MAJOR SPOLIGOTYPE FAMILIES OF *Mycobacterium tuberculosis* STRAINS ISOLATED FROM TUBERCULOSIS PATIENTS IN PORT ELIZABETH, EASTERN CAPE, SOUTH AFRICA

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Together in Excellence

A DISSERTATION

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ABSTRACT

South Africa is burdened with tuberculosis (TB) which is aggravated by the concurrent epidemic of HIV as well as the emergence of drug resistance. In most developed countries molecular techniques have been used to look at the dynamics of the TB epidemic however, despite the prevalence that is high in sub-Saharan Africa, there is little data on strain types that are available in Port Elizabeth. This study aims to find the major clades of *M. tuberculosis* that are circulating in Port Elizabeth. Two hundred MDR-TB DNA samples were obtained from the National Health Laboratory Services TB laboratory in Port Elizabeth. Spoligotyping and MIRU-VNTR were used to genotype the strains. Two hundred strains were sent to the University of Stellenbosch for spoligotyping and 179 of those were typed. Spoligotype defined families were further typed by MIRU-VNTR typing, so as to further differentiate and assess clonal diversity within the spoligotype families. The Beijing family was the dominant family and the MANU family being the least dominant, with percentages of 71% and 0.5% respectively. A comparison of spoligotyping results with the international spoligotyping database (SITVIT2) showed a total of 15 shared international types. Forty four percent (44%) of the isolates that were typed by MIRU-VNTR showed similarities, suggesting epidemiological relatedness. Thirty eight percent of isolates from spoligotyping were from the same family, the Beijing family, with the same shared international type STI1, but when typed by 12 MIRU-VNTR they showed no epidemiological relatedness and 18% of the isolates showed no relatedness when typed by 12 MIRU-VNTR but spoligotyping showed that they were from the LAM family. Results from our study illustrate the effectiveness of MIRU-VNTR typing together with spoligotyping in epidemiological studies in the region of Port Elizabeth.

DECLARATION

I, Babalwa Jennifer Nqini hereby declare that the project titled "Major Spoligotype Families of *Mycobacterium tuberculosis* Strains Isolated from Tuberculosis Patients in Port Elizabeth, Eastern Cape, South Africa, contained in this dissertation submitted for the award of MSc (Biochemistry) degree at the University of Fort Hare, is my own original work and has not previously been submitted at any university for a degree.

Signature: ……..

DEDICATION

I dedicate this work to my dearest mother Linda Jennifer Nqini for her unwavering support.

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CHAPTER 1

INTRODUCTION

1.1 HISTORY

Tuberculosis (TB) has been an infectious disease of humans for many years. There is evidence that dates back to many thousand years of tuberculosis-compatible lesion (Salo, et al., 1994). Based on the perspective of the disease's historical and contemporary burden, TB is one of the things that have brought about human suffering. At the beginning of the 19th century, the mortality ranged between 200 and 300 per 100 000 of the population in Europe (Kato-Maeda et al., 2001). Then in the 1880s things started to get better; there was an improvement in the general living conditions, also, there were specific TB control measures as well as public health measures that came into place. In 1882 the causative agent of TB was discovered by Robert Koch (Zumla et al., 1999), but there were still no antibiotics that were available for the treatment of TB until 1944 when streptomycin was discovered (Chopra and Brennan, 1998). In the 1950s there was a development of multidrug chemotherapy regimens by Sir John Crofton and his colleagues. When rifampicin was discovered, there was then a development of the present short course regimes (Zumla et al., 1999). Since then, tuberculosis has developed into an epidemic that is fuelled by HIV, poor management of the disease as well as non-compliance to treatment, and this has caused an increase in reactivation rates, re-infection of patients that had been cured, and the multidrug resistance development (Mokhethi, 2004).

Despite the global efforts to control and eradicate the disease, Tuberculosis (TB) remains a major cause of illness and death globally, more so in Asia and Africa (WHO, 2011). Twenty-two countries that were rated as the countries with high TB burden account for 80% of all new cases worldwide (WHO, 2011). Since 2008, South Africa was rated fourth among these countries, with an incidence rate of 940 cases per 100 000 people (WHO, 2011). Due to the lack of adherence to TB treatment or inappropriate use of drug regimes, the epidemic of TB has been largely aggravated by the emergence of multi-drug resistant (MDR) TB (Drobniewski, 2004).

By tradition, it has been assumed that TB is caused by an infection with a single strain and that recurrences result from the reactivation of the strain which causes the first episode (de Boer et al., 2000; Richardson et al., 2002; Warren et al., 2004). However, other studies have shown recently that patients in high-incidence locations may have more than one strain in the same sputum sample (Richardson et al., 2002; Warren, 2004) and that if a patient is found to be infected with both a sensitive and a resistant *Mycobacterium tuberculosis* isolate, mixed infections may cause complications in the treatment of the disease (Warren, 2004). Multidrug-resistant TB emerged as a threat to global TB control in the 1990s an since then, it has been reported in all countries throughout the world. The World Health Organisation (WHO, 2008), representing data from 116 countries, showed that new cases of MDR-TB had almost tripled since 2006. Global TB data from 198 countries in 2009 gave an estimated report of 0.5million cases of MDR-TB worldwide, with 27 countries (15 of them in the European Region) accounting for 85% of all the cases (WHO, 2009b). Extensively drug resistant (XDR) TB strains, which are defined as non-responsive to Isoniazid [INH] and rifampin [RIF], any fluoroquinolones, as well as at least one injectable anti-TB drug (CDC, 2006), were first reported in South Africa in 2006 and were later shown to be prevalent in at least 17 countries and all of the continents (Gandhi et al., 2006). The use of the molecular technique such as Spoligotyping in other studies has shown that the majority of these cases of XDR-TB were caused by *M. tuberculosis* that belongs to the KZN family (ST60), which has been known to be prevalent in this area since 1994 (Pillay, 2007). Studies like these highlight the need for accurate susceptibility testing together with high level molecular genotyping in order to carefully monitor new and emerging MDR and XDR TB strains (Stavrum et al., 2009). This is why the objective of this study was to find the major clades that are circulating in Port Elizabeth, Eastern Cape, South Africa.

1.2 THE *MYCOBACTERIUM TUBERCULOSIS* COMPLEX

M. tuberculosis is a member of *M. tuberculosis* complex (MTBC), which is made up of *M. tuberculosis, M. bovis, M. africanum, M. pinnipedii, M .caprae, M. microti and M. canetti.* These members are closely related, with 99.9% sequence similarity on the nucleotide level (Sreevatsan et al., 1997). But even with this close relatedness, they still have different phenotypes, they bring about different pathologies and they demonstrate different host specificities. The precise origin of the evolution of *M. tuberculosis* however, is still unknown,

but it has been suggested previously that is derived from *M. bovis* (which is an organism infecting cattle), as a result of cross-species jump (Stead et al., 1995). However, there was new evidence that was later found which suggested that *M. bovis* actually derived from *M. tuberculosis* (Hewinson et al., 2006; Mostowy et al., 2002).

1.2.1 Mycobacterium tuberculosis

Mycobacterium tuberculosis (*M. tuberculosis*) is the main cause of TB in humans, and debatably the most successful bacterial pathogen. It was first described by Robert Koch in 1888 and is thus sometimes referred to as Koch's bacillus (Koch, 1882). There have been speculations about *M. tuberculosis* evolving from *M. bovis* by specific adaptation of an animal pathogen to the human host (Stead et al., 1995). This was proposed before the availability of the whole genome sequence of *M. Tuberculosis* (Cole et al., 1998) and before several variable genomic regions in the members of the MTBC was uncovered by comparative genomics. The degree of relatedness to the last common ancestor of the MTBC was proposed based on the presence or absence of such conserved regions. It showed that the lineages of *M. tuberculosis* and *M. bovis* separated before the occurrence of the *M. tuberculosis* deletions TbD1. This analysis therefore, made it clear that it is impossible for *M. bovis* to have been the ancestor of *M. tuberculosis*, but rather, appears either to be descended from *M. tuberculosis* or to have emerged independently (Brosch et al., 2002, Cole, 2002, Huard et al., 2003).

1.2.2 Mycobacterium africanum

Mycobacterium africanum (M. africanum) is predominantly isolated in different parts of Africa (David et al., 1978). Up to 60% of isolates obtained from patients with pulmonary TB are represented by *M. africanum* in certain regions (Haas et al. 1997). This species was first isolated from a Senegalese patient suffering from pulmonary TB in 1968 (Castets et a., 1968). It was then discovered that it has characteristics that seem to lie in between *M. tuberculosis* and *M. bovis*. *M. africanum* species are subdivided into two major subgroups, based on their biochemical properties as well as their geographic origin: Subtype I are closer to *M. bovis* and are from West Africa; Subtype II are from East Africa and are closer to *M. tuberculosis*

(Niemann et al., 2002). There are differences in the distribution of *M. africanum* in various regions of Africa, the different prevalence of this species were Ivory Coast 5%, Guinea-Bissau 60%, Burkina Faso 18% and Uganda 49-60% (Castets et al 1968; Kallenius et al., 1999, Niemann et al., 2002). The issue of whether *M. africanum* subtype II is a genetically well-defined subspecies of the MTBC has been quite a considerable debate (Mostowy, 2002; Sola et al.; 2003).

1.2.3 Mycobacterium bovis and Mycobacterium bovis BCG

The host range of *M. bovis* is broad, and it can cause TB in different domestic or wild animals such as goats or cattle, but can also cause Tb in humans (Wayne et al., 1986). Bovine TB remains a significant disease in a number of countries of the world, as it is a cause of high rate of economic losses in certain countries. The risk factors for *M. bovis* infection in both animals and humans have been observed to be close, food hygiene practices, such as the consumption of dairy and meat products that have not been processed, and HIV/ AIDS, and this is more so in African settings where domestic animals are a part of human social life (Cosivi et al., 1998). *M. bovis BCG* (Bacillus Calmette-Guerin) is from a virulent *M. bovis* strain, where 230 in vitro passages of *M. bovis* were performed by Calmette and Guerin until the organism lost its virulence (Calmette, 1927). Even though this strain has been used as an attenuated vaccine, it is possible that it may cause disease, but only after vaccination with BCG.

1.2.4 Mycobacterium microti

Mycobacterium microti (*M. microti)* had earlier been considered non-pathogenic for humans, and only causes TB mainly in small rodents such as voles (Kremer et al., 1998, Wells et al., 1937). This species was proved to be a member of the MTBC based on the DNA sequence of the 16S rRNA gene and the 16S-to-23S internal transcribed spacer region. *M. microti* is very slow to grow and this has complicated the primary isolation and differentiation of this species using biochemical tests, however, spoligotyping has allowed the simultaneous detection and typing of *M. microti* (van Soolingen et al., 1998). This technique was applied in two studies of *M. microti* infections the Netherlands and England, allowing the identification of cases in Ilamas, cats and ferrets, and in the four humans that were used in the study only one was

described as immunocompetent. Another study was done by Niemann et al (2000) and he reported cases of *M. microti* infections that caused pulmonary TB in Germany, and the isolates, according to spoligotyping patterns, were identified as *M. microti* of the ilama and vole types, which displayed reactions with only two of the 43 spacers (spacers 37 and 38).

1.2.5 Mycobacterium caprae

A test was done in Spain where isolates from the MTC were cultured from caprine pathological tissue samples. These isolates were genetically and biochemically characterized, and the result was that they segregated from the members of the MTB complex. Based on the results of the test as well as the repeated relationship of this microorganism with goats, it was suggested that a new member of the complex had been identified, which does not match any of the already existing classical species. These isolates where then given the proposed name *M. tuberculosis subsp. Caprae subsp.nov* (Aranaz et al., 1999). Strains of this unusual member of the MTC have shown a unique combination of *pncA, axyR, katG* and *gyrA* gene polymorphisms, while the analysis of the sequence of the *gyrB* gene in the same strains revealed nucleotide substitutions that are not found in other members of the MTC that could be used in the differentiation of caprine mycobacterial strains from *M. bovis* and other members of the MTC. Based on this, it was then proposed that *M. tuberculosis subsp caprae* be elevated to species status, as *Mycobacterium caprae comb.nov, sp.nov* (Aranaz et al., 1999).

1.2.6 Mycobacterium pinippedii

In 1993, Tuberculosis was diagnosed in Australia in seals (pinnipeds) (Brosch, 2002). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and Western blotting techniques were once used to determine the similarity of mycobacterium sp strains that were isolated from the lungs of 2 seals, the secretory protein MPB70 that is present in *M. bovis* was not detected in the wild seal isolates. When further analysis of protein and DNA fragment profiles was done, it indicated that TB isolates that were found in seals formed a special cluster within the MTC. Another test was done where MTC isolates from seals in Australia, Argentina, Uruguay, Great Britain and New Zealand were compared to one another, in an effort to determine their relationships to each other and their taxonomic

position within the complex (Cousins et al., 2003), and using biochemical tests, it was confirmed that the seal isolates belonged to the MTB complex. When grown on media it was noted that, in many cases the isolates grew preferentially on media containing sodium pyruvate and there were minor differences that were noted (Asiimwe, 2008). Further analysis was done which showed all isolates contained the sequences IS6110, IS1081, mpb70 and mtp40 however, they failed to produce detectable MPB70 antigen. The pncA gene in *Mycobacterium pinippedii* contained CAC (His) at codon 57 and the oxyR gene showed G at position 285, and this was noted to be similar to *M. tuberculosis, M. microti* as well as *M. africanum.* When spoligotyping was done on the seal isolates, the spoligotypes were noted to from a cluster that differed from those of other MTC members. The proposed name for this novel member of the MTC was *Mycobacterium pinnipedii* sp. nov. (Cousins et al., 2003)

1.2.7 Mycobacteruim canettii

An unusual mycobacterial strain was once isolated from a 2 year old patient with lymphadenitis in Somali. Van Sooligen et al (1997), in an attempt to characterize the strain, applied various molecular methods. It was noted that the bacillus produces smooth and glossy colonies, this characteristic was exceptional this species. Another study was conducted by Miltgen et al (2002), and they identified the same smooth, glossy bacillus from an unusual strain of mycobacteria from two different patients who had pulmonary TB. Spoligotyping and IS6110 RFLP revealed that it was *M. canettii.* All known cases of TB caused by *M. canettii* are said to have been contracted in the Horn of Africa (Pfyffer et al., 1998). *M. canettii* and *M. tuberculosis* have some close similarities, and because of that the prevalence of *M. canettii* taxon isolates may be underestimated in the routine microbiology laboratory (Asiimwe, 2008).

