in this and other studies are limited in terms of identifying a time-line of infection (Warren *et al*, 2004).

To identify additional mixed infections in the population, the first and last isolate of the 129 patients were screened and an additional 15 patients were indicated as harbouring mixed W-Beijing and non-W-Beijing isolates. These patients include 20TM, 54GR, 61WM, 79RC, 80GI, 85SA, 86AF, 87GK, 95AM, 101GP, 104MM, 105AD, 114VJ, and 121VS. This increases the estimation of recovering mixed infections in the population to 16.3%, which approximates the previous study conducted in this setting (Warren *et al*, 2004).

The limiting factor with this PCR assay is that in certain patients with mixed isolates, such as patients 24NC, 46JP and 118AG, the assay does not delineate the non-W-Beijing isolates (Warren *et al*, 2004). A PCR algorithm, consisting of 3 separate PCR assays (RoD1, RoD2, and RoD3), was designed to differentiate potential mixed non-W-Beijing isolates. Primers

were designed to detect lineage-specific regions of difference in the DRs of *M. tuberculosis* strain families. RoD1, RoD2 and RoD3 all correctly amplified the targeted regions of the DR in the mixed samples, and confirmed the results obtained by spoligotyping. No undetected non-W-Beijing strains were observed in patients with mixed non-W-Beijing isolates (patients 24NC, 46JP and 118AG).

The RoD assays were then used to screen the first and last isolate of 129 patients included in this study. Of the 15 patients, 6 (61WM, 80GI, 85SA, 101GP, 105AD, and 117TS) harboured dual infections with X isolates, 5 patients (20TM, 54GR, 87GK, 95AM, and 114VJ) harboured dual infections with LAM isolates, and 4 patients (79RC, 86AF, 104MM, and 121VS) harboured dual infections with T isolates. However, the RoD assays do not discriminate the specific sub-family of the lineages detected, and thus additional genotyping tools will be required. As such, it would be of interest if the X isolates harboured by the additional 6 patients belong to the X3 subgroup, and furthermore if they are drug resistant. Again the association of X strains with W-Beijing strains in a relatively large proportion (38%) of the patients harbouring mixed infections is noteworthy. No additional patients harbouring mixed non-W-Beijing isolates were detected in the first and last isolate from the 129 patients tested, and confirmed the spoligotyping data obtained for each patient. This suggests that the proportion of patients harbouring a mixed infection of only non-W-Beijing isolates is very small.

Following the screen of the first and last isolate of all patients included in this study, it is worth screening the rest of the samples from the additional 15 patients, with both PCR assays, to observe when the change in spoligotype pattern occurred, especially in those patient samples where the non-W-Beijing isolate was not detected in the first isolate. Additionally, it would be of interest to perform DST on the same isolates to observe if a change in spoligotype coincides with a change in drug susceptibility. This is especially important in terms of the X3 dual infections detected in the additional 15 patients. Similarly, as mentioned previously, it would be valuable to follow up on these patients to ascertain whether drug resistance emerges and when, and also to determine whether changes in the infecting population had also occurred.

The RoD assays, in some instances, such as with patients 73NC, 81SM, and 107MD, did not readily detect the additional isolates early in the first few samples. This may be due to the W-Beijing DNA being over-represented in the sample and masking what little T4 or X3 DNA present, suggesting a low population of the latter strains. However it may be that these assays are not optimized to adequately detect mixed isolates early on in the infection when numbers of isolates is low. To this end, it would be valuable to titrate various template concentrations, thereby simulating a mixed infection, in order to detect the lowest concentration of DNA detected in a mixed sample using this assay. However, the assays did detect the underlying infecting strain early on in patients 24NC, 46JP, and 118AG, as well as patients 79RC, 80GI, 87GK, 104MM, 105AD, and 121VS, suggesting that spoligotyping was not as sensitive as the RoD assays in these instances.

