SPREAD OF MULTI DRUG RESISTANT TUBERCULOSIS (MDR) INCLUDING EXTENSIVELY DRUG RESISTANT TUBERCULOSIS (XDR TB), IN RURAL KWAZULU-NATAL

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ABSTRACT

Mycobacterium tuberculosis (MTB) is an airborne pathogen that is easily transmitted from person to person. An intact immune system prevents the organism from causing disease in most individuals. In South Africa, the prevalence of human immunodeficiency virus (HIV) has reached astronomical levels and is now fuelling the tuberculosis (TB) epidemic. Drug resistant MTB strains combined with a weakened host immune system is a lethal combination. Multi-drug resistant (MDR) including extensively drug resistant (XDR) tuberculosis is on the increase, with Tugela Ferry in KwaZulu-Natal South Africa, reporting the largest cluster of XDR cases in the world. It is unknown whether a single clone of the drug resistant strain is circulating in this area or whether there are multiple strains at play. Using 2 complementary genotyping methods, we showed that the MDR strains present are the result of clonal spread associated with the F28 family, as well as *de novo* resistance which manifests as unique patterns. The XDR epidemic in Tugela Ferry is the result of clonal spread of a strain belonging to the F15/LAM4/KZN family.

PREFACE

This study represents original work by the candidate and has not been submitted in any other form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

All routine and experimental work (growth of isolates from storage into liquid and solid media, biochemical tests for identification, culture-based drug susceptibility testing as well as the molecular genotyping), described in this dissertation was carried out by the candidate in the Department of Infection Prevention and Control, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa under the supervision of Professor P. Moodley.

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DECLARATION

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bps	Base pairs
cfu/ml	colony forming units per ml
CoSH	Church of Scotland Hospital
CDC	Centers for Disease Control and Prevention
CAS	Central Asian Strain
cm	centimetres
CTAB-NaCl	Cetyltrimethylammonium bromide – Sodium chloride
DNA	Deoxyribonucleic Acid
DR	Direct Repeat
DST	Drug Susceptibility Testing
DVR	Direct variable repeat
EAI	East African Indian
ECL	Enhanced chemi-luminescence
EDTA	Ethylenediamine Tetra-acetic Acid
et al.	and others
g	grams
HCl	Hydrochloric acid
HIV	Human Immunodeficiency Virus
i.e.	that is
INH	Isoniazid
IS	Insertion sequence
IU	International Units
LJ	Lowenstein Jensen
kbps	kilo base pairs

KZN	KwaZulu-Natal
LAM	Latin American Mediterranean
L	Litres
MDR	Multi Drug-Resistant
mg	Milligrams
MGIT	Mycobacterial Growth Indicator Tube
ml	Millilitres
mM	milli-Molar
MIRU-VNTR	Mycobacterial Interspersed Repetitive Units- Variable Number Tandem Repeats
MTB	*Mycobacterium tuberculosis*
OADC	Oleic acid-albumin-dextrose-catalase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
PGRS	Polymorphic GC-rich Repetitive Sequence
pmol	picomoles
RFLP	Restriction Fragment Length Polymorphism
rpm	revolutions per minute
SAF1	Southern Africa Family 1
SIT	Shared International Type

SDS	Sodium Dodecyl Sulfate
SNP	Single nucleotide polymorphism
SSC	Sodium chloride and sodium citrate
SSPE	Sodium chloride, Sodium Phosphate and EDTA
TB	Tuberculosis
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
USA	United States of America
µg	Micrograms
µl	micro-litres
UPGMA	Unweighted pair group method with arithmetic mean
UV	ultra-violet
V	Volts
viz	Namely
WHO	World Health Organisation

DEFINITIONS

MDR-TB: MTB strains which are resistant to the two most potent first lines drugs which are isoniazid and rifampicin.

PRE-XDR: MTB strains which are MDR and resistant to either a fluoroquinolone or a 2nd line injectable agent but not both.

XDR-TB: MTB strains which are MDR and have additional resistance to one of the fluoroquinolones and any of the second-line injectables which include kanamycin, amikacin and capreomycin.

CLUSTER: 2 or more isolates which have identical IS*6110* RFLP banding patterns are grouped into a cluster.

FAMILY: A group of isolates which have IS*6110* RFLP banding patterns with ≥65% similarity are classified as a family of strains.

ORPHAN: A spoligotype pattern which is not present on the international database.

UNIQUE: Isolates which have IS*6110* RFLP patterns which are not identical to another isolate in the collection.

SHARED INTERNATIONAL TYPE: Spoligotype patterns which are present on the international database, isolates are classified as corresponding to a SIT if their spoligotype pattern is identical to a spoligotype pattern present on the SpolDB4 database.

CHAPTER 1

INTRODUCTION

Tuberculosis (TB) continues to be a major cause of human morbidity and mortality in the developing world (Ahmed *et al*., 2011; Rodriguez *et al*., 2010). Human immunodeficiency virus (HIV) associated immune suppression increases susceptibility to co-infection with *Mycobacterium tuberculosis* (MTB). The KwaZulu-Natal (KZN) Province in South Africa is the epicenter of the global HIV epidemic (KZN LED Report, 2005; York *et al*., 2000). TB in this province is fuelled by the increasing number of immune-suppressed individuals.

In 2005 a cluster of 53 HIV-infected patients with extensively drug-resistant tuberculosis (XDR-TB) was detected in, the catchment area for the Church of Scotland Hospital (CoSH) in Tugela Ferry, in the KZN province, South Africa. Tugela Ferry is a rural town that is inhabited by approximately 300 000 Zulu people. This 'outbreak' caused global alarm since this was not only the largest reported cluster of patients with XDR TB, but the majority of patients had demised before the drug susceptibility testing (DST) results became available (Gandhi *et al*., 2006).

In March 2006 XDR TB was reported to have a worldwide distribution, and was deemed a potential global threat by the Centers for Disease Control and Prevention (CDC) (CDC MMWR, 2006). Between November 2004 and November 2005, the World Health Organization (WHO) and CDC conducted a survey by obtaining data from an international network of TB laboratories. The supranational TB laboratory reference network consisted of 25 reference laboratories, on six continents. A total of 17690 TB isolates from the period 2000-2004 were tested for susceptibility to three second line drugs. The results

revealed that of the 17690 TB isolates 20% (3520/17690) were multidrug resistant tuberculosis (MDR-TB) and 2% (347/17690) were XDR TB. Of the 3520 MDR TB isolates, 1298 were from South Korea of which 15% (200/1298) were XDR TB. The remaining laboratories reported a combined total of 2222 MDR cases of which 7% (147/222) were XDR TB. Population based data on MTB drug susceptibility were obtained from United States of America (USA) for the period 1993-2000, Latvia 2000-2002 and South Korea 2004. Data from USA during 1993-2000 reported on a total of 169654 patients, of which 1.6% were MDR TB cases. Of the MDR cases, 4.1% were XDR TB cases. In Latvia, of the 605 MDR TB cases who initiated therapy during 2000-2002, 19% were XDR TB cases. The proportion of XDR TB in these regions increased over the years 2000-2004 (CDC MMWR, 2006).

Of the XDR TB patients who sought treatment at the CoSH for their current TB illness, 30 of the 53 were detected in the outpatient department. Fourteen of these gave no previous history of hospitalisation. There was no history of previous TB treatment in 55% of the XDR TB cases. This was the first indication that the Tugela Ferry "outbreak" was not an isolated nosocomial outbreak at CoSH. All 44 patients tested for HIV were found to be positive. An analysis of the isolates using IS*6110* restriction fragment length polymorphism (RFLP) and spoligotyping revealed that 85% (39/46 tested) patients were infected with a genetically similar XDR strain of MTB which belonged to the KZN family. The 7 other isolates were genotyped subsequent to publication and were also classified as belonging to the KZN family. This implied that possible transmission of the XDR TB strains may have occurred in the community, during clinic visits and/or on admission to the hospital wards (Gandhi *et al*., 2006; Dorman and Chaisson, 2007).

The aim of this study was to therefore genotype a selection of TB isolates from patients in the Tugela Ferry area to ascertain the genetic relatedness of locally circulating strains. This was accomplished by evaluating and comparing the usefulness of IS*6110* RFLP and spoligotyping in describing the epidemiology of MTB in Tugela Ferry.

CHAPTER 2

BACKGROUND

2.1 Tuberculosis (TB)

2.1.1 Epidemiology

Approximately one third of the world's population is infected with TB (WHO, 2005). Every year, approximately 3 million deaths and 8 million new active cases occur. The global estimates of TB in 2009 were 9.4 million new cases with 1.7 million deaths which equated to 4700 deaths per day. Among the estimated 9.4 million new cases reported 3.3% had MDR TB. A recent WHO survey reported that the former Soviet Union with 28% had the highest incidence of MDR TB. XDR TB cases were confirmed in 58 countries (WHO, 2010a; 2010b).

South Africa ranks fourth on the WHO list of high TB burden countries (Schmidt, 2008; WHO, 2008). Since 1998 TB incidence in South Africa has increased. In KZN the number of TB cases reported in 1998 was 28637, this increased over the years to 39586 cases reported in 2001 (DOH TB Fact Sheet, 2003). In 2006 the incidence of TB in KZN was 780 per 100 000 population (DOH, 2007).

2.1.2 HIV co-infection

Approximately 40 million people are infected with HIV worldwide and about one third are co-infected with TB. The majority of HIV-TB co-infected cases occur in sub Saharan Africa (WHO, 2005). In 2009 the global estimate of new HIV-TB co-infected cases, was 1.1 million with 380 000 deaths (WHO, 2010a; 2010b). Tuberculosis is known as the

leading cause of death among HIV infected individuals. The dual epidemics of HIV and TB pose a major public health problem in South Africa. Whilst South Africa constitutes 0.7% of the world's population, it contributed to 17% of the global burden of HIV infection in 2007 (Abdool Karim *et al*., 2009; Bimbaum *et al*., 2011; DOH, 2007). In South Africa between 60% and 80% of new TB cases occur in people who are co-infected with HIV (Mattheyse, 2007; Weyer, 2007).

2.1.3 Physical characteristics of *Mycobacterium tuberculosis*

Tuberculosis is caused by the organism *Mycobacterium tuberculosis.* MTB belongs to the order Actinomycetales, family Mycobacteriacea, and genus Mycobacterium (Encyclopaedia of Infectious Diseases, 2007). It is an acid fast bacillus, classified as an obligate aerobe. The organism is non-encapsulated, curved, beaded, rod-shaped and non-spore forming, measuring approximately 0.5µm in width and 3µm in length. It has a unique cell wall structure that contains fatty acids and mycolic acids which are covalently attached by the interwoven polysaccharide arabinogalactan to an underlying peptidoglycan layer (Knechel, 2009).

2.1.4 Transmission

Transmission of MTB occurs via the airborne or droplet routes (Mathema *et al*., 2006; Knechel, 2009). It is therefore easily spread from person to person especially in overcrowded and poorly ventilated environments such as prisons, clinics and hospitals. Higher incidence rates of TB are usually found in such environments as compared to the general population (Haugen, 2007). Nosocomial transmission is enhanced by inadequate triage and infection control practices (Basu *et al*., 2007). Increased poverty with poor access to medical care especially in rural and poorly resourced areas also influences

transmission of MTB (Haugen, 2007; Murphy, 2008). Overcrowding associated with poverty potentiates the transmission of TB. Higher rates of TB amongst immigrants have been linked to poverty. The movement of populations due to natural disasters with it ensuing crowding and malnutrition have also been associated with TB transmission (Glynn, 1998; Lienhardt, 2001). Factors affecting access to medical care is multifactorial and delays in access results in delayed diagnosis and treatment of TB, thus increasing the risk of transmission of TB in the community (CDC MMWR, 2005; Lienhardt, 2001). In KZN over 80 % of reported cases of tuberculosis are not culture-confirmed in keeping with the policy on diagnosis of tuberculosis in South Africa. Consequently, drug susceptibility testing is not done. This raises major concern since a significant proportion of patients with MDR/XDR TB are likely to die before it is realised that drug susceptibility tests are needed to guide their treatment. A recent study reported that more than 65 % of MDR, (including XDR) cases of culture-confirmed tuberculosis did not reach the referral centre in KZN and therefore remained untreated. The disease in these patients was therefore inappropriately managed and they represent a pool of potential transmitters of drug-resistant tuberculosis, both in the community and institutional settings (Moodley *et al*., 2011).

2.2 Genotyping of *Mycobacterium tuberculosis*

2.2.1 Genome characteristics

The genome of MTB has approximately 4.4 mega base-pairs (bps) and contains 3924 open reading frames (Cole *et al*., 1998). Although mobile insertion sequences (IS) and polymorphic regions are found, the genome remains highly conserved (Mathema *et al*., 2008). Many genetic markers within the MTB genome have been identified. However, only a few offer sufficient discrimination and reliable reproducibility (Kremer *et al*., 1999).

Eisenach *et al.* (1988) and Zainuddin and Dale (1989) recognised the existence of repetitive deoxyribonucleic acid (DNA) elements in the organism and its potential contribution to strain typing of MTB. The IS*6110* element was first reported by Thierry *et al.* (1990a, 1990b). IS*6110* is a member of the IS3 family (Otal *et al*., 1991; Thierry *et al*., 1990a; Thierry *et al*., 1990b). It is a small mobile genetic element which encodes its own transposase but does not encode a phenotypic marker (Mathema *et al*., 2006). It comprises 1355bps and contains an imperfect 28bp inverted repeat at the ends (Thierry *et al*., 1990a; Thierry *et al*., 1990b). The IS*6110* is present in different copy numbers and integrated at various regions within the MTB genome (Mathema *et al*., 2006). The gold standard for MTB genotyping is restriction fragment length polymorphism (RFLP) using IS*6110* as a genetic marker (Van Embden *et al*., 1993).

Another extensively studied region within the MTB genome is the polymorphic direct repeat region (DR) which is the basis for another MTB genotyping method viz: spoligotyping. The DR region has a unique structure as it contains directly repeated sequences interspersed with unique sequences. The DR region is 36bps and the interspersed sequences range from 34 to 41bps (Hermans *et al.*, 1991; Kamerbeek *et al*., 1997; van Embden *et al*., 2000; Warren *et al*., 2002). The spoligotyping technique was developed and is used as an epidemiological tool which targets the DR regions (Kamerbeek *et al*., 1997; Streicher *et al*., 2007).

2.2.2 Genotyping methods available for *Mycobacterium tuberculosis*

Key factors in controlling spread of TB include rapid detection, adequate treatment and contact tracing to stop further transmission. Genotyping of MTB facilitates identification of disease transmission routes, differentiation of re-infection from reactivation and

detection of laboratory cross contamination. IS*6110* RFLP and spoligotyping are the most frequently used techniques.

2.2.2.1 Roles of genotyping methods

IS6110 RFLP is well suited to study molecular epidemiology of MTB strains and can also be used for evolutionary and phylogeny studies (Mathema *et al*., 2006). Using spoligotyping more conserved genetic information is visualised and this method can be used to classify MTB complex into taxons and subspecies (Van Soolingen, 2001). MIRU:VNTR is also well adapted to study molecular epidemiology of MTB strains. It has the potential for real time genotyping and high throughput typing. Single nucleotide polymorphism (SNP) based genotyping is considerably more powerful for examining the population based structure of MTB. It is also used for phylogenetic classification of strains (Mathema *et al*., 2006).

2.2.2.2 IS*6110* Restriction Fragment Length Polymorphism (RFLP)

IS*6110* RFLP remains the gold standard, which targets the IS*6110* transposable element located in the MTB genome (Van Embden *et al*., 1993). RFLP is useful for detecting outbreaks and conducting population based studies of recent transmission. Epidemiologically unrelated strains of TB can be distinguished by different fingerprint patterns whilst linked strains have identical patterns (Barnes and Cave, 2003).

Although IS*6110* RFLP is highly reliable and remains the gold standard it also has many limitations. This genotyping technique is labour intensive and requires weeks of incubation to obtain sufficient amounts of DNA (DeRiemer and Daley, 2004; Kanduma *et al*., 2003). Isolates which have a low copy number of IS*6110* insertions are insufficiently

discriminated since there is a limited degree of polymorphism. Hence supplementary methods of genotyping have to be used (Barnes and Cave, 2003; DeRiemer and Daley, 2004; Kanduma *et al*., 2003; Mathema *et al*., 2006). Genetic variability of MTB exists worldwide and IS*6110* RFLP alone cannot distinguish among strains with too low or too high copy numbers of insertion sequences since poorly informative or complex patterns are generated. Alternative genotyping methods have been developed to circumvent these problems (Sola *et al*., 2003).

2.2.2.3 Spoligotyping

Spoligotyping is a polymerase chain reaction (PCR) based technique which is simple, robust and highly reproducible. It targets spacer sequences which are present between repetitive segments located in the direct repeat locus, by PCR and reverse dot-blot analysis (Kamerbeek *et al*., 1997).

MTB strains differ by the presence or absence of spacers and patterns of spacers are used to analyse the genotype. Results generated are highly reproducible and binary data are easily interpreted, computerized and amendable to inter-laboratory comparisons. Spoligotyping requires small amounts of DNA and can also be performed on isolates of MTB shortly after a clinical specimen has been inoculated into liquid culture (Barnes and Cave, 2003; DeRiemer and Daley, 2004; Mathema *et al*., 2006).

The discriminatory power is inferior as compared to IS*6110* RFLP analysis (Kremer *et al*., 1999; Mathema *et al*., 2006). Identical spoligotype patterns but differing IS*6110* RFLP patterns are often encountered. In endemic areas transmission events are unable to be discerned but low copy number strains can be discriminated. Discriminatory power of

spoligotyping when used alone is limited since a single locus which accounts 0.1% of the MTB genome is being targeted (Kanduma *et al*., 2003; Kremer *et al*., 1999; Mathema *et al*., 2006).

2.2.2.4 Polymorphic GC-rich repetitive sequences (PGRS)

PGRS is a southern blot hybridization technique which utilizes a PGRS specific probe cloned in plasmid pTBN12. This technique is resource intensive and patterns generated are too complex to computerize for standardization and analysis (Mathema *et al*., 2006; Ross *et al*., 1992).

2.2.2.5 Pulse Field Gel Electrophoresis (PFGE)

PFGE is used to simplify IS*6110* RFLP. It uses a less frequently cutting enzyme and produces high molecular weight fragments which are separated under special conditions. However, small polymorphism differences for different strains are observed and do not always produce sufficient discrimination (Kanduma *et al*., 2003).

2.2.2.6 Mycobacterial interspersed repetitive units - Variable number-tandem repeat (MIRU-VNTR) Typing

An alternative PCR based technique has recently been described called MIRU-VNTR which targets 12 to 24 loci containing, a variable number of tandem repeats of genetic elements named mycobacterial interspersed repetitive units. These are located mainly in intergenic regions interspersed in the MTB genome (Alonso-Rodriguez *et al*., 2008; Supply *et al*., 2006). The automation of the technique via ABI-gel based sequencing or Beckman capillary based sequencing has improved its rapidity and convenience. MIRU-VNTR genotyping is simpler than IS*6110* RFLP and can be applied directly to MTB

cultures without DNA purification. The data generated are highly amendable to inter and intra-laboratory comparisons. Analysis can be automated and used to evaluate a large number of strains. The discriminatory power of MIRU-VNTR analysis is proportional to the number of loci evaluated. When 12 loci are used MIRU-VNTR is less discriminate relative to IS*6110* RFLP for isolates with a high IS*6110* copy number and more discriminate for isolates with a low IS*6110* copy number. When more than 12 loci are used or MIRU-VNTR and spoligotyping analysis are combined the discriminatory power approximates that of IS*6110* RFLP analysis (Barnes and Cave, 2003; Marzars *et al*., 2001; Mathema *et al*., 2006; Supply *et al*., 2001).

2.2.3 Genotyping and Drug Resistance

MDR and XDR TB cases are the result of primary transmission of already resistant strains and/or *de novo* development of resistance within an individual. Primary MDR or XDR TB occurs via transmission of an already resistant strain and is by definition clonal because the same strain is responsible for two or more cases. In contrast, *de novo* acquisition of drug resistance occurs in a single patient during treatment by the outgrowth of minor populations of drug resistant organisms in a largely susceptible population under selective pressure of exposure to drug. MTB strains which develop *de novo* resistance are not related to clonal spread, but may contribute to strain diversity in an area. The spread of already resistant MTB strains in an area is often clonal and may be linked to an outbreak (Bifani *et al*., 1996; Edlin *et al*., 1992; Gandhi *et al*., 2006). Multiple drug resistant clusters have also been described in some areas implying that more than one outbreak occurred concurrently. MTB strains which acquire de novo resistance in an individual may be transmitted to others in an already resistant form resulting in clonal spread.

The MDR and XDR susceptibility profiles of strains of MTB have been linked to certain families. Studies have shown that MDR strains were associated with the Beijing family (Almeida *et al*., 2005; Johnson *et al*., 2006; Mistry *et al*., 2002). In KZN, the F15/LAM4/KZN family were found to be dominant amongst the MDR and XDR isolates (Gandhi *et al*., 2006; Pillay and Sturm, 2007). A study by Mlambo *et al.*, (2008) found a high diversity in the genotypes of XDR MTB strains from different geographical locations throughout South Africa. These findings indicated that a significant number of the XDR TB cases were due to *de novo* acquisition of drug resistance rather than primary transmission. XDR TB in South Africa is not in general due to the spread of a single outbreak strain but more often the result of *de novo* acquired resistance. Two different mechanisms of evolution of XDR TB may be operating in Tugela Ferry and the country as a whole is not a mutually exclusive hypotheses.

Acquired drug resistance by the spontaneous insertion of IS*6110* elements into genes associated with pyrazinamide activity has been reported (Lemaitre *et al*., 1999). A study by Purwar *et al.*, (2011) attempted to define an association between drug resistance and IS*6110* copy numbers. They reported that isolates with identical IS*6110* copy numbers had different susceptibility profiles and vice versa. Although resistant strains have been associated with specific strain families, the relationship between specific patterns generated by genotyping analysis and susceptibility profiles remains unclear.

2.3 Genotyping and Outbreaks of *Mycobacterium tuberculosis*

Strains of W/Beijing strain family of MTB have been responsible for many large outbreaks in correctional institutes and nosocomial environments (Bifani *et al*., 2002; Glynn *et al*., 2002). The Haarlem strain family has also been documented on different continents such as

Europe, Asia and Africa. The Haarlem strain family has been responsible for a few outbreaks which have been reported in Argentina, Czech Republic and Tunisia (Kubin *et al*., 1999; Mardassi *et al*., 2005; Ritacco *et al*., 1997).

