Strain differentiation of South African clinical isolates of *Mycobacterium tuberculosis* by restriction and amplified fragment length polymorphisms

by

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ABSTRACT:

DNA fingerprinting of *Mycobacterium tuberculosis* strain has been used in combination with conventional epidemiologic investigation, which has improved the understanding of tuberculosis transmission. Restriction Fragment Length Polymorphism (RFLP) based on IS6110 probe has become a standard method of fingerprinting of *M. tuberculosis*. Since the technique is labour intensive and the discriminatory power of IS6110 fingerprinting method for strains habouring only one to five copies is poor, other typing methods for typing *M. tuberculosis* should be evaluated. In this regard, Amplified Fragment Length Polymorphism (AFLP) has the potential to overcome many of the RFLP problems.

The first objective was to determine the suitability of the RFLP and AFLP techniques and to study the extent of transmission of tuberculosis in a referral hospital in South Africa. A total of 47 *M. tuberculosis* isolates were differentiated using RFLP technique. The same samples were typed using the PCR- based AFLP technique and results were compared. The second objective was to determine the prevalence of isoniazid (INH) resistance and estimate the incidence of recent transmission of the disease in the Eastern-Cape (EC) and North-West province (NW) by using the best suited technique.

RFLP grouped the 47 typed *M. tuberculosis* isolates into five families and four clusters. AFLP grouped the analyzed isolates (previously typed by RFLP) into two groups based on the banding patterns observed. As a result of the low degree of genotypic variation among the AFLP band pattern of *M. tuberculosis* isolates, AFLP seemed less promising for individual strain differentiation of *M. tuberculosis*. This technique can be used in future for differentiation of Mycobacterial species and

RFLP appears to be better suited than AFLP for differentiation of *M. tuberculosis* strains.

The prevalence of INH resistance was found to be 6.7% in the EC and 8.4% in the NW province. The magnitude of recent transmission in the Eastern Cape studied by RFLP method, was found to be at 22% among the positive tuberculosis isolates identified. Transmission of TB in NW province was associated with reactivation rather than recent transmission due to lack of clustering of strains in that region.

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TABLE OF CONTENTS

LIST OF FIGURES

FIGURE NUMBER **PAGE**

LIST OF TABLES

TABLE NUMBER **PAGE**

LIST OF ABBREVIATTIONS

AFLP	amplified fragment length polymorphism
°C	degrees Celcius
dH_2O	distilled water
DNA	Deoxyribonucleic acid
dNTP	2-deoxyribonucleoside-5 triphosphate
DOTS	directly observed short- course therapy
H_2O	water
EC	Eastern Cape
ETH	ethambutol
INH	isoniazid
M	molar
M. tuberculosis	*Mycobacterium tuberculosis*
mA	milliampere
$MgCl_2$	magnesium chloride
min	minutes
ml	milliliter
Mm	millimolar
NW	North West
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PZA	pyrazinamide

CHAPTER 1. GENERAL INTRODUCTION

Tuberculosis (TB) is a long-term bacterial infection caused by the acid-fast bacillus *Mycobacterium tuberculosis*. Aerosolized droplets of liquid containing tubercle bacilli are the primary means of tuberculosis transmission. Infection usually affects the lungs, although infection of the organ system also occurs. Extra-pulmonary TB is rarely contagious (except for laryngeal TB), however, transmission from extra-pulmonary sites has been reported during aerosol-producing procedures, such as autopsis and tissue irrigation (Lundgren *et al.*, 1987). Usually, individuals who are infected with *Mycobacterium tuberculosis* are asymptomatic and do not spread the disease. However, they may develop TB disease at some time in the future.

Currently, therapy for tuberculosis involves multidrug chemotherapy for a period of several months. The two most effective drugs available at the moment are isoniazid (INH) and rifampicin (R). It is well accepted that the most effective treatment regimen would include the combined use of these drugs at least for part of the treatment period (Vareldzis *et al.*, 1994). The incidence of tuberculosis in South Africa is increasing, and a most disturbing aspect of the problem is the increase in multiple drug resistant (MDR) tuberculosis caused by strains resistant to both isoniazid and rifampin, which poses a threat to individual patients (prolonged, toxic and expensive treatment with poor cure rates), to communities and to control programme efforts (World Health Organization (WHO) 1997). With the incipient HIV explosion in South Africa, extensive spread of drug resistance could make tuberculosis uncontrollable. Once

infectious particles are aerosolized, they spread throughout a room or building by air currents and can be inhaled by other individuals. The ease of transmission of tuberculosis is dependent on the resistance of the organism to conditions in the environment, particularly temperature and humidity. It is therefore both timely and appropriate to appraise the problem of drug resistance in order to learn more about the source of infection and delineate outbreaks due to these resistant strains. Circumstantial evidence leads us to the hypothesis that a significant proportion of drug resistant tuberculosis in North West and Eastern Cape Province are as a result of recent rather than acquired transmission.

DNA fingerprinting of *Mycobacterium tuberculosis* has complemented conventional epidemiological methods in the investigation of outbreaks and nosocomial transmission of both drug sensitive and drug resistant tuberculosis and laboratory cross-contamination (Wilkinson *et al.*, 1997; Aznar *et al.*, 1995; Van Soolingen *et al.*, 1995 and Small *et al.*,1993). In addition, many investigators have used *Mycobacterium tuberculosis* DNA fingerprinting in population-based cohorts of tuberculosis patients to determine the proportions of tuberculosis disease attributable to recent transmission versus reactivation, to delineate risk factors for recent acquisition of *Mycobacterium tuberculosis* infection and TB disease, and to describe the sites of the greatest transmission of infection (Lockman *et al.*, 2001). Among the various genetic elements that have been found to assist in the typing of *M. tuberculosis* strains, insertion element IS6110 has been studied extensively (Thierry

et al., 1990; van Embden *et al.*, 1993 and van Soolingen *et al.*, 1991). The technique however, requires a relatively large amount of genomic DNA and laborious laboratory proceedings. The relatively new PCR-based fingerprinting technique called Amplified Fragment Length Polymorphism (AFLP), may offer a less time consuming and more efficient option for the typing and epidemiological study of *Mycobacterium tuberculosis*.