In the initial screening of the mixed samples it was discovered that S strains also amplify a 1529bp product, which is a shortfall of the assay, as both strains yield products of 1529bp in RoD1, they both yield products from all 3 assays. Thus the RoD assay requires further design to include an S-specific region of difference that will allow the identification of S strains. Furthermore, the RoD assays have only been designed to differentiate out a limited but major group of non-W-Beijing isolates, and therefore the possibility exists of strains not being detected and differentiated. Additionally, it is worth noting, the potential difficulties in using the DR region of *M. tuberculosis* as means of differentiating non-W-Beijing isolates. Although a generalized consensus spoligotype pattern for each non-W-Beijing lineage can be determined, there exists isolates within that lineage that deviates slightly from that consensus, and as such may not be detected, or more importantly may be identified as belonging to another lineage erroneously.

Perhaps, a more specific and reliable method of differentiating non-W-Beijing isolates is to utilize lineage-specific polymorphisms, such as the *Rv1519* deletion defining EAI strains (Newton *et al*, 2006), or the *mgtC* codon 182 SNP that defines the Haarlem genotype (Alix *et al*, 2006). However the benefit of the RoD assay described here is that the majority of non-W-Beijing strains circulating in the Western Cape are identified in an algorithm format, making interpretation of the results simpler. Thus the clinical implications of such an assay are highly

beneficial. Once fully tested on a larger group of isolates, and the specificity and sensitivity of the assay are optimized, then this assay could be used in the clinical setting, at point of care. The materials and reagents needed for PCR are minimal and cost effective, and many samples can be tested at one time, ideal for resource-limited clinics and laboratories.

This data confirms that the rate of mixed infections in the Western Cape is of a relatively high proportion. This has great implications on the epidemiology of *M. tuberculosis* studied in this region. Of greater importance are the subsequent implications that such data has on the way one treats TB patients in this country. Accordingly 17% of newly diagnosed patients are infected or will acquire a mixed infection during treatment (Warren *et al*, 2004), and the presence of a drug resistant isolate within the mixed population could go unnoticed, resulting in a greater chance of the spread of drug resistant strains in the communities. As South Africa has one of the highest incidences of TB in the world (WHO, 2007), the importance of considering mixed infections in treatment regimes and vaccine trails is vital.

Conclusion

This study investigated the frequency of mixed *M. tuberculosis* infections in the Delft community of the Western Cape.

The sequential samples collected from each patient during the study period allowed for the identification of a relatively high proportion (16.3%) of mixed infections in the population. Spoligotyping was shown to underestimate the frequency (4.3%) of isolating mixed infections. This was thought to be due to the similarities observed in the non-W-Beijing spoligotyping profiles. Spoligotyping, therefore, needs to be carried out in conjunction with a PCR assay to allow for a more accurate representation of the frequency of mixed infections in the population.

Although at the time of DST these isolates were susceptible to both RIF and INH, the association of W-Beijing and X3 isolates in dual infections, strains previously identified to be associated with MDR and INH mono-resistance, respectively, suggests a high predisposition of these isolates to become drug resistant in the future. The need for further follow up to confirm this, was highlighted.

Analysis of the genetic relatedness of the strains identified mostly clonal homogeneity within patients, and between patients, but that inter-patient strain differences can and did occur. A clonal complex was formed by the T lineage strains and W-Beijing lineage strains. Although MIRU-VNTR analysis identified these strains to be related, spoligotyping separates these strains out into distinct lineages. Thus, even within a relatively close community, different subpopulations of strain families are circulating and causing disease.

The PCR assay developed to detect mixed infections caused by W-Beijing and non-W-Beijing isolates in the same sample, offers a reliable and modest estimation of the rate of mixed infections in the Western Cape, but was limited in the differentiation of mixed non-W-Beijing isolates.

The RoD assay was developed for the purposes of differentiating the non-W-Beijing isolates present in mixed infections. It was able to confirm the results obtained from spoligotyping, but detected no mixed non-W-Beijing infections in the study population, without the presence of an infecting W-Beijing isolate. Thus it was concluded that the proportion of patients harbouring a mixed infection of non-W-Beijing isolates alone, is very small. This assay represents a novel means of differentiating the major circulating non-W-Beijing isolates in the Western Cape, in an easy to follow format that allows for the rapid interpretation of results. Further optimizations are required, however, before it can be used routinely at the point of care facilities.