2.3.1 New York City

In the early 1990's a multi-drug resistant MTB strain was identified in New York City. It was resistant to isoniazid, rifampin, ethambutol, streptomycin and in most instances kanamycin. Analysis of the strain revealed that it was a member of the Beijing strain family. This strain spread rapidly among a HIV positive population and 357 cases were reported from prisons and hospitals. DNA fingerprinting revealed that 253 of the cases shared an identical 18 banded pattern or similar IS*6110* pattern and were therefore associated with clonal spread (Bifani *et al*., 1996; Bifani *et al*., 2002; Glynn *et al*., 2002).

2.3.2 Argentina

In Buenos Aires, during January 1994 – June 1995 an increase of MDR TB was identified at a referral treatment centre among HIV infected patients. A total of 124 HIV positive patients with MDR TB were identified of which 101 TB isolates had resistance to 5 drugs. IS*6110* RFLP analysis was done on 77 isolates. Of the 77 patients 74 were hospitalised in the previous year. An additional 3 MDR TB isolates from HIV positive patients and 35 TB isolates of HIV negative patients, who were present in the hospital during 1992-1993 were also analysed. IS*6110* RFLP analysis revealed that 68 of the 74 isolates from 1994-1995 clustered and 2 of the 3 isolates from 1992 belonged to the M genotype. The IS*6110* RFLP patterns resembled those of the Haarlem family. The strain involved in the outbreak was traced to HIV positive patients hospitalised in 1992. The clinical records of both patients

suggested nosocomial infection. It was likely that this strain or an ancestor had been brought into the hospital prior to the occurrence of an outbreak (Ritacco *et al*., 1997).

2.3.3 Italy

In Europe the largest nosocomial outbreak during October 1991- July 1995 occurred in Milan. The MTB strains were resistant to 4 first line drugs and were present among HIV positive patients in 2 large urban hospitals in Milan. A total of 116 MDR TB cases were identified among 2 urban hospitals. In one hospital, (A) 85 cases were identified and 31 cases in a second hospital (B). A patient infected at hospital A had introduced the strain into hospital B. IS*6110* RFLP analysis was done on 92 of the 116 MDR TB isolates and 82 of these 92 isolates clustered in one genotype. The results revealed that nosocomial transmission occurred in hospital A among 39 of the 56 patients and 24 of the 26 in hospital B. For 8 patients with the outbreak strain, exposure was reported to have occurred in other hospitals, in the community or in prison. IS*6110* RFLP patterns in this study differed from the commonly found Beijing strain associated with numerous outbreaks (Moro *et al*., 1998).

2.3.4 Iran

The existence and transmission of XDR TB among MDR TB patients was evaluated in Iran among patients that were referred for treatment and diagnosis during 2003-2005. A total of 12 XDR TB isolates were identified among 113 MDR TB isolates. Spoligotyping and IS*6110* RFLP analysis were performed on the XDR TB isolates. Retrospective analysis revealed that 2 clusters were present. In cluster I, 8 isolates were present and these isolates were reported as being involved in an outbreak among a family and community members associated with this family. The first patient had infected 2 of the siblings and 5

other patients who had shared a room with this patient. In cluster II, the outbreak strain was found among 4 family members. Spoligotyping and IS*6110* RFLP results revealed that the MTB strains belonged to the Haarlem 1 and the East African Indian (EAI) 3 families of strains (Masjedi *et al*., 2006).

2.4 Genotyping and Prevalent *Mycobacterium tuberculosis* strains

2.4.1 China

The Beijing family of MTB strains are described as endemic in China and dominant in neighbouring countries of Mongolia, South Korea and Thailand, while relatively low in prevalence but emerging on other continents. While drug resistance has been associated with Beijing strains in other parts of the world, including New York, Cuba, Estonia and Vietnam, these strains have not been significantly associated with drug resistance in other countries. In China the Beijing family of strains are the most prevalent MTB strains circulating in the population, indicative of a long history of endemicity. Drug resistant Beijing strains are relatively uncommon in this part of the world. The Beijing MTB strain possesses similar molecular epidemiological characteristics to those MTB strains reported during the New York City outbreak (Bifani *et al*., 2002; Glynn *et al*., 2002; Van Soolingen *et al*., 1995).

2.4.2 Mozambique

A study conducted in Mozambique by Viegas *et al.* (2010) analysed 445 MTB isolates using spoligotyping. The four predominant genotypes which were identified included the Latin-American Mediterranean (LAM) lineage which accounted for 37%, the East African-Indian (EAI) which accounted for 29.7%, the T lineage which accounted for 11.6% and the

Beijing lineage which accounted for 7%. A great diversity of spoligotyping patterns was found within the LAM, EAI and T lineages. The spread of MTB in Mozambique is facilitated by various circulating strains with different spoligotype patterns and 4 predominant lineages (Brudey *et al*., 2006; Viegas *et al*., 2010).

2.4.3 Zambia

In a study conducted by Chihota *et al*. (2007), MTB isolates from Zambia were analysed by spoligotyping. The results revealed that 63.2% of the isolates had a characteristic spoligotype pattern. The results also showed that 6 isolates shared a spoligotype pattern with isolates from Zimbabwe and 4 isolates shared a spoligotype pattern with isolates from Cape Town.

In a study done by Mulenga *et al.* (2010) in the Ndola district of Zambia 273 MTB isolates were genotyped using spoligotyping to establish the different lineages of MTB circulating in this area. Of the 273 isolates 177 isolates belonged to a lineage present in the international SpolDB4 database and 96 isolates did not match any of the spoligotype patterns present on the SpolDB4 database. These isolates were referred to as orphans. The predominant spoligotype family was the LAM lineage. The majority of the isolates in the LAM lineage belonged to the LAM11-ZWE sub-lineage which is designated as the Southern Africa Family 1 (SAF1). Other lineages which were common among the isolates included the T lineage and X lineage. A small proportion of the isolates also belonged to the following lineages CAS, EAI, H, S, X1-LAM9 and U.

2.4.4 Zimbabwe

In a study done by Chihota *et al.* (2007) the genetic diversity of MTB isolates were analysed using spoligotyping and IS*6110* RFLP. Isolates were obtained from patients presenting to referral hospitals in Zimbabwe. A predominant group was identified among the isolates which had a characteristic IS*6110* banding pattern and a unique spoligotype signature which had spacers 21-24, 27-30 and 33-36 absent. This indicated a clonal expansion from a common progenitor. This group of strains were designated the Southern Africa family 1 (SAF1). Previous studies have shown that SAF1 is a member of the LAM lineage (Brudey *et al*., 2006). Comparison between the IS*6110* RFLP patterns among the Zimbabwean isolates and IS*6110* RFLP patterns of Cape Town isolates on a database showed a close relation to a strain family in Cape Town with 2.3% of Cape Town TB isolates related. Four IS*6110* RFLP patterns and 3 spoligotype patterns were shared between Zimbabwe and Cape Town. Among the Cape Town isolates 8 had the characteristic SAF1 spoliogtype signature. The unique and predominant SIT59 pattern was compared to the SpolDB3 database. This pattern was found to be rare in other parts of the world but common in Southern Africa (Chihota *et al*., 2007).

2.4.5 Botswana

In a study by Lockman *et al*. (2001) patterns of TB transmission were analysed using IS*6110* RFLP. MTB isolates were obtained from patients from four communities in Botswana during January 1997-June 1998. IS*6110* RFLP patterns which had >5 bands were considered to be clustered if they had identical patterns or differed by 1 band. Patterns which had ≤5 bands were considered clustered if all bands were identical. Of the 301 MTB isolates, 125 isolates belonged to 20 different clusters.

2.4.6 South Africa

In South Africa different families of strains have been described in different areas of the country.

2.4.6.1 Western Cape strains

In the Western Cape in South Africa in 2004 over 44000 people had been diagnosed with TB of whom 450-600 had MDR TB (Johnson *et al*., 2006). MDR TB was 1st identified in 1985 in the Western Cape (Streicher *et al*., 2004). The TB epidemic in the Western Cape Province is primarily driven by four MTB strains. These strains include the Beijing strain which predominates among those responsible for drug resistance. An outbreak with a specific cluster of the Beijing strain occurred in Cape Town in 2005. Analysis of isolates of 4 school children identified with MDR TB revealed that the cluster contained a specific mutation in the *inh*A gene conferring isoniazid resistance. These isolates belonged to the Beijing cluster 220. Archived MTB drug resistant strains were also analysed and 28% belonged to the Beijing cluster 220 and an additional 42% had a mutation in the *rpo*B gene. The presence of these transmissible MDR TB strains poses a threat to the community (Johnson *et al*., 2006).

The F11 and F28 genotypes are also present in the Western Cape. The F11 genotype has been identified as the most successful strain in this province and is also present on four other continents and in 25 different countries. The F11 genotype can be identified by a unique spoligotype pattern and dot blot screening for a specific rrs491 polymorphism (Streicher *et al*., 2004; Victor *et al*., 2004). The DRF 150 genotype was detected during an outbreak of an emerging resistant non-Beijing strain. This genotype is identified by 5

IS*6110* insertions, a specific spoligotype and high levels of resistance to isoniazid, rifampicin and streptomycin (Victor *et al*., 2007).

2.4.6.2 KwaZulu-Natal

In a study by Pillay and Sturm (2007) the strain that was found in KZN was classified as belonging to the F15/LAM4/KZN family. The F15 family forms part of the Latino-American and Mediterranean family and corresponds with the LAM4 sub-lineage (Streicher *et al*., 2007). These strains were found to have a spoligotype pattern which had been reported previously (Brudey *et al*., 2006; Filliol *et al*., 2003). The pattern was known to have spacers 21-24, 33-36 and 40 absent but the IS*6110* RFLP fingerprint pattern was found to be unique. The F15/LAM4/KZN family strain has been present in KZN as early as 1994 and has been associated with many MDR TB cases which had been genotyped during 1994-2002. In 2001 the first XDR TB isolate was identified in one patient and possessed the F15/LAM4/KZN fingerprint pattern (Pillay and Sturm, 2007). During 2005 in Tugela Ferry isolates from the XDR TB cases also displayed the F15/LAM4/KZN fingerprint (Gandhi *et al*., 2006).

During 1994-1997 a study in patients from the community in Durban revealed that the Beijing strain was predominant and all isolates were fully drug susceptible (Pillay and Sturm, 2010).

2.5 Why Study The Molecular Epidemiology of Strains in KZN?

KZN being a region with high endemicity of HIV has an increasing population of immune suppressed individuals. This compounds the ease with which MTB is transmitted in. Additionally, these immune suppressed patients often require frequent hospital admissions

for other opportunistic infections. They therefore represent a conduit between the community and health care institutions allowing for nosocomial pathogens to be transmitted at community level and vice versa. MTB strains isolated from in-patients do not exclude the possibility of similar strains circulating in the community. Genotyping techniques allow the possibility of studying the molecular epidemiology of MTB within a community and between community and health care institutions. This provides useful information in the analysis of outbreaks. It also facilitates analysis of complex transmission routes which are often undetected in routine contact screening.

CHAPTER 3

MATERIALS AND METHODS

3.1 Geographical Location of Source of Isolates

The Msinga sub-district is a rural area situated in KwaZulu-Natal and comprises a predominantly indigent Zulu population. Tugela Ferry is the only town in this sub-district which is home to the Church of Scotland Hospital (CoSH). This hospital is a district hospital with 355 inpatient beds and in addition serves as a referral facility for 14 clinics. CoSH attends to patients which are referred by the clinics. Isolates for this study were collected from patients seeking in-patient or outpatient health services at this hospital.

3.2 Ethics Approval

The study was approved by the Biomedical Research Ethics Committee at the University of KwaZulu-Natal (#BCA274/09) for bacterial isolates that were grown from specimens sent for routine clinical care. Patients were not asked to give informed consent at the time of the clinical encounters. Additionally, specimens that were collected that were part of clinical studies were covered under ethics numbers E313/05 and BFC 061/07.

3.3 *Mycobacterium tuberculosis* isolates

The selected isolates were from 2 time periods viz: 2005/2006 and 2008/2009. These were isolated from specimens obtained from in-patients and out-patients attending CoSH.

2005/2006

The isolates were obtained from patients participating in the prevalence study by Gandhi *et al.* (2006) and from patients who had been routinely tested for the purpose of further

clinical management. Isolates from all new cases of TB presenting to CoSH that were part of a prevalence study done by Gandhi *et al.* (2006) were selected; additional isolates from this period were obtained from patients presenting to CoSH for management of tuberculosis.

2008/2009

The isolates were obtained from patients participating in the Microscopic Observation Drug Susceptibility (MODS) Assay study by Shah *et al*. (2011). Isolates from new cases presenting to CoSH during 2008-2009 who were recruited into a MODS study were also selected.

The isolates from these studies were stored in the laboratory at UKZN. Viable cultures were selected for further processing in this study. Access to clinical information for isolates used in this study was limited.

3.4 Culture of stored isolates

3.4.1 Retrieval of isolates

All stored isolates were retrieved from the -70 degrees Celsius (°C) freezer and thawed to room temperature. The thawed isolates were revived by inoculation into Middlebrook 7H9 (Becton Dickinson and Company, Difco Laboratories, USA) broth or mycobacterial growth indicator tubes (MGIT) (BD, USA).

3.4.2 Inoculation of Middlebrook 7H9 broth

Sterile free-standing tubes containing 5ml of Middlebrook 7H9 (BD, Difco Laboratories, USA) broth supplemented with oleic acid-albumin-dextrose-catalase (OADC) (BD, USA)

and made selective by adding the following antibiotics: Polymixin B (200.000 IU/L) (Sigma-Aldrich, USA), Amphotericin B (20mg/L) (Sigma-Aldrich, USA), Carbenicillin (100mg/L) (Sigma-Aldrich, USA) and Trimethoprim (20mg/L) (Sigma-Aldrich, USA) were added. Approximately 100µl of the isolate was inoculated into Middlebrook 7H9 (BD, Difco Laboratories, USA) broth using a glass pasteur pipette. Tubes were incubated for 3-6 weeks at 37°C until growth was observed.

3.4.2.1 Culture onto Middlebrook 7H11 agar plates

Upon observation of growth in Middlebrook 7H9 (BD, Difco Laboratories, USA) broth, culture was aspirated, using a sterile glass pasteur pipette and inoculated onto Middlebrook 7H11 (BD, Difco Laboratories, USA) agar plates. A sterile quadloop was used to streak the inoculum across the entire plate. Plates were heat sealed in gas permeable bags and incubated for 3 weeks at 37°C in a regular incubator. Plates were viewed after 3 weeks for the presence of MTB colonies.

These cultures were used to perform drug susceptibility testing (DST) and to extract DNA for spoligotyping.

3.4.2.2 Culture onto Lowenstein Jensen (LJ) slants

Upon observation of growth in Middlebrook 7H9 broth, culture was aspirated using a sterile glass pasteur pipette and inoculated onto LJ slants (BD, USA). The LJ slants were inverted a few times to allow culture to spread across the surface of the slant. The caps of the LJ slants were loosened before being incubated for 3-6 weeks at 37°C in a CO_2 incubator.

These cultures were used to extract DNA for IS*6110* RFLP.

3.5 Culture Confirmation

Identification to species level of the growth on Middlebrook 7H11 (BD, Difco Laboratories, USA) agar plates was done using two biochemical tests.

3.5.1 Nitrate test

Sterile tubes were labelled with isolate numbers and 500µl of nitrate broth was aliquoted into each tube. Using a sterile quadloop a loopful of culture was scraped off the Middlebrook 7H11 (BD, Difco Laboratories, USA) agar plate and suspended into the tubes containing nitrate broth. Tubes were incubated at 37°C for 2 hours. Following incubation, 1 drop of concentrated hydrochloric acid (HCl) (Merck, SA) followed by 2 drops of 0.2% sulfanilimide (Sigma-Aldrich, USA) (nitrite1) solution and 2 drops of 0.1% N-(naphthyl) ethylenediamine dihydrochloride (Sigma-Aldrich, USA) (nitrite2) solution was added to all tubes. Tubes were observed for a colour change, any shade of pink indicated a positive result (Clinical Microbiology procedures handbook volume 2, 2004a).

H37Rv laboratory strain was used as a positive control and a tube containing no culture was used as a negative control.

3.5.2 Niacin test

This was performed as described in the Clinical Microbiology procedures handbook volume 2 (2004a) with a few modifications.

A drop of cyanogen bromide (Sigma-Aldrich, USA) was added onto a lawn of confluent growth followed by a drop of aniline (Sigma-Aldrich, USA) on top of the cyanogen bromide. Plates were left to stand for 5 minutes and thereafter observed for the presence of a colour change, a shade of yellow indicated a positive result.

H37Rv was used as a positive control and uninoculated culture medium as negative control.

If both nitrate and niacin tests were positive, this confirmed the culture as being MTB.

3.6 Drug Susceptibility Testing

3.6.1 1% Modified Proportion method

This was done as described in the Clinical Microbiology procedures handbook volume 2 (2004b) using the indirect method, with slight modifications.

This method is the gold standard for susceptibility testing of MTB. Colonies from a 3 week culture were used and a bacterial suspension in PBS with, 0.05% Tween-80 (Merck, SA) and 4 glass beads (diameter: 5mm) was prepared by vortexing tubes. The remaining clumps were allowed to settle after which the top layer was siphoned off. The harvest was matched to a McFarland standard 1 by adding PBS. Three 10-fold serial dilutions were done by adding 0.5ml of the suspension to 4.5ml of sterile triple distilled water to obtain an inoculum suspension of 10^{-4} colony forming units per ml (cfu/ml). Of these suspensions, 100µl was inoculated on quadrants of the Middlebrook 7H10 (BD, Difco Laboratories, USA) agar plates containing different drugs and a drug free control. This resulted in a final

inoculum of 10^{-3}cfu/quadrant. Plates were allowed to dry for 30 minutes and sealed in gas permeable bags and incubated at 37°C in a CO_2 incubator.

Susceptibility tests were read after 3 weeks. The concentrations of anti-TB drugs used were: Isoniazid (INH) (Sigma-Aldrich, USA): 1µg/ml, Rifampicin (Sigma-Aldrich, USA): 1µg/ml, Ethambutol (Sigma-Aldrich, USA): 7.5µg/ml, Streptomycin (Sigma-Aldrich, USA): 2µg/ml, Ofloxacin (Sigma-Aldrich, USA): 2µg/ml, Kanamycin (Sigma-Aldrich, USA): 5µg/ml, Ethionamide (Sigma-Aldrich, USA): 5µg/ml, Capreomycin (Sigma-Aldrich, USA): 10µg/ml.

A strain was classified as susceptible to a drug if <1% of growth was present on drug containing quadrants as compared to the growth present on the drug free control quadrant and resistant if the number of colonies on the drug containing quadrant was >1% of colonies that were present on the control quadrant.

Laboratory strains with known susceptibilities were used as controls. H37Rv was used as a susceptible control and A169 was used as a resistant control.

3.7 Spoligotyping

Spoligotyping was performed as previously described by Kamerbeek *et al.* (1997) and following the procedures described in the Spoligotyping Manual version 4 (2002).

3.7.1 DNA Isolation

This method was done as described previously by Van Soolingen *et al*. (1991) and following the procedure outlined in the Spoligotyping Manual version 4 (2002), with slight modifications.

3.7.1.1 Cetyltrimethylammonium bromide – Sodium chloride (CTAB-NaCl) method

Growth from Middlebrook 7H11 (BD, Difco Laboratories, USA) agar plates was harvested using a sterile quadloop and suspended into 2ml micro-centrifuge tubes (Eppendorf) containing 500µl of 1xTris-EDTA (TE) buffer (Appendix 1.1). Cultures were heat killed at 80°C for 30 minutes. 50µl of lysozyme (Sigma-Aldrich, USA) of concentration 10mg/ml (Appendix 1.2) was added to each sample which was then incubated in a 37°C water bath for 1 hour. Following this, 75µl of proteinase K (Roche Diagnostics, Mannheim, Germany) of concentration 10mg/ml (appendix 1.3) and 10% Sodium Dodecyl Sulfate (SDS) (Sigma-Aldrich, USA) solution (appendix 1.4) were added to each sample, vortexed and incubated at 65°C for 10 minutes. Then 100µl of 5M Sodium Chloride (NaCl) (Sigma-Aldrich, USA) solution (appendix 1.5) was added and the tube was vortexed for few seconds after which 100µl of pre-warmed CTAB-NaCl (Sigma-Aldrich, USA) solution (appendix 1.6) was added. The tube was vortexed until its contents turned milky. This was followed by incubation at 65°C for 10 minutes. Chloroform (Sigma-Aldrich, USA): Isoamyl alcohol (Sigma-Aldrich, USA) (24:1) solution (appendix 1.7) was added followed by vortexing and centrifugation for 20 minutes at 12000 rpm. The supernatant was transferred into a clean 1.5ml micro-centrifuge tube (Eppendorf) and 500µl of Isopropanol (Sigma-Aldrich, USA) was added to each tube. Samples were mixed by inverting tubes and the contents, was observed for thread formation. If a thread was visible, tubes were centrifuged for 30 minutes, the supernatant discarded and the pellet washed with 1ml of 70% ethanol (Merck,

SA) (appendix 1.8). The tubes were then inverted, centrifuged for 5 minutes and the ethanol was discarded. The pellet was washed again in 500µl of 70% ethanol (Merck, SA) and the steps described for the previous wash were repeated. Pellets were left to dry for 10 minutes at room temperature and the size was measured visually. Appropriate amounts of 1xTE buffer were added to each pellet and stored at 4°C overnight or until further use.

3.7.1.2 Gel Electrophoresis

Estimation of DNA concentration and assessment of DNA quality

A 1% agarose gel (appendix 1.9) was made using 1x Tris-Borate-EDTA (TBE) buffer (appendix 1.10). The agarose (Lonza, USA) was dissolved in 1xTBE buffer by heating in a microwave. The solution was allowed to cool and ethidium bromide (Sigma-Aldrich, USA) solution (appendix 1.11) was added. A casting tray and a 20 well comb were wiped with 70% ethanol. The casting tray was taped around the edges and the agarose solution poured in and allowed to set at room temperature. Once the gel had solidified the tape and comb were removed and the casting tray containing the gel was placed into an electrophoresis tank (Hoefer) containing 1xTBE buffer. A 3-5µl volume of sample loading dye (appendix 1.12) was added to a piece of parafilm for each DNA sample and 1µl of each DNA sample was mixed with the dye and loaded into a well of the gel. Electrophoresis was performed at 100 volts (V) for 30-45 minutes.

The Syngene gel documentation system and Gene Snap software package were used to photograph and print pictures of the gels. The pictures were used to estimate the concentration of each DNA sample and to assess their quality.