Therefore, these two molecular techniques were compared for their suitability in our study of the epidemiology of TB in South Africa. The aims of this study were 1) To evaluate the usefulness of Amplified Fragment Length Polymorphism (AFLP) for epidemiological typing of South African *Mycobacterium tuberculosis* strains and to compare this method with Restriction Fragment Length Polymorphism (RFLP) in which the IS6110 probe is used for typing. 2) To study the epidemiology of drug resistant tuberculosis in the Eastern and Northern Cape Provinces.

CHAPTER 2. LITERATURE REVIEW

2.1 General characteristics of *Mycobacterium tuberculosis*

The *Mycobacterium tuberculosis* complex consists of *Mycobacterium tuberculosis, Mycobacterium africanum, Mycobacterium bovis and Mycobacterium microti.* The latter three species are now considered to be the sub-species of *Mycobacterium tuberculosis*. *M. tuberculosis* is a fastidious, slowly growing, non-motile, non-encapsulated, strictly aerobic, lipid rich, hydrophobic, acid-fast bacterial rod varying from 1μm to 4μm long by 0.3μm to 0.5μm in diameter. It is resistant to drying and chemical disinfectants and sensitive to heat (pasteurization) and UV radiation (Bloom 1994).

Tubercle bacilli are best isolated from clinical specimens on rich and fairly complex media, but the apparent fastidiousness of such isolates may be a consequence of their injury by the treatment employed for processing of the clinical specimen (Wayne 1977). Once isolated, *M. tuberculosis* is capable of adapting to growth on an extremely simple medium, containing a simple source carbon, nitrogen, buffer salts and trace elements. Such a medium is, however, not suitable for primary isolation because clinical specimens may contain a very low number of bacterial cells. Two kinds of media are customarily used for culturing specimens; (1) a complex medium containing eggs or egg yolks and potato extract, e.g Löwenstein-Jensen medium and (2) a synthetic medium containing oleic acid and albumin, such as the Middlebrook 7H-11 agar (Hoeprich 1983). Under optimal conditions, *M. tuberculosis* requires 16

to 18 hours to undergo one cycle of replication (Wayne 1977). With a generation time in that range, a single bacillus can yield a visible colony on solid medium within about two weeks after inoculation. The excessively long time of 6 to 10 weeks required for detection of colonies on media plated with some clinical specimens is probably the result of a need to repair injury of the bacilli in the specimen (Krasnow and Wayne 1977).

2.2 Infection and Transmission

Tuberculosis typically affects the lungs, in up to one third of the patients, but the illness also involves other areas of the body, especially the lymph nodes, genito-urinary tract, bone joints, meninges (membranes covering the brain), and peritonium (membrane covering the digestive organs) (Bloom 1994). TB bacteria are transmitted primarily by airborne droplets each of which contains 1-3 organisms; infection occurs when susceptible persons inhale infectious droplets produced by exhalations (coughs and sneezes) of persons with pulmonary tuberculosis (Bloom 1994). The organisms travel through the breathing passages to the deepest portion of the lungs, where they establish a site of primary TB infection. In almost all healthy adults, this primary infection is controlled by the body's immune system within 2-10 weeks. These patients do not develop active TB disease, although some TB bacteria remain dormant in the lungs for life.

Active TB disease occurs in several different forms: primary pulmonary, post-primary pulmonary, and extra-pulmonary tuberculosis. Primary infection, the initial phase, occurs in people without specific immunity, generally normal children and young adults who have not previously been exposed to *Mycobacterium tuberculosis.* The initial infection can occur at any time during childhood, but adolescence is the period of risk. Primary disease develops within 5 years of the initial infection, which stimulates specific immunity as demonstrated by the development of a positive skin response to purified protein derivative of tuberculin (Milburn 2001). Postprimary pulmonary tuberculosis is another type of active TB. Is also called "adult type TB", "reactivation TB", or "secondary tuberculosis". It is caused by the reactivation of dormant TB bacteria, and is most common in adults. Postprimary pulmonary tuberculosis can destroy large areas of the lungs, eventually forming cavities filled with TB bacteria and lung debris (Ortona and Federico 1998). Extrapulmonary tuberculosis is also called "disseminated tuberculosis", and it is commonly seen in children and in patients with weakened immune defenses. In extrapulmonary tuberculosis, TB bacteria enter the bloodstream and spread from lungs to other areas of the body. In children, this bacterial spread usually occurs within 2-6 months after primary TB infection (Ortona and Federico 1998).

Dormant TB bacteria have the potential to cause active TB disease in later years, especially when the patient's immune defences are severely weakened by illness (i.e cancer, Human Immunodeficiency Virus infection, malnutrition) or by drugs which

suppress the immune system (Bloom 1994). Globally, new infections of tuberculosis occur at a rate of one person every second. Left untreated, each person with active tuberculosis will infect on average between 10 and 15 people in each year. But people infected with tuberculosis will not necessarily get sick with the disease. The immune system "walls off" the tuberculosis bacilli which, protected by a thick waxy coat, can lie dormant for many years. Only 5-10% of people who are infected with tuberculosis become sick or infectious at some time during their life (Bloom 1994).