This data has great implications into how one interprets clinical and epidemiological data. Of concern is the growing proportion of drug resistant isolates particularly MDR *M. tuberculosis* isolates. Thus the field of mixed infections requires further study to identify reliable methods in distinguishing between mixed isolates, particularly those samples that have mixed drug susceptibilities, as this has considerable consequences in the manner in which patients are treated. Furthermore, from this and other studies, it can be clearly established that no immune protection is afforded by the initial infecting strains towards subsequent infecting strains. Therefore further studies into protective immunity, and particularly into vaccine design, need to be mindful of this eventuality.

TB is one of mankind's oldest diseases, and still is considered an emerging disease, with thousands of people becoming infected or dying as a result of this affliction. Apart from HIV infections, TB is one of Africa's most pressing concerns. Our understanding into the various aspects of TB disease progression, the vaccines designed to protect against the pathogen, as well as our antibiotics utilised in treating patients, should be revised in terms of the frequency of mixed infections, if we are to have any chance in combating this disease.

Appendix A

Solutions and Reagents

1% Agarose

2g Agarose (Whitehead Scientific)

200ml 1X TAE Buffer

2µl EtBr

Boil agarose and 1X TAE in microwave on high power for 3 – 4 minutes. Add EtBr when agarose has cooled slightly.

1.5% Agarose

3g Agarose (Whitehead Scientific)

200ml 1X TAE Buffer

2µl EtBr

Boil agarose and 1X TAE in microwave on high power for 3 – 4 minutes. Add EtBr when agarose has cooled slightly.

24:1 Chloroform / Isoamyl Alcohol (50ml)

48ml chloroform (Saarchem, Merck)

2ml isoamyl alcohol (Saarchem, Merck)

Add 48 parts chloroform with 2 parts isoamyl alcohol. Store at room temperature.

10% CTAB (100ml)

10g CTAB (Sigma)

80ml distilled water

Add CTAB slowly while heating to 60°C. Make up to 100ml with distilled water.

20mM EDTA (100ml) pH 8

0.74g EDTA (Saarchem, Merck)

70ml distilled water

Dissolve EDTA in water and adjust the pH with concentrated NaOH. Make up to 100ml with distilled water.

80% Ethanol

80ml 100% Ethanol (Saarchem, Merck)

20ml distilled water

5M NaCl (100ml)

29.22g NaCl (Saarchem, Merck)

100ml distilled water

Dissolve NaCl in distilled water.

10% SDS (100ml)

10g SDS (Sigma)

80ml distilled water

Dissolve SDS in water while heating to 60°C. Make up to 100ml with distilled water.

20X SSPE (1000ml) pH 7.4

35.6g $Na_2HPO_4*2H_2O$ (0.2M) (Saarchem, Merck)

210.24g NaCl (3.6M) (Saarchem, Merck)

7.4g EDTA (20mM) (Saarchem, Merck)

700ml distilled water

Dissolve all components in distilled water. Adjust pH to 7.4. Make up to 1 litre with distilled water.

2X SSPE (500ml)

50ml 20X SSPE

450ml distilled water

2X SSPE / 0.5% SDS (1000ml)

100ml 20X SSPE

50ml 10% SDS (Sigma)

850ml distilled water

2X SSPE / 0.1% SDS (1000ml)

100ml 20X SSPE

10ml 10%SDS (Sigma)

890ml distilled water

50X TAE Buffer

242g Tris (Trizma)

57.1ml Glacial acetic acid (ProAnalyst, Merck)