3.7.2 Polymerase Chain Reaction (PCR) of the Direct Repeat (DR) region

Genomic DNA isolated from the MTB cultures was used as target DNA, to amplify the DR region. PCR was done a day before hybridisation and PCR products were stored at 4°C. The PCR mastermix contained the following reagents: nuclease free water, colourless GoTaq flexi buffer (Promega, Madison, WI, USA), PCR nucleotide mix (Roche Diagnositics, Mannheim, Germany), 20 pmol of each primer DRa (biotinylated) and DRb (Whitehead Scientific, IDT, Iowa) and GoTaq DNA Polymerase (Promega, Madison, WI, USA). The mastermix was aliquoted into PCR tubes and DNA added to each tube. The tubes were centrifuged using a micro-centrifuge (Eppendorf) and placed into the thermocycler (Applied Biosystems) for amplification. The following cycling conditions were used: 3 minutes at 96°C, 1 minute at 55°C and 30 seconds at 72°C for 20 cycles.

3.7.3 Hybridisation

For hybridisation, 20µl of each PCR product was diluted in 150µl 2xSSPE/0.1% SDS (appendix 2.1). The diluted PCR products were heat-denatured for 10 minutes at 99°C and thereafter immediately cooled on ice. The membrane (Ocimum Biosolutions, India) was washed for 5 minutes at 60°C in 250ml of 2xSSPE/0.1% SDS. The membrane and a support cushion (Ocimum Biosolutions, India) were placed onto the mini-blotter, so that the slots were perpendicular to the applied oligonucleotides present on the membrane. The residual fluid in the slots of the mini-blotter was removed by aspiration. The diluted PCR products were pipetted into the slots in such a way that the mini-blotter channels were perpendicular to row of the oligonucleotides. Hybridisation was done for 1 hour at 60°C. Samples were removed by aspiration and plastic forceps were used to remove the membrane from the mini-blotter and to place this into a plastic container.

3.7.4 Washing of the membrane and incubation with the conjugate

Following hybridisation the membrane was washed twice using 250ml of 2xSSPE/0.5% SDS (appendix 2.2) for 10 minutes at 60°C. The membrane was allowed to cool in a plastic container to prevent inactivation of the peroxidase that followed. Streptavidin-peroxidase conjugate (Roche Diagnostics, Mannheim, Germany) was added to 20ml of 2xSSPE/0.5% SDS. The membrane was incubated in this solution for 55 minutes at 42°C in its plastic container. The membrane was washed twice using 250ml of 2xSSPE/0.5% SDS for 10 minutes at 42°C and thereafter washed twice using 250ml of 2xSSPE (appendix 2.3) for 5 minutes at room temperature.

3.7.5 Detection

This was performed in the darkroom. For detection of the hybridised DNA, the membrane was submerged for 1 minute in 10ml of enhanced chemi-luminescence (ECL) detection solution (Amersham, Buckinghamshire, UK). The membrane was wrapped in plastic and wiped with a tissue to remove bubbles and placed into a hypercassette (Amersham, Buckinghamshire, UK), followed by exposure of a hyperfilm (Amersham, Buckinghamshire, UK) to it for 30 minutes. The hyperfilm was removed from the hypercassette and placed into developer (Agfa-Gevaert, Belgium) until spoligotype patterns appeared. Thereafter the film was placed into fixer (Agfa-Gevaert, Belgium) until the hyperfilm had a clear background and rinsed with tap water.

3.7.6 Regeneration of the membrane

For repeated use of the membrane, the membrane was washed twice by incubation in 1% SDS solution (appendix 2.4) for 30 minutes at 80°C. The membrane was washed in 20mM EDTA (Sigma-Aldrich, USA) (appendix 2.5) pH8, for 15 minutes at room temperature on

an orbital shaker (Stuart). The membrane was sealed in plastic to prevent dehydration and stored at 4°C until further use.

3.7.7 Analysis of Spoligotying Patterns

Spoligotyping patterns were compared to the international SpolDB4 database. The patterns were entered in a binary format onto the SITVIT database (Pasteur Institute of Guadeloupe: http://www.pasteur-guadeloupe.fr:8081/SITVIT/DEMO) which is an updated version of the SpolDB4 database. The corresponding shared international type (SIT) was identified for each isolate. The SpolDB4 database was also used to classify isolates into lineages and sub-lineages (Brudey *et al*., 2006). Isolates which had a unique spoligotype pattern and were not found on the international database were classified as 'orphans' (Mathuria *et al*., 2007; Mulenga *et al*., 2010). Dendrograms were constructed using the Bionumerics version 3.5 software (Applied Maths, Kotrijk, Belgium). The dendrogram showed how spoligotyping patterns clustered and their degree of similarity.

3.8 IS*6110* RFLP

Genotyping by IS*6110* RFLP was performed as previously described by Van Embden *et al.* (1993) with a few modifications.

3.8.1 DNA Isolation

This was done using the CTAB-NaCl method, as previously described by Van Soolingen *et al*. (1991) with slight modifications.

MTB colonies were scraped off LJ slants using a sterile swab and suspended into 2ml micro-centrifuge tubes (Eppendorf) containing 500µl of sterile distilled water. The bacteria

were heat killed at 80°C in a water bath for 30 minutes following which 70µl of 10% SDS and 50µl of proteinase K (10mg/ml) were added to each to tube. Tubes were incubated in a water bath at 60°C for 1 hour. The 5M NaCl and CTAB-NaCl solutions were prewarmed at 60°C and 100µl of each solution was added to each tube and mixed thoroughly with its contents by inverting the tubes a few times. The tubes were then incubated at 60°C for 15 minutes and its contents frozen at -20°C for 15 minutes. The reaction mixtures were then defrosted at 60°C and 700µl of Chloroform-Isoamyl alcohol (24:1) solution was added to each tube and inverted several times until the contents turned milky. Tubes were centrifuged for 10 minutes at 12000 rpm. The upper aqueous supernatant was transferred to 1.5ml micro-centrifuge tubes (Eppendorf) which contained 700µl of isopropanol. Tubes were inverted several times and placed in a -20°C freezer for 30 minutes, followed by centrifugation for 10 minutes at room temperature at 12000 rpm. The isopropanol was discarded and the pellet was washed with 70% ethanol and centrifuged for 10 minutes at 12000 rpm. The ethanol was discarded and the pellets were dried in a DNA concentrator for 4 minutes. Approximately 35-55µl of 1xTE buffer was added to each tube depending on the pellet size. Tubes were rotated on an orbital shaker for 30 minutes to facilitate dissolving of the pellets. A 5µl volume of each DNA sample was electrophoresed on a 1% agarose gel as previously described, to estimate DNA concentration (3.7.1.2).

3.8.2 Restriction

The genomic DNA was digested with *Pvu*II restriction endonuclease that cleaves the IS*6110* sequence once.

The approximate DNA concentration was estimated visually from pictures obtained and the amount of sample and water to be used were calculated to have a final volume of 22µl.

Appropriate volumes of sterile distilled water were added to each sterile 1.5ml micro-centrifuge tube. Thereafter 2.5µl of buffer (Roche Diagnostics, Mannheim, Germany) was added to each tube, followed by the appropriate volume of each DNA sample, followed by 1.5µl of *Pvu*II enzyme (Roche Diagnostics, Mannheim, Germany). Each sample was mixed using a pipette and then placed onto a floater and incubated in a water bath at 37°C for 4 hours.

3.8.2.1 Gel Electrophoresis

Separation of DNA fragments

A 1% agarose gel was prepared using 1xTBE buffer as described previously (3.7.1.2) but without adding ethidium bromide. The gel was allowed to cool. A casting tray and a comb were wiped with 70% ethanol. The casting tray was taped around the edges and the comb was placed at an appropriate level from the base of the tray. The agarose solution was poured into the casting tray and allowed to solidify at room temperature.

On completion of the incubation period samples were removed from the water bath and a volume of 5µl of sample loading dye was added to each tube. Samples were quick-spun in a micro-centrifuge and loaded into wells 2-18 and 20. The Jacks standard molecular weight marker was loaded into wells 1 and 19, this marker served as the external reference. The gel was run at 100V for 45 minutes and at 36V overnight.

3.8.3 Southern Blotting

This was performed using the vacuum method. It involved the transfer of electrophorectically separated fragments to a Hybond N^+ membrane (Amersham, Buckinghamshire, UK).

The membrane was cut to the size of 15x20cm and placed into a glass container containing sterile distilled water. This was followed by a 5 minute washing step of the membrane in 10xSSC solution (appendix 3.1) on an orbital shaker at room temperature. The vacuum blotting unit (Vacugene) was set up by placing the porous support (Amersham, Buckinghamshire, UK) onto the blotting unit correctly, followed by placing the membrane on top. The plastic mask (Amersham, Buckinghamshire, UK) was then aligned accordingly over the edges of the membrane. The gel was removed from the electrophoresis tank and gently slid onto the membrane. The vacuum pump was started and once it reached 55cm bar a 1:100 HCl solution (appendix 3.2) was poured over the gel and the vacuum applied for 20 minutes. The HCl solution was removed by tilting the blotting unit and aspirated using a suction device. The next solution called soak I (appendix 3.3) was poured over the gel and the vacuum applied again for 20 minutes. The aspiration step was repeated as previously and the soak II solution (appendix 3.4) was poured over the gel followed by application of the vacuum for 20 minutes and aspiration. The next solution 10xSSC (appendix 3.1) was poured over the gel, the vacuum was increased to 65cm bar and applied for 1 ½ hours. At all times the gel was completely covered with the appropriate solution used.

On completion of blotting, the last solution was removed by aspiration and the gel removed and discarded. The membrane was allowed to air dry on paper towel for 5 minutes and thereafter placed into the UV cross-linker and exposed by using a preset setting of 1200 Joules. The membrane was removed and rolled and placed into a hybridisation bottle.

3.8.4 Hybridisation

Enhanced chemi-luminescence (ECL) direct system was used, the method is based on horse-radish peroxidase labelling of the probe.

The membrane was pre-hybridised using 25-30ml of hybridisation buffer (Amersham, Buckinghamshire, UK) (appendix 3.5), rotating for 30 minutes in a hybridisation oven at 42°C. In a micro-centrifuge (Eppendorf) tube 10µl of the probe was mixed with 5µl of sterile distilled water, boiled for 5 minutes and placed on ice for 10 minutes. Equal volumes of DNA labelling reagent (Amersham, Buckinghamshire, UK) and glutaraldehyde (Amersham, Buckinghamshire, UK) were added to the probe mixture and incubated in a water bath at 37°C for 10-15 minutes. Hybridisation buffer covering the membrane was mixed with the probe and this mixture was then poured back into the hybridisation bottle containing the membrane. The hybridisation took place overnight at 42°C in a hybridisation oven (Stuart) on a bottle rotisserie.

The hybridisation buffer was discarded the next day and the membrane was rinsed with primary wash buffer (appendix 3.6) which was discarded. Another volume of primary wash buffer was added to the membrane followed by incubation for 30 minutes at 42°C. The membrane was rinsed with 2xSSC solution (appendix 3.7) and transferred to a glass container. Two 5 minute washes with 2xSSC solution were applied at room temperature on an orbital shaker (Stuart). The final solution was discarded and the membrane was transferred to a plastic container.

3.8.5 Detection

The banding patterns were detected by ECL.

This was performed in the darkroom, following the same procedure described for spoligotyping detection process. For preparation of the membrane, it was placed into the hypercassette. The hypercassette was closed and the hyperfilm was exposed for 2 minutes. The hyperfilm was removed and placed into developer until all bands appeared and then placed into fixer until the background was clear. It was then rinsed with tap water. A second exposure was done and the timing depended on the intensity of the bands from the first exposure. The membrane was stored in plastic upon completion.

3.8.6 Polymerase chain reaction (PCR)

This was performed to prepare the DNA probe. It involved preparation of PCR fragments to be used as a probe.

The mastermix for the PCR contained the following: 470µl nuclease free water, 10µl INS1 and 10µl INS2 primers (Whitehead Scientific, IDT, Iowa), 10µl template DNA and 500µl Choice Taq mastermix (Denville Scientific, New Jersey, USA). Each PCR tube contained 100µl of template DNA and mastermix. The cycling conditions were 5 minutes at 94°C, 30 seconds at 94°C, 30 seconds at 55°C and 1 minute at 72°C, for 30 cycles. The PCR products were examined by electrophoresis using a 1% agarose gel as described previously for DNA estimation (3.7.1.2). If the correctly sized fragments were obtained the probe was stored at 4°C until use.

3.8.7 Analysis of IS*6110* RFLP patterns

The banding patterns were analysed visually and by means of a computer assisted program. The Bionumerics version 3.5 software (Applied Maths, Kortijk, Belgium), was used for the analysis of IS*6110* RFLP patterns. The construction of dendrograms was done using the unweighted pair group clustering method of averages (UPGMA) and the dice coefficient. The dendrogram showed how fingerprints clustered and their degree of similarity.

A cluster was defined by two or more isolates which had IS*6110* RFLP banding patterns that were identical (Glynn *et al*., 1999; Murase *et al*., 2010). Isolates with unique patterns were considered to be non-clustered. A family of RFLP patterns is defined as a group of patterns with more than 4 IS*6110* copies, differing by less than 1/3 of the hybridising bands or sharing 2/3 of hybridising bands which is equivalent to a similarity index of $\geq$ 65% (Razanamparany *et al*., 2009; Streicher *et al*., 2007; Warren *et al*., 2002).

CHAPTER 4

RESULTS

4.1 Antimicrobial susceptibility of isolates

A total of 67 isolates were retrieved from storage for the period 2005/6. Using the 1% proportion method, these isolates were classified into categories as shown in Tables 1a, b, c. There were 2 isolates that were resistant to INH only (Table 1a). Of the MDR isolates (Table 1b), the susceptibility profile varied for the antimicrobials tested other than INH and rifampicin. There were no pre-XDR isolates identified. Most of the XDR isolates were resistant to all tested antimicrobials, there were 2 isolates which were susceptible to capreomycin. (Table 1c)

Table 1a: Antimicrobial susceptibility profile of isolates from Tugela Ferry: 2005/6

Isolate #	Susceptibility Category	DST results per antibiotic							
		I	R	E	S	O	K	Et	C
61551	Susceptible	S	S	S	S	S	S	S	S
01118	Susceptible	S	S	S	S	S	S	S	S
62410	Susceptible	S	S	S	S	S	S	S	S
62411	Susceptible	S	S	S	S	S	S	S	S
29800	Susceptible	S	S	S	S	S	S	S	S
71350	Susceptible	S	S	S	S	S	S	S	S
01420	Susceptible	S	S	S	S	S	S	S	S
42541	Susceptible	S	S	S	S	S	S	S	S
75811	Susceptible	S	S	S	S	S	S	S	S
72433	Susceptible	S	S	S	S	S	S	S	S
49330	Susceptible	S	S	S	S	S	S	S	S
64944	INH mono-resistant	R	S	S	S	S	S	S	S
51350	INH mono-resistant	R	S	S	S	S	S	S	S

Abbreviations:
I: Isoniazid **R**: Rifampicin **E**: Ethambutol **S**: Streptomycin
O: Ofloxacin **K**: Kanamycin **C**: Capreomycin **Et**: Ethionamide

Table 1b: Antimicrobial susceptibility profile of isolates from Tugela Ferry: 2005/6

Isolate #	Susceptibility Category	DST results per antibiotic							
		I	R	E	S	O	K	Et	C
34415	MDR	R	R	S	S	S	S	S	S
69446	MDR	R	R	S	S	S	S	S	S
64747	MDR	R	R	S	S	S	S	R	S
44949	MDR	R	R	S	R	S	S	S	S
72441	MDR	R	R	S	R	S	S	S	S
78838	MDR	R	R	S	R	S	S	S	S
67563	MDR	R	R	S	R	S	S	S	S
47928	MDR	R	R	S	R	S	S	S	S
69183	MDR	R	R	S	R	S	S	R	S
14899	MDR	R	R	S	R	S	S	R	S
64145	MDR	R	R	S	R	S	S	R	S
62910	MDR	R	R	S	R	S	S	R	S
74541	MDR	R	R	R	R	S	S	S	S
23209	MDR	R	R	R	R	S	S	S	S
36480	MDR	R	R	R	R	S	S	S	S
47380	MDR	R	R	R	R	S	S	R	S
74536	MDR	R	R	R	R	S	S	R	S
54412	MDR	R	R	R	R	S	S	R	S
47381	MDR	R	R	R	R	S	S	R	S
74542	MDR	R	R	R	R	S	S	R	S
09105	MDR	R	R	R	R	S	S	R	S
26663	MDR	R	R	R	R	S	S	R	S

Abbreviations:
I: Isoniazid **R**: Rifampicin **E**: Ethambutol **S**: Streptomycin
O: Ofloxacin **K**: Kanamycin **C**: Capreomycin **Et**: Ethionamide

Table 1c: Antimicrobial susceptibility profile of isolates from Tugela Ferry: 2005/6

Isolate #	Susceptibility Category	DST results per antibiotic							
		I	R	E	S	O	K	Et	C
66680	XDR	R	R	R	R	R	R	R	R
17783	XDR	R	R	R	R	R	R	R	R
31065	XDR	R	R	R	R	R	R	R	R
31066	XDR	R	R	R	R	R	R	R	R
30033	XDR	R	R	R	R	R	R	R	R
42586	XDR	R	R	R	R	R	R	R	R
50998	XDR	R	R	R	R	R	R	R	R
51336	XDR	R	R	R	R	R	R	R	R
69696	XDR	R	R	R	R	R	R	R	R
17765	XDR	R	R	R	R	R	R	R	R
36661	XDR	R	R	R	R	R	R	R	R
48006	XDR	R	R	R	R	R	R	R	R
65787	XDR	R	R	R	R	R	R	R	R
38831	XDR	R	R	R	R	R	R	R	R
58836	XDR	R	R	R	R	R	R	R	R
80198	XDR	R	R	R	R	R	R	R	R
66175	XDR	R	R	R	R	R	R	R	R
36461	XDR	R	R	R	R	R	R	R	R
62912	XDR	R	R	R	R	R	R	R	R
63545	XDR	R	R	R	R	R	R	R	R
47396	XDR	R	R	R	R	R	R	R	R
37806	XDR	R	R	R	R	R	R	R	R
49127	XDR	R	R	R	R	R	R	R	R
69396	XDR	R	R	R	R	R	R	R	R
61719	XDR	R	R	R	R	R	R	R	S
45291	XDR	R	R	R	R	R	R	R	R
65623	XDR	R	R	R	R	R	R	R	R
55125	XDR	R	R	R	R	R	R	R	R
62723	XDR	R	R	R	R	R	R	R	S
55126	XDR	R	R	R	R	R	R	R	R
61563	XDR	R	R	R	R	R	R	R	R
65622	XDR	R	R	R	R	R	R	R	R

Abbreviations:
I: Isoniazid **R**: Rifampicin **E**: Ethambutol **S**: Streptomycin
O: Ofloxacin **K**: Kanamycin **C**: Capreomycin **Et**: Ethionamide

A total of 32 isolates were retrieved from storage for the period 2008/9. Using the 1% proportion method, these isolates were classified into susceptibility categories as shown in Table 1d.

Table 1d: Antimicrobial susceptibility profile of isolates from Tugela Ferry: 2008/9

Isolate #	Susceptiblity Category	DST results per antibiotic							
		I	R	E	S	O	K	Et	C
586	Susceptible	S	S	S	S	S	S	S	S
628	Susceptible	S	S	S	S	S	S	S	S
632	Susceptible	S	S	S	S	S	S	S	S
641	Susceptible	S	S	S	S	S	S	S	S
662	Susceptible	S	S	S	S	S	S	S	S
685	Susceptible	S	S	S	S	S	S	S	S
689	Susceptible	S	S	S	S	S	S	S	S
776	Susceptible	S	S	S	S	S	S	S	S
777	Susceptible	S	S	S	S	S	S	S	S
811	Susceptible	S	S	S	S	S	S	S	S
824	Susceptible	S	S	S	S	S	S	S	S
825	Susceptible	S	S	S	S	S	S	S	S
830	Susceptible	S	S	S	S	S	S	S	S
844	Susceptible	S	S	S	S	S	S	S	S
860	Susceptible	S	S	S	S	S	S	S	S
682	MDR	R	R	S	S	S	S	S	S
320	MDR	R	R	S	R	S	S	S	S
11	MDR	R	R	S	S	S	S	R	S
644	MDR	R	R	R	R	S	S	S	S
47	MDR	R	R	R	R	S	S	S	S
141	XDR	R	R	R	R	R	R	R	R
143	XDR	R	R	R	R	R	R	R	R
181	XDR	R	R	R	R	R	R	R	R
195	XDR	R	R	R	R	R	R	R	R
334	XDR	R	R	R	R	R	R	R	R
338	XDR	R	R	R	R	R	R	R	R
39	XDR	R	R	R	R	R	R	R	R
370	XDR	R	R	R	R	R	R	R	R
387	XDR	R	R	R	R	R	R	R	R
513	XDR	R	R	R	R	R	R	R	R
642	XDR	R	R	R	R	R	R	R	R
667	XDR	R	R	R	R	R	R	R	R

Abbreviations:
I: Isoniazid **R**: Rifampicin **E**: Ethambutol **S**: Streptomycin
O: Ofloxacin **K**: Kanamycin **C**: Capreomycin **Et**: Ethionamide

4.2 SITs and Drug Susceptibility Profiles

Among the susceptible isolates for the periods 2005-2006 and 2008-2009, 4 SITs were predominant. SIT53 accounted for 25% (7/28) of the isolates, SIT34 for 7.1% (2/28), SIT1 for 21.4% (6/28) and SIT33 for 10.7% (3/28). There was one single isolate in the 2005-06 group which belonged to SIT59. The 2008/9 group contained 9 such isolates which fell into SIT21, SIT73, SIT39, SIT136, SIT137, SIT563, SIT811, SIT815 and 1 orphan pattern (Tables 2a and 2b).

Among the MDR MTB isolates, SIT34 was the predominant pattern in 2005/6 and 2008/9: 68% (14/22) and 40% (2/5) respectively. Nine percent (2/22) of MDR MTB isolates in 2005/6 and 20% (1/5) in 2008/9 displayed the SIT53 pattern. The SIT60 accounted for 23% (5/22) of isolates for 2005/6 and 20% (1/5) 2008/9 group. The fifth isolate from 2008/9 and 5% (1/22) from 2005/6 showed the SIT831 pattern. (Tables 2a and 2b)

The SIT60 pattern accounted for 94% (30/32) of XDR TB isolates from 2005/6 and 58% (7/12) of XDR TB isolates from 2008/9 (Table 2a and 2b). The other 2 XDR isolates from 2005/6 belonged to SIT53 (Table 2a). The five XDR isolates from 2008/9 not belonging to SIT60 displayed the SIT1750 (2/12; 17%) or an orphan pattern (3/12; 25%).