2.3 Epidemiology

2.3.1 History of tuberculosis

Tuberculosis probably occurred as an endemic disease among animals long before it infected humans (Steele and Ranney 1958). *Mycobacterium bovis,* which causes bovine tuberculosis and is otherwise known as the bovine tubercle bacillus, was the most likely species involved, thus the first human infections may have been with *M. bovis*. Since *M. tuberculosis* infects all primate species it is also possible that this bacterium infected non human primates before it became established in human populations.

Early on, tuberculosis probably occurred as a sporadic and an unimportant disease of humans. With urbanization, the increased population density provided the necessary environmental conditions for the epidemic spread of tuberculosis (Bloom 1994). The epidemic slowly expanded world-wide because of infected Europeans travelling to

and colonising distant regions (Diamond 1992). In the 1700s and 1800s tuberculosis prevalence peaked in Western Europe and the United States and was the largest cause of death among these populations (Bloom and Murray 1992). One to two centuries later, tuberculosis had spread in full force to Eastern Europe, Asia, and South America (Daniel *et al.*, 1994).

Tuberculosis is well documented to have been in Egypt from as far back as 300 BC (Donald 1997). Reports by many reliable observers show that it is unlikely that tuberculosis occurred to any great extent among indigenous peoples of South Africa, but was rather established following the colonization of Africa by the European settlers and was then perpetuated through the rapid urbanization that followed for purposes of commerce and trade (Livingstone 1857). Besides the explosion of tuberculosis in indigenous people, the turn of the century saw a large influx of tuberculosis sufferers from Europe to South Africa. By the 1920s, tuberculosis was showing endemic features in indigenous rural population of South Africa and beyond. About that time, tuberculosis was recognized as a common health problem also affecting the black and coloured people of South Africa (Donald 1997).

2.3.2 The recent epidemic of tuberculosis in the world

In industrialized and developing countries, the steady drop in tuberculosis incidence began to level off in the mid-1980s and then stagnated or even began to increase (Fig.2.1). Much of this rise can be at least partially attributed to a high rate of

immigration from countries with a high incidence of tuberculosis. It is also difficult to perform epidemiological surveillance and treatment in immigrant communities due to various cultural differences (Harries 1994).

A great influence in the rising tuberculosis trend is HIV infection. Chances are that only one out of ten immunocompetent people infected with *M. tuberculosis* will succumb to the disease. However, among those with HIV, one in ten per year will develop active tuberculosis, while one in two or three tuberculin test positive (usually defined as an induration of >10mm in immunocompetent persons and >5mm in persons with HIV infection) AIDS patients will develop active tuberculosis. In many industrialized countries this is a tragedy for the patients involved, but these cases make up only a small minority of tuberculosis cases. In developing countries, the impact of HIV infection on the tuberculosis situation, especially in the 20-35 age. Moreover, there has recently been a disturbing increase in the number of TB cases that are caused by organisms that are resistant to the two most important drugs, isoniazid (INH), and rifampicin (RIF). A survey in 72 countries suggested that the multidrug-resistant TB is more wide-spread than previously thought and the situation is likely to worsen (O'Brien 2001).

The registered number of new cases of tuberculosis worldwide roughly correlates with economic conditions: the highest incidences are seen in those countries of Africa, Asia, and Latin America with the lowest gross national income. The WHO estimates

that about 32% of the world's population or 1.86 billion people are infected with TB. The WHO also states that every year approximately 8 million of these infected people develop active TB, and almost 3 million of these will die from the disease (O'Brien 2001). In terms of numbers of cases, the biggest burden of TB is in south east Asia, in India alone one person dies of TB every minute (WHO 2000).

While wealthy industrialized countries with good public health care systems can be expected to keep tuberculosis under control, in much of the developing world a catastrophe awaits. It is crucially important that support be given to research efforts devoted to developing an effective tuberculosis vaccine, shortening the amount of time required to ascertain drug sensitivities, improving the diagnosis of tuberculosis and creating new, highly effective anti-tuberculosis medications. Without support for such efforts, we run the risk of losing the battle against tuberculosis (WHO 1997).

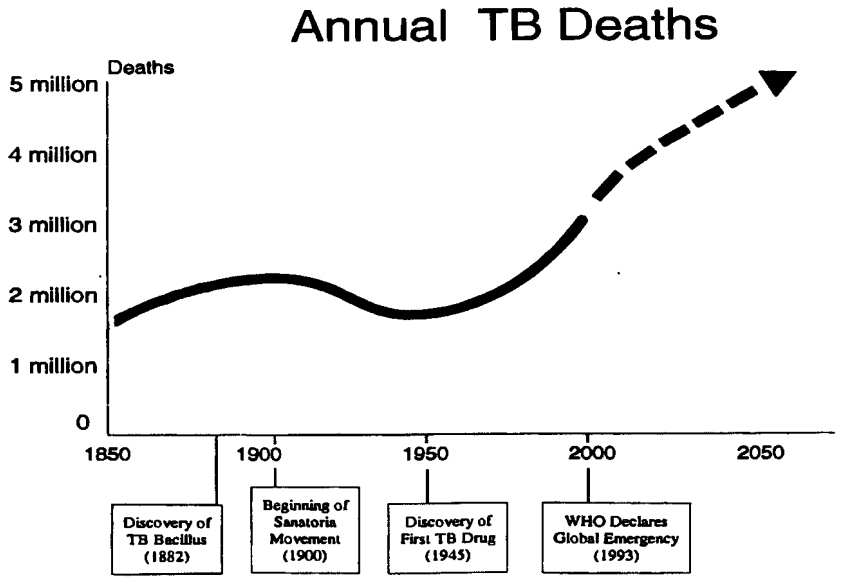

Figure 2.1: Estimated trends of mortality wold-wide due to tuberculosis into the twenty-first century (Pilheu 1998).