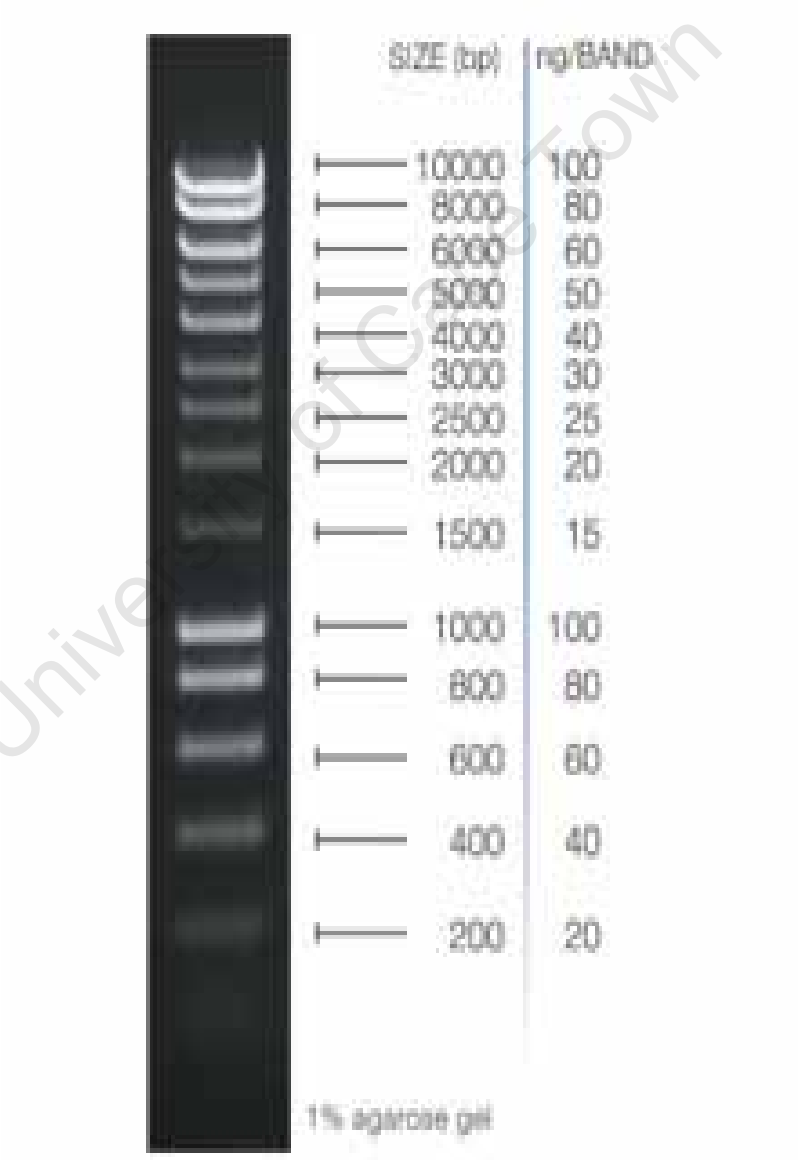
100ml EDTA (Saarchem, Merck)