Table 2a: Spoligotyping results for isolates from Tugela Ferry: 2005/6

Susceptibility	Spoligotyping: Shared international Type (SIT)							
	SIT1	SIT33	SIT34	SIT53	SIT59	SIT60	SIT831	Total
Susceptible	4	1	1	6	1			13
MDR			14	2		5	1	22
XDR				2		30		32
Total	4	1	15	10	1	35	1	67

Table 2b: Spoligotyping results for isolates from Tugela Ferry: 2008/9

Susceptibility	Spoligotyping: Shared international Type (SIT)																
	SIT1	**SIT21**	**SIT33**	**SIT34**	**SIT39**	**SIT53**	**SIT60**	**SIT73**	**SIT136**	**SIT137**	**SIT563**	**SIT811**	**SIT815**	**SIT831**	**SIT1750**	**Orphan 1**	**Orphan 2**
Susceptible	2	1	2	1	1	1		1	1	1	1	1	1				1
MDR				2		1	1							1			
XDR							7								2	3	
Total	**2**	**1**	**2**	**3**	**1**	**2**	**8**	**1**	**1**	**1**	**1**	**1**	**1**	**1**		**3**	**1**

4.3 Spoligotypes and Lineages

Figure 1 is a scanned copy of the hyperfilm of the predominant spoligotypes obtained in this study. Hyperfilms showing additional spoilgotypes are contained in appendix 4.

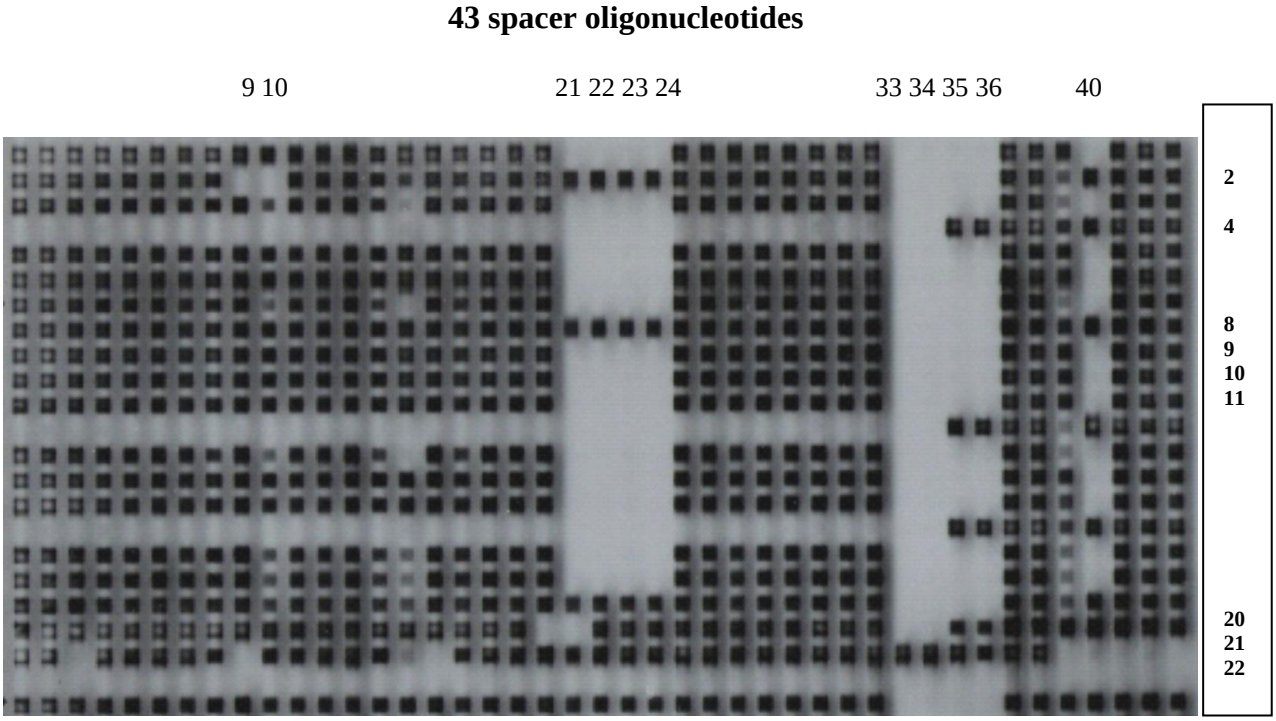


Figure 1: Predominant Spoligotype patterns among the isolates

Lane 2: SIT34

Lane 4: SIT1

Lane 8: SIT53

Lane 9-11: SIT60

Positive controls: Lane 20: H37Rv, Lane 21: *M.bovis*

Negative control: Lane 22: water

Figure 2: SIT, Lineage and corresponding Spacer Patterns of Isolates

* SIT#[a]	*Lineage/ Sub-lineage[b]	*Binary spoligotype[c]
1	Beijing	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■
33	LAM3	■■■■■■■■□□□■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■
34	S	■■■■■■■■□□■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
53	T1	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
60	LAM4	■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■□■■■
21	CAS1-KILI	■■■□□□□■■□■■■■■■■■■□□□□□□□□□□□□□□□□■■■■■■■■
39	T4-CEU1	■■■■■■■■■■■■■■■■■□■■■□□■■■■■■■■□□□□■□□■■■■
59	T5-MADZ	■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■■□□□□■■■■■■■
73	T2-T3	■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■
136	T1	■■■■■■■■■■■■□□□□□□■■■□□□□□□■□■■■■■■□□□□■□□■■■■
137	X2	■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■□□□□■■□□□□■
563	U	■■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■■□□□□■■■■■■
811	LAM4	■■■■■■■■■■■■■■■■■■■■■□□□□■□□□□□□■■□□□□■■■□■■■
815	LAM11-ZWE	■■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■■□□□□■■■□■■■
831	S	■■■■■■■■□□■■■■■□■■■■■■■■■■■■■■■■□□□□■■■■■■■
1750	LAM4	■■■■■■■■■■■■■■■□■■■■■□□□□■■■■■■■■□□□□■■■□■■■
Orphan1		■■■■■■■■■■■■■■□■■■■■□□□□■■■■■■■■□□□□■■□□■■■
Orphan 2		■■■■■■■■■■■■■□□□□□■■■■■□■□□□□□□■□□□□■■■■■■■

*Analysis done using the international spoligotype database SpolDB4 http://www.pasteur-guadeloupe.fr:8081/SITVIT/DEMO

[a] Shared international type number as describe in the spoligotype database SpolDB4

[b] Strain family as assigned in the international spoligotype database SpolDB4

[c] Spoligotype pattern with solid blocks indicating the presence of a spacer and empty blocks indicating the absence of a spacer.

Figure 2 depicts the classification of the spoligotypes obtained in this study as compared to the international SpolDB4 database. Based on the spoligotype patterns, the isolates could be classified into lineages or sub-lineages. The S lineage is represented by SIT34 and SIT831 with the latter differing by omission of spacer 15 as compared to the former. The LAM4 sub-lineage contains SIT60, SIT811 and SIT1750. While the missing spacers between SIT60 and SIT811 consist of a block of 5, SIT1750 differs with only one missing spacer from SIT60.

Figure 3: Dendrogram of Spoligotype Patterns

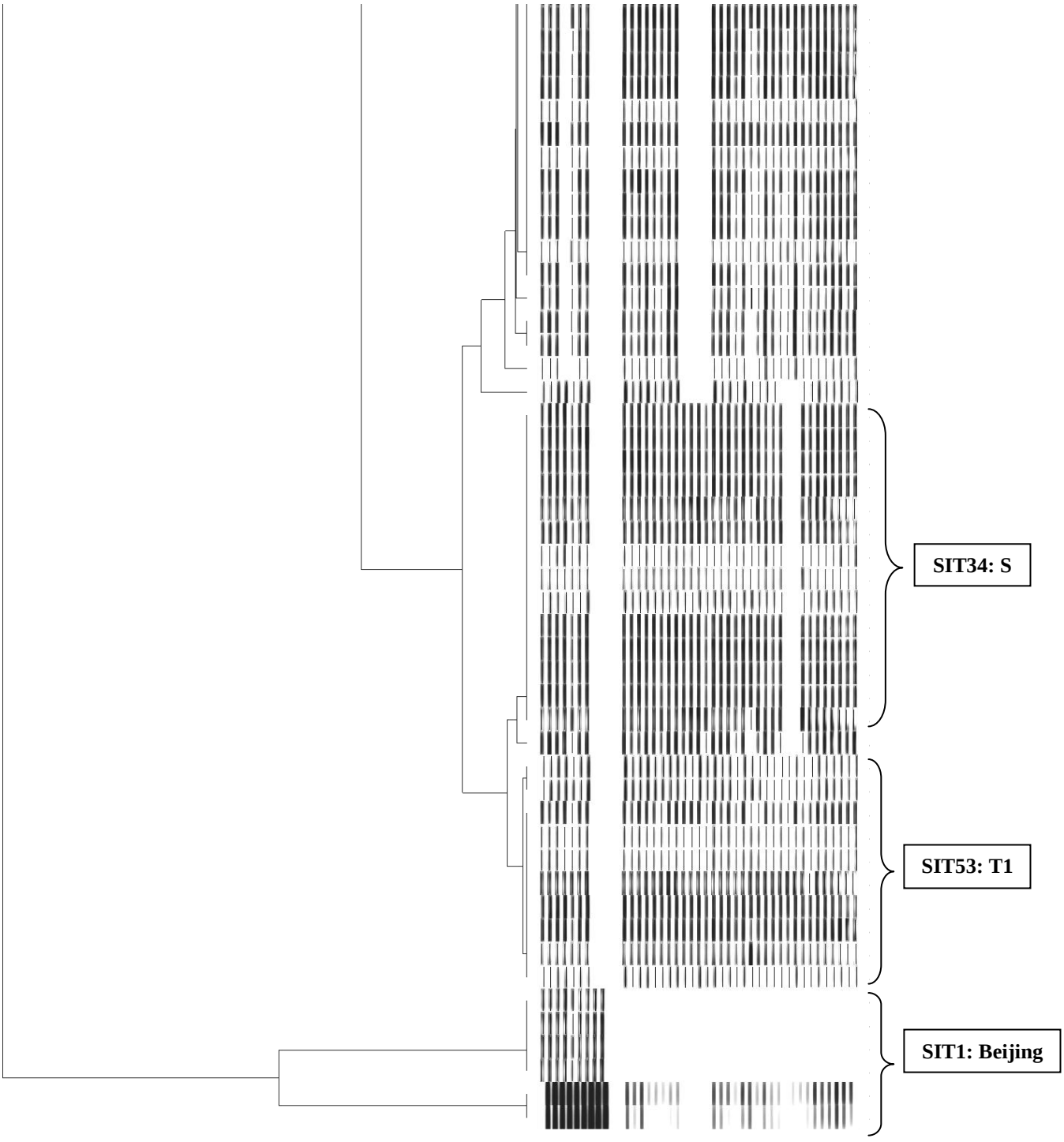


Figure 3 shows the spoligotype dendrogram for all isolates in the study. The predominant patterns were SITs 60, 53, 34, 33 and 1.

4.4 Differentiation of Isolates by IS*6110* RFLP

All sensitive and resistant strains were considered when clustering analysis was performed. A cluster was defined as two or more isolates with an identical *IS6110* RFLP pattern. Strain clusters indicate clonal transmission of MTB.

Among the susceptible isolates in this study, only (2/13) from 2005/6 clustered but did not belong to one of the families dominant in KZN (Table 3a). Of the 11 non-clustering isolates 4 belonged to the Beijing family of strains. None of the 15 susceptible isolates from 2008/9 belonged to an IS*6110* RFLP cluster. Two belonged to the Beijing family (Table 3b).

A total of 22 MDR isolates from 2005/6 were analysed and 5 from 2008/9. Of the first group, 14 (64%) clustered. Of these, 3 showed the F15/LAM4/KZN family fingerprint while 11 belonged to the F28 family. The other 8 MDR TB isolates all had unique patterns, 2 belonging to the F15/LAM4/KZN family and 3 to the F28 family. (Table 3a) Two of the 5 MDR isolates from 2008/9 formed a cluster and that cluster belonged to the F28 family (Table 3b).

All 44 XDR TB isolates (32 from 2005/6 and 12 from 2008/9) belonged to the F15/LAM4/KZN family. Of these 43/44 (98%) clustered and the remaining 1 XDR (from 2008/9) isolate differed by 1 band from the cluster. (Tables 3a and 3b)

Table 3a: IS6110 RFLP results from Tugela Ferry 2005/6

	Strain Families							
	Other		F15/LAM4/KZN		F28		Beijing	
Susceptibility	**Clustering**	**Non-clustering**	**Clustering**	**Non-clustering**	**Clustering**	**Non-clustering**	**Clustering**	**Non-clustering**
Susceptible	2	7						4
MDR		3	3	2	11	3		
XDR			32					
Total	**2**	**10**	**35**	**2**	**11**	**3**		**4**

Table 3b: IS6110 RFLP results from Tugela Ferry 2008/9

	Strain Families							
	Other		F15/LAM4/KZN		F28		Beijing	
Susceptibility	**Clustering**	**Non-clustering**	**Clustering**	**Non-clustering**	**Clustering**	**Non-clustering**	**Clustering**	**Non-clustering**
Susceptible		13						2
MDR		1		1	2	1		
XDR			11	1				
Total		**14**	**11**	**2**	**2**	**1**		**2**

Figure 4 is a scanned hyperfilm showing the IS*6110* RFLP patterns obtained in the study. Hyperfilms showing additional IS*6110* RFLP patterns are contained in appendix 5.

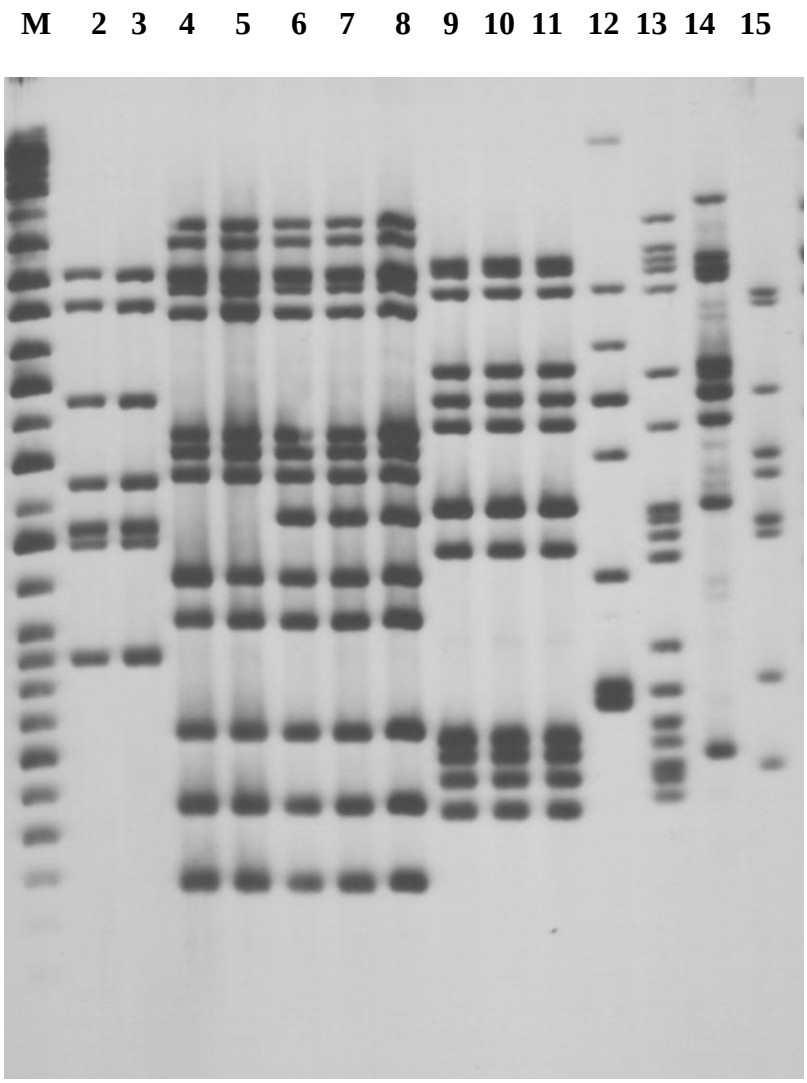


Figure 4: IS*6110* RFLP patterns for (2005-2006) and (2008-2009)

Lane 1: Jack's standard molecular weight marker (0.7-15kbps)

(2005-2006): Lane 2-3: unique cluster

Lane 4-8: F15/LAM4/KZN family patterns

Lane 9-11: F28 family patterns

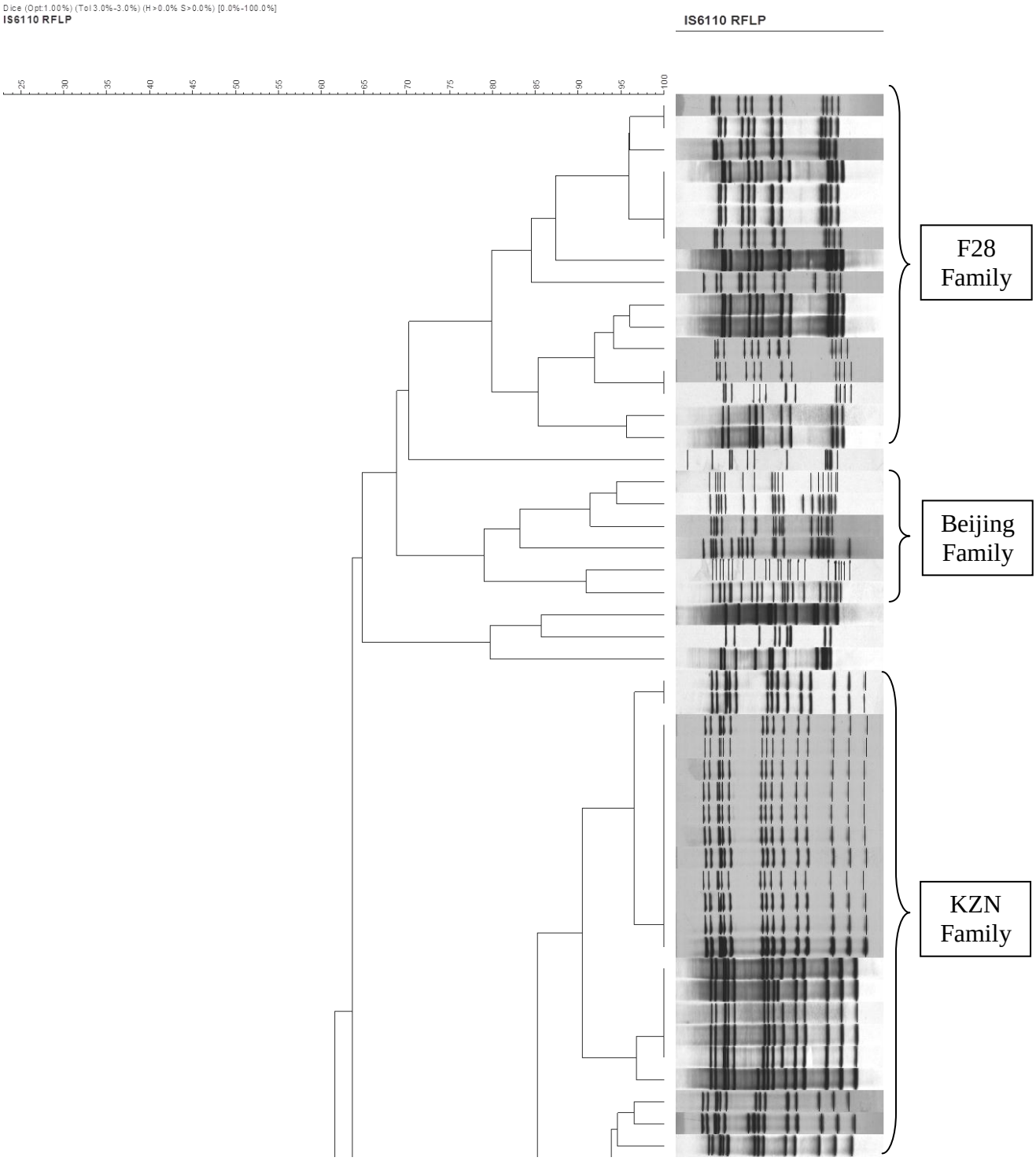
Lane 12: unique patterns

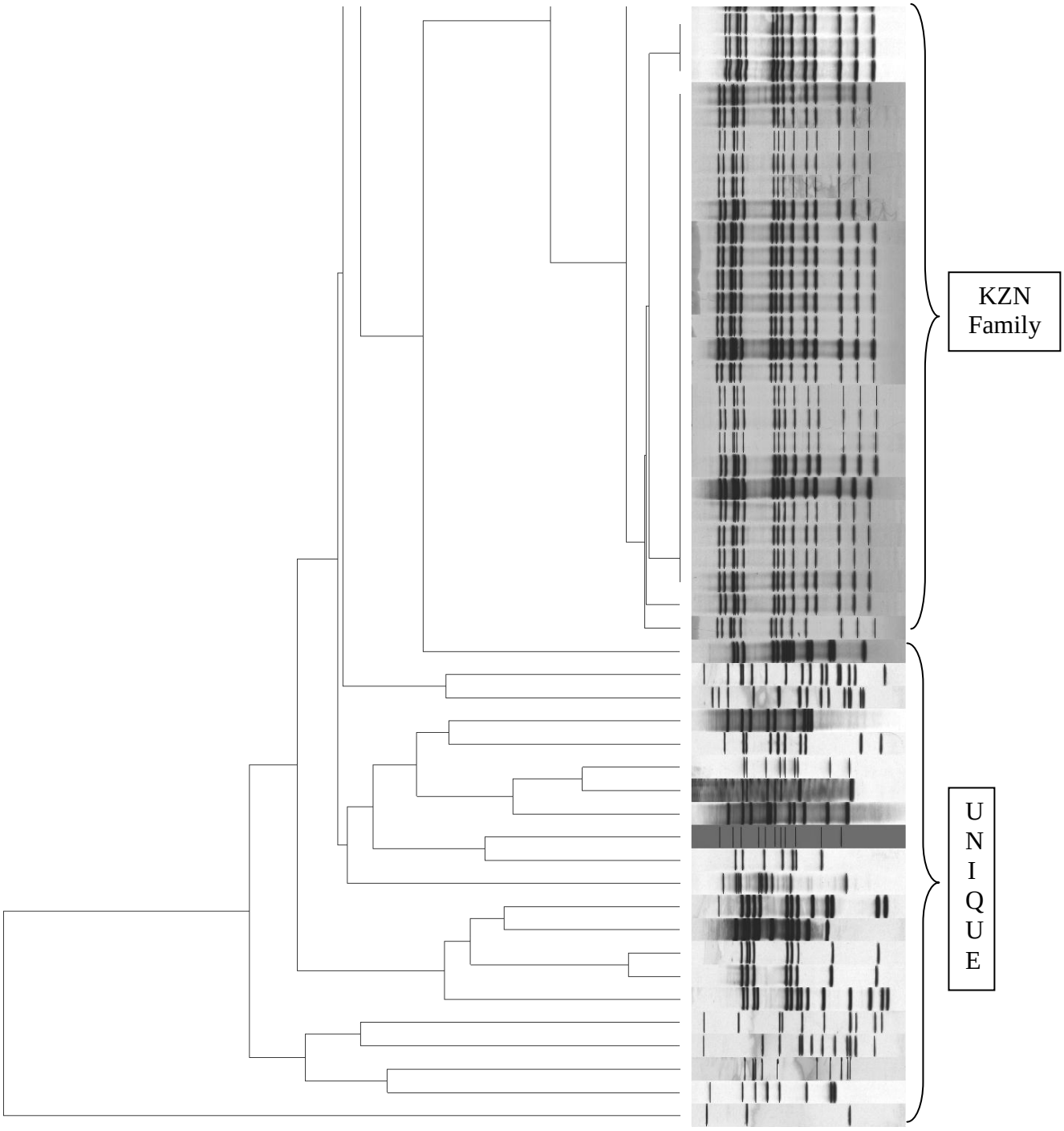
Lane 13: Beijing family pattern

(2008-2009): Lanes 14-15: unique patterns

Figure 5 shows the dendrogram that was generated for the IS*6110* RFLP patterns. The predominant families and unique patterns are labelled accordingly.