CHAPTER 2

LITERATURE REVIEW

2.1 EPIDEMIOLOGY

Epidemiologic information on tuberculosis has been recorded in detail since 1953 by the US Centres for Disease Control and Prevention. Since the early 20th century the incidence of TB started to decline due to various factors such as basic infection-control practices. However, in 1985, the incidence of TB was noted to resurge again. This increase was especially noted in persons infected with HIV and as a result TB has since been associated with HIV infections. Worldwide, co-infection with HIV is highest in South Africa, India and Nigeria (Assam-Assam et al., 2010).

Mycobacterium tuberculosis is the single leading global cause of infectious disease. In 2008 it was estimated that there were 9.4 million incident cases of TB globally and 80% of those cases were in 22 countries (Evans, 2011). The highest total number of cases, which is 35% of the global total is in South-East Asia, whereas rates in Sub-Saharan African nearly doubles the number of cases in South-East Asia, with over 350 cases per 100 000 population. Thirteen of the fifteen countries with highest estimated TB incidence rates are in Africa, and half of all new cases are in six Asian countries- Bangladesh, China, India, Indonesia, Pakistan and Philippines (Evans, 2011). Globally, there were 1.3 million deaths that were caused by TB in 2008, with more than 2 billion people infected. Very slowly, the overall global TB incidence rate is decreasing at a rate of less than 1% per year. However, the global population growth brings about an offset in the slow decline in incidence rates (WHO, 2009). The World Health Organisation, because of the increase in the global burden, in 1993 declared tuberculosis a 'Global emergency' (Blumberg, 1995).

South Africa has relatively attractive economic destinations, it is committed to combating the awful TB disease, but because it has poor and under-serviced rural areas it still remains troubled with one of the worst tuberculosis epidemics (Mokhethi, 2004). Approximately 400 000 new cases are reported yearly within a population of 50 million (WHO, 2011). After HIV, tuberculosis is the second leading cause of death from an infectious disease in humans, it infects almost more than one-third of the population of the world (WHO, 2011).

2.2 DRUG RESISTANT TUBERCULOSIS

South Africa has made considerably a lot of effort to fight its TB epidemic, and this is evident in the attention that was devoted to TB in the National Strategic plans (NSP) on HIV/AIDS, sexually transmitted infections (STIs) and TB (South African Ministry of Health, 2000, 2007). The NSP has placed so much devotion on TB such that it has been critisezed for devoting a lot of attention to TB at the expense of HIV/AIDS and other STIs (Daku, 2011).

In addition to the large TB problem, the country is also heavily burdened with Multi Drug-Resistant TB (MDR-TB) and Extensively Drug-Resistant TB (XDR-TB), and this has complicated the response to TB (Daku, 2011). In 2010, approximately 1.8% of all new TB infections were multi-drug resistant, and for those cases of TB that were being re-treated, this number jumps to 6.7% (WHO, 2011). There are no official numbers on XDDR-TB available, however, in 2010, five hundred and eleven cases were found to be XDR-TB in South Africa, and this is the highest absolute number of infections that has been recorded worldwide to date (Daku, 2011).

Multi-drug resistance (MDR) is the resistance to at least rifampicin (RIF) and isonizid (INH), which are the two drugs that form the backbone of the modern short course therapy. In *Mycobacterium tuberculosis* it is caused by the mutations that accumulate in the genes that encode the targets of rifampicin and isoniazid. Extensively drug resistant TB (XDR-TB) is defined as resistance to isoniazid, rifampicin, as well as resistance to a flouroquinolone anmid any of the injectable second line drugs (amikacin, capreomycin, or kanamycin) (Sougakoff, 2011).

Since the emergence of MDR strains in the 1990s, the prevalence of MDR-TB has been slowly but constantly increased, such that it now accounts for almost 5% of global TB cases. (Sougakoff, 2011). According to the Multidrug and extensively drug-resistant TB 2010 Global report on surveillance and response, it can be estimated that 440 000 cases of MDR TB emerged globallly in 2008, and this caused an estimated 150 000 deaths (Daku, 2011). More troublesome is the emergence of XDR-TB, which is a nearly untreatable form of the disease which has an estimated rate of 15% among the MDR strains (Sougakoff, 2011).

There are number of reason why South Africa in particular, would be vulnerable to an extensive epidemic of drug-resistant TB. South Africa has over 4.4 million people living in urban informal settlements (Misselhorn, 2010), and because TB is spread with ease in unventilated, unsanitary or overcrowded spaces, approximately 10% the population is at a high risk of TB infection (SANAC, 2011). More importantly however, is South Africa's large HIV-positive population of about 5.6 million people and worldwide, TB is the most common opportunistic infection that is associated with HIV/AIDS (Chaisson and Martinson, 2008). Because HIV- positive patients have a short incubation period for TB, outbreaks in this group may be seen as "canaries in the mine shaft" for a larger epidemic (Basu and Galvani, 2008; Wise, 2006).

2.3 DIAGNOSIS AND DETECTION OF TUBERCULOSIS

2.3.1 Microscopy

This examination is used all over the world as a diagnostic test for suspected pulmonary tuberculosis. Ziehl-Neelsen staining method is the popular method that is used to demonstrate the bacilli of mycobacteria, however, there is another method that is also used, which is the fluorochrome staining method (Mokhethi, 2011). When acid-fast bacilli is found in sputum a presumptive diagnosis of tuberculosis is establish, and this serves as an alarm that the patient is capable of transmitting the the infection and thus, necessary measure and precautions must be taken in order to prevent infection (Mokhethi, 2011). The detection limit for any microscopic diagnostic method is between 10^4 and 10^5 bacilli per millilitre of specimen. If a patient then has organisms that are fewer than 10^4 per ml, it means that the patient will be smear-negative and less has less chances of infecting other people. The microscopic examination method is quick, cheap and it is comparatively easy to perform. It has a sensitivity of about 60 - 70% but, the sensitivity of acid-fast smears of sputum from a patient that is HIV positive is even lower (50% in adults). There are other organisms that also exhibit acid-fastness, like *Nocardia asteroides* and this may lead to false smear-positive results (Blumberg, 1995).

2.3.2 Culture

Culture of sputum samples, because it can detect 10 - 100 organisms per ml, it is more sensitive than the microscopic examination method. Because of this high sensitivity, cases are detectable earlier, and most often before they become highly infectious. *M. tuberculosis* however, grows slowly, a period of about 8 weeks and specimens have to be held for over a month before they can be recorded as negative (Mokhethi, 2004). Lowenstein-Jensen and the BACTEC liquid medium are the two most commonly used culture media. In order to get a definite diagnosis of tuberculosis, bacilli must be isolated from the patient and identified as *M. tuberculosis* (Blumberg, 1995).

Sputum is not the only specimen that can be used for culturing, if a patient cannot produce sputum, like in the case of pulmonary tuberculosis, gastric aspiration can also be a method of choice as a specimen. Other specimens that can be used are liver biopsies, cerebrospinal fluid, as well as bone marrow (Blumber, 1995).

The identification of species is initially based on the rate of growth during culturing and then the production of pigments, which then classifies the *Mycobacterium* species into four groups. Species within the four groups can then be further identified by biochemical tests. To identify the growing organism as *M. tuberculosis*, the NAP test in the BACTEC liquid-culture system is used. High-performance liquid chromatography (HPLC) can also be used in the identification of each *Mycobacterium* species, but because this method is highly expensive, it is seldom used. There are also commercial kits that are available like the serodiagnostic test kits for anti-tuberculosis antibodies but there have been concerns that have been raised in the South African setting with regard to the interpretation of the results from these tests (Blumber, 1995).

2.3.3 Genotypic Identification

2.3.3.1 Identification of *Mycobacterium tuberculosis* Complex by Analysis of the Regions of Difference (RD)

Traditional methods such as culture, smear microscopy and biochemical tests are most widely used in differentiating the members of the *Mycobacterium tuberculosis* complex. While these methods are inexpensive, they however have proven to be labour intensive, with low sensitivity, they are time consuming and are not practical for surveillance purposes (Warren et al., 2006). Despite the 99.95% homogeneity of *M. tuberculosis* and *M. bovis*, the results from comparative genomic analyses that were carried out showed that there were certain genes that were identified in the *M. tuberculosis* genome that were not found in *M. bovis* genome. These regions were then referred to as regions of difference (RD) and 16 regions have been identified, RD1-RD16 (Parsons et al., 2002).

The presence or absence of these genes was then used as a tool to differentiate the *Mycobacterium tuberculosis* complex species by using a PCR based technique. In 2002, Parsons and co-workers recommended the Polymerase Chain Reaction (PCR) amplification of the 6 regions of difference: RD1, RD3, RD5, RD9, RD10, and RD11 to identify the *Mycobacterium tuberculosis* complex (MTC) isolates. A two-step multiplex PCR approach was then suggested in a more recent study, where two sets of primers were used. The first set of primers was to target the RD1, RD4, RD9 and RD12 and this was found to discriminate between *M. tuberculosis, M. bovis, M. canetti, M. caprae and M. bovis* BCG and the second set targeted the $RD1^{mic}$ and $RD2^{seal}$, and this set differentiated between *M. africanum, M. microti and M. pinnipedii.* The *M. bovis* BCG, which is the vaccine strain, differs from the pathogenic *M. bovis* strain by the presence of RD1 in the pathogenic *M. bovis* strains (Warren et al., 2006).

It is important to differentiate the members of the MTC so as to get an accurate diagnosis of mycobacterial disease, for public health surveillance as well as appropriate case management. There is only limited data on the total incidence or prevalence of mycobacterial disease in most countries due to specific MTC members it has.(Cosivi et al., 1998; Bonard et al., 2000).

The absence of simple test in routine diagnostic laboratories accounts for the lack of surveillance (Warren et al., 2006).

Differentiation of MTC members has become particularly important in patients with HIV related immune suppression. PCR based methods have been widely used to differentiate TB from bacilli Calmette-Guerin (BCG) disease in infants that are HIV infected, and accurate diagnoses has repercussions for both clinical management as well as policy (Hesseling, 2003).

2.4 MOLECULAR EPIDEMIOLOGY

One of the aspects of TB research is the epidemiology of *M. tuberculosis*, with the aim to document the dynamics of the disease in different groups of individuals (Coggon et al., 1997). This makes it possible for epidemiologist to assume the reason for the disease occurring in a particular setting (Falmer, 2008). In recent years, molecular epidemiological methods have been used comprehensively in transmission studies of *M. tuberculosis*. The release of the *M. tuberculosis* genome has greatly increased the knowledge of researchers about the organism significantly, so much that focus can now be directed towards its evolution and genetics (Falmer, 2008).

In the past years, molecular typing approaches have made a great enhancement in the understanding of TB epidemiology by indicating possible epidemiological links between TB patients and also by detecting suspected outbreaks (WHO, 1998). In the West bank, Palestinian territories (population of approximately 2.44 million individuals), the incidence rate of TB is low. This can be explained as representing the specific incidence of the disease or a result of a misdiagnosis due to the low sensitivity of the diagnostic methods which are primarily based on microscopic examination of self- expectorated sputum which is stained for acid fast bacilli (AFB) (Ereqat et al., 2012).

A number of cases of multi drug resistance (MDR) and lack of adherence to therapy were reported in the last few years (Ereqat et al., 2011a; Ereqat et al., 2011b). This recognized that the deterioration in the economical, nutritional and health situations caused by regional conflict increases the likelihood of an increase in misdiagnose patients and the rise of MDR TB case (Ereqat et al., 2012).

In the Palestinian territories, TB diagnostic criteria are well established and practiced, but although conventional microbiology (microscopic examination of sputum and culture) still constitutes the principal tool for the diagnosis of TB and appropriate for laboratories with minimal infrastructure, direct microscopy has low sensitivity (Ereqat et al., 2011b), and culturing requires a long period of time, which then leads to delay in diagnosis and treatment, which gives rise to development of drug resistance (Gopinath et al., 2009).

Early diagnosis, effective treatment as well as identification of factors influencing disease dynamics are therefore considered as major factors in the control of TB in the region West Bank region. A detailed genetic study of the regional MTB isolates, identification of the genotypes associated with drug resistance and evaluation of the appearance of new MDR-TB strains in any studied population can facilitate both a more effective drug therapy regime and also give information at the molecular epidemiological level (Ereqat et al., 2012).