Make up to 1000ml with distilled water

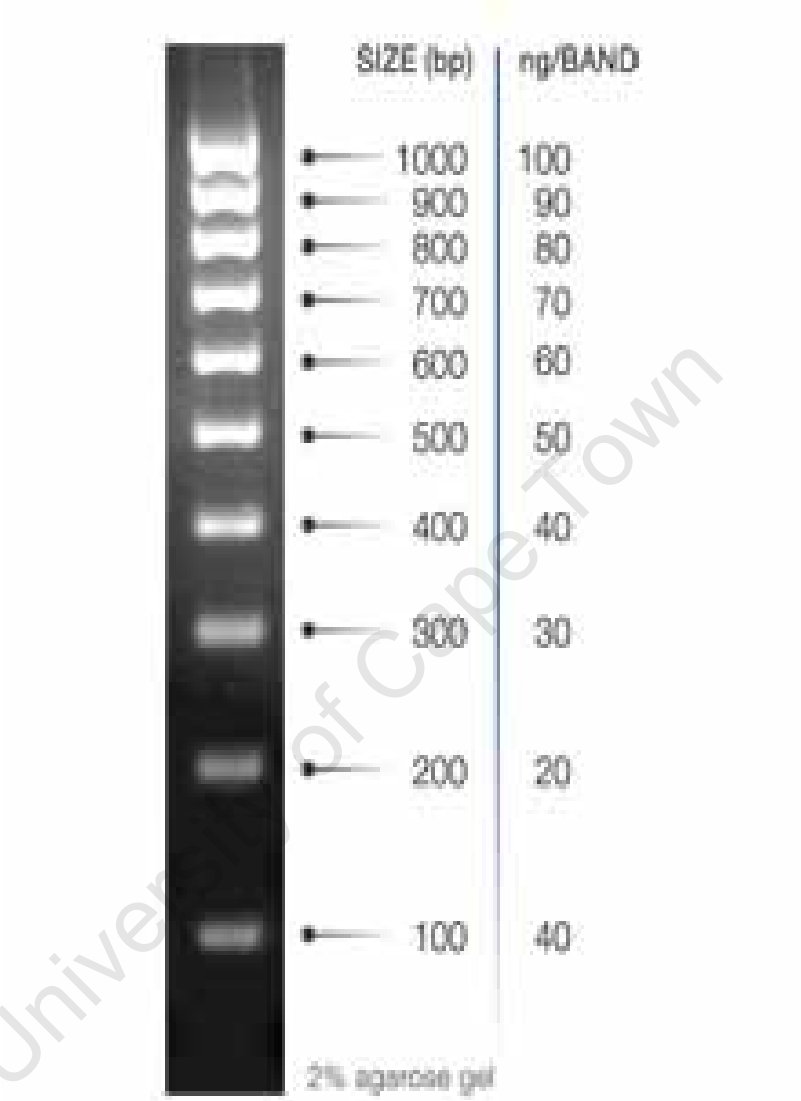
Appendix B

Molecular Weight Markers

(i) Hyperladder I (Bioline)



(ii) Hyperladder IV (Bioline)



Appendix C

List of patients and samples included in this study

Patient Number	Patient Name	Sample Number	Date of Isolation
2	FA	1234421	2005/06/17
4	UO	1223737	2005/06/09
		1260616	2005/06/30
		1269351	2005/07/07
5	GS	1255768	2005/06/29
6	RB	1246392	2005/06/23
7	XT	1281542	2005/07/14
		1293114	2005/07/21
8	RJ	1265215	2005/07/05
		1277144	2005/07/12
9	OA	1265212	2005/07/05
		1289163	2005/07/19
		1307762	2005/07/26
		1312157	2005/08/02
10	MO	1251338	2005/06/27
		1262949	2005/07/04
11	DP	1251339	2005/06/27
		1262971	2005/07/04
		1277114	2005/07/11
		1329408	2005/08/01
		1343923	2005/08/23
12	TM	1267623	2005/07/06
		1279425	2005/07/13
		1314276	2005/08/03
13	BB	1265207	2005/07/05
		1277147	2005/07/12
		1307768	2005/07/26
		1312165	2005/08/02
		1329397	2005/08/11
		1359908	2005/08/31
14	MM	1307754	2005/07/29
		1312146	2005/08/02
		1324741	2005/08/10
		1334015	2005/08/16
15	JS	1307737	2005/07/28
		1316335	2005/08/03

		1329383	2005/08/11
		1349874	2005/08/25
		1373431	2005/09/08
16	DM	1349872	2005/08/25
		1367291	2005/09/01
		1373444	2005/09/08
		1400869	2005/09/22
		1412610	2005/09/30
17	MM	1334036	2005/08/16
18	WJ	1359916	2005/08/31
		1383382	2005/09/15
		1395446	2005/09/21
		1407244	2005/09/28
		1432842	2005/10/12
		1458241	2005/10/27
19	BB	1367311	2005/09/01
		1373435	2005/09/08
		1385616	2005/09/15
		1400856	2005/09/22
		1408412	2005/09/29
20	TM	1367330	2005/09/05
		1383361	2005/09/13
		1395430	2005/09/20
		1405193	2005/09/27
		1415619	2005/10/03
		1453396	2005/10/25
		1465991	2005/10/31
21	CJ	1367317	2005/09/05
		1379060	2005/09/12
		1390854	2005/09/19
		1415616	2005/10/03
22	JE	1367303	2005/09/05
		1383342	2005/09/13
		1390857	2005/09/19
		1403194	2005/09/26
		1415602	2005/10/03
		1428216	2005/10/10
		1440829	2005/10/17
		1453398	2005/10/24
		1465977	2005/10/31
23	FM	1383351	2005/09/14
		1408414	2005/09/29
		1420420	2005/10/05
		1432846	2005/10/12
		1445748	2005/10/20
24	NC	1371553	2005/09/07
		1383366	2005/09/14
		1395436	2005/09/21
		1408416	2005/09/29