Figure 5: Dendrogram of IS*6110* RFLP Patterns

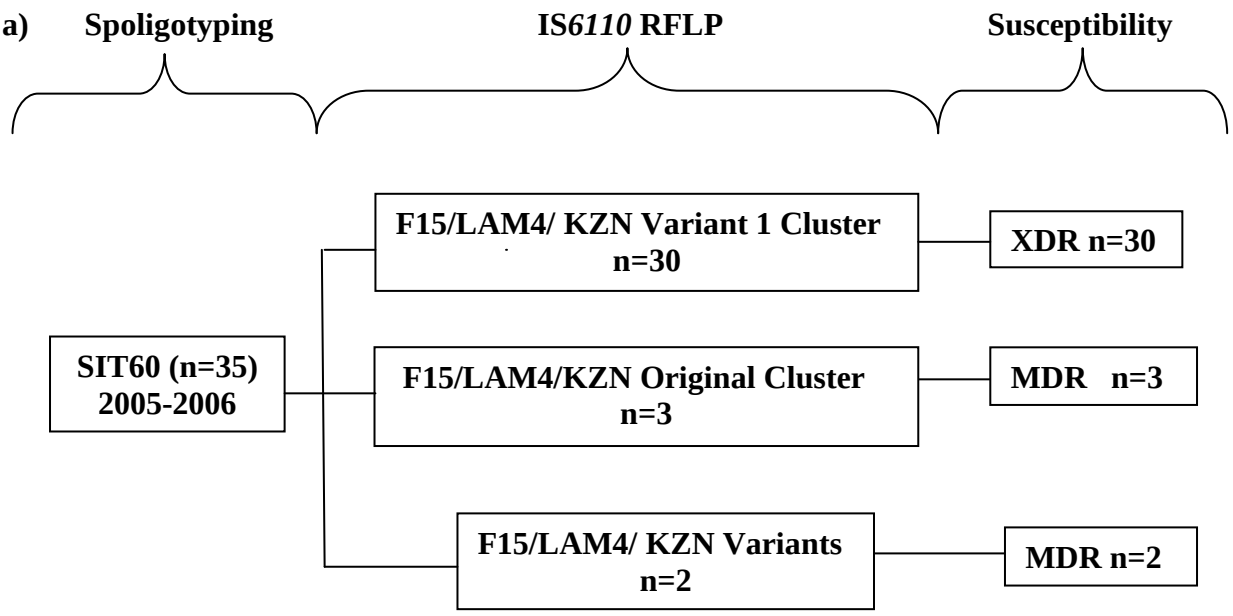



KZN
Family
UNIQUE

4.5 Linking of Spoligotyping and IS*6110* RFLP results

Figure 6a and 6b shows further differentiation of isolates belonging to SIT60 by IS*6110* RFLP. All isolates in SIT60 belonged to the F15/LAM4/KZN family by IS*6110* RFLP. The majority of XDR (36/37, 97%) and MDR (3/6, 50%) isolates were classified into 1 of 2 clusters and the remaining IS*6110* RFLP patterns differed by 1-3 bands from those in clusters.

Figure 6a (2005/6) and 6b (2008/9): Further differentiation of isolates belonging to SIT60, by IS*6110* RFLP and linked to Susceptibility profiles.



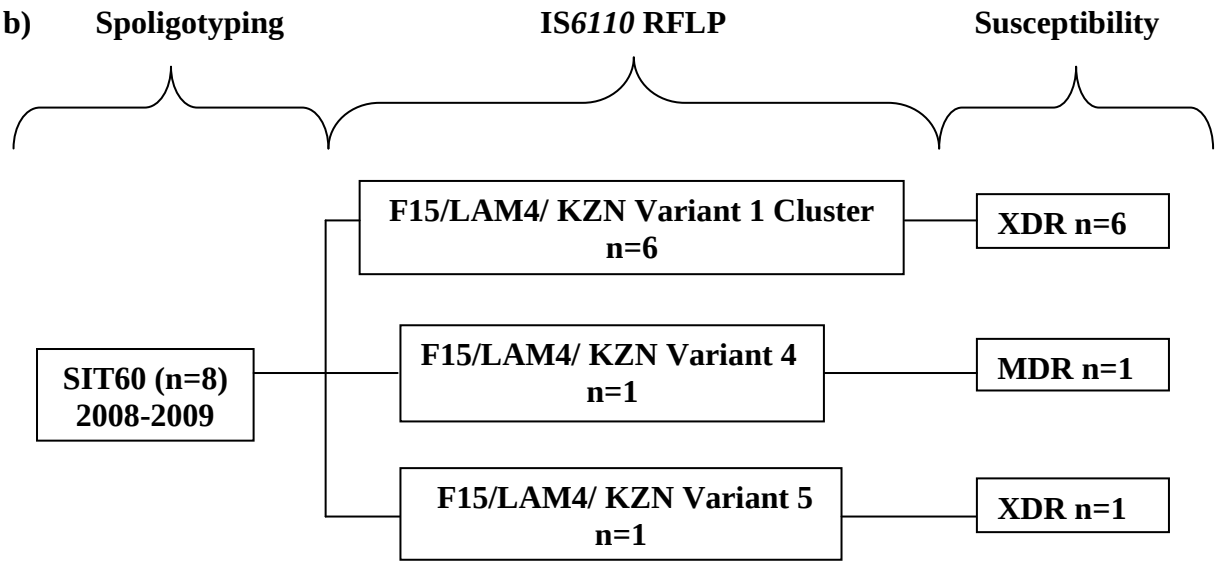


Figure 7 (2008/9): Further differentiation of isolates belonging to SIT1750 and orphan1, by IS*6110* RFLP and linked to Susceptibility profiles

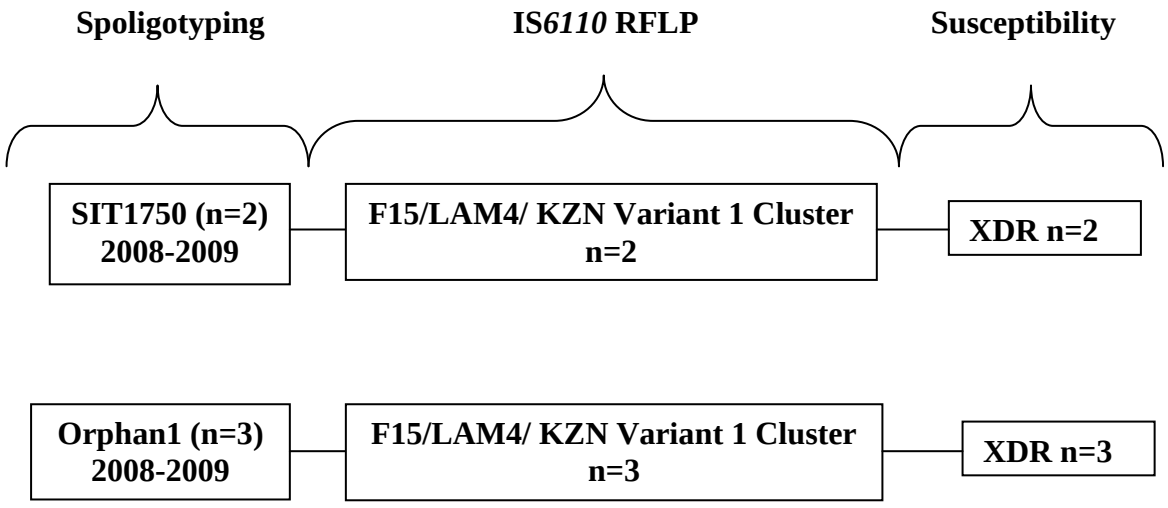
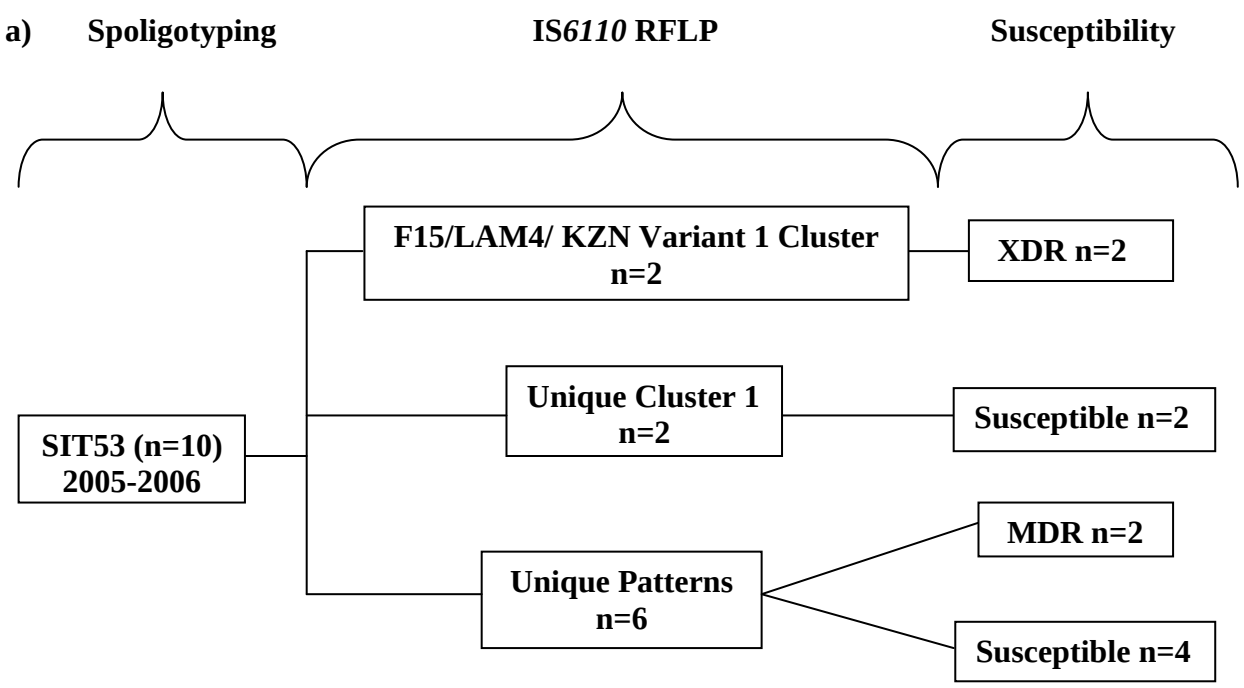


Figure 7 shows further differentiation of isolates belonging to SIT1750 and an orphan group by IS*6110* RFLP which were all XDR isolates from 2008/9. All 5 isolates showed IS*6110* RFLP patterns belonging to the F15/LAM4/KZN family and all 5 were part of the dominant cluster of XDR TB isolates (Fig 6a).

Figure 8a (2005/6) and 8b (2008/9): Further differentiation of isolates belonging to SIT53, by IS*6110* RFLP and linked to Susceptibility profiles



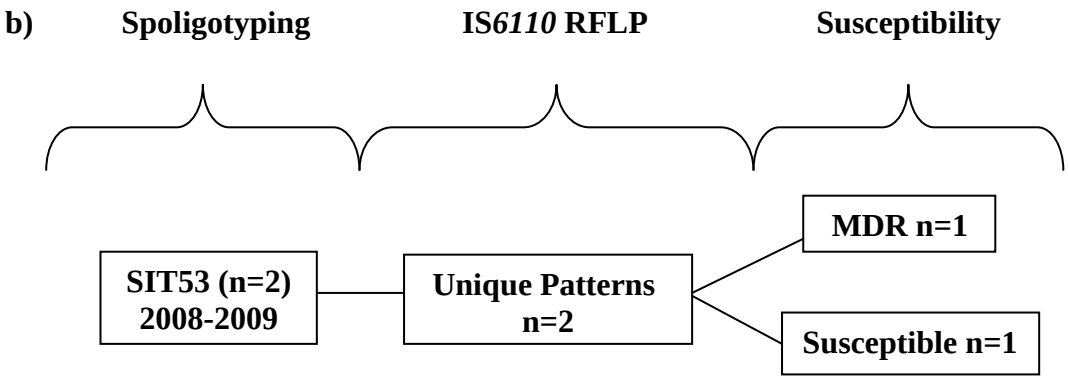


Figure 8a and 8b displays the further differentiation by IS*6110* RFLP of isolates that belonged to SIT53. A total of 12 isolates (10 from 2005/6; 2 from 2008/9) with varying susceptibility profiles belonged to this spoligotype. The 2 XDR TB isolates, both from 2005/6 clustered by IS*6110* RFLP with patterns belonging to the F15/LAM4/KZN family.

All 3 MDR TB isolates had unique IS*6110* RFLP patterns. Within the 7 susceptible isolates from 2005/6 there was an IS*6110* RFLP cluster of 2 while the remaining 5 susceptible isolates all had unique IS*6110* RFLP patterns.

Figure 9a (2005/6) and 9b (2008/9): Further differentiation of isolates belonging to SIT34 by IS*6110* RFLP and linked to Susceptibility profiles

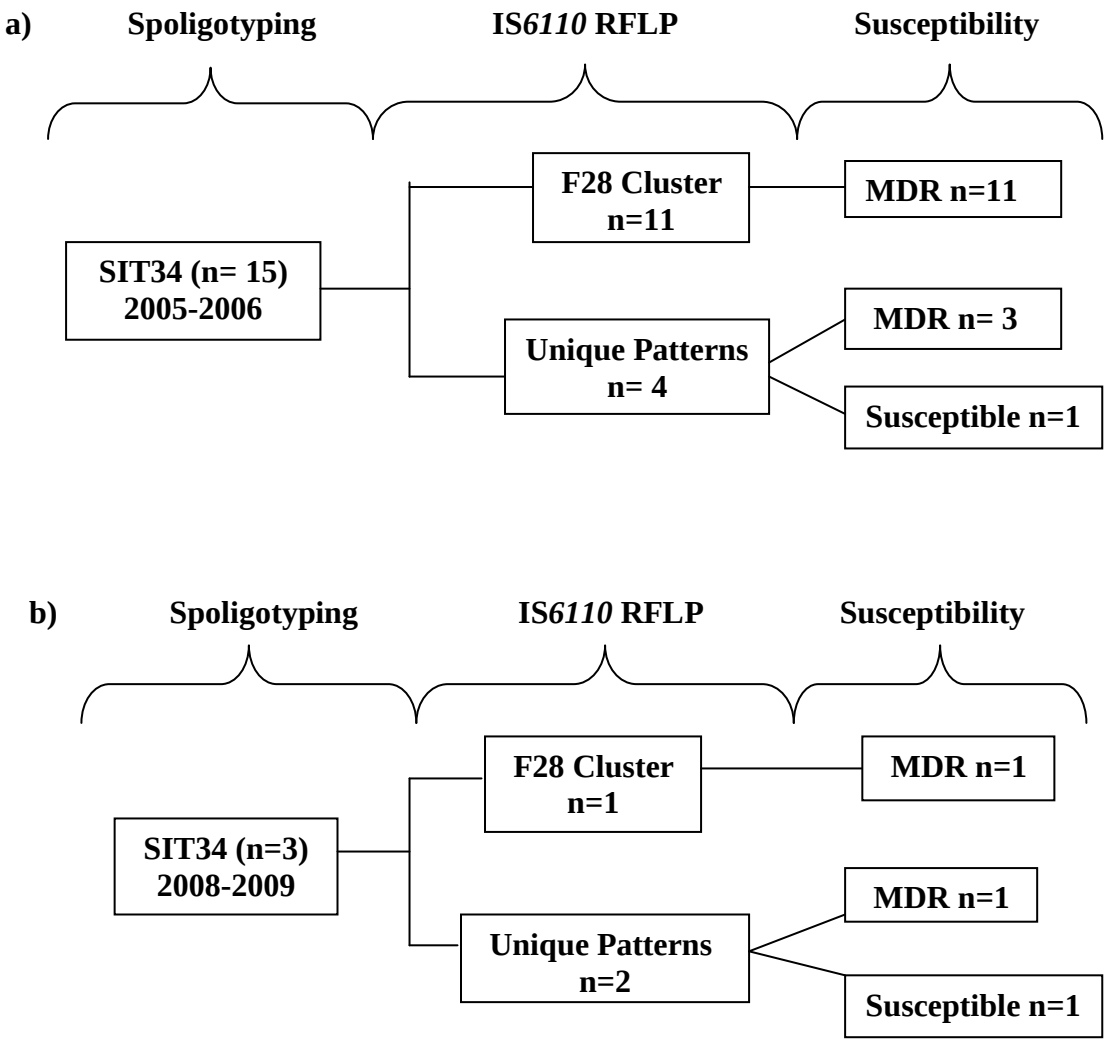


Figure 9a and 9b show the differentiation by IS*6110* RFLP of isolates that belonged to SIT34. A total of 16 MDR and 2 susceptible TB isolates were present in SIT34. IS*6110*

RFLP analysis showed that 12 (70%) of the MDR TB isolates belonged the F28 cluster while the remaining 4 MDR TB isolates were unique, but 3 of the 4 had IS*6110* RFLP patterns which also belonged to the F28 family. Three of the 4 unique MDR isolates differed by 1-3 bands from those in the F28 cluster. The 2 susceptible isolates and the remaining 1 MDR isolate had totally unique IS*6110* RFLP patterns from those in the cluster.

Figure 10a (2005/6) and 10b (2008/9): Further differentiation of MDR TB isolates belonging to SIT831 by IS*6110* RFLP

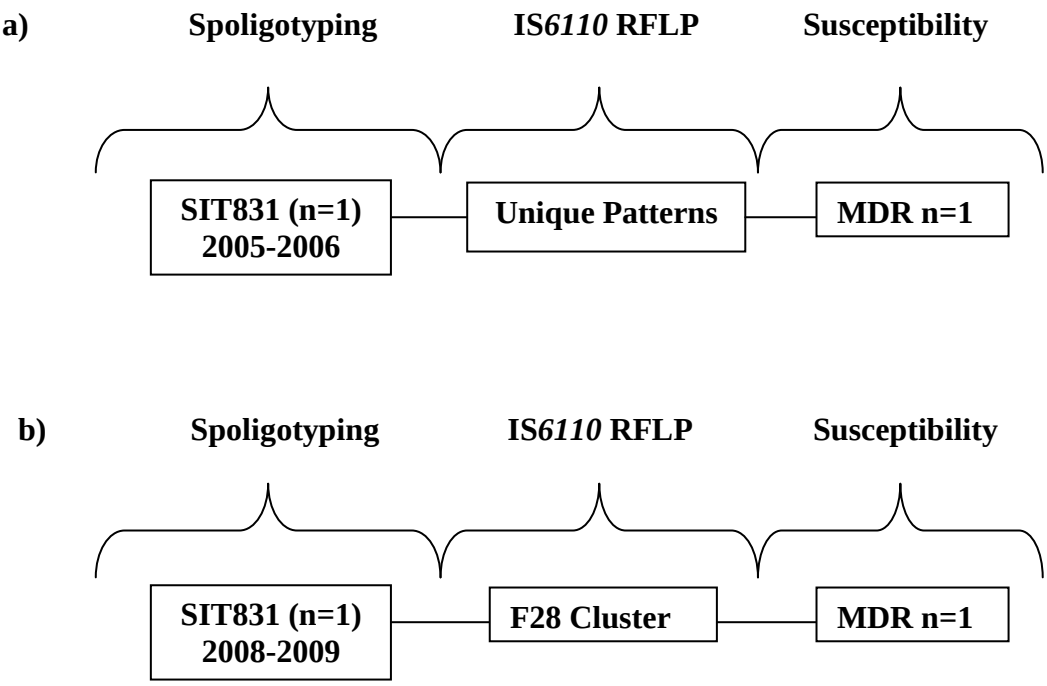


Figure 10a and 10b shows 2 MDR TB isolates belonging to SIT831 which had IS*6110* RFLP patterns which belong to the F28 family. The 1 MDR TB isolate from 2008-2009 belonged to the cluster in the F28 family.