2.3.3 The burden of TB in South Africa

In 1997 South Africa had an estimated 180 507 cases (less than 50% reported) or 419 per 100 000 of the total population (WHO 1997). Of these patients, 32.8% (73 679 cases) were also infected with HIV. Estimates by the Medical Research Council put the expected burden of tuberculosis in South Africa for the year 2000 at 273 365 new cases, of whom 113 945 will be infectious and 46,7% will also be HIV positive. Excess cases of tuberculosis directly attributable to the HIV epidemic will amount to 123 616, or 42% of the expected total caseload. Given these figures, tuberculosis and HIV are the most important infectious disease problems facing South Africa, internationally ranking the country second only to Zimbabwe in terms of the

combined burden of these overlapping epidemics (WHO 1997).

The burden of tuberculosis is not distributed evenly throughout South Africa and rates continue to vary considerably among the nine provinces. The highest rates are recorded from the Western, Eastern and Northern Cape, where incidence figures are more than double those reported from the other provinces. Due to population size differences, the provinces most severely affected by actual patient numbers are KwaZulu-Natal (65 695 cases expected), the Eastern Cape (56 495 cases expected), and Gauteng (45 598 cases expected) (Fig 2.2) (Fourie 2000).

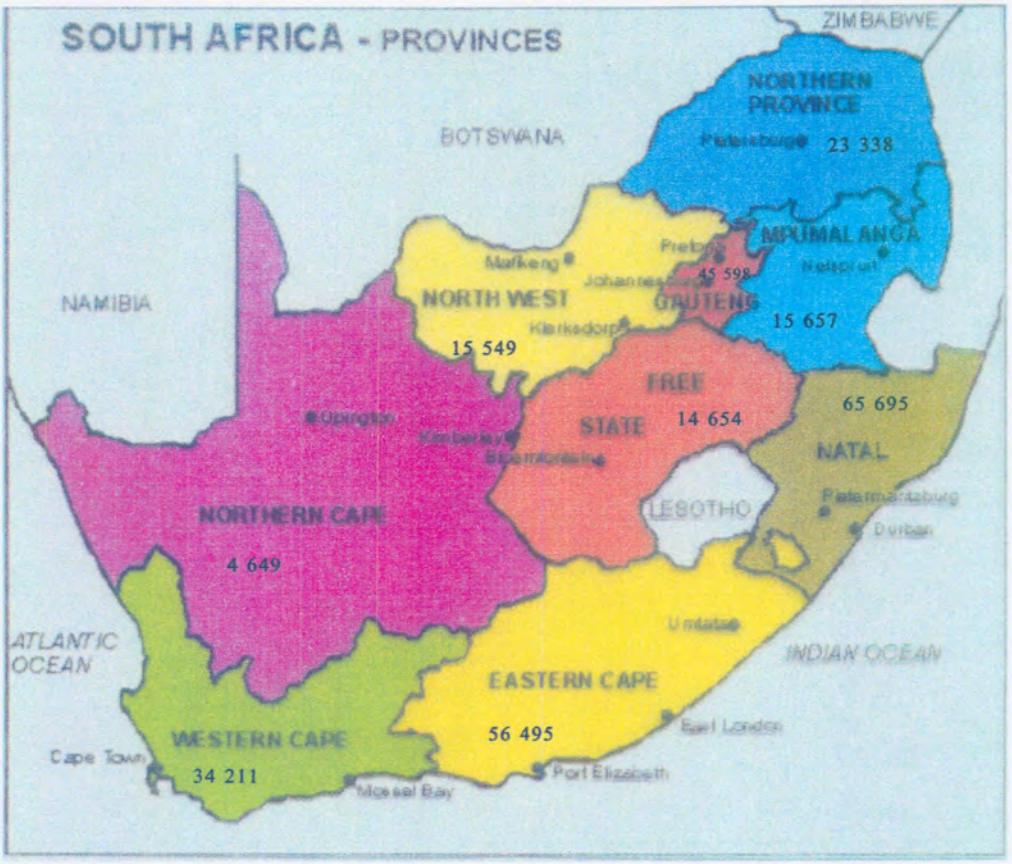

Figure 2.2: Map of South Africa showing the distribution of TB cases in all nine provinces listed as follows: Northern Province (23 338); Mpumalanga (15 657); North West (15 549); Gauteng (45 598); Free State (14 654); KwaZulu-Natal (65 659); Eastern Cape (56 495); Northern Cape (4 649); Western Cape (34 211); **Total=273 365** (Fourie 2000).

2.4 Treatment of tuberculosis

2.4.1 Chemotherapy

The backbone of TB therapy has been a set of special antibiotics that are effective primarily against mycobacteria: isoniazid, ethambutol, and pyrazidamide. The bacterial populations within lesions of human tuberculosis vary considerably in the speed with which they grow and metabolize, depending upon the particular environment of the bacilli. The bacilli located in the cavity wall are actively

multiplying because of the abundance of oxygen, whereas those located in the closed lesions deprived of oxygen are likely to be more slowly metabolizing. In this regard, different drugs act on the special populations of tubercle bacilli in the lesions (Mitchison 1979). Isoniazid kills 90% of the total population of bacilli during the first few days of treatment. It is most effective against the metabolically active, continuously growing bacilli. This population is responsible for the positive smears and cultures seen in the laboratory and most of the drug-resistant mutants that arise during or after therapy. Unlike the case with isoniazid, rifampicin can kill the semi dormant bacilli (these occasionally display short periods of active growth). Pyrazinamide kills bacilli in an acidic environment inside cells, e.g in macrophages (Mitchison 1979).