		1420396	2005/10/05
		1432832	2005/10/13
		1470151	2005/11/03
		1408411	2005/09/29
		1420409	2005/10/06
		1470138	2005/11/03
26	YK	1415610	2005/10/03
		1465961	2005/10/31
28	JJ	1420405	2005/10/06
		1470119	2005/11/03
29	FJ	1420418	2005/10/06
		1445737	2005/10/20
		1458226	2005/10/26
		1482453	2005/11/10
		1507276	2005/11/24
		1519359	2005/11/30
30	AV	1494783	2005/11/17
		1539261	2005/12/13
32	WC	1470273	2005/11/02
		1494798	2005/11/17
33	WH	1470135	2005/11/02
		1494790	2005/11/16
		1507295	2005/11/23
		1543475	2005/12/14
34	CI	1494793	2005/11/17
		1507273	2005/11/24
		1568275	2006/01/04
35	NN	1482475	2005/11/10
		1507264	2005/11/23
		1543473	2005/12/15
36	BQ	1494784	2005/11/17
		1507268	2005/11/24
37	KM	1568273	2005/01/05
38	TP	1579214	2006/01/12
		1641282	2006/02/15
		1653902	2006/02/22
39	JD	1579217	2006/01/12
		1603044	2006/01/26
		1615749	2006/02/02
		1661615	2006/02/27
40	SD	1588560	2006/01/16
		1598645	2006/01/22
		1623700	2006/02/07
		1649304	2006/02/20
		1661614	2006/02/27
		1673416	2006/03/06
41	SM	1588562	2006/01/16
		1610958	2006/01/31
42	MM	1610936	2006/01/30

		1628716	2006/02/06
		1661618	2006/02/27
		1673381	2006/03/06
43	XM	1623695	2006/02/07
		1636638	2006/02/13
		1661611	2006/02/28
		1685858	2006/03/13
		1710042	2006/03/28
		1610954	2006/01/31
44	DL	1623688	2006/02/07
		1649308	2006/02/21
		1685846	2006/03/13
45	LG	1641334	2006/02/16
		1653910	2006/02/23
		1664849	2006/03/02
		1678125	2006/03/09
		1695690	2006/03/17
		1702154	2006/03/23
		1715014	2006/03/30
46	JP	1641320	2006/02/16
		1653908	2006/02/23
		1678107	2006/03/09
		1690893	2006/03/15
47	ZM	1636639	2006/02/13
		1649274	2006/02/20
		1661608	2006/02/27
		1678080	2006/03/06
		1696795	2006/03/20
		1710014	2006/03/27
48	ML	1636635	2006/02/14
		1649265	2006/02/21
		1661620	2006/02/28
		1673413	2006/03/07
		1685832	2006/03/13
		1702184	2006/03/22
		1710019	2006/03/28
		1722731	2006/04/04
49	DN	1623691	2006/02/07
		1641298	2006/02/15
		1661616	2006/02/28
		1685850	2006/03/14
		1702161	2006/03/22
50	SD	1628703	2006/02/09
		1641293	2006/02/16
		1653906	2006/02/23
		1664836	2006/03/02
		1678129	2006/03/09
		1690895	2006/03/16
		1702164	2006/03/22

		1715023	2006/03/30
51	SM	1653904	2006/02/22
		1664832	2006/03/02
		1678086	2006/03/09
		1690881	2006/03/16
		1727539	2006/04/06
52	SM	1664845	2006/03/02
		1678067	2006/03/08
		1690897	2006/03/16
		1702159	2006/03/23
		1715026	2006/03/30
		1727541	2006/04/06
		1740707	2006/04/13
		1750132	2006/04/20
		1760497	2006/04/26
53	TN	1678117	2006/03/09
		1690888	2006/03/15
		1702182	2006/03/22
54	ER	1690891	2006/03/16
		1715028	2006/03/30
		1740711	2006/04/13
		1760498	2006/04/25
		1772240	2006/05/04
55	BM	1702179	2006/03/23
		1715015	2006/03/30
		1735554	2006/04/10
		1750127	2006/04/20
		1760502	2006/04/26
		1780684	2006/05/08
		1785629	2006/05/11
56	TZ	1745403	2006/04/18
		1767931	2006/05/02
57	SM	1785633	2006/05/11
		1798432	2006/05/18
		1810230	2006/05/25
		1822840	2006/05/31
		1836616	2006/06/08
		1849019	2006/06/15
		1860258	2006/06/22
		1885012	2006/07/06
58	NM	1794023	2006/05/15
		1810235	2006/05/25
		1818053	2006/05/29
		1831075	2006/06/06
		1844175	2006/06/13
		1855694	2006/06/20
		1872720	2006/06/28
		1880528	2006/07/04
59	AM	1798436	2006/05/16