Figure 11a (2005/6) and 11b (2008/9): Further differentiation of isolates belonging to SIT1 by IS*6110* RFLP and linked to Susceptibility profiles

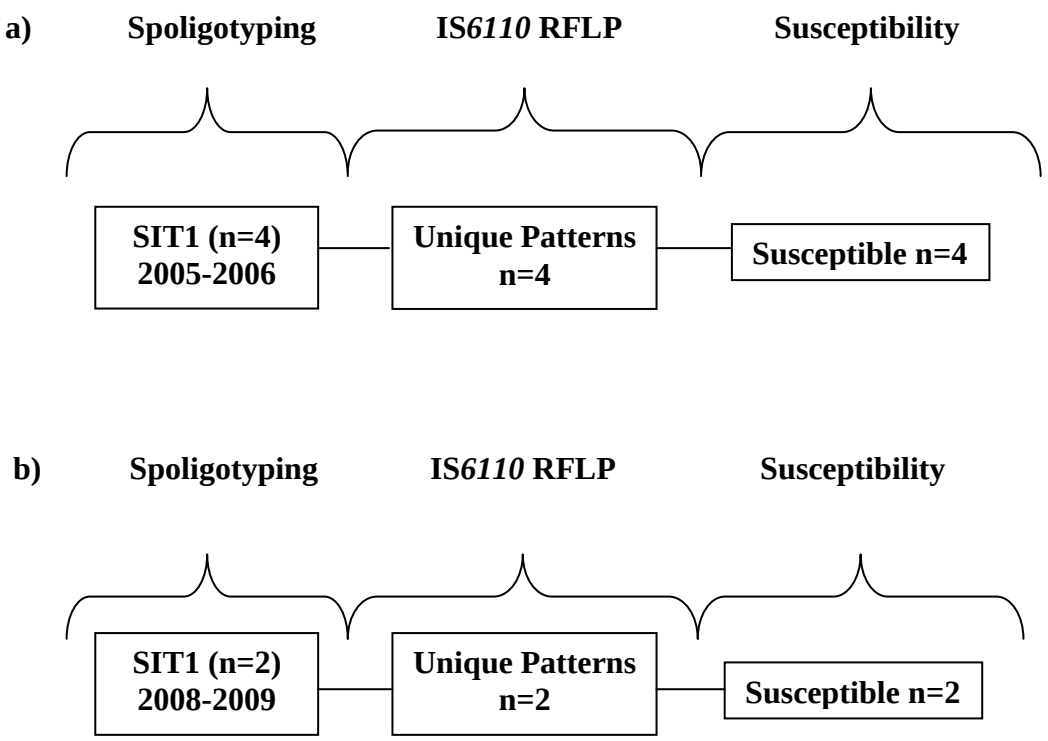


Figure 11a and 11b shows the further differentiation by IS*6110* RFLP of isolates that belonged to SIT1 which were all susceptible. IS*6110* RFLP patterns for these isolates were all unique but belonged to the Beijing family.

Figure 12a (2005/6) and 12b (2008/9): Further differentiation of susceptible isolates belonging to SIT33 by IS*6110* RFLP

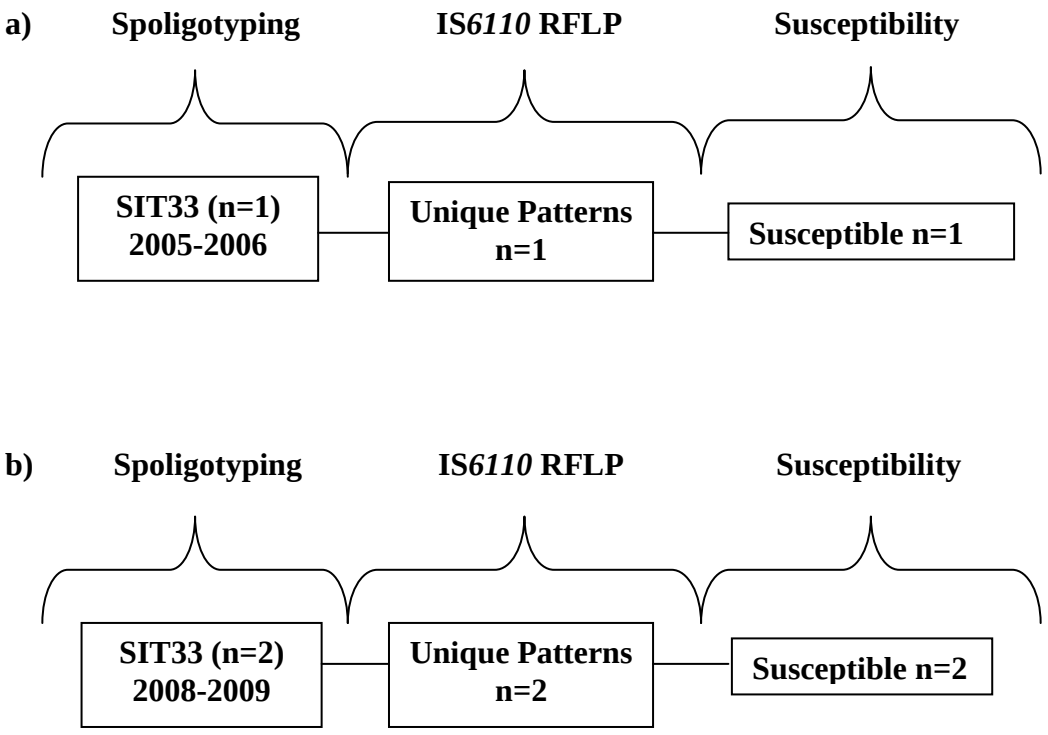
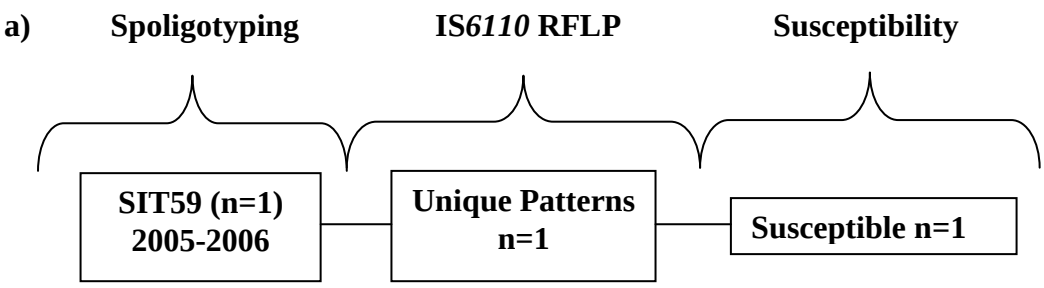


Figure 12a and 12b shows the further differentiation by IS*6110* RFLP of isolates that belonged to SIT33 which were all susceptible. IS*6110* RFLP patterns for these isolates were all unique.

Figure 13a (2005/6) and 13b (2008/9): Further differentiation of single susceptible TB isolates belonging to various SITs by IS*6110* RFLP



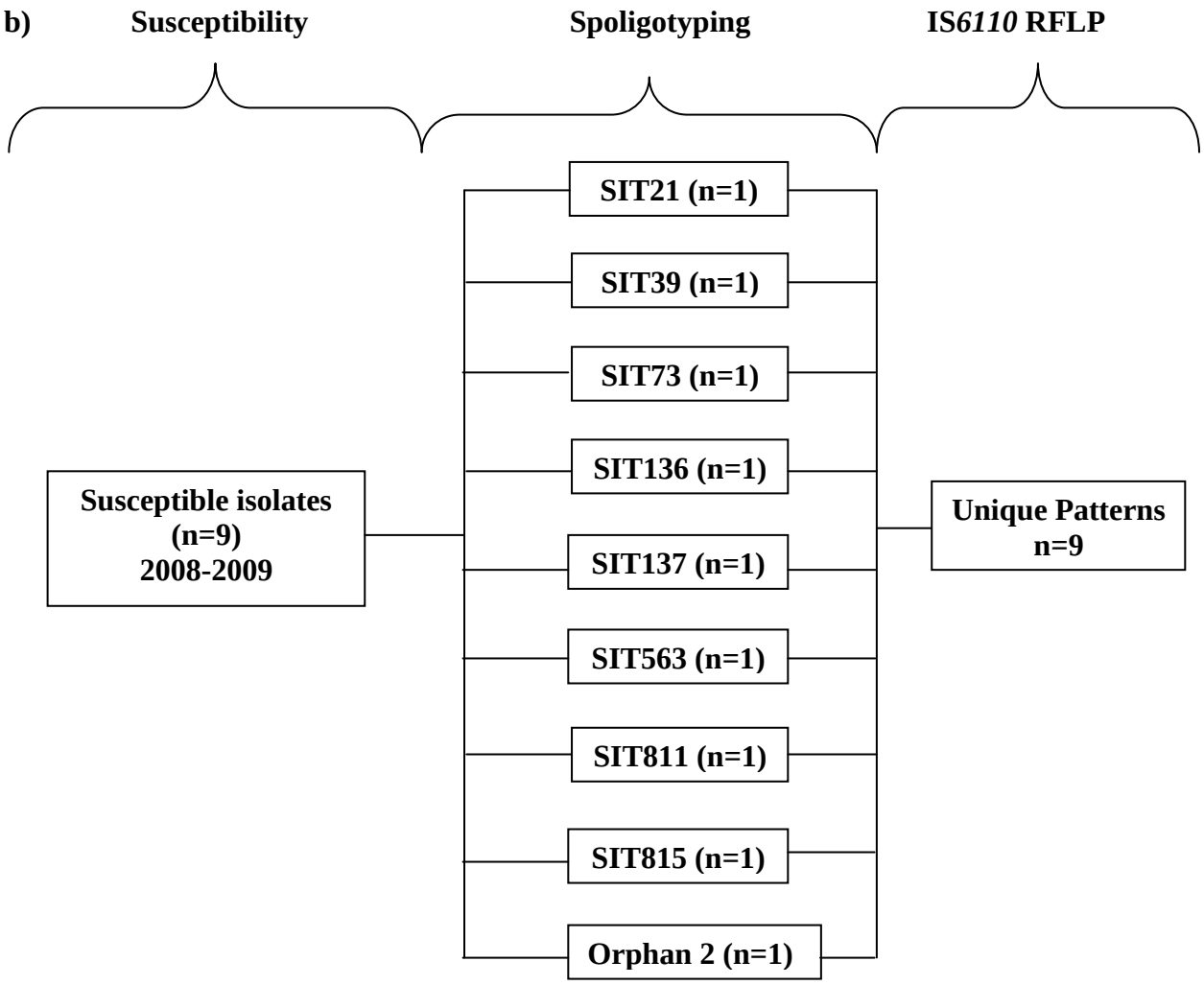
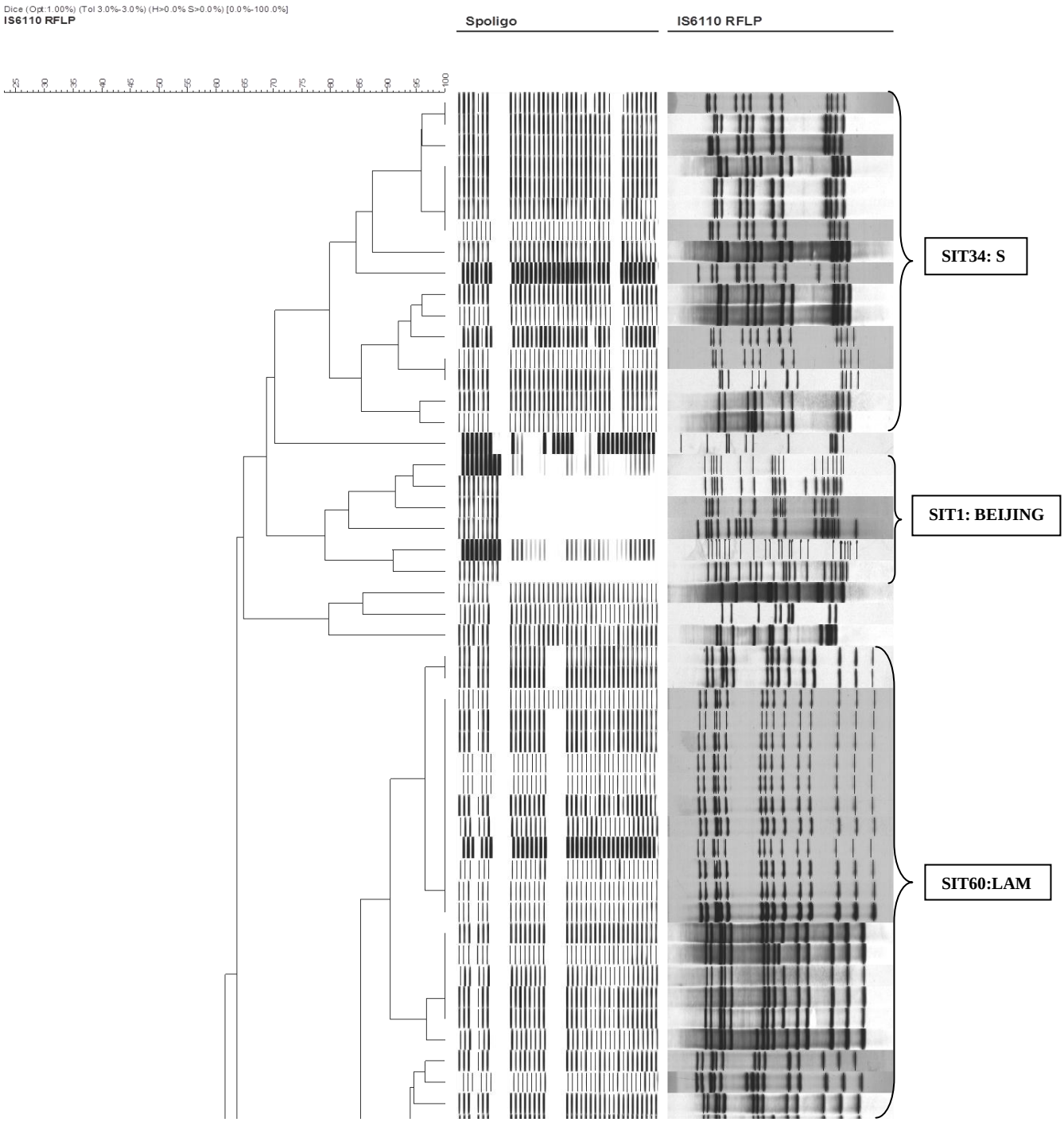


Figure 13a and 13b shows further differentiation by IS*6110* RFLP of single susceptible TB isolates belonging to various SITs. All these isolates had unique IS*6110* RFLP patterns.

Figure 14: Dendrogram linking spoligotypes and IS*6110* RFLP patterns



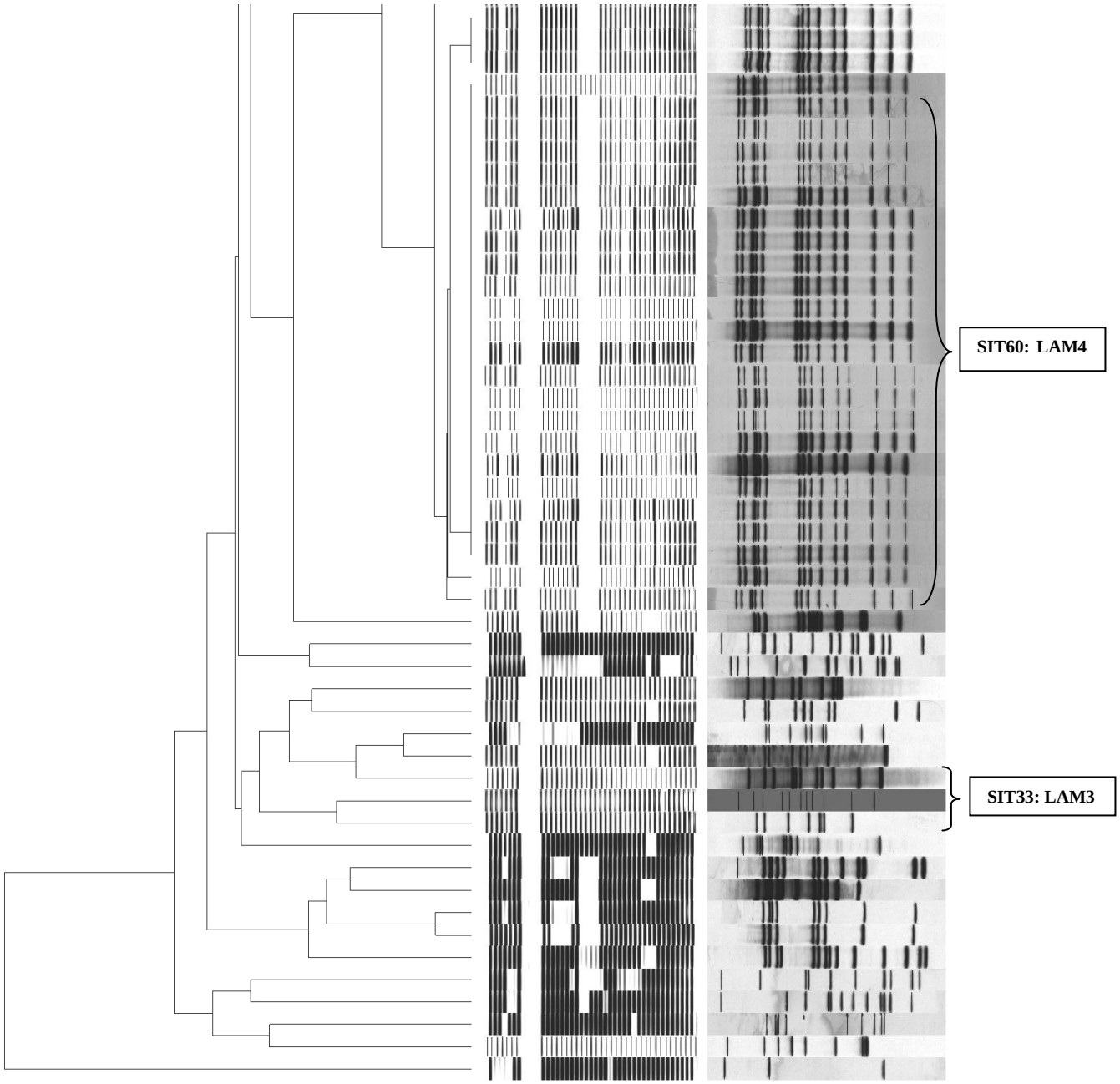


Figure14 shows the dendrogram that was generated for the combined spoligotypes and IS*6110* RFLP patterns.

CHAPTER 5

DISCUSSION

Studies describing the frequency and distribution of MDR and XDR MTB genotypes in South Africa are limited (Stavrum *et al*., 2009). In this study we investigated the molecular epidemiology of MTB in Tugela Ferry in KZN, to ascertain whether the cases of MDR including XDR TB are the result of clonal spread or simultaneous *de novo* development of resistance in different strains. Two genotyping methods were employed viz: spoligotyping and IS*6110* RFLP. A total of 99 isolates, 67 from 2005/6 and 32 from 2008/9, with varying susceptibility profiles were analysed. Our results show that the spread of XDR TB in Tugela Ferry was clonal during both study periods. The strain responsible for transmission was a strain belonging to the F15/LAM4/KZN family, which has been responsible for about 1/3 of all MDR TB cases in KZN since 1994 (Pillay and Sturm, 2007). Our conclusion that this XDR TB epidemic in Tugela Ferry is due to clonal spread, rather than *de novo* amplification of drug resistance in different MTB strains is supported by the cluster analysis of the isolate genotypes. This analysis showed that 43 out of the 44 XDR TB isolates analysed were clustered and belonged to the same F15/LAM4/KZN genotype, while the remaining isolate was closely related, differing by only 1 band on the *IS6110* RFLP. In contrast, only 2 out of the 28 susceptible or INH mono-resistant isolates were clustered, while 16 out of the 27 MDR isolates were clustered, belonging to either F15/LAM4/KZN or F28 genotypes.

The isolates that were genotyped in this study were from inpatients and outpatients. We do not have enough clinical, epidemiological and contact tracing information to determine the source of the XDR strain circulating in Tugela Ferry. It is unclear whether the isolate first

appeared in the community or in CoSH. Despite the relatively small numbers, this study adds to information on the molecular epidemiology of MTB strains circulating in the Tugela Ferry area and provides insight as to which genotypes are primarily responsible for infections amongst the local population.

Spoligotyping of the XDR TB strains revealed that the LAM4 sub-lineage which is part of the LAM lineage and represented by SIT60, was the predominant genotype. Although the first XDR isolate was found in 2001 (Pillay and Sturm, 2007), the first spoligotyping of such isolates in South Africa was performed on the isolates from the first XDR TB patients identified in Tugela Ferry in 2005 (Ghandi *et al*, 2006). The spoligotyping results revealed that all the XDR isolates belonged to the KZN family which corresponded to the spoligotype pattern SIT60 (Ghandi *et al*., 2006). A retrospective analysis of isolates obtained between 1994 and 2002 from patients in KZN, revealed that strains with the SIT60 pattern have been present in KZN since at least 1994. These strains had a unique RFLP fingerprint belonging to the F15/LAM4/KZN genotype; at this time point, the strains with the SIT60 pattern was most prevalent amongst the MDR isolates from patients in the province of KZN (Pillay and Sturm, 2007). Isolates with the SIT60 spoligotype pattern in our study accounted for the vast majority of XDR TB cases and this correlates with previous studies in Tugela Ferry, in which this spoligotype was responsible for the majority of re-infections with XDR-TB (Andrews *et al*., 2008). In this study all of XDR cases were infected with one of the IS*6110* genotypes that evolved out of the original genotype reported from 1994. In contrast, almost half of the MDR isolates with the SIT60 spoligotype still belonged to the original IS*6110* genotype.

A study by Mlambo *et al*. (2008) provides genotypic information on XDR TB strains in 7 of the 9 provinces in South Africa. The study found XDR TB in 4 of the 7 provinces. The study showed that XDR TB strains in South Africa belong to seven different lineages. In Gauteng province and Limpopo province 3 of the 41 XDR TB isolates had the SIT60 spoligotype pattern and belonged to the LAM4 sub-lineage. This spoligotype pattern has also been found in 12 countries in Europe, North and South America (Brudey *et al*., 2006; Filliol *et al*., 2003). The study concluded that *de novo* drug resistance rather than transmission was the predominant source of XDR TB cases (Mlambo *et al.,* 2008). Our study implies that the factors driving the XDR TB epidemic locally in Tugela Ferry may differ from those in other regions of South Africa.

Additional spoligotype patterns found in this study among the XDR TB isolates included the SIT53 spoligotype pattern which is representative of the T1 sub-lineage. In this study, the XDR isolates with the SIT60 and 53 patterns clustered with an identical RFLP pattern. Patterns which differ from those that cluster by 1 of the 2 techniques may be associated with mixed infections or may be the result of evolution of the DR region. Alternatively, our results may indicate that isolates with the SIT53 pattern are evolving and acquiring more resistance mechanisms.

Our findings may be explained by the presence of multiple strains in the specimen. As with similar studies, we have not been able to rule out mixed infections with more than one MTB strain. This has the potential to result in false genotypes. The fact that this is a reality has been shown in several studies. Matsumoto *et al*. (2005) analysed a sweep of colonies from initial culture, as well as individually separated colonies using IS*6110* RFLP and spoligotyping. Genotyping of the single colonies showed different patterns with varying

susceptibility to rifampicin. Analysis on the sweep of colonies did not detect differences. This suggested that dual infection with different MTB strains may occur. Stavrum *et al*. (2009) showed that 53.6% isolates with the SIT53 pattern had a mixed MTB infection when analysed by 15 loci MIRU-VNTR. A recent study that included all culture confirmed cases of TB in KZN over an 18-month period, showed that mixed infections occurred in almost 7% of patients in 2006/2007 (Moodley *et al*., 2011). A limitation of the study was the inability to detect mixed infections since genotyping was performed on a sweep of colonies as opposed to single colonies.