Just about a decade ago, the only molecular markers available for the epidemiology study of TB were drug susceptibility profiles and phage types (Falmer, 2008). However, there were some limitations to these methods and in recent years, quite a number of genotyping methods have been developed that are based on DNA polymorphisms in the genome of the organism *M. tuberculosis*. The discovery of polymorphic regions within the genome has led to a whole new era of epidemiology based on molecular methods using these polymorphic regions as markers (Falmer, 2008). Generally, these polymorphisms are located in non-coding regions with different frequencies shown between different strain families. As a consequence, molecular epidemiology of *M. tuberculosis* uses specific genetic markers within the *M. tuberculosis* genome to study the distribution of *M. tuberculosis* strains as well as how the strain distribution changes over a period of time. Compared to traditional methods, molecular

methods give more information and because molecular methods are rapid, they are suitable for use in the diagnosis of TB (Falmer, 2008). It is imperative when conducting epidemiological studies that the marker chosen for a specific method must be suitable for the study setting. Below are some molecular methods that are used in different studies to understand the epidemiology of Tuberculosis.

2.4.1 IS*6110* RFLP ANALYSIS

In 1990 Thierry et al, described a 1.36 kb insertion sequence (IS), which is only found within the MTBC. This sequence which was given the term (IS) 6110, belongs to the IS3 family and is characterized by unique 28 bp imperfect terminal inverted repeats (TIRs). IS6110 possesses a transposase that enables it to extract itself from the genome and re-insert into another genomic region and is also capable of copying itself for insertion at another position. This ability of the sequence to frequently jump from one location to another results in IS6110 displaying both positional and numerical polymorphisms (Falmer, 2008). Restriction fragment length polymorphisms (RFLP) is a method based on the this feature of IS6110. In this method, the restriction enzyme, *PvuII* is used to cut the DNA, which is then dissolved for gel electrophoresis to separate the cleaved DNA. This is then followed by southern transfer of DNA to a specialized membrane, followed by detection and visualization of RFLP patterns by autoradiography. The DNA bands represent the IS6110 insertion elements within the genome of different isolates of *M. tuberculosis* (Falmer, 2008).

These events of transpositions of IS6110 are frequent enough to allow differentiation between strains that are more distantly evolved, but at the same time they are rare enough to show stability within the strains that are more closely related. Therefore, this method not only differentiates between strains, but can also determine whether epidemiological events were either recent (transmission) or distant (reactivation) (Dale et al., 1997). The use of this DNA fingerprinting method has proved to be critical in differentiating between endogenous reactivation and exogenous re-infection (Heldal et al., 2000), detecting outbreaks in hospitals (Coronado et al., 1993; Edlin et al., 1992; Moro et al., 1998), in prisons (Valway et al., 1994), among health care workers (Jereb et al., 1995; Beck-Sague, 1992) and within communities (Van Rie et al., 1999). Although this method is suitable for an epidemiological research, it has its short comings. One of the shortcomings of this method is that discrimination is much

lower for strains with less 5 IS6110 copy numbers than strains with more than 5 IS6110 copy numbers, therefore, for low copy strains (<5 copy number) other genotypic methods are preferred (Falmer, 2008).

2.4.2 SPOLIGOTYPING

Spacer Oligotyping (Spoligotyping) is a PCR-based typing method that was first published in 1997 (Kamerbeek et al., 1997). This was the first genotypic method that allowed for the detection and differentiation of *M. tuberculosis* complex strains simultaneously in clinical samples, without the need for culture. Spoligotyping relies on identifying polymorphisms in the spacer units in the direct repeat (DR) region of the genome. This region contains multiple, conserved 36 base pairs (bp) DRs that have distinctive, individual spacer sequences that range from 34-41 bp in length spread between each DR. Strains of *M. tuberculosis* differ in the number and presence or absence of DRs (Groenen et al., 1993; Hermans et al., 1991). These variations are thought to be caused by homologous recombination between adjacent or distinct DRs or by transformation due to the transposition of IS6110, which is almost consistently present in the DR region (Groenen et al., 1993).

The analysis of the DR locus by PCR was initially by "Direct Variable Repeat PCR". The development of detection through reverse hybridization to probes against 43 selected individual spacer regions bound, covalently to a nylon membrane, not only enhanced the technique but also made the analysis of data simple (Evans, 2008). The variation in presence or absence of spacer regions provides different hybridization patterns. The technique is called spacer oligotyping or spoligotyping because each spacer region has a complementary oligonucleotide probe on the nylon membrane (Evans, 2008).

Spoligotyping is a fast and vigorous genotyping technique hence it has been applied in a number of studies of *M. tuberculosis* complex strains globally. Spoligotyping data from a number of previous studies have been merged into the "fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology" (Brudey et al., 2006). The database consists of spoligotyping data from 39 295 strains from 122 countries. In

total, there are 5309 individual patterns with 1939/5309 shared by more than one isolate and 3370 orphan types. Sixty two global clades have been acknowledged with the use of a mixed expert-based and bioinformatics approach. This database plays a huge role in the construction of a global phylogenetic relationship for *M. tuberculosis* and has given insight into strain transmission as well as migration (Evans, 2008).

To date, there are eight chief *M. tuberculosis* spoligotyping clades that have been identified, these are Beijing, Central Asian Strain (CAS), East-African Indian (EIA), Haarlem, Latin American and Mediterranean (LAM), X, and T. Each clade seems to be linked with prevalence in particular geographical continents. The Beijing strain represents about 50% of the strains that are found in Far-East Asia and is also noted to be emerging in Russia; CAS is mainly found in the Middle-East or South Asia and precisely in India; EAI is prevalent in Central/South-East/Far-East-Asia, in the Middle East, as well as in Oceania. Haarlem is prevalent in Europe as well as Central America/Caribbean, and the fact that it is present in both areas suggests that there is an association with historical European colonisation. LAM is present in South America, coastal regions in the Mediterranean and the Caribbean. The X family in North America and Central America could be associated with the Anglo-Saxon ancestry but at present it is interrelated with African-Americans. Lastly, the T family is prevalent in all continents (Brudey et al., 2006).

To obtain spoligopatterns, PCR amplification of the DR region, then the amplification products are hybridized on the 43 spacer DNA containing membrane. To visualize the pattern, a second antibody is used which leads to a fluorescent emission (Kamerbeek et al., 1997).

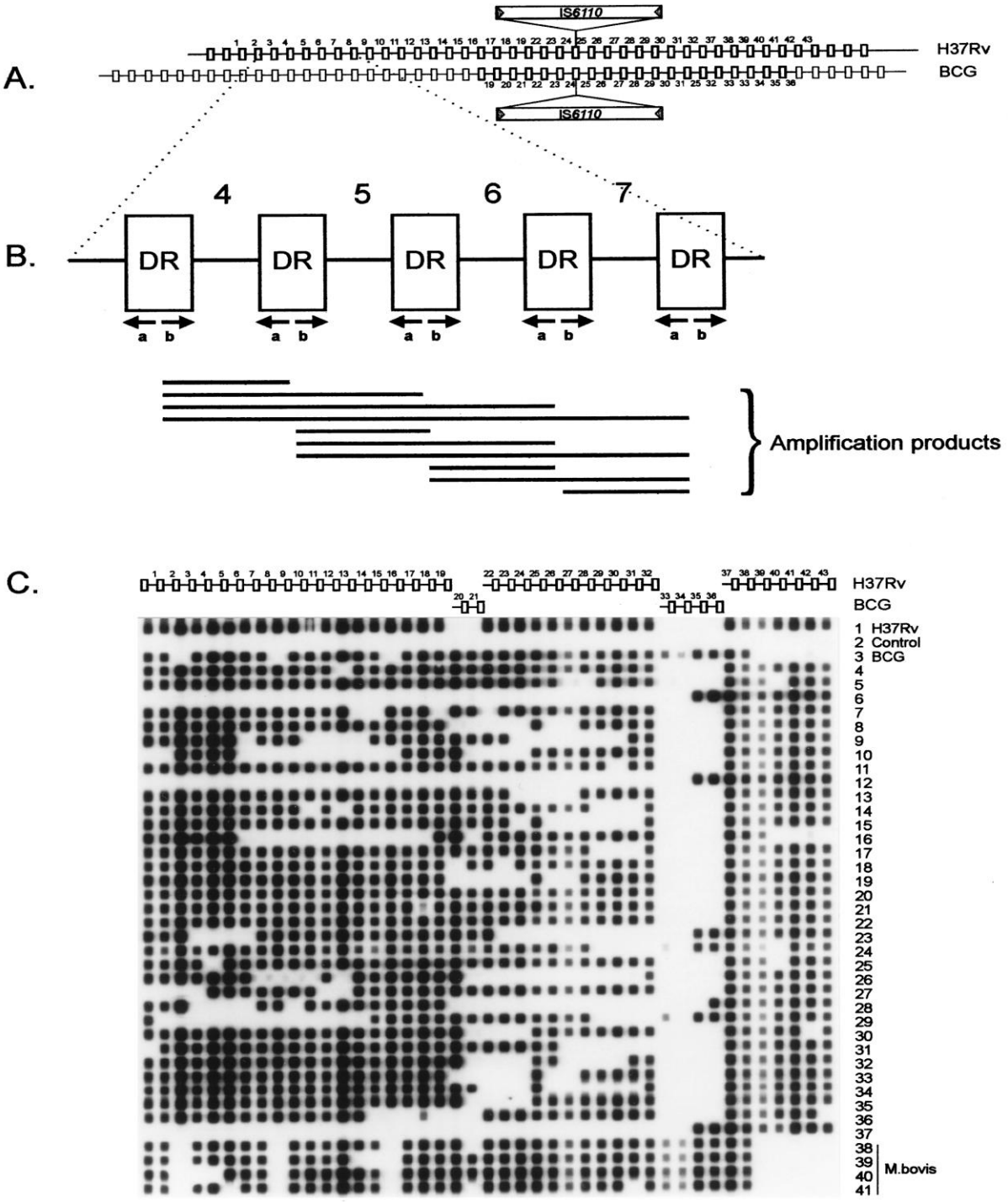


Figure 2.4.2.1 (A) Structure of the DR locus in the mycobacterial genome. *M. tuberculosis* H37Rv contains 48 DRs and *M. bovis* BCG contains 41 DRs (shown as rectangles). They are interspersed with distinctive spacers differing in length from 35 to 41 pb. The spacers that are numbered relate to 37 spacers from *M. tuberculosis* H37Rv and 6 from *M. bovis* BCG. The position of incorporation of insertion element IS6110 is illustrated. (B) Principles of in vitro amplification of the DR region by PCR. Any DR in the DR region may function as a target for these primers. The amplified DNA is therefore composed of a mixture of a large number of different-size fragments. A combination of fragments is presented that would be produced by in vitro amplification of a DR target containing only five contiguous DRs. (C) Hybridzation patterns (spoligotypes) of amplified mycobacterial DNAs of 35 *M. tuberculosis* and 5 *M. bovis* strains. The order of the spacers on the filter corresponds to their order in the genome. The spoligotypes of strains 6, 12, and 37 correspond to the strains from the Beijing genotypic group (Kamerbeek et al., 1997)

2.4.3 MIRU-VNTR ANALYSIS

Genotyping of *M. tuberculosis* helps in the investigation of confirmed or assumed outbreaks; it helps to identify cross contamination that might have taken place in the laboratory; it helps facilitate the estimation of the degree of recent transmission; and also it distinguishes between reactivation and re-infection. Identifying clusters and associated lineages can be of assistance in investigating virulence, transmissibility, as well as drug resistance. In principle, genotyping should be used to measure genetic markers that represent the whole genome. The changes in these markers may then be used in the hyphothesis of the degree of relatedness and evolutionary paths between strains (Shorten, 2008). Mycobacterial Interspersed Repetitive Units- Variable Number Tandem Repeat (MIRU-VNTR) is one of the basic methodologies that are widely as genotyping tools and will be used in this study.

Repetitive units of DNA in organisms are well described and have been used in the genotyping of a large range of bacteria such as *Escherichia coli, Clostridium difficile, Bacillus anthracis*, *Yersinia pestis, Listeria monocytogen*, *Shigella* spp. VNTRs have also been used in mapping human genes (Nakamura et al. 1987). This method evaluates the number of repeats at different loci distributed throughout the entire genome. Normally PCR amplification followed by the comparison of the product sizes with a molecular size marker on an agarose gel, is sufficient as the size differences are within a range of 30-100 base pairs, Kremer and others (1999) have described the VNTR typing of isolates of MTC. The development of polymorphic markers is now enabled by the availability of whole genome sequences for bacterial genomes which show that the members of MTC randomly contain 41 MIRUs (Supply et al., 2000).

This method is reasonably easy and straight forward and the resulting data can be coded and exchanged between laboratories with ease, independently of the technology that is used to measure PCR fragment size (Le Fleche et al., 2002). Moreover, the resolution of tandem repeats typing is cumulative, that means if more markers are included in typing assay that can increase the resolution of identification (Le Fleche et al., 2002). The density of tandem repeat in bacterial genome however, differs from species to species and not all tandem repeats are polymorphic (Le Fleche et al., 2001).

In a sample of 47 isolates from Northern Ireland, these markers were proven to be more discriminatory than spoligotyping (Roring et al., 2002). Savine and others (2002) and Skuce and others (2005) did a study and the results they obtained suggested that VNTR-type loci are satisfactorily steady and robust and allow transmission chains to be traced effectively. Variable number of tandem repeats analysis are high-throughput PCR based techniques. It works well from boiled cell lysates without the need for to sub-culture (Le Fleche et al., 2002). In addition to that, VNTR typing has the potential for further automation of PCR product analysis and allele calling (Supply et al., 2000; Lindstedt, 2005).