Tuberculosis drug regimens are divided into two phases: the intensive phase of treatment, during which the aim is to kill the population that are dividing, and the continuous phase, during which the aim is to kill bacilli that are more dormant. More drugs are commonly used in the intensive phase, which lasts about 2 months, than in continuation phase, which lasts 4 to 6 months. However, the use of these drugs in the continuous phase may ensure elimination of all remaining organisms, including the so-called persisters. Such treatment will prevent the relapse after completion of the treatment schedule (Bloom 1994). When exposed to a single effective anti-tuberculosis medication, the predominant bacilli, sensitive to that drug, are killed; the few drug resistant mutants, likely to be present if the bacterial population is large,

will continue to multiply (WHO 1997). Since it is very unlikely that a single bacillus will spontaneously mutate to resistance to more than one drug, giving multiple effective drugs simultaneously will inhibit the multiplication of these resistant mutants. This is why it is absolutely essential to treat tuberculosis patients with the recommended four drug regimen (Bloom 1994).

2.4.2. Sites of action and molecular mechanisms of resistance of *M. tuberculosis* to antituberculosis drugs

Most antibacterial agents inhibit biosynthetic pathways involved in the production of macromolecules (proteins, nucleic acids, or cell wall polymers) (Bloom, 1994). The mechanisms of *M. tuberculosis* resistance to many anti-TB drugs is unknown. Isoniazid (INH) is a prodrug that requires oxidative activation by the mycobacterial catalase peroxidase kat G. The active form of INH subsequently interacts with the enzymatic machinery that synthesizes mycolic acids, essential components of the cell wall. Many INH resistant organisms have mutations in the kat G gene encoding catalase-peroxidase that result in altered enzyme structure. These structural changes apparently result in decreased conversion of INH to a biologically active form (Musser 1995). Some INH resistant organisms also have mutations in the inhA locus or a recently characterized gene (kasA) encoding beta-ketoacyl-acyl carrier protein. These mutations reduce the affinity of InhA enzyme for the NADH cofactor (Quemard *et al.*, 1995). Sequence analysis of InhA in resistant clinical isolates found that most amino acid substitutions are located within the enzyme's NADH-binding site. These

substitutions may confer resistance by lowering the enzymes affinity to isonocotinic acyl-NADH or by altering the kinetic mechanism of the enzyme (Rozwarski *et al.*, 1998).

In the great majority of organisms, pyrazinamide resistance is caused by mutations in the gene encoding pyrazinamidase (pncA). This enzyme degrades pyrazinamide to a bactericidal substance, pyrazinoic acid, and the mutation results in a decrease in this activity (Ramaswamy and Musser 1998). Streptomycin is one of the family of amionoglycosides that acts by inhibiting protein synthesis. Resistance to streptomycin is due mainly to mutations in the 16SrRNA gene or the rpsL gene encoding ribosomal protein S12 (Ramaswamy and Musser 1998). Rifampicin interferes directly with the bacterial machinery for transcribing ribonucleic acid (RNA) from DNA. Its resistance is caused by mutations in the gene encoding the beta subunit of the DNA dependent RNA polymerase (rpoB) (Musser 1995). Ethambutol interferes with the construction of the arabinogalactan layer of the mycobacterial cell wall. Resistance to this drug in approximately 60% of organisms is due to amino acid replacements at position 306 of an arabinosyltransferase encoded by the embB gene (Ramaswamy and Musser 1998). The function of arabinosyl transferase is to catalyze cell wall synthesis.

2.5 Global prevalence of drug resistant tuberculosis

As mentioned in the previous section, a major factor contributing to the resurgence of tuberculosis is the emergence of multi-drug resistance. Drug resistance in tuberculosis occurs as a result of spontaneous mutations of genomic DNA (Riska *et al.*, 2000). The prevalence of drug resistant tuberculosis varies considerably throughout the world and the extent of primary resistance in a community is an important indication of the effectiveness of treatment schedules. Drug resistance (DR) and multidrug resistant (MDR) tuberculosis is a consequence of human activity and did not exist before chemotherapeutic drugs were introduced. Monotherapy with various drugs in sequence or other inadequate drug regimens have strongly contributed to the creation of MDR-TB (Petrini and Hoffner 1999). Therefore resistance to antituberculosis drugs is directly caused by poor management of tuberculosis control. It gives rise to treatment failure, relapse, further transmission of resistant tuberculosis, and multidrug-resistant tuberculosis. Patients infected with multiple drug resistant strains require prolonged chemotherapy with very expensive medication that will at best cure only half of them. Such treatment costs at least ten times as much as the cost of curing an ordinary tuberculosis patient infected with drug-sensitive bacteria (Fourie 2000).

Drug-resistant tuberculosis is increasing day by day and is a significant threat to tuberculosis control because there are only a few drugs effective against *M. tuberculosis* (Zwolska *et al.*, 2000). Information about susceptibility patterns of *M. tuberculosis* isolates against antituberculosis drugs is an important aspect of

tuberculosis control, and surveillance and analysis of local rates of tuberculosis drug resistance is helpful in the detection and monitoring of the extent of multiple drug resistant (MDR) strains, indicating the quality of tuberculosis control in the country. Knowledge of the prevalence of primary drug resistance is helpful in guiding the selection of drugs used in initial treatment of tuberculosis and early diagnosis of MDR-tuberculosis is essential to prevent its transmission in the community (Zwolska *et al.*, 2000).