We report SIT53 to be responsible for a small proportion 6% (2/32) of XDR TB cases in 2005-2006. These 2 XDR TB isolates were confirmed to be SIT53 on repeat testing. XDR TB re-infections with the SIT53 pattern were formerly reported in 17% of the cases in Tugela Ferry (Andrews *et al*., 2008).

Among the XDR TB isolates obtained during the period 2008-2009, two (16.7%) had the SIT1750 spoligotype pattern which like SIT60, belongs to the LAM4 sub-lineage and has only one spacer difference with SIT60. Another three (25%) showed an orphan pattern *i.e.*: this pattern was not found on the international SpolDB4 database. However this pattern has major similarity with the SIT60 and SIT1750 spoligotype patterns, it has spacers 15, 21-24, 33-36, 39 and 40 absent. The orphan pattern has a difference of 2 spacers as compared to the SIT60 spoligotype pattern, which are absent and a difference of 1 spacer as compared to the SIT1750 spoligotype pattern is absent.

XDR TB isolates from the period 2005/6 which had the SIT53 pattern and from 2008/9 with the SIT1750 pattern and the orphan pattern which were similar to SIT60, these

patterns could be associated with evolution of the direct repeat (DR) region which occurs over time. Different mechanisms are involved which include IS*6110* mediated mutations, point mutations, homologous recombination and duplication of direct variable repeat (DVR) sequences. In a study by Warren *et al.* (2002) the DR region was analysed for three independent evolving strain families. IS*6110* mediated mutations and homologous recombination were responsible for changes in the DVR sequences. The frequency of change within the DR region seemed to be strain family specific and the mechanisms were dependent either on the DR region structure or the mutation rate of the strain family. Isolates in each strain family had specific DVR sequences deleted. These spoligotype signatures appeared to be unique for a specific strain family. Insertion of IS*6110* into the DR region or spacer sequences results in deletion of the DVR sequence and influences the spoligotype pattern. IS*6110* has the ability to prevent hybridisation of spacer sequences and inhibit PCR amplification. The spacers may be present and can be identified by sequencing. IS*6110* has preferential integration loci in the DR region which are also strain family specific and enables the DR structure to influence evolution. Among the XDR TB isolates in this study which had great similarity with SIT60 and the characteristic spacers 21-24, 33-36 and 40 absent, IS*6110* could be responsible for the additional spacers being absent and evolution of the DR region may have occurred.

Whilst the spoligotype patterns for the 44 XDR MTB isolates in this study differed as discussed above, IS*6110* RFLP analysis showed that 43 isolates clustered with an identical IS*6110* RFLP banding pattern corresponding to the F15/LAM4/KZN family, whilst 1 XDR TB isolate had an IS*6110* RFLP banding pattern which differed by 1 band as compared to those in the predominant cluster. This could be associated with a time lapse and the evolution of the MTB genome over time since IS*6110* elements have the ability to relocate

within the genome (McEvoy *et al.*, 2007). Isolates from more recent years should be evaluated to establish if IS*6110* patterns in XDR isolates have changed over time.

Our results show that SIT34 which is representative of the S lineage was the predominant spoligotype among MDR TB isolates. The SIT53 (T1 sub-lineage), SIT831 (S lineage) and the SIT60 (LAM4 sub-lineage) spoligotypes were also present among the MDR MTB isolates.

MDR TB genotypes belonging to 10 different lineages were identified in a study by Stavrum *et al.* (2009). The LAM lineage was the most common in the Eastern Cape, KZN, Mpumalanga and North West provinces.

In a study by Chihota *et al.* (2010) the S lineage was most common in South Africa and was identified in 7% of the isolates. Information regarding MTB genotypes in South Africa is restricted to those areas where genotyping is performed. In a study by Streicher *et al*. (2004) which was conducted in the Western Cape the F28 family and SIT34 were associated with MDR TB cases as well as INH and rifampicin mono-resistant cases. In our study, the majority of the MDR TB strains belonged to the F28 family which implies that the spread of MDR may be clonal with respect to this strain family. While up till 2002 the F15/LAM4/KZN strain dominated amongst MDR cases (Pillay and Sturm, 2007), the data presented here suggest that F28 has taken over this role with F15/LAM4/KZN now dominating amongst the XDR cases.

Further analysis of the genotype patterns of the MDR MTB isolates in this study revealed that a large F28 cluster and 4 unique patterns were present among the isolates that belonged to SIT34. The IS*6110* RFLP patterns for isolates belonging to the F28 cluster and

those with unique patterns (3/4) had a high degree of similarity with the F28 patterns reported by Warren *et al*. (2002).

The 6 MDR TB isolates which belonged to the SIT60 were further differentiated by IS*6110* RFLP. IS*6110* RFLP analysis revealed that 50% (3/6) still clustered but differed from the XDR TB cluster by 1 band. The other 50% (3/6) of MDR TB isolates in the SIT60 cluster had IS*6110* RFLP patterns which differed by 1 band from each other and from the MDR and XDR TB cluster. All these isolates had IS*6110* RFLP patterns that belonged to the F15/LAM4/KZN family.

MDR TB isolates which belonged to SIT53 were further differentiated into unique patterns by IS*6110* RFLP. Our genotyping results, suggest that most of the MDR TB cases in the Tugela Ferry area, result from clonal spread. A case for *de novo* resistance is more difficult to make since one cannot be sure whether isolates with a unique pattern among those studied are not part of a cluster of isolates of which have not been included. However, the evolution of, in particular F15/LAM4/KZN from MDR to XDR can only be explained by such *de novo* development followed by clonal spread. The F28 pattern was predominant amongst MDR TB strains circulating in this area. In addition another cluster which had IS6*110* RFLP patterns belonging to the KZN family was present. The many non- clustering MDR TB isolates may be linked with *de novo* resistance. This challenges the concept that the use of fixed drug combinations like Rifafour prevent such events.

In our study the majority of XDR isolates were of the KZN family. Previous reports have shown that the MDR form of the KZN family of strains is present in our area (Pillay and Sturm, 2007). The proportion of XDR TB in the Tugela ferry area has been reported to be

at least 3 times higher than any other district in the KZN Province (Moodley *et al.*, 2011). This could represent an outbreak of XDR TB in the Tugela Ferry area which might explain the disproportionate number of KZN family XDR isolates compared with MDR isolates.

The susceptible isolates in this study also had spoligotype patterns similar to those strains which were responsible for MDR and XDR TB spoligotypes *i.e*. SIT34 and SIT53. No SIT60 pattern was found among the susceptible isolates tested. One possibility is the fact that the sample size was limited and not representative of the entire community. Secondly, SIT60 may reflect an already mutated resistant isolate. The SIT1 is representative of the Beijing lineage. The Beijing lineage has worldwide distribution and has been responsible for both susceptible and drug resistant MTB infections. In our setting we found SIT1 only amongst susceptible isolates.

Several SITs were present as single isolates (Figure 13a and 13b). Most of these SITs have also been reported from other African countries.

The Latin-American Mediterranean (LAM) lineage can be divided into sub-lineages LAM1-LAM11 (Stavrum *et al*., 2009). The LAM3 (SIT33), LAM4 (SIT811) and LAM11-ZWE (SIT59 and SIT815) sub-lineages have been found in susceptible TB isolates in this study. The LAM3 sub-lineage has been responsible for TB cases in the Western Cape Province, South Africa (Victor *et al*., 2004). The LAM11-ZWE sub-lineage has accounted for 57.8% of isolates from patients in Zimbabwe and it is likely to be identical to the recently described Meru family found in Tanzania (Brudey *et al*., 2006; Easterbrook *et al*., 2004; McHugh *et al*., 2005).

The CAS1-KILI (SIT21) lineage is found in Tanzania (Kibiki *et al*., 2007). The X lineage (SIT137) which can be divided into X1-X3 sub-lineages is prevalent in United Kingdom, USA and former British colonies (Brudey *et al*., 2006).

The ill-defined T lineage has been found on all continents (Brudey *et al*., 2006). In a study by Chihota *et al*. (2010) the T1 sub-lineage was predominant within the T lineage. The T1 strains were found in different proportions among the following countries, 74.5% in Madagascar, 37.9% in Mozambique, 43.5% in South Africa, 57.5% in Tanzania, 85.7% in Zambia, 86.7% in Zimbabwe and all isolates from Namibia and Malawi. In this study the following SITs: SIT21 representative of the CAS-KILI lineage, SIT39 representative of the T4-CEU1 sub-lineage, SIT33 representative of the LAM3 sub-lineage, SIT73 representative of the T2-T3 sub-lineage, SIT137 representative of the X2 sub-lineage, SIT563 representative of the U lineage, SIT811 representative of the LAM4 sub-lineage and SIT815 representative of the LAM11-ZWE have been identified and have also been found in a study conducted in South Africa by Stavrum *et al.* (2009).

The SITs found in South Africa have been found in neighbouring countries. The spread of these strains may be facilitated by cross border migration. A study conducted in Zimbabwe, Zambia and South Africa identified a predominant group of strains with a unique spoligotype pattern with spacers 21-24, 27-30 and 33-36 absent (Chihota *et al*., 2007). Other studies in surrounding countries of South Africa have identified strains corresponding with those in this present study.

Further differentiation by IS*6110* RFLP was done for all susceptible isolates, but very few clustered. This is likely the result of the very small sample out of many patients infected

with susceptible isolates. A total of 16 unique patterns were found among the susceptible isolates which belonged to one of the four predominant SITs. In our study the Beijing strains have been associated with susceptible isolates and belong to the SIT1 and have a multi-band IS*6110* RFLP fingerprint. Susceptible isolates which belonged to the SIT1 had differing IS*6110* RFLP banding patterns but which had a similarity of ≥65% and therefore still belonged to a common family with their corresponding SIT1. Susceptible isolates within the respective SITs *i.e.* SIT33, SIT34 and SIT53 had IS*6110* banding patterns which were similar.

A total of 99 isolates which were classified into 18 SITs (8 SITs containing 2 or more isolates and 10 SITs containing only 1 isolate) were further genotyped by IS*6110* RFLP. This revealed that 29 isolates had unique patterns and 61 had identical patterns to at least 1 other isolate in this collection. Additionally 9 isolates had IS*6110* RFLP patterns with approximately 95% similarity (*i.e.*1 band difference) to another isolate. A total of 42 different RFLP patterns were seen amongst the 99 isolates. IS*6110* RFLP analysis enabled further differentiation of MTB strain clusters identified by spoligotyping.

Spoligotyping revealed that the LAM4, T1, S and Beijing families were predominant among this TB isolate collection. Out of 89 isolates that clustered by spoligotyping, 28 could be further differentiated as unique strains by IS*6110* RFLP. It is recommended that both techniques be used simultaneously to genotype MTB. The use of spoligotyping alone in this study would have resulted in assigning more isolates to clusters and underestimate the number of isolates with unique patterns. The use of IS*6110* RFLP alone would distinguish unique patterns however isolates with less than 6 IS*6110* copies are difficult to discriminate and supplementary genotyping techniques are required to analyse these.

Previous studies have shown that isolates which clustered by spoligotyping could be further differentiated into unique patterns by RFLP (Mathuria *et al*., 2007). Combining two genotyping methods reduced the fraction of clustering among the MDR and XDR TB isolates in our study with the IS*6110* RFLP being more discriminatory.

Further differentiation of the spoligotyping SITs by IS*6110* RFLP revealed that within each SIT more distinct banding patterns among isolates were present, however although IS6*110* RFLP patterns differed among isolates, the similarity of fingerprints were ≥65% among isolates sharing a common susceptibility profile, suggesting that the majority of strains still belonged to a common family within their corresponding SIT.

In a study by Warren *et al*. (2002) it was shown that different isolates from the same strain family classified by IS*6110* RFLP and isolates from different strain families were observed to converge to the same spoligotype pattern. Homologous recombination of IS*6110* elements led to deletion of DVR sequences and influenced spoligotype patterns. This evolution mechanism resulted in identical spoligotype patterns among different strain families.

Tugela Ferry is known to have a high HIV TB co-infection prevalence. Patients in high TB incidence settings have shown to be more prone to mixed infections as a result of repeated re-infections (Richardson *et al*., 2002; Stavrum *et al*., 2009; Warren *et al*., 2004). Our study population is susceptible to contracting concurrent infections with different TB strains and our results suggest that this may occur. Further studies are required to evaluate the extent and implications of mixed bacterial populations in management of patients and in interpreting genotyping results.

There appears to be a substantial decrease in the proportion of XDR strains, comparing 2005/6 to 2008/9. This may indeed reflect a decline in the prevalence in Tugela Ferry since increased TB control has intensified case finding and screening. TB treatment teams have also been recruited to monitor treatment in the community. By strengthening TB control programs, the TB default rate has decreased and the treatment success rate has increased. Household contact tracing has also been implemented for MDR and XDR TB, trace teams visit households and screen contacts for sign and symptoms of TB. A community surveillance system monitors TB transmission, trace teams collect gps co-ordinates of all households which have MDR and XDR patients, which enables the mapping of the distribution and number of households affected. Intensive case finding programs in hospitals and the community have been established. Patients entering healthcare facilities are screened to identify TB suspects. Infection control policies are being carefully monitored and a decline in MDR and XDR TB in CoSH wards has been observed. Community based treatment programs help ensure successful completion of treatment, nurses visit those infected with MDR TB and administer 2nd line agents by injection and also monitor doses of oral medication (TF CARES/Philanjalo).

Spoligotyoing has been shown to be less discriminate than *IS6110* RFLP in previous studies. Our study confirms this observation since strains with identical spoligotypes were further discriminated by *IS6110* RFLP genotyping. However, *IS6110* RFLP is labour intensive and has a slow turnaround time since prolonged incubation periods are required to generate high quality DNA. The resolution of this technique is inversely proportional to the copy number of *IS6110* elements with strains having less than six copies of *IS6110* being indiscriminate. Inter-laboratory analysis of RFLP patterns is tedious and specialised

software is required (Mathema *et al.,* 2006). In this study 2 complementary genotyping methods were used in an attempt to overcome the limitations when used individually.

Further studies to confirm the clonal spread of MDR and XDR TB strains described in this study should use genotyping methods such as MIRU:VNTR. A study by Murase *et al.* (2010) evaluated RFLP clusters of MDR and XDR TB using MIRU:VNTR. The results between RFLP and MIRU:VNTR correlated since 7 of the 9 RFLP clusters had identical MIRU:VNTR profiles. The remaining 2 clusters had a single locus variant i.e. 1 strain had 1 locus difference in each cluster. The newly proposed standard which utilises 24 loci of tandem repeats has a comparable specificity to *IS6110* RFLP and is faster and cheaper. Since the MIRU:VNTR method has demonstrated an ability similar to IS6110 RFLP method to assess clonality in previous studies, it may be used as an alternative method to confirm clonality.

CONCLUSION

While the Beijing strain shows a tendency to resistance in other geographic areas, in the Tugela Ferry area of KwaZulu-Natal, this family of strains is circulating in a susceptible form. The F15/LAM4/KZN strain family has dominated amongst MDR isolates from at least 1994 till 2002. The dominant MDR strain family is now F28, similar to the situation in the Western Cape. The F28 strain currently circulating as an MDR TB outbreak may have some parallels with the evolution of the F15/LAM4/KZN strain. Similarly, the MDR F28 strain has the potential to acquire resistance to additional drugs to become XDR in the future. The vast majority of XDR isolates belongs to the F15/LAM4/KZN family; IS*6110* RFLP patterns suggest clonal spread with evolving genotypes. Spoligotyping alone cannot

be used for epidemiological purposes. Different SITs have also been found in isolates belonging to the same IS*6110* RFLP family.

CHAPTER 6

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Appendix 1

DNA Extraction Solutions

1.1 10x TE Buffer

Trizma base (Sigma-Aldrich, USA) 1.21g

EDTA (Sigma-Aldrich, USA) 0.37g

Weigh out the required amounts of reagent powders and dissolve the trizma base in 80ml of distilled water, pH to 8 using concentrated HCl (Merck). Add the EDTA and dissolve. Check the final pH and adjust to a final volume of 100ml. Autoclave at 121°C for 15 minutes.

1x TE Buffer

Dilute 10ml of 10x TE Buffer into 90ml of triple distilled water. Store at room temperature.

1.2 Lysozyme (10mg/ml) (Sigma-Aldrich, USA)

Add 1ml of distilled water to 10mg of lysozyme powder. Store at 4°C until

1.3 Proteinase K (10mg/ml) (Roche Diagnostics)

Add 10ml of distilled water to 100mg of proteinase K powder. Store at 4°C until.

1.4 10% Sodium Dodecyl Sulfate (SDS) (Sigma-Aldrich, USA)

Weigh 10g of SDS powder and dissolve in 100ml of distilled water.

1.5 5M Sodium Chloride (NaCl) (Sigma-Aldrich, USA)

Weigh 14.6 g of NaCl powder and dissolve in 50ml of distilled water, autoclave at 121°C for 15 minutes.

1.6 CTAB-NaCl Solution

NaCl (Sigma-Aldrich, USA) 4.1g

CTAB (Sigma-Aldrich, USA) 10g

Weigh out the required amounts of reagent powders and dissolve in 100ml of distilled water, the solution is heated to 65°C until powders are completely dissolved.

1.7 Chloroform: Isoamyl alcohol (24:1)

Add 1ml of isoamyl alcohol (Sigma-Aldrich, USA) to 24ml of chloroform (Sigma-Aldrich, USA).

1.8 70% ethanol

Add 35ml of absolute ethanol (Merck, SA) to 15ml of distilled water. Store at -20°C.

1.9 1% agarose gel (140ml)

Agarose powder (Lonza, USA)	1.4g
1x TBE buffer	140ml
Ethidium bromide	140µl

The agarose powder was weighed and added to a flask containing 1x TBE buffer. The mixture was boiled using a microwave until the powder dissolved. It was allowed to cool and ethidium bromide was added.

1.10 10xTBE Buffer

Trizma base (Sigma-Aldrich, USA)	108g
Boric acid (Sigma-Aldrich, USA)	55g
EDTA (Sigma-Aldrich, USA)	9.3g

Weigh out the required amounts of the reagents and dissolve in 1000ml of distilled water.

1x TBE

Add 100ml of 10x TBE to 900ml of distilled water.

1.11 Ethidium Bromide (5µg/ml)

Weigh out 10mg of ethidium bromide powder and dissolve in 20ml of distilled water. Store at 4°C in a dark bottle.

1.12 Sample loading dye

1% Double dye

Bromphenol Blue (Sigma-Aldrich, USA) 1g

Xylene cyanole (Sigma-Aldrich, USA) 1g

Weigh out the required amounts of reagent powders and dissolve in 100ml of distilled water.

50 ml of Loading Dye from 1% Double Dye (DD) stock:

10x TBE 5 ml

Glycerol (Merck, SA) 25 ml

1% Double dye 5 ml

Measure the required volumes of reagents and dissolve in 15ml of distilled water.

Appendix 2

Spoligotyping Solutions

2a 20x SSPE Solution

0.2M $Na_2HPO_4.2H_2O$ (Sigma-Aldrich, USA)	35.6g
3.6M NaCl (Sigma-Aldrich, USA)	210.24g
20mM EDTA (Sigma-Aldrich, USA)	7.4g

Weigh out the required amounts of the reagents and dissolve in 800ml of distilled water using a magnetic stirrer, adjust pH to 7.4 using 4M NaOH. Add distilled water to get a final volume of 1000ml.

2b 0.5M EDTA

Dissolve 93.05g of EDTA (Sigma-Aldrich, USA) powder in 450ml of distilled water, adjust pH to 8 using NaOH pellets (Sigma-Aldrich, USA). Add distilled water to get a final volume of 500ml.

2c 10% SDS solution

Weigh 10g of SDS powder (Sigma-Aldrich, USA) and dissolve in 100ml of distilled water.

2.1 2x SSPE 0.1% SDS

Add 26ml of 20x SSPE and 2.6ml of 10% SDS to 231.4ml of distilled water.

2.2 2x SSPE 0.5% SDS

Add 102ml of 20x SSPE and 51ml of 10% SDS to 867ml of distilled water.

2.3 2x SSPE

Add 50ml of 20x SSPE to 450ml of distilled water.

2.4 1% SDS

Add 40ml of 10% SDS to 360ml of distilled water.

2.5 20mM EDTA

Add 2ml of 0.5M EDTA to 48ml of distilled water.

Appendix 3

IS*6110* RFLP Solutions

3a 20x SSC Solution

3M NaCl (Sigma-Aldrich, USA)	175g
0.3M Tri-sodium citratedihydrate (Merck, SA)	88g

Weigh out the required amounts of the reagents and dissolve in 800ml of distilled water, pH to 7.0 using concentrated HCl (Merck, SA) and adjust to a final volume of 1000ml.

3.1 10x SSC Solution

Add 500ml of 20x SSC to 500ml of distilled water.

3.2 1:100 HCl

Dissolve 5ml of concentrated HCl (Merck, SA) in 495ml of distilled water.

3.3 Soak I Solution

0.5M NaOH pellets (Sigma-Aldrich, USA)	20g
1.5M NaCl (Sigma-Aldrich, USA)	87.66g

Weigh out the required amounts of the reagents and dissolve in 1000ml of distilled water.

3.4 Soak II Solution

0.5M Trizma base (Sigma-Aldrich, USA)	62.6g
1.5M NaCl (Sigma-Aldrich, USA)	87.67g
Conc. HCl (Merck, SA)	40ml

Weigh out the required amounts and volume of the reagents and dissolve in 800ml of distilled water, pH to 7.2 using concentrated HCl (Merck, SA) and adjust to a final volume of 1000ml.

3.5 Hybridisation buffer

Hybridisation buffer (Amersham, UK)	270ml
5M NaCl (Sigma-Aldrich, USA)	30ml
Blocking agent (Amersham, UK)	15g

Weigh out the required amount and volumes of reagents. Dissolve NaCl in hybridisation buffer using a stirrer bar on a magnetic stirrer, add small amounts of blocking agent slowly and heat solution at 60°C to enable blocking agent to dissolve. Buffer is aliquoted into 50ml falcon tubes and stored at -20°C.