Two methods are currently adopted for VNTR typing: Multiplex PCR approach which has three or more sets of primers followed by separation by capillary electrophoresis (Allix et al., 2006); in the other approach only one pair of primers amplifies a locus, then separation is done by agarose gel electrophoresis (Le Fleche et al., 2002). The second method is considered less expensive but is as discriminatory as the first, however, there is not enough data to support its discriminatory ability as compared to the first method.

Variable number tandem repeat sequences are multiple independent genetic loci that are found in at least 40 location throughout the entire genome, with each locus containing repetitive DNA sequences that vary in the number of repeats between the strains (Figure 2.4.3.1and Figure 2.4.3.2) (Supply et al., 2000).

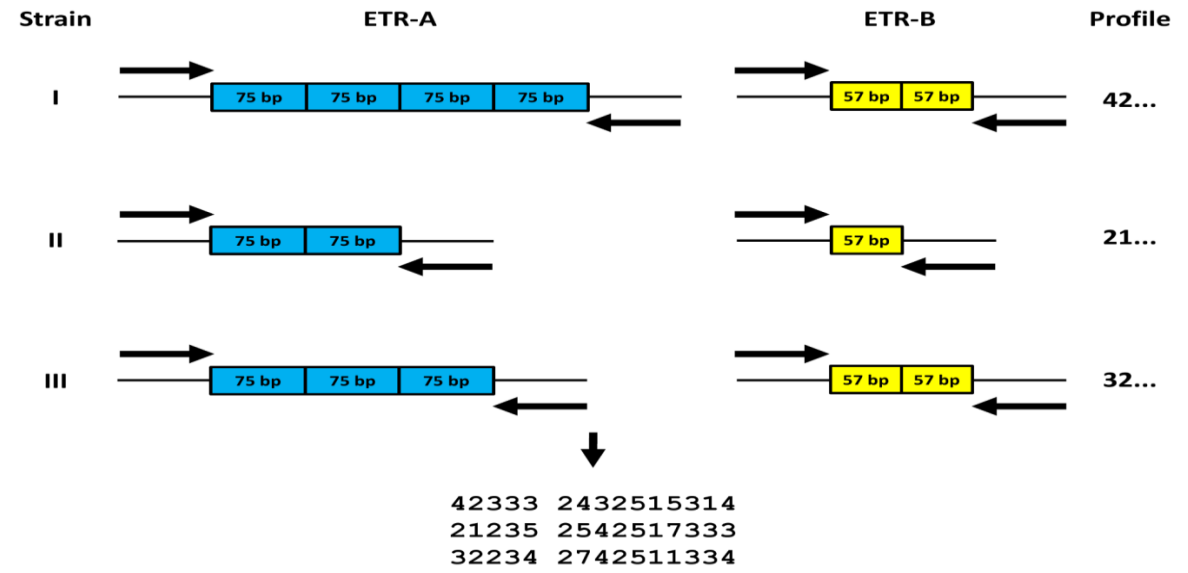


Figure 2.4.3.1 MIR-VNTR typing: Two examples of MIRU-VNTR are shown (ETR-A and ETR-B) in three example strains. Each locus is amplified by PCR using primers that are precise for the flanking region up- and dowm-stream of the repetitive DNA sequences. The Varying sizes of PCR amplicons is as a result of the variation in the number of repeats (Evans, 2011).

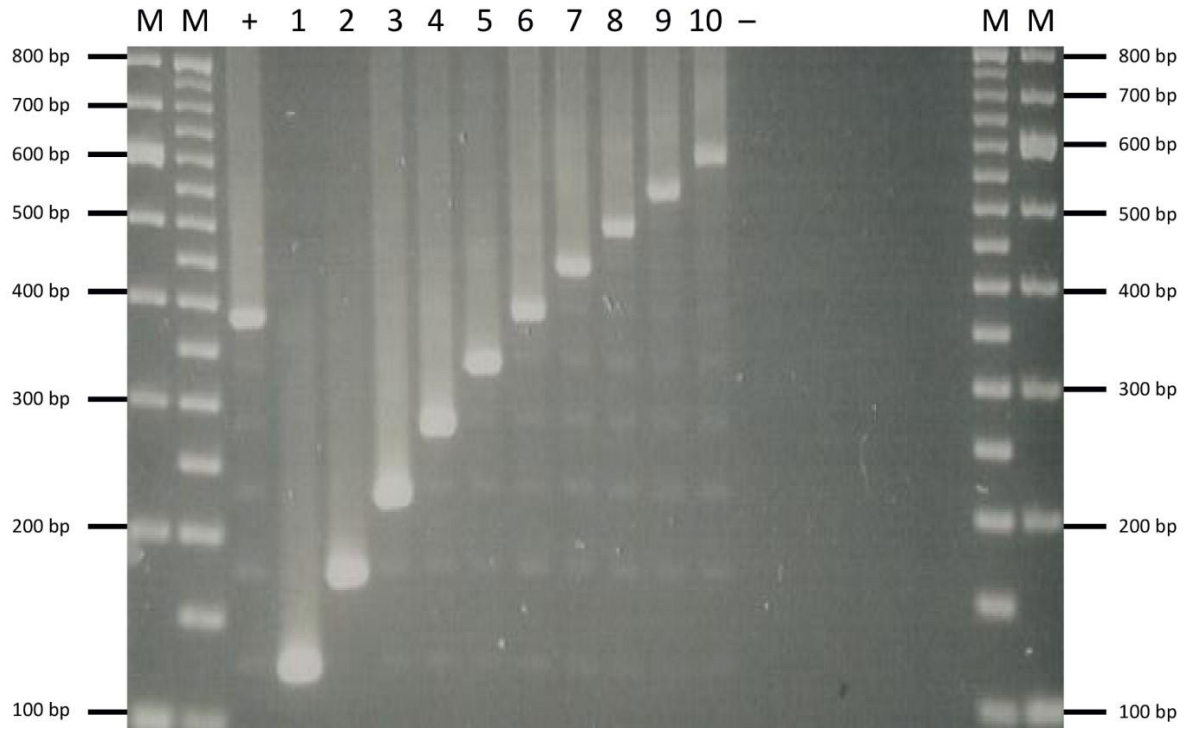


Figure 2.4.3.2 MIRU-23 Analysis of alleles by agarose gel electrophoresis. The MIRU-23 locus in ten different strains was amplified by PCR and was separated by agarose gel electrophoresis. Each strain strain had different allele value ranging from 1-10 repeats. 50 bp and 100bp molecular markers were used, with the positive control being H37Rv (Evans, 2011).

Today, molecular methods are increasingly applied to the control of TB and more methods are developed as more information on the *M. tuberculosis* becomes available. Consequently, molecular methods have become an extremely valuable tool in the detection of drug resistance and in the understanding of the transmission dynamics of different *M. tuberculosis* strains including those that possess drug resistance (Falmer, 2008).

3. CHAPTER3

3.1 PURPOSE OF THE STUDY

Tuberculosis persists as one of the most important public health concern worldwide, despite the global efforts to control and eliminate the disease (Ereqat, 2012). The World Health Organization (WHO, 2011) has declared TB as a global epidemic with high morbidity and mortality, especially in low-income countries, where most cases occur. Rapid detection and adequate therapy to prevent *Mycobacterium tuberculosis* (MTB) transmission are the key factors in TB control programs. However, in the last years, molecular typing approaches have greatly enhanced our understanding of TB epidemiology by indicating possible epidemiological links between TB patients and by detecting suspected outbreaks (Supply et al., 2006). These methods are also rapid in detecting causative TB pathogens, providing epidemiological information on strain identities and have high discriminatory power of distinguishing between MTB strains (Supply et al., 2012). Some mutations which have been described in MTB genes that are associated with resistance to rifampin (RIF) and isoniazide (INH) were found to be specific to particular strain families (Lipin et al., 2007), therefore the use of these molecular typing methods will aid in the linking of resistant strains to their particular strain families.

3.2 Hypothesis

There is an association between MDR-TB and a particular *M. tuberculosis* genotype in Port Elizabeth. Also, the application of molecular epidemiological methods will help in understanding the general population structure of *M. tuberculosis* isolates in Port Elizabeth, irrespective of the drug susceptibility patterns.

3.3 Objectives

3.3.1 Overall Objective

The overall objective of this study was to find the major clades that are circulating in Port Elizabeth.

3.3.2 Specific Objectives

The specific objectives of this study were:

- To determinine the genotypes of *M. tuberculosis* complex in Port Elizabeth using Spoligotyping and VNTR

3.4 MATERIALS AND METHODS

3.4.1 Ethical Clearance

This study was approved by the Research and Ethical Committees of both the University of Fort Hare and the Nelson Mandela Metropolitan University.

3.4.2 The NHLS TB Referral Laboratory

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

3.4.3 Sample Collection

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

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

The current study sample reflects resistance patterns to first-line anti-TB drugs, since most of the samples do not have DST results for the second-line anti-TB drugs (kanamycin,

ofloxacin, and ethionamide). This is because second-line testing was not routinely performed in the NHLS TB referral lab.

3.4.4 Sample Processing

Samples were obtained from the NHLS TB referral lab as Mycobacteria Growth Indicator Tube (MGIT) culture (BD BioSciences, Sparks, MD, USA). Two aliquots were taken from each tube. The first was used for subculture in new MGITs (if growth in the original MGIT was very low). The second was heat-killed in preparation for spoligotyping and MIRU-VNTR. The original MGIT tubes were stored at 4^{o}C.

3.4.5 Culture in MGITs

The materials provided by the manufacturer for culture in MGIT tubes included BBL Mycobacteria Growth Indicator Tube (MGIT), BACTEC^{TM}MGIT 960 supplement kit, containing BACTEC MGIT Growth Supplement and BBL MGIT^{TM}PANTA (Polymyxin, Amphotericin, Nalidixic acid, Trimethoprim, Azlocillin) antibiotic mixture (BD BioSciences, Sparks, MD, USA). Fifteen millilitres of the growth supplement was added to a lyophilized vial of the antibiotic mixture. Eight hundred microliters of the growth supplement and PANTA mixture was then added to each MGIT tube, followed by 300µl of the specimen. The tubes were tightly closed and incubated at 37^{o}C. Growth was detected by the presence of small 'grains' or flakes in the culture medium, usually after 3-6 weeks in culture. An unopened, un-inoculated MGIT tube was used as a control. Spoligotyping, described below in section 3.5, was used as a 'positive control' method to investigate possible cross contamination. Random samples were chosen and aliquots from the original MGIT and the subcultures were used to test for laboratory cross contamination of results.

3.4.6 Culture of LJ Slants

LJ slants (BD BioSciences, Sparks, MD, USA) were inoculated with 300µl of culture from the MGIT. The slants were incubated (positioned sideways) at 37^{o}C overnight, then turned upright. The excess culture media from the original inoculum was poured out after 7 days in culture. The slants were aerated after every 3 days for 2 weeks, thereafter every week for 6 to 8 weeks depending on the growth observed. Growth was indicated by formation of granular white/cream colonies on the slants.

3.5 Spoligotyping

3.5.1 *In Vitro* Amplification of Spacer DNA by PCR

Spoligotyping was done at the University of Stellenbosch for and the method was done according to Kamerbeek et al. (1997). The chromosomal DNA of *M. tuberculosis* strain H37Rv and *M. bovis* BCG were included as positive controls and water was used as a negative control. The reaction mixture consisted of 4µl of primers DRa and DRb, 4 µl of dNTP mixture, 5 µl of 10x Super T buffer, 0.1 µl of Super T polymerase, nuclease free water and template DNA (20 ng). A drop of mineral oil was added to the tubes to prevent evaporation of the PCR-mix during amplification. The tubes were then placed in a PCR-apparatus for amplification and the following temperature cycling was performed: 1x cycle of 96^{o}C for 3 min, followed by 20 cycles of 96^{o}C for 1 min; 55^{o}C for 1 min and 72^{o}C for 30 sec; then lastly 1x cycle of 72^{o}C for 5 min.

3.5.2 Hybridization with PCR Product and Detection

All buffers were pre-warmed before use. The following buffers were prepared from concentration stocks using de-mineralised water for dilution: 250 ml of 2x SSPE/0.1% SDS at 60^{o}C; 250 ml of 2x SSPE/0.5% SDS at 60^{o}C; 250 ml of 2x SSPE/0.5% SDS at 42^{o}C; 250 ml of 2x SSPE at room temperature (all these quantities are for one membrane). Twenty microlitres of the PCR products were added to 150 µl of 2x SSPE/ 0.1% SDS. The diluted PCR product was then heat-denatured for 10 min at 99^{o}C and was then immediately cooled on ice. The membrane was washed for 5 min at 60^{o}C in 250 ml of 2x SSPE/ 0.1% SDS. The membrane and support cushion was placed into the mini-blotter in such a way that the slots were perpendicular to the line pattern of the applied oligonucleotides. The residual fluid was then removed from the slots of the mini-blotter by aspiration. The slots were filled with diluted PCR product and hybridization was performed at 60^{o}C for 60 min on a horizontal surface. The samples were removed from the mini-blotter by aspiration and the membrane was taken using forceps. The membrane was washed twice in 250 ml of 2x SSPE/0.5% SDS for 10 min at 60^{o}C. The membrane was then placed in a rolling bottle and was allowed to cool down to prevent inactivation of the peroxidase in the next step. 2.5 µl of streptavidin-peroxidase conjugate (500 U/ml) were added to 10 ml of 2x SSPE/0.5% SDS and the membrane was incubated in this solution for 45 to 60 min at 42^{o}C. The membrane was then rinsed with 250 ml of 2x SSPE for 5 min at room temperature. For chemiluminiscent detection of the hybridizing DNA, the DNA was incubated for 1 min in 20 ml ECL detection liquid. The membrane was then covered with a transparent plastic sheet or Saran-wrap and a light sensitive film was exposed to the membrane for 20 min.