2.6 The control of tuberculosis

Current TB control strategies include the following components: case finding and treatment, chemoprophylaxis, vaccination with an avirulent strain of *M. bovis* (BCG) and the improvement of socio-economic conditions (Rodrigues and Smith 1990). BCG vaccine is named after Calmette and Guérin, the discoverers of the strain (Slayers and Whitt, 1994). This tuberculosis vaccine, is usually given into the shoulder soft tissue shortly after birth and leaves a scar.

The biomedical focus for TB control concentrates on reducing the transmission of pulmonary tuberculosis by targeting the most contagious persons (sputum-positive cases), by case finding of sputum-positive TB and treating them until they become sputum-negative and less likely to transmit the disease (Hurtig *et al.*, 1999). In a study by Behr *et al.*, 1999, it is, however suggested that patients who test sputum negative for acid fast bacilli can still transmit *M. tuberculosis* (Behr *et al.*, 1999) because the

threshold for detecting bacilli on light microscopy is about 5000-10000 bacilli/ml, while the infecting dose of *M. tuberculosis* is estimated to be fewer than ten organisms (Hobby *et al.*, 1973).

WHO has developed an effective control strategy to provide short-course chemotherapy to all identified smear-positive TB cases under direct observation (DOTS) (WHO 2001). The direct observation of treatment contained within the DOTS strategy is designed to enhance patient compliance. Compliance is an important part of TB control and can be defined as the extent to which a person's health related behavior coincides with medical advice. The strategy requires that the patient take his or her medications in the presence of a health worker or other responsible third party (Snider 1982).

Although DOTS is highly effective; 82% of patients managed under DOTS in 1997 in the 22 countries with the highest TB burden were successfully treated, implementation has been slow and overall coverage low, estimated at only 28% worldwide in 1998. Moreover, DOTS is cumbersome and labour intensive, particularly because currently available anti-TB drugs require a minimum treatment duration of 6 months. Even if WHO achieves its treatment targets under DOTS by the year 2010, it will have prevented only 23% (48 million) of the TB cases predicted between 1998 and 2020 (O'Brien 2001).

2.7 Molecular epidemiology

2.7.1 Introduction

Molecular epidemiology is the integration of molecular techniques with conventional epidemiologic approaches, in order to track specific strains of pathogens towards understanding the distribution of disease in populations (van Embden *et al.*, 1992). One

of the approaches to tackling tuberculosis today is the control of transmission of the disease (van Soolingen and Hermans 1995). The demonstration of lack of transmission of drug-resistant tuberculosis is good news for chemotherapy and drug development, because it suggests that the current treatment protocol works well and that under conditions of compliance, acquisition of drug resistance is rare (van Helden and Hoal-van Helden 2000).

Phenotypic markers that distinguish between strains of *M. tuberculosis,* such as unusual antibiotic resistance patterns and mycobacterial phage susceptibility (bacteriophage typing), have long been employed in the investigation of point source outbreaks of *M. tuberculosis*. The use of anti-resistant patterns is limited by the low variability of sensitivity patterns and the fact that a sensitivity pattern may change when exposed to anti-tuberculous drugs. Bacteriophage typing is laborious, time consuming and relatively few different phage types can be distinguished (Snider *et al.*, 1984). This phenotypical method of typing poorly reflects the genetic relationship of strains (van Soolingen and Hermans 1995).

In recent years, novel approaches to studying the epidemiology of tuberculosis have been provided by molecular biological techniques based on deoxyribonucleic acid (DNA) fingerprinting (van Soolingen and Hermans 1995). DNA fingerprinting was established by the selection of genetic markers from the chromosome of *M. tuberculosis*, which allow the detection of DNA polymorphism among epidemiologically unrelated clinical isolates. The major advantage of DNA fingerprinting compared to the phage typing method is the detection of a high degree of genetic polymorphism, which has significantly increased the predictive epidemiological value. Moreover, from the similarity of fingerprints, conlusions can be drawn concerning the degree of the relationship between two *M. tuberculosis* strains (van Soolingen and Hermans 1995).

The requirements for an ideal DNA fingerprinting technique for detecting relations between strains would be the following:

1) Sensitivity: The technique should not require large amounts of material.

2) Reproducibility: Results should be reproducible or repeatable.

3) Stability: Fingerprint pattern should remain the same over an extended period.

4) Discriminatory power: It should be able to discriminate or differentiate between the strains.

5) Typeability: Should be able to type a particular organism.

6) Production of clear banding patterns suitable for computerized analysis.

2.7.2 Ribotyping

Ribosomal RNA genes have been shown to be present in multiple copies in many bacterial species. Since part of their sequence is conserved among very distantly related species, they have been used as probes for generating RFLP fingerprinting. This method, which has been named ribotyping is of limited use with pathogenic mycobacteria, since it possess a single ribosomal RNA gene copy which makes differentiation between strains difficult (Poulet and Cole 1995).

2.7.3 Pulse field gel electrophoresis

Pulse field gel electrophoresis (PFGE), in which the entire genome can be represented as a distinct pattern of DNA restriction fragments, is a particularly powerful tool in epidemiology for the determination of clonal identity of bacteria providing information for understanding and controlling the spread of disease. Application of PFGE to the study of mycobacterial diseases has been limited because isolation of high quality genomic DNA from mycobacterial sources has proved problematic.