		1810237	2006/05/25
		1822851	2006/05/31
		1836623	2006/06/08
		1849023	2006/06/14
		1860266	2006/06/21
		1885016	2006/07/05
		1897788	2006/07/12
60	JS	1805913	2006/05/22
		1818059	2006/05/29
		1831073	2006/06/05
		1844174	2006/06/12
		1855683	2006/06/19
		1867927	2006/06/26
61	WM	1805900	2006/05/23
		1831081	2006/06/06
		1844176	2006/06/13
		1855688	2006/06/20
		1867952	2006/06/27
		1885028	2006/07/05
		1893056	2006/07/11
		1905871	2006/07/18
62	CG	1818055	2006/05/29
63	MC	1836612	2006/06/07
		1860254	2006/06/21
		1885026	2006/07/05
		1910319	2006/07/19
64	BV	1855692	2006/06/20
		1867961	2006/06/27
		1885032	2006/07/05
		1923227	2006/07/26
65	RR	1867947	2006/06/21
		1880519	2006/07/03
		1893049	2006/07/10
		1905870	2006/07/17
		1931626	2006/08/01
66	RP	1880507	2006/07/04
		1893053	2006/07/11
		1905867	2006/07/18
		1931651	2006/08/01
		1955253	2006/08/15
		1967936	2006/08/22
		1980993	2006/08/29
67	CE	1905875	2006/07/17
		1931637	2006/08/01
		1943975	2006/08/08
		1980998	2006/08/29
		1993557	2006/09/04
68	NM	1905886	2006/07/17
		1918533	2006/07/24

		1931656	2006/08/01
		1967887	2006/08/21
		1980985	2006/08/29
69	BD	1936392	2006/08/03
		1985554	2006/08/30
		1998477	2006/09/07
70	JB	1931645	2006/08/01
		1959613	2006/08/17
71	NV	1936389	2006/08/02
		1959624	2006/08/16
		1985556	2006/08/30
		1998472	2006/09/06
		2013120	2006/09/13
72	VF	1980989	2006/08/29
		1993553	2006/09/04
73	NC	1993545	2006/09/05
		2006928	2006/09/11
		2025035	2006/09/21
		2031330	2006/09/20
		2031350	2006/09/26
		2044964	2006/10/03
		2057353	2006/10/09
		2072165	2006/10/16
		2084284	2006/10/24
		2096927	2006/10/31
74	SE	1998486	2006/09/06
		2025032	2006/09/20
		2036235	2006/09/27
		2049406	2006/10/04
75	CK	2011461	2006/09/14
		2025027	2006/09/20
		2036254	2006/09/27
76	BD	2011453	2006/09/14
		2036242	2006/09/28
78	NM	2011445	2006/09/14
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		3254518	2008/09/16

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Appendix D

MIRU-VNTR Allele Tables

Table D1. Main MIRU-VNTR allele table

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

The numbers represented in this table correspond to the sizes of the amplified MIRU loci and the equivalent VNTR alleles. (Adapted from Supply *et al*, 2000)

Table D2. MIRU-VNTR alleles of isolates obtained from patients identified by spoligotyping as harbouring mixed infections

Cluster	Lineage†	MIRU02	MIRU04	MIRU10	MIRU16	MIRU20	MIRU23	MIRU24	MIRU26	MIRU27	MIRU31	MIRU39	MIRU40
Cluster 1	T4	2	2	4	3	2	5	1	5	3	3	2	3
Cluster 2	S	2	2	3	3	2	3	1	5	3	3	3	2
Cluster 3	WB	2	3	4	3	2	5	1	7	3	5	3	3
Cluster 4	LAM 3	2	3	4	3	2	6	1	5	3	5	4	3
Cluster 5	WB + X3	2	3	4	3	2	5	1	7	3	3	2	3
Cluster 6	WB	2	2	3	3	2	5	1	7	3	5	3	3
Cluster 7	T1	2	2	3	4	2	5	1	5	3	3	2	3

†WB: W-Beijing