3.6 Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats Analysis (MIRU-VNTR)

Sixteen isolates were type by MIRU-VNTR due to small amounts of DNA that had remained. The reaction mixture was made up of 12.5 µl of DreamTaq mastermix, 2 µl forward and reverse primers, 2 µl of DNA template and was filled up to volume with nuclease free water. The following temperature cycling parameters were used: Incubation at 95^{o}C for 15 minutes, followed by 40 cycles at 95^{o}C for 1 minute, then annealing at 72^{o}C for 1 minute followed by incubation at 72^{o}C for 10 minutes, then the temperature was held at 4^{o}C until the amplicon

were ready for electrophoresis. The PCR products were then separated by agarose gel electrophoresis. 3% agarose was used in 0,5X TBE buffer of pH 8 and was run at 50V for 3 hours. Below is a table with the primers sets that were used.

TABLE 3.6.1 MIRUs and primer sets used in the PCR reaction

MIRUs	**PRIMERS**
MIRU 2- Forward	5'-TGGACTTGCAGCAATGGACCAACT-3'
Reverse	5'-TACTCGGACGCCGGCTCAAAAT-3'
MIRU 4- Forward	5'-GCGCGAGAGCCCGAACTCG-3'
Reverse	5'-GCGCAGCAGAAACGCCAGC-3'
MIRU 10- Forward	5'-GTTCTTGACCAACTGCAGTCGTCC-3'
Reverse	5'-GCCACCTTGGTGATCAGCTACCT-3'
MIRU 16- Forward	5'-TCGGTGATCGGGGTCCAGTCCAACTA-3'
Reverse	5'-CCCGTCGTGCAGCCCTGGTAC-3'
MIRU 20- Forward	5'-TCGGAGAGATGCCCTTCGAGTTAG-3'
Reverse	5'-GGAGACCGCGACCAGGTACTTGTA-3'
MIRU 23- Forward	5'-CTGTCGATGGCCGCAACAAAACG-3'
Reverse	5'-AGCTCAACGGGTCGCCCTTTTGTC-3'
MIRU 24- Forward	5'-CGACCAAGATGTGCAGGAATACAT-3'
Reverse	5'-GGGCGAGTTGAGCTCACAGAA-3'
MIRU 26- Forward	5'-TAGGTCTACCGTCGAAATCTGATGAC-3'
Reverse	5'-CATAGGCGACCAGGCGAATAG-3'
MIRU 27- Forward	5'-TCGAAAGCCTCTGCGTGCCAGTAA-3'
Reverse	5'-GCGATGTGAGCGTGCCACTCAA-3'
MIRU 31- Forward	5'-ACTGATTGGCTTCATACGGCTTTA-3'
Reverse	5'-GTGCCGACGTGGTCTTGAT-3'

MIRU 39- Forward	5’-CGCATCGACAAACTGGAGCCAAAC-3’
Reverse	5’-CCGGAAACGTCATCGCCCCACACAT-3’
MIRU 40- Forward	5’-GGGTTGCTGGATGACAACGTGT-3’
Reverse	5’-GGGTGATCTCGGCAGAAATCAGATA-3’

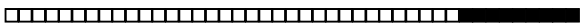
CHAPTER 4

RESULTS

4.1 SPOLIGOTYPING

In this study, 200 DNA samples from MTB isolates obtained from NHLS in Port Elizabeth were sent to the University of Stellenbosch for typing however, only 179 isolates were typed. The results of the typing are summarised in the table below with Spoligotypes ranging from 71% of Beijing family to 5,5% of the MANU family type

Table 4.1.1 A summary of Spoligopatterns of representative strains from human MDDR-TB isolates

PATTERN	SIT	FAMILY	%
	1	BEIJING	127 (71%)
	60	LAM_4	10 (6%)
	1750	LAM_4	1 (0.5%)
	33	LAM_3	5 (3%)
	42	LAM_9	1 (0.5%)
	2271	LAM_2	1 (0.5%)
	Orphan	LAM_9	1 (0.5%)
	1329	X1	1 (0.5%)
	2286	X3	6 (3.3%)
	92	X3	2 (1.1%)
	70	X3	1 (0.5%)
	Orphan	X3	1 (0.5%)
	53	T1	1 (0.5%)

[redacted]			
[redacted]	34	S	1 (0.5%)
[redacted]	1247	MANU2	1 (0.5%)
[redacted]	Not in SITVIT	-	11 (6%)
[redacted]	Not in SITVIT		
[redacted]	Not in SITVIT		
[redacted]	Not in SITVIT		
[redacted]	Not in SITVIT		
[redacted]	Not in SITVIT		
[redacted]	Not in SITVIT		
[redacted]	Not in SITVIT		
[redacted]	Not in SITVIT		
[redacted]	Not in SITVIT		
[redacted]	Not in SITVIT		
[redacted]	Not in SITVIT		
[redacted]	451	H37rV	
[redacted]	482	Bovis	

4.2 MIRU-VNTR

The isolate B161 was amplified and figure 4.2.1 below is a gel showing the different size bands of the different MIRUs that were used

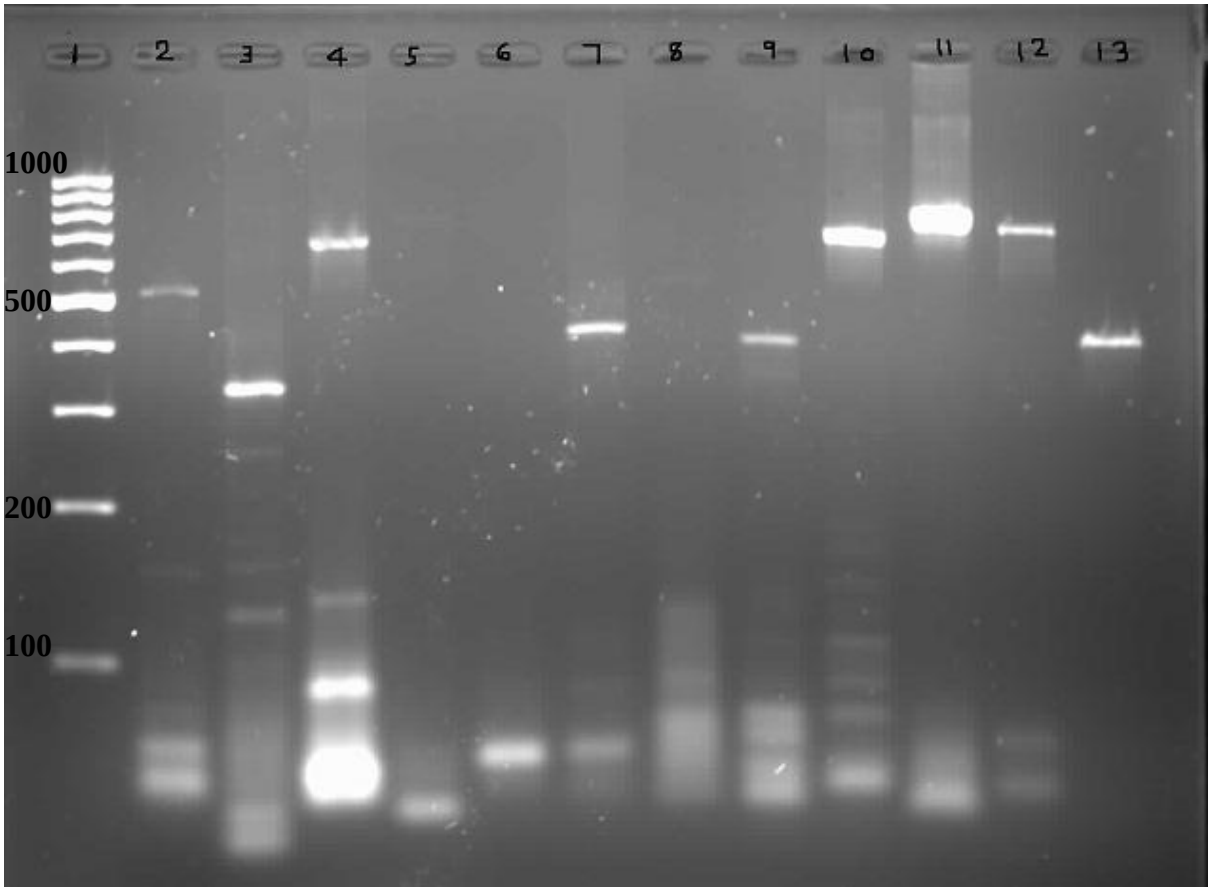


Figure 4.2.1 The same strain B161 of *M. tuberculosis* was amplified using different MIRU alleles. On lane 1 is a 100 bp molecular marker; Lane 2: MIRU2; Lane 3: MIRU4; Lane 4: MIRU10; Lane 5: MIRU16; Lane 6: MIRU20; Lane 7:MIRU23; Lane8: MIRU24; Lane 9: MIRU26; Lane 10:MIRU27; Lane 11: MIRU31; Lane 12: MIRU 39; Lane 13: MIRU40

The isolate B103 was amplified and figure 4.2.2 below is a gel showing the different size bands of the different MIRUs that were used

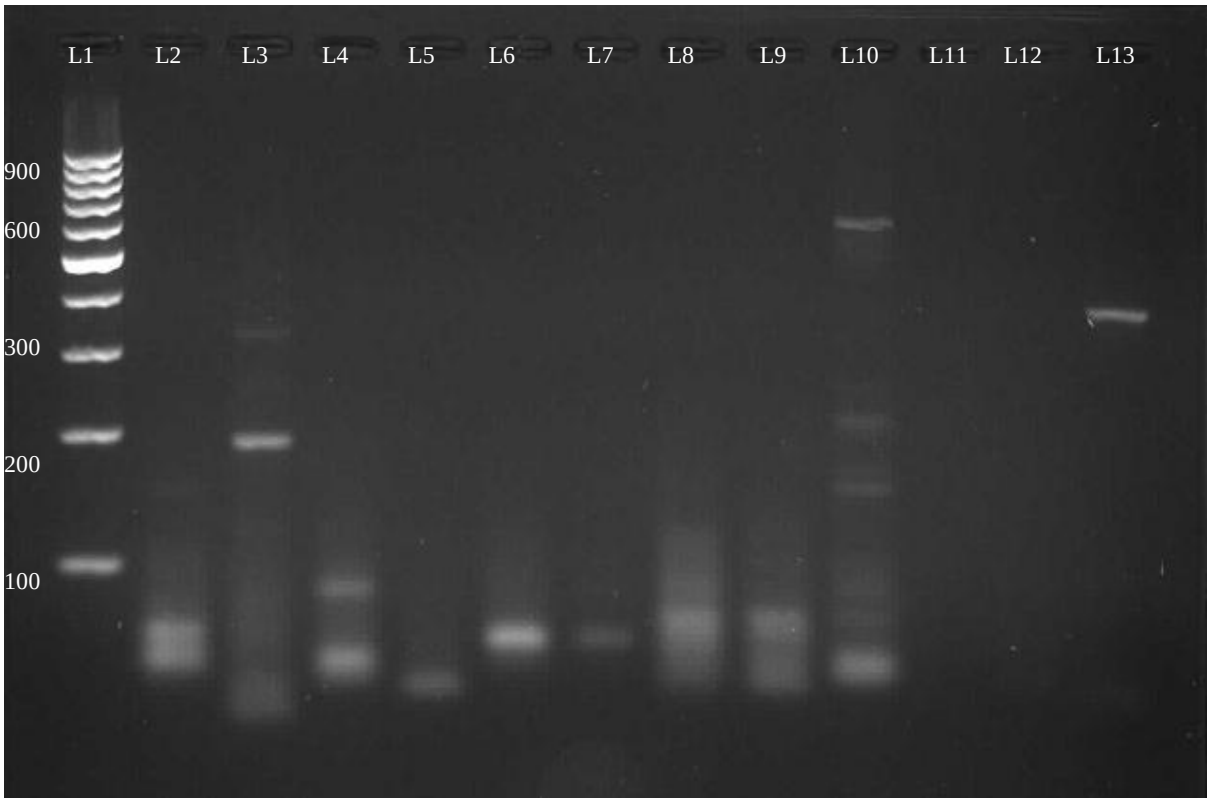


Figure 4.2.2 The same strain B103 of *M. tuberculosis* was amplified using different MIRU alleles. On lane 1 is a 100 bp molecular marker; Lane 2: MIRU2; Lane 3: MIRU4; Lane 4: MIRU10; Lane 5: MIRU16; Lane 6: MIRU20; Lane 7:MIRU23; Lane8: MIRU24; Lane 9: MIRU26; Lane 10:MIRU27; Lane 11: MIRU31; Lane 12: MIRU 39; Lane 13: MIRU40

The isolate B000 was amplified and figure 4.2.3 below is a gel showing the different size bands of the different MIRUs that were used.