2.7.4 Repetitive sequences in *M. tuberculosis*

2.7.4.1 Insertion sequence IS6110

The insertion sequences IS6110, IS986 and IS987, collectively referred to as IS6110, have been the most extensively used for strain differentiation (McAdam *et al.*, 1990). These three sequences differ in only a few base pairs and therefore can be considered

essentially the same element. It has been recommended to use the term IS6110 for these elements, except when referring to a specific copy (Dale 1995). Sequence comparison shows that IS6110 is related to the IS3 family of insertion elements; a family initially discovered in gram-negative bacteria (McAdam *et al.*, 1990).

The IS6110 elements have been found exclusively in the *M. tuberculosis* complex, commonly with 10-20 copies in various positions in genomes of the clinical isolates of *M. tuberculosis.* The polymorphism of IS6110 is presumed to be due to its ability to transpose within the genome with little target sequence specificity (Butcher *et al.*, 1996), therefore it might be expected that the profiles would change over time. However, the patterns appear to be stable during prolonged growth *in vivo* or *in vitro* and the development of drug resistance does not appear to alter the fingerprint (Yuen *et al.*, 1993).

Although most *M. tuberculosis* strains carry multiple copies of IS6110, a significant minority of strains carry only one or two copies (Fomukong *et al.,* 1994; Gillespie *et al.,* 1995; van Soolingen *et al.,* 1993; Yuen *et al.,* 1993). These strains show much less polymorphism, in this case the use of other probes, especially polymorphic GC-rich repetitive sequences (PGRS) and direct repeat locus (DR), enables differentiation of epidemiologically unrelated strains. Furthermore, a small number of *M. tuberculosis* isolates lack IS6110 altogether, which is especially significant in relation to the use of IS6110 as a target for PCR in DNA fingerprinting.

2.7.4.2 Insertion sequence IS1081

This 1.324bp long sequence with 15bp inverted repeat ends was discovered in 1991 (Collins and Stephens 1991). It is found in all species of the *M. tuberculosis* complex as well as in *M. xenopi* (van Soolingen and Collins, personal communication). In contrast to IS6110, IS1081 is distributed rather homogeneously in different *M. tuberculosis* strains, with five to seven copies frequently present in the same sites. Therefore, IS1081 is not a useful marker for distinguishing between strains of *M. tuberculosis*. However, because IS1081 is present on a characteristic *Pvu* II fragment in *M. tuberculosis*, it may be used to differentiate the vaccination strain BCG from other strains of *M. tuberculosis* (van Soolingen *et al.*,1992)

2.7.4.3 The direct repeat sequence (DR)

The insertion site of IS6110 in BCG is found in an unusual chromosomal region containing a variable number of directly repeated sequences (DR), 36 bp in length, interspersed with unique spacer sequences of 36 to 41 bp (Hermans *et al.*, 1992). This typing method is known as spacer oligonucleotide typing (spoligotyping) (Refer to 2.7.5.4). The number of copies of the DR sequence has been determined to be approximately 10 to 50 in a variety of *M. tuberculosis* complex strains (Cousins *et al.*, 1998). DR-based probes are valuable for differentiation of strains of *M. tuberculosis* for which IS6110 does not provide sufficient discrimination (van Soolingen *et al.*, 1993, 1994).

2.7.4.4 The major polymorphic tandem repeats (MPTRs)

Major polymorphic tandem repeats are 10-bp repeating sequences invariably separated by unique 5-bp spacer sequences. The 10-bp repeat is heterogenous in sequence, with at least 5 bases conserved. The MPTR copy number has been estimated at about 80 per genome (Hermans *et al.*,1992), although mapping studies with ordered cosmid suggest that they are confined to nine loci (Poulet and Cole 1994). Unlike the previously described elements, MPTR have been detected in species other than those of the M. tuberculosis complex, including *M. gordonae* and *M. kansasii.*

2.7.4.5 The polymorphic GC-rich repetitive sequence (PGRS)

The PGRS elements appear to be the most abundant repetitive sequences in the *M. tuberculosis* complex, occurring at 26 to 30 loci. They display significant polymorphism (Ross *et al.*, 1992; Poulet and Cole 1994; Van Soolingen *et al.*, *1993* and van Embden *et al.*, 1993), which makes them potentially useful in epidemiological studies. Several copies have been cloned and sequenced (De Wit *et al.*, 1990; Doran *et al.*, 1992; Poulet and Cole 1994; Ross *et al.*, 1992) and these vary both in size (0.7 to 1.3 kb) and nucleotide sequence, exhibiting about 70% sequence identity. A characteristic feature of PGRS is its GC-richness (about 80%). The host range of PGRS extends from the *M. tuberculosis* complex to other mycobacteria species, such as *M. kansasii*, *M. gordanae*, *M. marinum, M. microti, M. gastri and M. szulgai* (Poulet and Cole 1994; Ross *et al.*, 1992). MTPR and PGRS elements share some

similarities in their nucleotide consensus sequence, host range and high copy number (Doran *et al.*, 1992; Ross *et al.*, 1992)

2.7.5 PCR- based methods

Numerous amplification-based methods are being developed for typing mycobacteria, as they do not require the extraction of large amounts of DNA and potentially enable typing to be done from clinical strains.

There are many different DNA fingerprinting techniques which have been used to study the transmission of the disease, random amplified polymorphic DNA (RAPD), amplified ribosomal DNA restriction analysis (ARDRA), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and spoligotyping (Koeleman *et al.*, 1998).