3.6 Primary wash buffer

Urea (Sigma-Aldrich, USA)	360g
20xSSC	25mL
SDS (Sigma-Aldrich, USA)	4g

Weigh out the required amounts and volume of the reagents and dissolve in 800ml of distilled water using a magnetic stirrer bar, adjust to a final volume of 1000ml.

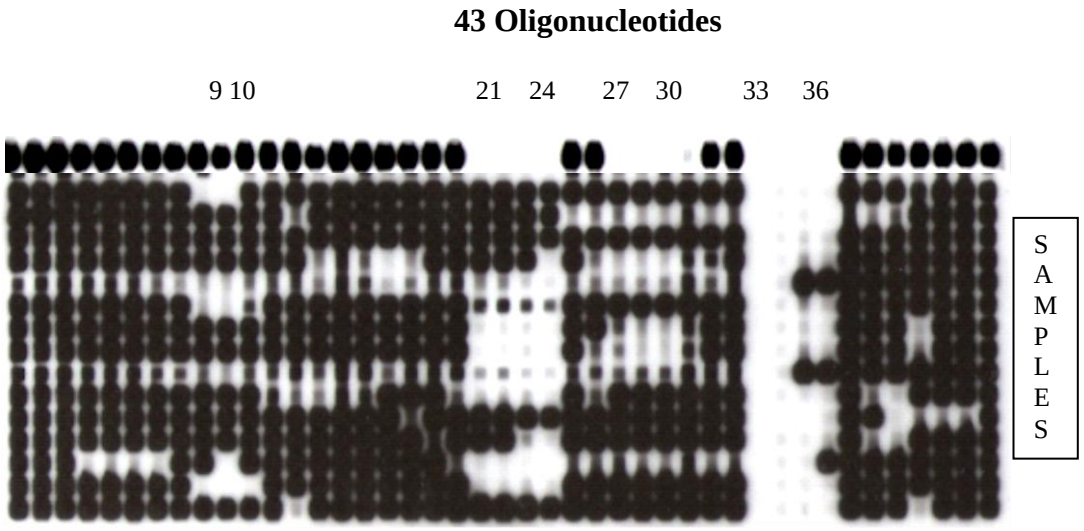
3.7 2x SSC Solution

Add 100ml of 20x SSC to 900ml of distilled water.

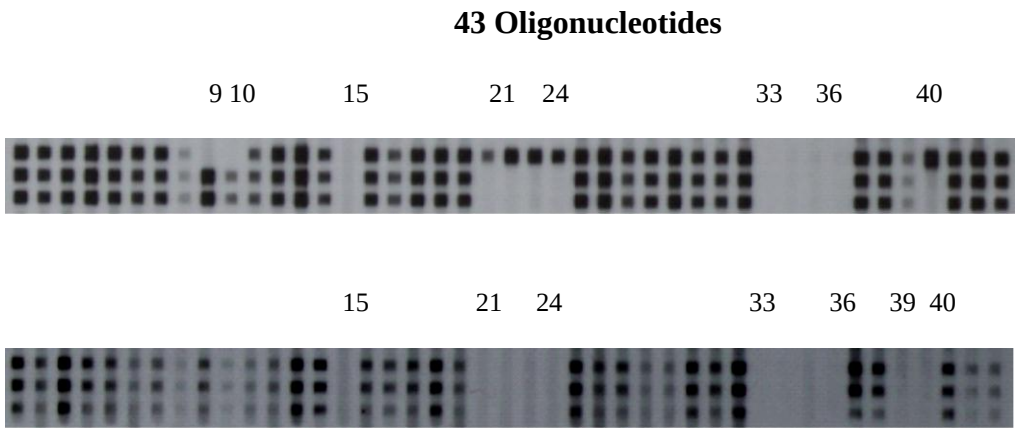
Appendix 4

Additional spoligotype patterns obtained in this study.

1. Spoligotype patterns obtained among the susceptible isolates.



2. Additional spoligotype patterns among MDR and XDR TB isolates.

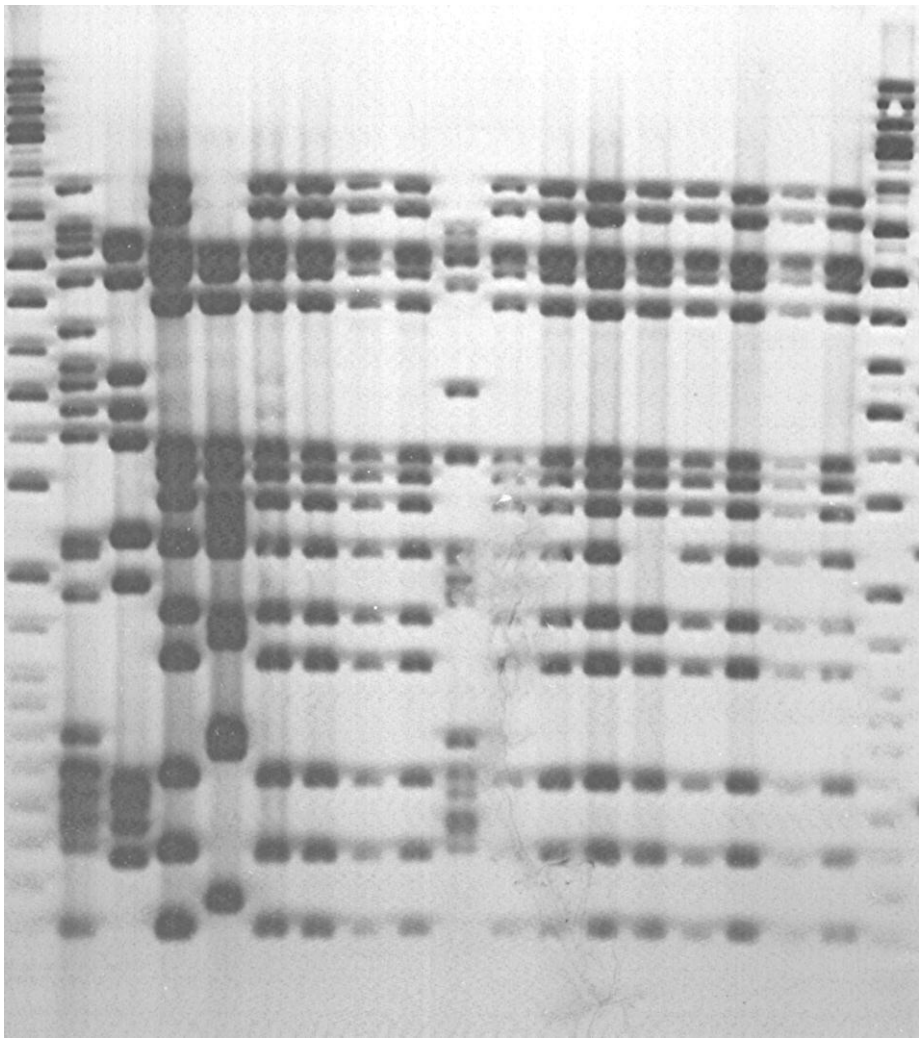


3. Control spoligotype patterns: H37Rv and *M.bovis.*

Appendix 5

IS*6110* RFLP patterns

M 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 M



IS6110 RFLP patterns (2005-2006)

Lane 1 & 19: Jack's standard molecular weight marker (0.7-15 kbps)

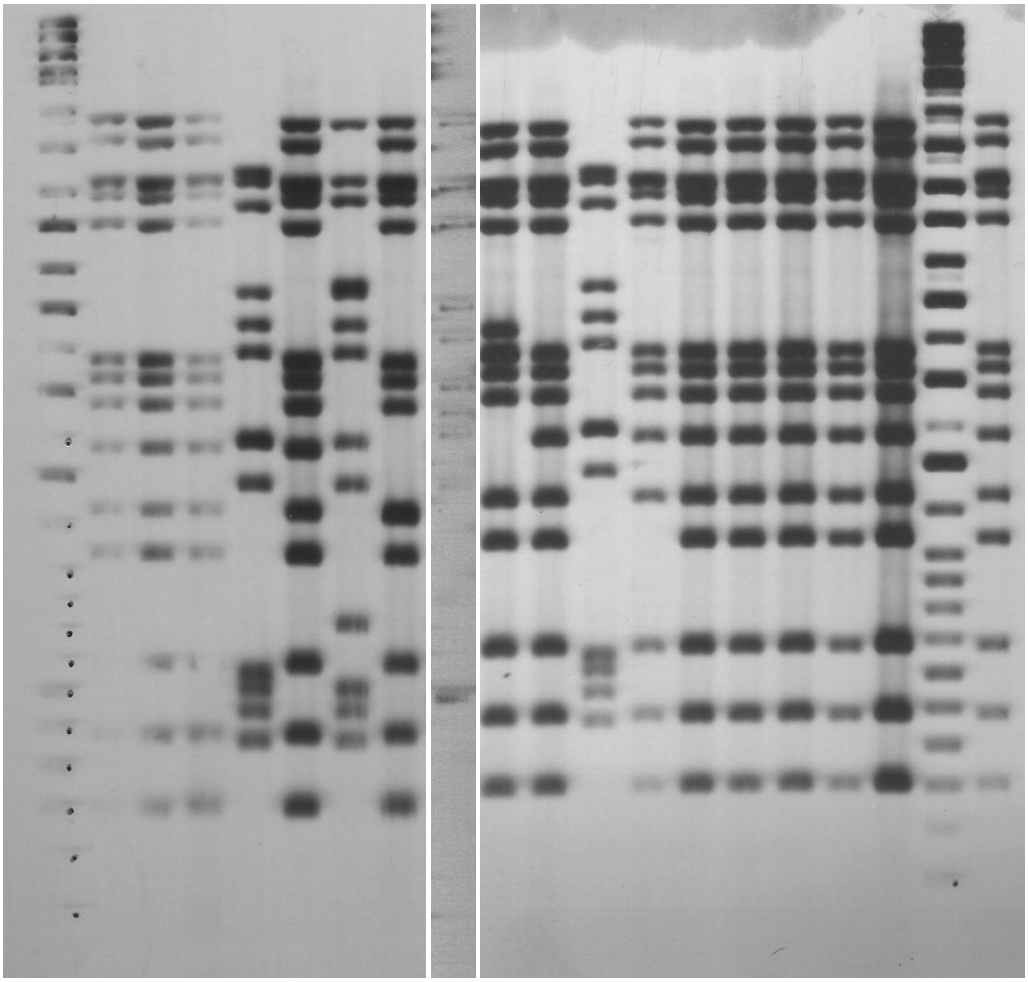
Lane 2 & 10: Beijing family patterns

Lane 3: F28 family pattern

Lane 5: unique pattern

Lane 6-9, 11-18: F15/LAM4/KZN family patterns

M 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 M 20



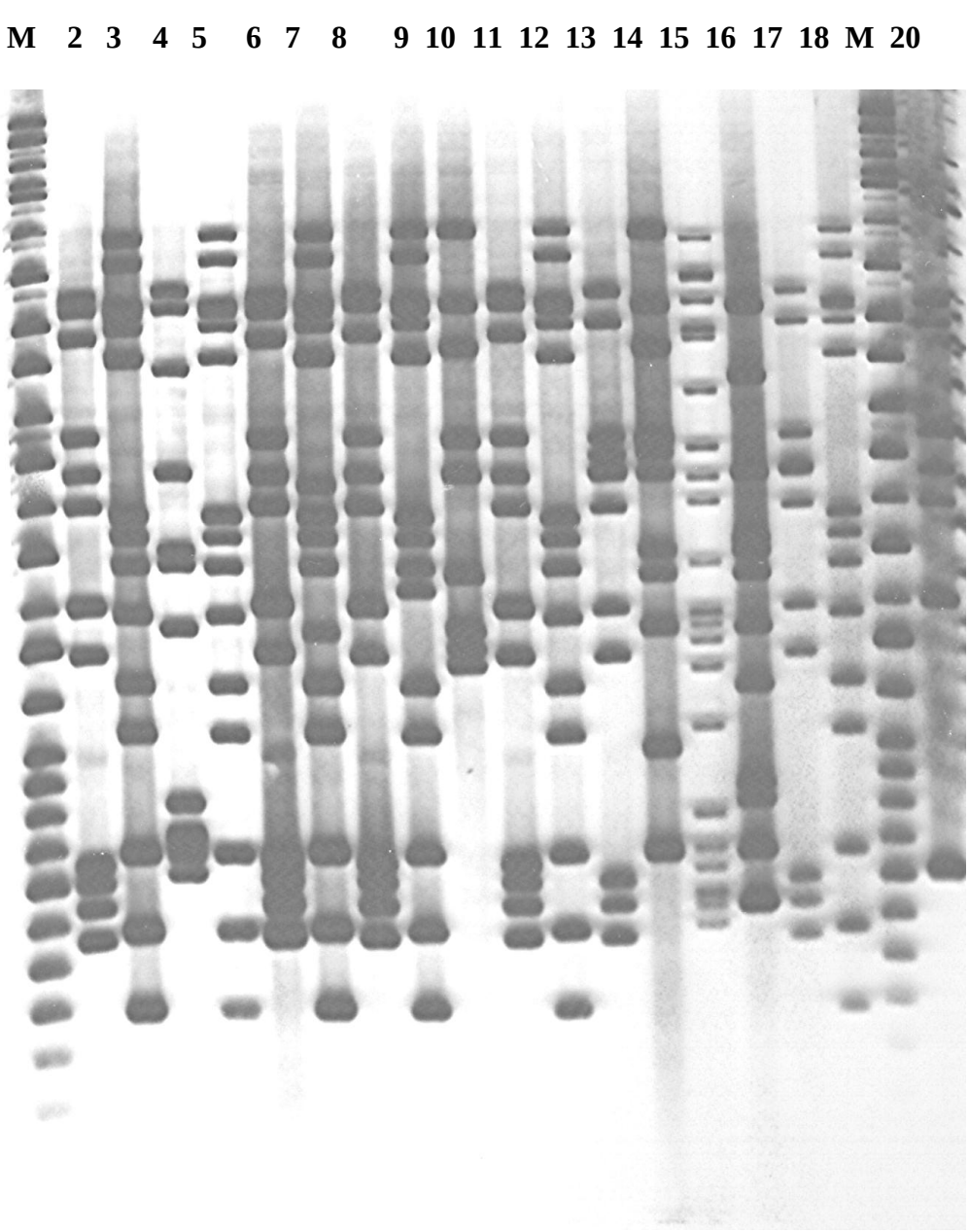
IS*6110* RFLP patterns (2008-2009)

Lane 1 & 19: Jack's standard molecular weight marker (0.7-15 kbps)

Lane 2-4, 6, 8, 10-11, 13-18, 20: F15/LAM4/KZN family patterns

Lane 5, 7, 12: F28 family patterns

Lane 9: unique pattern



IS*6110* RFLP patterns (2005-2006)

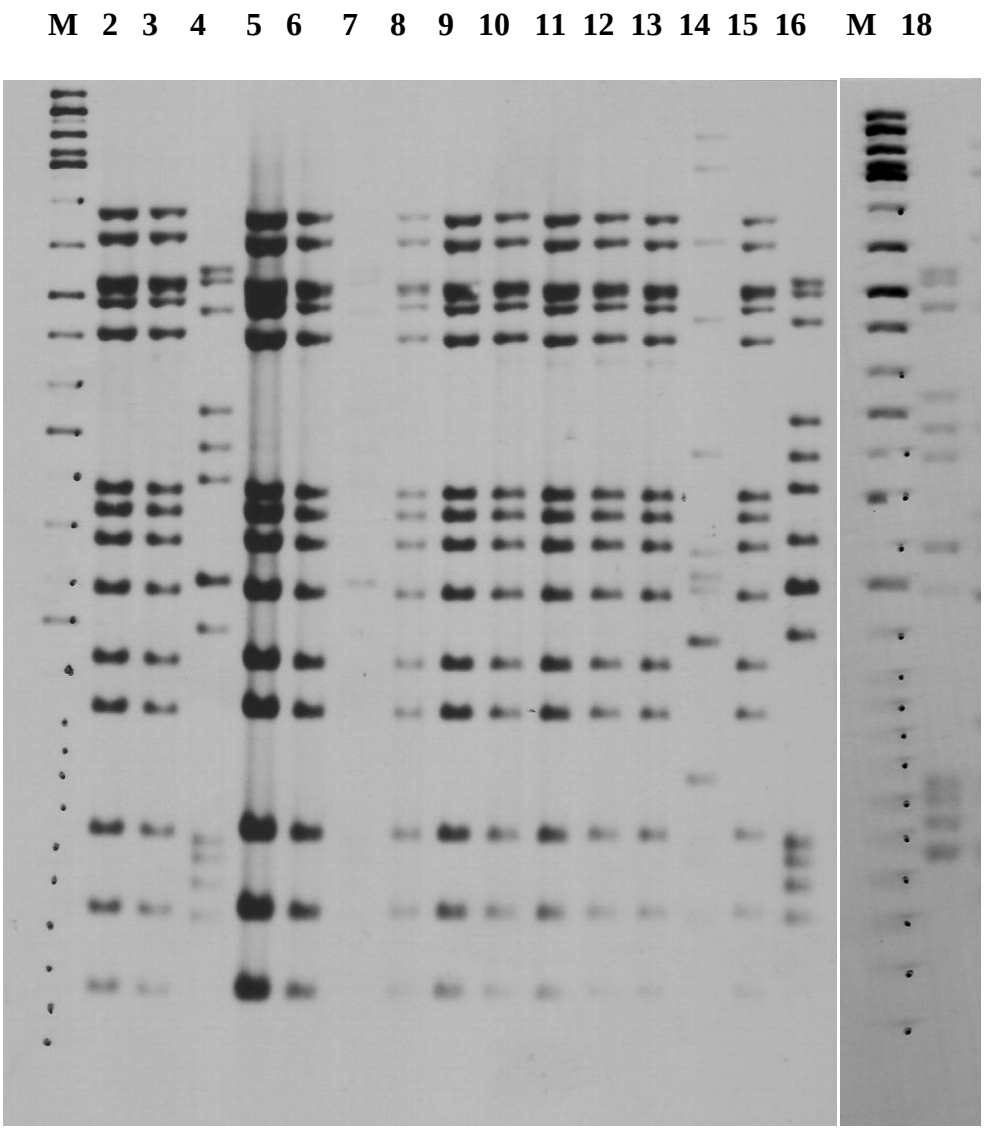
Lane 1 & 19: Jack's standard molecular weight marker (0.7-15 kbps)

Lane 2, 6, 8, 11: F28 family patterns

Lane 3, 5, 7, 9, 12, 18: F15/LAM4/KZN family patterns

Lane 4, 10, 13, 14, 16, 17, 20: unique patterns

Lane 15: Beijing family patterns.



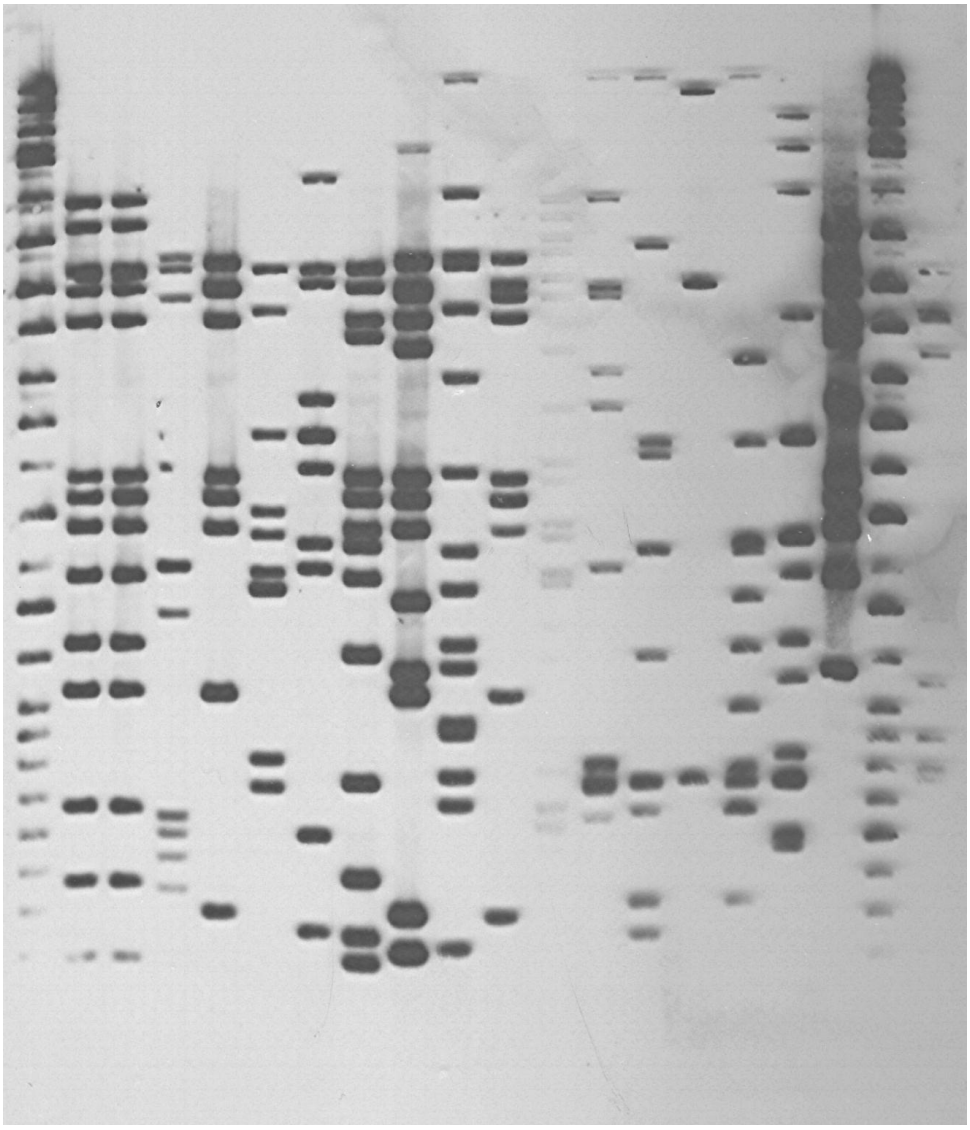
IS*6110* RFLP patterns (2005-2006)

Lane 1& 17: Jack's standard molecular weight marker (0.7-15 kbps)

Lane 2-3, 5-6, 8-13, 15: F15/LAM4/KZN family patterns

Lane 4 & 16, 18: F28 family patterns

M 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 M 20



IS*6110* RFLP patterns for (2005-2006) and (2008-2009)

Lane 1 & 19: Jack's standard molecular weight marker (0.7-15 kbps)

(2005-2006): lane 2-3 F15/LAM4/KZN family patterns

Lane 4: F28 family patterns,

Lane 5-7: unique patterns

(2008-2009):

Lane 8-18, 19: unique patterns