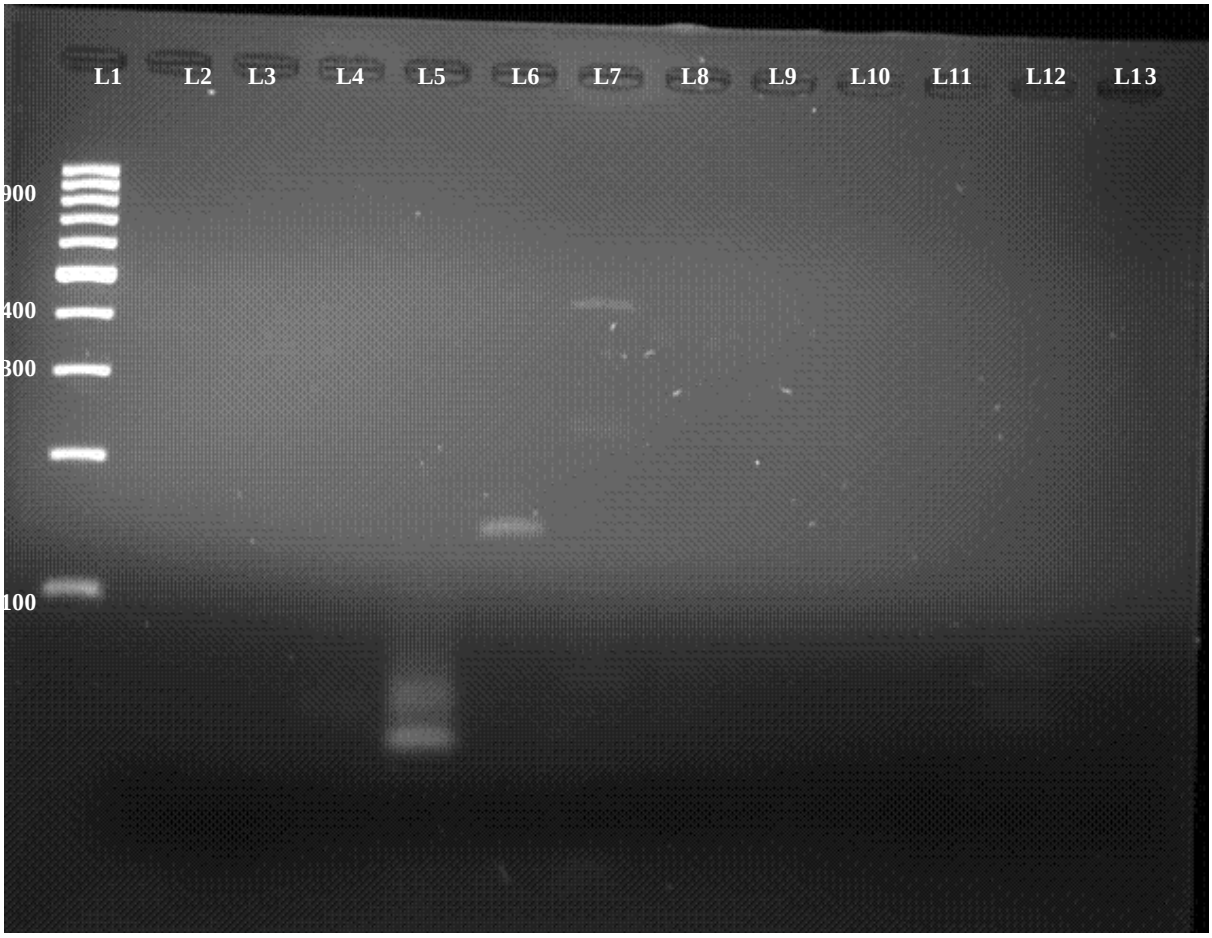


Figure 4.2.3 The same strain B000 of *M. tuberculosis* was amplified using different MIRU alleles. On lane 1 is a 100 bp molecular marker; Lane 2: MIRU2; Lane 3: MIRU4; Lane 4: MIRU10; Lane 5: MIRU16; Lane 6: MIRU20; Lane 7:MIRU23; Lane8: MIRU24; Lane 9: MIRU26; Lane 10:MIRU27; Lane 11: MIRU31; Lane 12: MIRU 39; Lane 13: MIRU40

The isolate B482 was amplified and figure 4.2.4 below is a gel showing the different size bands of the different MIRUs that were used

Figure 4.2.4 The same strain B482 of *M. tuberculosis* was amplified using different MIRU alleles. On lane 1 is a 100 bp molecular marker; Lane 2: MIRU2; Lane 3: MIRU4; Lane 4: MIRU10; Lane 5: MIRU16; Lane 6: MIRU20; Lane 7:MIRU23; Lane8: MIRU24; Lane 9: MIRU26; Lane 10:MIRU27; Lane 11: MIRU31; Lane 12: MIRU 39; Lane 13: MIRU40

The isolate B022 was amplified and figure 4.2.5 below is a gel showing the different size bands of the different MIRUs that were used

Figure 4.2.5 The same strain B022 of *M. tuberculosis* was amplified using different MIRU alleles. On lane 1 is a 100 bp molecular marker; Lane 2: MIRU2; Lane 3: MIRU4; Lane 4: MIRU10; Lane 5: MIRU16; Lane 6: MIRU20; Lane 7:MIRU23; Lane8: MIRU24; Lane 9: MIRU26; Lane 10:MIRU27; Lane 11: MIRU31; Lane 12: MIRU 39; Lane 13: MIRU40

The isolate B792 was amplified and figure 4.2.6 below is a gel showing the different size bands of the different MIRUs that were used.

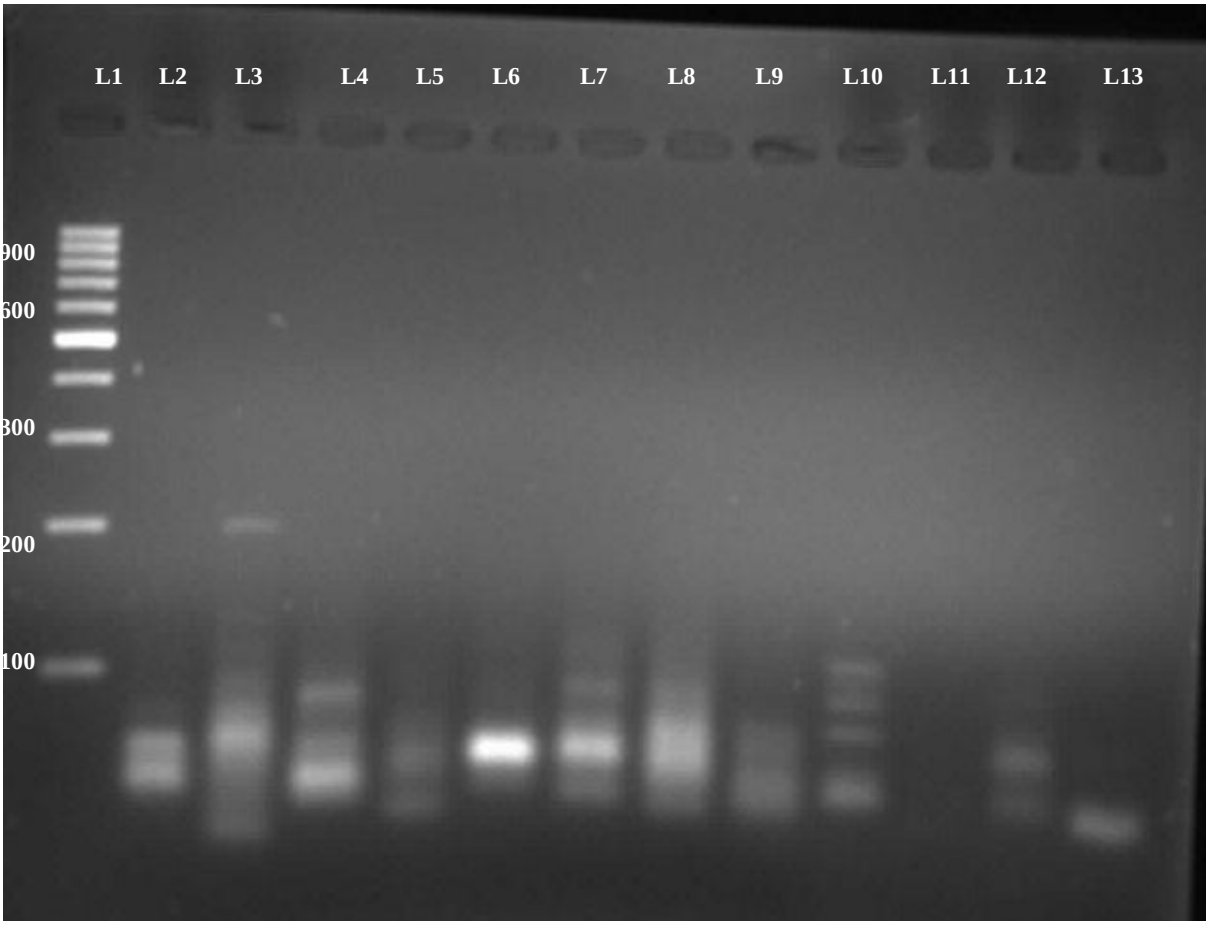


Figure 4.2.6 The same strain B792 of *M. tuberculosis* was amplified using different MIRU alleles. On lane 1 is a 100 bp molecular marker; Lane 2: MIRU2; Lane 3: MIRU4; Lane 4: MIRU10; Lane 5: MIRU16; Lane 6: MIRU20; Lane 7:MIRU23; Lane8: MIRU24; Lane 9: MIRU26; Lane 10:MIRU27; Lane 11: MIRU31; Lane 12: MIRU 39; Lane 13: MIRU40

The isolate B541 was amplified and figure 4.2.7 below is a gel showing the different size bands of the different MIRUs that were used

Figure 4.2.7 The same strain B541 of *M. tuberculosis* was amplified using different MIRU alleles. On lane 1 is a 100 bp molecular marker; Lane 2: MIRU2; Lane 3: MIRU4; Lane 4: MIRU10; Lane 5: MIRU16; Lane 6: MIRU20; Lane 7:MIRU23; Lane8: MIRU24; Lane 9: MIRU26; Lane 10:MIRU27; Lane 11: MIRU31; Lane 12: MIRU 39; Lane 13: MIRU40

The isolate B141 was amplified and figure 4.2.8 below is a gel showing the different size bands of the different MIRUs that were used

Figure 4.2.8 The same strain B141 of *M. tuberculosis* was amplified using different MIRU alleles. On lane 1 is a 100 bp molecular marker; Lane 2: MIRU2; Lane 3: MIRU4; Lane 4: MIRU10; Lane 5: MIRU16; Lane 6: MIRU20; Lane 7:MIRU23; Lane8: MIRU24; Lane 9: MIRU26; Lane 10:MIRU27; Lane 11: MIRU31; Lane 12: MIRU 39; Lane 13: MIRU40

The isolate B017 was amplified and figure 4.2.9 below is a gel showing the different size bands of the different MIRUs that were used

Figure 4.2.9 The same strain B017 of *M. tuberculosis* was amplified using different MIRU alleles. On lane 1 is a 100 bp molecular marker; Lane 2: MIRU2; Lane 3: MIRU4; Lane 4: MIRU10; Lane 5: MIRU16; Lane 6: MIRU20; Lane 7:MIRU23; Lane8: MIRU24; Lane 9: MIRU26; Lane 10:MIRU27; Lane 11: MIRU31; Lane 12: MIRU 39; Lane 13: MIRU40

The isolate B804 was amplified and figure 4.2.10 below is a gel showing the different size bands of the different MIRUs that were used

Figure 4.2.10 The same strain B804 of *M. tuberculosis* was amplified using different MIRU alleles. On lane 1 is a 100 bp molecular marker; Lane 2: MIRU2; Lane 3: MIRU4; Lane 4: MIRU10; Lane 5: MIRU16; Lane 6: MIRU20; Lane 7:MIRU23; Lane8: MIRU24; Lane 9: MIRU26; Lane 10:MIRU27; Lane 11: MIRU31; Lane 12: MIRU 39; Lane 13: MIRU40

The isolate B1753 was amplified and figure 4.2.11 below is a gel showing the different size bands of the different MIRUs that were used

Figure 4.2.11 The same strain B1753 of *M. tuberculosis* was amplified using different MIRU alleles. On lane 1 is a 100 bp molecular marker; Lane 2: MIRU2; Lane 3: MIRU4; Lane 4: MIRU10; Lane 5: MIRU16; Lane 6: MIRU20; Lane 7:MIRU23; Lane8: MIRU24; Lane 9: MIRU26; Lane 10:MIRU27; Lane 11: MIRU31; Lane 12: MIRU 39; Lane 13: MIRU40

The isolate B238 was amplified and figure 4.2.12 below is a gel showing the different size bands of the different MIRUs that were used

Figure 4.2.12 The same strain B238 of *M. tuberculosis* was amplified using different MIRU alleles. On lane 1 is a 100 bp molecular marker; Lane 2: MIRU2; Lane 3: MIRU4; Lane 4: MIRU10; Lane 5: MIRU16; Lane 6: MIRU20; Lane 7:MIRU23; Lane8: MIRU24; Lane 9: MIRU26; Lane 10:MIRU27; Lane 11: MIRU31; Lane 12: MIRU 39; Lane 13: MIRU40

The isolate B492 was amplified and figure 4.2.13 below is a gel showing the different size bands of the different MIRUs that were used

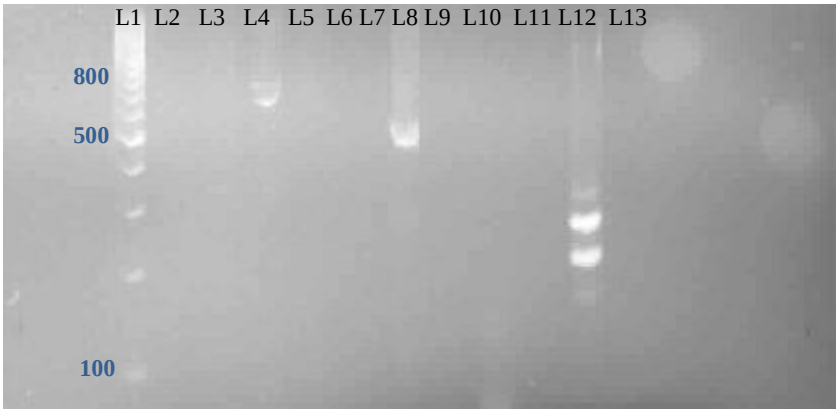


Figure 4.2.13 The same strain B492 of *M. tuberculosis* was amplified using different MIRU alleles. On lane 1 is a 100 bp molecular marker; Lane 2: MIRU2; Lane 3: MIRU4; Lane 4: MIRU10; Lane 5: MIRU16; Lane 6: MIRU20; Lane 7:MIRU23; Lane8: MIRU24; Lane 9: MIRU26; Lane 10:MIRU27; Lane 11: MIRU31; Lane 12: MIRU 39; Lane 13: MIRU40

The isolate B446 was amplified and figure 4.2.14 below is a gel showing the different size bands of the different MIRUs that were used

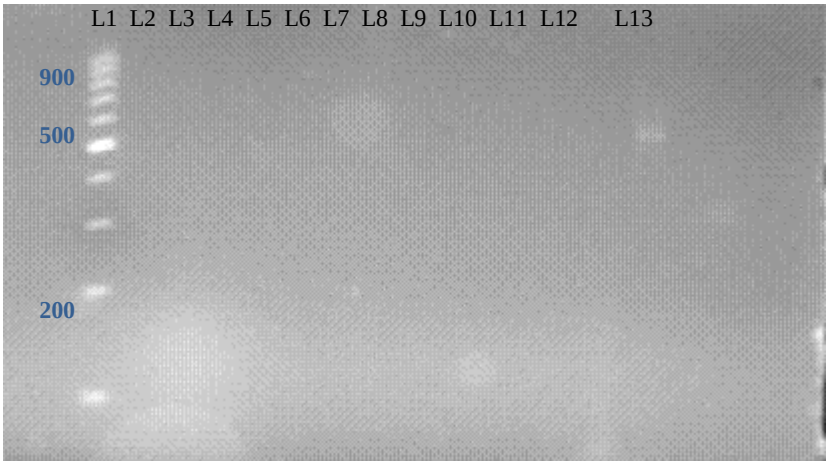


Figure 4.2.14 The same strain B446 of *M. tuberculosis* was amplified using different MIRU alleles. On lane 1 is a 100 bp molecular marker; Lane 2: MIRU2; Lane 3: MIRU4; Lane 4: MIRU10; Lane 5: MIRU16; Lane 6: MIRU20; Lane 7:MIRU23; Lane8: MIRU24; Lane 9: MIRU26; Lane 10:MIRU27; Lane 11: MIRU31; Lane 12: MIRU 39; Lane 13: MIRU40

The isolate B378 was amplified and figure 4.2.15 below is a gel showing the different size bands of the different MIRUs that were used

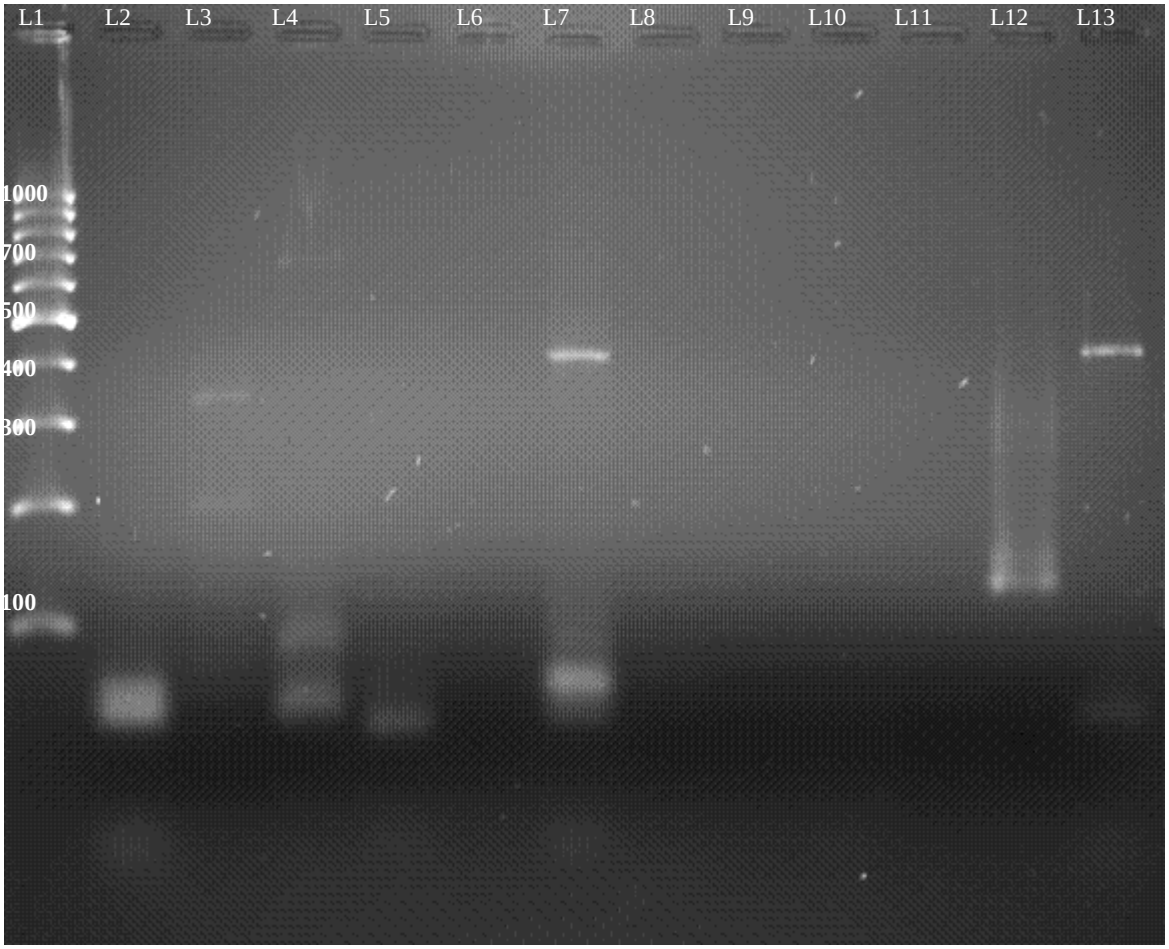


Figure 4.2.15 The same strain B378 of *M. tuberculosis* was amplified using different MIRU alleles. On lane 1 is a 100 bp molecular marker; Lane 2: MIRU2; Lane 3: MIRU4; Lane 4: MIRU10; Lane 5: MIRU16; Lane 6: MIRU20; Lane 7:MIRU23; Lane8: MIRU24; Lane 9: MIRU26; Lane 10:MIRU27; Lane 11: MIRU31; Lane 12: MIRU 39; Lane 13: MIRU40

The isolate B030 was amplified and figure 4.2.16 below is a gel showing the different size bands of the different MIRUs that were used

Figure 4.2.16 The same strain B030 of *M. tuberculosis* was amplified using different MIRU alleles. On lane 1 is a 100 bp molecular marker; Lane 2: MIRU2; Lane 3: MIRU4; Lane 4: MIRU10; Lane 5: MIRU16; Lane 6: MIRU20; Lane 7:MIRU23; Lane8: MIRU24; Lane 9: MIRU26; Lane 10:MIRU27; Lane 11: MIRU31; Lane 12: MIRU 39; Lane 13: MIRU40

All the band sizes for each isolate that was amplified were summarized in the table 4.1.17 below.

Table 4.1.17 Isolates and band sizes obtained after amplification from each isolate and MIRU.

ISOLATES	**MIRU's**											
	2	**4**	**10**	**16**	**20**	**23**	**24**	**26**	**27**	**31**	**39**	**40**
B103	-	12	-	-	-	-	-	-	4	-	-	1
B000	-	-	-	-	-	7	-	-	-	-	-	-
B482	0	12	--	-	-	-	-	-	-	-	-	-
B022	-	-	-	-	-	-	-	-	-	-	-	-
B792	-	1	-	-	-	-	-	-	-	-	-	-
B030	-	-	-	-	-	-	-	-	-	-	-	-
B541	-	1	-	-	-	-	-	-	-	-	-	1
B141	-	1	-	-	-	7	2	3	4	-	-	1
B017	-	-	-	-	-	-	-	-	-	-	-	-
B804	-	11	-	-	-	-	-	-	-	-	-	1
B1753	-	11	-	-	-	6	-	-	4	-	-	2
B238	-	-	-	-	-	-	-	-	-	-	-	3
B492	-	-	2	-	-	7	-	-	-	-	-	-
B161	-2	1012	2	-	-	5	-	2	4	4	3	1

B446	-	-	-	-	-	-	-	-	-	-	-	3
B378	-	1 2	-	-	-	5	-	-	-	-	-	1

CHAPTER 5

DISCUSSION

South Africa is one of the countries where TB is endemic and in such countries the identification of predominant strains is imperative so as to keep track of the changes in strain composition within communities. This will also help understand the epidemiology of the disease in the country. Spoligotyping is a quick and convenient method in which the identification of shared types and clades of strains is ensured (Sola et al., 2003; Yang et al., 1998).

In our study five families where identified and these identified, the Beijing, LAM, X, T, S and the MANU families. In a study done by Mlambo in 2011 in the Western Cape and Johannesburg areas, the families that were identified were the LAM family which was predominant in the Western Cape, the EIA family which was predominant Johannesburg, the X family which was also dominant in the Johannesburg and the most family with the highest dominance in their study was the Beijing family type (Mlambo, 2011).

The most prevalent strain in this study was the Beijing family (71%) and all the strains had the same shared type SIT1. The presence and dominance of the Beijing family in our study was expected because the strain family had been previously identified in a number of regions in the African continent (Kibikio et al., 2007; Homolka et al., 2008; Githui et al., 2004; Glynn et al., 2005), and in most cases the family has been associated with drug resistance (Stavrum et al., 2009; Streicher et al., 2004). A number of advantages have been associated with the success of the Beijing family to cause disease, these include a lower effectiveness of BCG for Beijing strains (Dormans et al., 2004; Manca et al., 2004), higher virulence (Lopez et al., 2003; Manca et al., 2001; van Crevel et al., 2001); the ability to induce a differential immune response (Dormans et al., 2004; Manca et al., 2004); a higher ability to transmit; a heightened capability to grow in human macrophages and monocytes (Li et al., 2002; Zhang et al, 1999) and lastly, an improved ability to obtain drug resistance (Rad et al., 2003).

There is a consistency with other studies from South Africa with regards to the diversity in the predominant strains that were observed in our study. The LAM family formed 10% of the total isolates that were typed and of that percentage there were seven different shared types, with LAM4 SIT 60 being the most dominant (53%). In the Western Cape, SIT 60 was not predominant but in Johannesburg it was the second predominant spoligotype (Mlambo et al., 2008, Stavrum et al., 2009, Streicher et al., 2004), and in our study, LAM SIT 60 was also found to be the second predominant spoligotype in Port Elizabeth. This spoligotype is referred to as the KZN strain because of the outbreak of XDR-TB in the KZN Province which it was reported to be responsible for. In KZN it was identified in 85% of the patients (Gandhi et al. 2006).

Twenty six (26%) percent of the 10% formed by the LAM family in our study is LAM3 SIT 33 and this SIT is one of the strains driving the drug resistant TB epidemic in the Western Cape (Mlambo, 2001). The global spoligotyping database showed a more extensive comparison, that the strain type was poorly represented in our study and all African regions except for Southern Africa, particularly, South Africa.

The LAM9 spoligotype SIT 42 which is prevalent in North Africa (Namouchi et al., 2008) was represented in our study sample by only 5,2% of the 10% of the LAM family, but LAM 11_ZWE (SIT 59), which is prevalent in Zambia and Zimbabwe, (Easterbrook et al., 2004, Chihota et al., 2007) was not represented in our sample. LAM_10 CAM (SIT 61, the Cameroon) family, which is mostly found in West Africa (Niobe-Eyangoh et al., 2003, Godreuil et al., 2007, Homolka et al., 2008) was also not identified in our sample study. These observations were however expected because Port Elizabeth is considered far to attract and receive a diverse population of both legal and illegal immigrants from the entire African continent.

Among the 10% of the LAM isolates was the SIT 1750 spoligotype pattern which like SIT60, belongs to the LAM4 sub-lineage and has only one spacer difference with SIT60 (Ramtahal, 2011). One isolate (5,2%) showed an orphan pattern. This pattern has major similarities with the SIT60 and SIT1750 spoligotype patterns, it has spacers 15, 21-24, 33-66, 39 and 40

absent. The orphan pattern has a difference of 2 spacers as compared to the SIT60 spoligopattern, which are not present and a difference of 1 spacer as compared to the SIT1750 spoligotype pattern is absent (Ramtahal, 2011).

In our Study the X family was also identified and formed 6% of the 179 isolates that were typed. In this study this family was sub-divided into X1 and X3. The X3 SIT 2286 was predominant in Port Elizabeth, forming 54% of the 6% of the total X family. However, according to the SITVIT 2 database, this family is not well represented in the rest of Africa. In the present study, from the X family other strains that were identified were STIs 1329 (9%), 92 (18%), 70 (9%) and an Orphan (9%), of which one of them (STI 1329) was represented in the study that was done with isolates from Cape town (STIs 336, 137, 1329, 119) (Mlambo, 2011). This family is considered to be linked with Anglo-Saxon ancestry (Sebban et al., 2002). There is an overrepresentation of the X family in the Western Cape and this could suggest previous colonization history as well as the use of Cape Town as the main port of entry.

Additional spoligotype patterns in this study among the isolates included SIT 53 (0.5%) spoligotype pattern which is representative of the T1 sub-lineage and the S (0.5%)Family with shared international type SIT 34. In a study by (Ramtahal, 2011), XDR isolates with the SIT53 pattern clustered with an identical RFLP pattern, and in that study it was suggested that patterns which differ from those that cluster by one of the two techniques may be associated with mixed infections or may be as a result of evolution of the DR region. Alternatively, their results could also have suggested that isolates with the SIT53 patterns are evolving and acquiring more resistance mechanisms. This study also reported that SIT53 was responsible for a small proportion 6% (2/23) of XDR TB cases in 2005-2006 in Tugela Ferry, KwaZulu-Natal (Andrews et al., 2008).

One of the isolates (0.5%) was from the MANU family with shared type SIT1247. MANU is a clade of strain that was identified in India as belonging to the ancestral family of principle genetic group (PGG) -1 and is more prevalent in Mumbai (Viegas et al., 2010). In a study by Viegas et al., 2010, 445 *M. tuberculosis* isolates from 7 different provinces in Mozambique

were characterized by spoligotying and a remarkable feature was the presence of the ancenstral MANU lineage strains of which some of the MANU 2 patterns appeared to result from mixed infections of the Beijing and Euro-American TB. This kind of a mixture has been described in adjacent South Africa (Viegas et al., 2010).

There are also other families that have been associated with drug resistance outbreaks in the region of Africa. These include the Haarlem family (SIT 50) which is a family that was associated with the outbreaks in North Africa (Mardassi et al., 2005); LAM (SIT60) - this family was associated with the outbreak of XDR-TB among primarily HIV-positive patients in KZN (Gandhi et al., 2006) and LAM3/F11- a strain driving the drug-resistant TB epidemic in eth Western Cape (Victor et al., 2004, Streicher et al., 2004). These *M. tuberculosis* strain families bring about a potential hazard for the outbreaks of MDR and XDR-TB in the Port Elizabeth region, more so among those persons with a compromised immune system.

Harmonized and reliable typing of bacteria that are pathogenic, such as *Mycobacterium tuberculosis* allows for easy identification of clones that are circulating both locally and internationally. This is especially true for diseases with a worldwide distribution like tuberculosis, and also diseases with a global emergence of drug resistance strains. Mycobacterial interspersed repetitive unit-variable number of tandem repeat (MIRU-VNTR) typing has become a popular method for fast and high-resolution PCR-based genotyping of *M. tuberculosis* complex isolates (Supply, 2000).

Further in the study, the spoligotype-defined families were typed by MIRU typing, so as to further differentiate and assess clonal diversity within the spoligotype families. Patients whose isolates have identical patterns are considered to have been infected recently in epidemiological studies, and because of this they can be targeted for further epidemiological investigations, so as to detect the sequence of transmission (van Soolingen, 2001).

However, in this study the 16 isolates that were differentiated by 12 MIRU-VNTR (Isolate no. B238 and B446; B141 and B161; B1753 and B378; B103 and B378) showed some

similarities, which then suggests that these isolates are epidemiologically related as they were all from the Beijing spoligotype family, all with the same shared type SIT1. Isolates B000, B022, B792, B541, B017, B804 and B446 did not show any relatedness even though they are all from the Beijing family with the same shared type SIT1. Three of the other isolates where all from the LAM family but with different shared types (SITs1750, 42, 60), and this suggested that they had no epidemiological relatedness.

Some researchers have encountered some complications when interpreting the proportion of disease due to a transmission that is recent and that is derived directly from a clustered proportion (Glynn et al. 1999). In urban areas, it is suggested that clustering that is observed in epidemiological data shows recent transmission of TB that is on-going, however, in rural populations that are geographically stable, clustering may result from simultaneous reactivation of infection that is acquired from the same source in the distant past (Braden et al., 1997).

The relationship between clustering and the proportion of disease that is attributable to recent transmission is dependent on a number of various factors, and this has been suggested from previous studies. Some of these factors are the age of the patient, the area of study, the duration of the study (van Soolingen, 2001) as well as the number of patients that are studied and the virulence and transmissibility of the strains (Glynn et al., 1999).

The clustering rates from this study were lower than the estimates of recent transmission and rates of clustering that are reported in other studies done in other African countries (Easterbrook et al., 2004; Verver et al., 2004; Godfrey-Faussett et al., 2000; Tudo et al., 2004; Lockman et al., 2001). This could suggest that there are differences in transmission dynamics or the molecular typing technique used in this study could have resulted in the clustering rates being lower than those reported in other studies. To further compare and analyse these isolates, a more discriminative typing technique such as 24 MIRU-VNTR typing could be used but nevertheless, the clustering rate that is observed in this study could imply an a transmission that is on-going, and since the strains transmitted are from families

that are associated with drug resistance, this observation has dreadful consequences for the control of TB. (Mlambo, 2011).

CONCLUSION

Results from our study illustrate the effectiveness of MIRU-VNTR typing together with spoligotyping in epidemiological studies in the region of Port Elizabeth.

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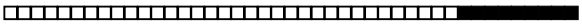
APPENDIX

Table 4.1.18 Spoligopatterns of representative strains from human MDDR-TB isolates

Isolate no.	Spoligotypes	SIT	SpolDB4 Family
B446		**1**	**BEIJING**
B614		**Not in SITVIT**	
B326		**1**	**BEIJING**
B444		**1**	**BEIJING**
B166		**1**	**BEIJING**
B492		**60**	**LAM4**
B440		**1**	**BEIJING**
B000		**1**	**BEIJING**
B705		**1**	**BEIJING**
B307		**1**	**BEIJING**
B299		**1329**	**X1**
BCG		**482**	**BOVIS1**
H37Rv		**451**	**H37Rv**
B802		**1**	**BEIJING**
B675		**1**	**BEIJING**
B686		**60**	**LAM4**
B459		**1**	**BEIJING**

B358		**1**	**BEIJING**
B639		**1**	**BEIJING**
B436		**1**	**BEIJING**
B437		**2286**	**X3**
B093		**60**	**LAM4**
B174		**1**	**BEIJING**
B479		**33**	**LAM3**
B433		**1**	**BEIJING**
B976		**Not in SITVIT**	
B137		**2286**	**X3**
B338		**NO RESULT**	
B833		**1**	**BEIJING**
B724		**53**	**T1**
B165		**1**	**BEIJING**
B723		**1**	**BEIJING**
B197		**Not in SITVIT**	
B678		**1**	**BEIJING**
B819		**1**	**BEIJING**
B023		**NO RESULT**	
B534		**34**	**S**
B706		**1**	**BEIJING**
B066		**1**	**BEIJING**

B030		**42**	**LAM9**
B368		**1**	**BEIJING**
B699		**1**	**BEIJING**
B316		**1**	**BEIJING**
B506		**1**	**BEIJING**
B267		**1**	**BEIJING**
BCG		**482**	**BCG**
H37Rv		**451**	**H37Rv**
B753		**1**	**BEIJING**
B905		**NO RESULT**	
B628		**60**	**LAM4**
B749		**NO RESULT**	
B153		**1**	**BEIJING**
B136		**2286**	**X3**
B017		**NO RESULT**	
B858		**1**	**BEIJING**
B103		**1**	**BEIJING**
B365		**92**	**X3**
B288		**1**	**BEIJING**
B539		**60**	**LAM4**
B019		**NO RESULT**	
B495		**1**	**BEIJING**
B176		**1**	**BEIJING**

B657		**1**	**BEIJING**
B393		**1**	**BEIJING**
B130		**1**	**BEIJING**
B796		**92**	**X3**
B207		**1**	**BEIJING**
B068		**1**	**BEIJING**
B663		**60**	**LAM4**
B045		**70**	**X3**
B313		**33**	**LAM3**
B149		**1**	**BEIJING**
B022		**1**	**BEIJING**
B541		**1**	**BEIJING**
B048		**1**	**BEIJING**
B284		**1**	**BEIJING**
B505		**Not in SITVIT**	
B070		**2286**	**X3**
B420		**1**	**BEIJING**
B659		**ORPHAN**	**X3**
H37Rv		**451**	**H37Rv**
BCG		**482**	**BOVIS1**

B692		**1**	**BEIJING**
B012		**1**	**BEIJING**
B704		**1**	**BEIJING**
B269		**1**	**BEIJING**
B047		**1**	**BEIJING**
B071		**1**	**BEIJING**
B337		**1**	**BEIJING**
B552		**1**	**BEIJING**
B578		**1**	**BEIJING**
B105		**1247**	**MANU2?**
B088		**Not in SITVIT**	
B928		**2286**	**X3**
B510		**1**	**BEIJING**
B418		**Not in SITVIT**	
B529		**NO RESULT**	
B941		**1**	**BEIJING**
B736		**Not in SITVIT**	
B340		**33**	**LAM3**
B682		**1**	**BEIJING**
B280		**1**	**BEIJING**
B167		**1**	**BEIJING**
B063		**1**	**BEIJING**

B584		**1**	**BEIJING**
B951		**1**	**BEIJING**
B550		**2286**	**X3**
B721		**1**	**BEIJING**
B328		**1**	**BEIJING**
B596		**60**	**LAM4**
B138		**1**	**BEIJING**
B161		**1**	**BEIJING**
B496		**Not in SITVIT**	
B921		**1**	**BEIJING**
H37Rv		**451**	**H37Rv**
BCG		**482**	**BOVIS1**
B238		**1**	**BEIJING**
B082		**1**	**BEIJING**
B001		**60**	**LAM4**
B060		**1**	**BEIJING**
B844		**1**	**BEIJING**
B300		**1**	**BEIJING**
B679		**1**	**BEIJING**
B669		**1**	**BEIJING**
B891		**NO RESULT**	
B209		**60**	**LAM4**

B275		**1**	**BEIJING**
B064		**33**	**LAM3**
B898		**1**	**BEIJING**
B466		**1**	**BEIJING**
B392		**1**	**BEIJING**
B795		**1**	**BEIJING**
B994		**1**	**BEIJING**
B221		**1**	**BEIJING**
B311		**1**	**BEIJING**
B624		**1**	**BEIJING**
B702		**1**	**BEIJING**
B591		**NO RESULT**	
B108		**33**	**LAM3**
B515		**1**	**BEIJING**
B794		**1**	**BEIJING**
B192		**1**	**BEIJING**
B180		**1**	**BEIJING**
B482		**1750**	**LAM4**
B251		**NO RESULT**	
BCG		**482**	**BOVIS1**
H37Rv		**451**	**H37Rv**
B453		**1**	**BEIJING**

B241		**1**	**BEIJING**
B095		**ORPHAN**	**LAM9**
B990		**1**	**BEIJING**
B959		**1**	**BEIJING**
B208		**1**	**BEIJING**
B792		**1**	**BEIJING**
B890		**1**	**BEIJING**
B038		**1**	**BEIJING**
B883		**1**	**BEIJING**
B531		**1**	**BEIJING**
B230		**1**	**BEIJING**
B270		**Not in SITVIT**	
B378		**1**	**BEIJING**
B483		**1**	**BEIJING**
B707		**1**	**BEIJING**
B958		**NO RESULT**	
B385		**1**	**BEIJING**
B081		**1**	**BEIJING**
B611		**1**	**BEIJING**
B100		**1**	**BEIJING**
B817		**1**	**BEIJING**
B597		**2271**	**LAM2**

B804		**1**	**BEIJING**
B141		**1**	**BEIJING**
B960		**1**	**BEIJING**
B367		**1**	**BEIJING**
B645		**1**	**BEIJING**
B227		**1**	**BEIJING**
H37Rv		**451**	**H37Rv**
BCG		**482**	**BOVIS1**
B205		**NO RESULT**	
B018		**NO RESULT**	
B021		**1**	**BEIJING**
B062		**1**	**BEIJING**
H37Rv		**451**	**H37Rv**
BCG		**482**	**BOVIS1**
B5166		**1**	**BEIJING**
B6657		**1**	**BEIJING**
B0976		**1**	**BEIJING**
B4197		**Not in SITVIT**	
B1819		**1**	**BEIJING**
B8368		**Not in SITVIT**	
B0368		**60**	**LAM4**
B1753		**1**	**BEIJING**

B8753		**1**	**BEIJING**
B2022		**1**	**BEIJING**
B1070		**1**	**BEIJING**
B6692		**1**	**BEIJING**
B1071		**1**	**BEIJING**
B6891		**1**	**BEIJING**