2.7.5.1 Random Amplified Polymorphic DNA (RAPD)

Although RAPD offers the advantages of simplicity and rapidity in distinguishing *M. tuberculosis* strains, a lack of reproducibility has been reported due to its high susceptibility to variation by primer and DNA concentration, DNA template quality, gel electrophoresis, and the type of DNA polymerase used (Rodriguez *et al.*, 2000).

2.7.5.2 Amplified Fragment Length Polymorphism (AFLP)

One of the newest and most promising methods is amplified fragment length polymorphism (AFLP) analysis which combines universal applicability with high powers of discrimination and reproducibility (Janssen *et al.*, 1996).

An increasing number of reports describe the use of AFLP analysis for plant and animal genetic mapping, medical diagnostics, phylogenetic studies and microbial typing. The patterns obtained from different strains are polymorphic due to (i) mutations in the restriction sites, (ii) mutations in the sequences adjacent to the restriction sites and complementary to the selective primer extensions, and (iii) insertions or deletions within the amplified fragments (Wilson *et al.*, 1998). AFLP overcomes many of the problems, the advantage of using it is that it is based on the total chromosome, has high discriminatory power, reproducibility and production of clear banding patterns suitable for computer analysis.

2.7.5.3 Spoligotyping

Spoligotyping analyzes a single locus to characterize tuberculosis strains (Kamerbeek *et al.*, 1997; van Soolingen and Hermans 1995). The method relies on the in vitro amplification of the DNA sequence of the highly polymorphic direct repeat (DR) locus in the chromosome of the mycobacteria of the *M. tuberculosis* complex (Refer. to 2.7.4.3). This region was described flanking an IS6110 sequence (Hermans *et al.*, 1990). It has a characteristic organization, with conserved 36-bp DR sequences

interspersed with variable spacers. The polymorphism is carried by these spacers, which are variable in length and sequence. The number of DR spacers is also variable. Although these spacers vary, each is common to a group of strains. This technique tests which spacers from a spacer catalog are present in the strains studied and allows each strain to be characterized by its spacers (Goguet de la Salmoniere *et al.*, 1997).

To study the potential of AFLP fingerprinting, *M. tuberculosis* clinical isolates were first typed by RFLP as described in Chapter 3 and thereafter by AFLP as described in Chapter 4 and results were evaluated. In Chapter 5 the prevalence of Isoniazid (INH) in the Eastern-Cape and North-West Province is discussed.

CHAPTER 3. CHARACTERIZATION OF MYCOBACTERIAL TUBERCULOSIS STRAINS USING RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

3.1 Introduction

Over the past few years the use of modern molecular biological techniques to determine the degree of sequence conservation between bacterial genomes has led to the development of methods solely based on the detection of naturally occurring DNA polymorphisms for studies of molecular epidemiology.

Strain differentiation by DNA restriction fragment length polymorphism (RFLP) has been used for studies of the epidemiology of *Mycobacterium tuberculosis* infections. Among the genetic elements that have been found to contribute to DNA restriction fragment length polymorphism in *Mycobacterium tuberculosis*, the insertion element IS6110 has been studied most extensively. The variability in both the number and genomic position, generate strain specific patterns. RFLP is a technique which enables distinct "fingerprints" which reflects the genetic constitution of the organism in question (van Embden *et al.*, 1993).

The development of restriction fragment length polymorphism analysis for the identification of mycobacteria has made it possible to evaluate RFLP analysis as an epidemiological tool. Using the IS6110 insertion sequence fragment, which is specific

to the *M. tuberculosis* complex, as a probe, variations among *M. tuberculosis* have been detected (Hermans *et al.,* 1990; Thierry *et al.,* 1990). A number of IS6110 sequences are scattered in the *M. tuberculosis* genome where they are arranged differently from strain to strain. Studies using (RFLP) analysis have shown that some *M. tuberculosis* strains cause disease in many patients, whereas others do not (Small *et al.*, 1994). This difference is generally attributed to characteristics of the source case and to environmental factors (Zhang *et al.*, 1999).

The objective of this study was to determine the extent of transmission of tuberculosis in a referral hospital (Nkqubela Hospital) situated in the East London district of the Eastern Cape Province, in South Africa by differentiating *M. tuberculosis* isolates using RFLP technique and also to compare the potential of RFLP and a PCR based technique (discussed in Chapter 4) in differentiating isolates. The ability of RFLP in differentiating *M. tuberculosis* isolates is limited because of the instability of insertion element IS6110 caused by its transposition (van Soolingen *et al.*, 1995). If IS6110-based genotypes change rapidly, it would obscure epidemiological links and underestimate transmission. On the other hand, if genotypes change too slowly, then IS6110-based genotypes RFLP analysis would link cases that are only distantly related, estimating transmission (Yeh *et al.*, 1998).

Most strains showed a high copy number of the IS6110 insertion element and 43 distinct patterns were identified. Eleven isolates were classified into four clusters of

identical banding patterns. One strain of *M. tuberculosis* was found which harboured only a single copy of this element and according to van Soolingen and Hermans (1995), strains with one to five copies of IS6110 element are not typable by IS6110 fingerprinting. An alternative typing method as proposed by Wilson was used to confirm low-copy-number isolates that were identified in this study (Wilson *et al.*, 1998).

3.2 MATERIAL AND METHODS

3.2.1 Bacterial strains used

Clinical isolates of M. tuberculosis were obtained from Nkqubela hospital in East London in the Eastern Cape province of South Africa. For all the isolates, the following information was recorded about the patient from whom the strain was obtained: (1) geographical region; (2) race; (3) previous tuberculosis history; (4) known contacts; (5) clinical specimen (sputum, blood, etc.); (6) pathology (pulmonary tuberculosis, non-pulmonary tuberculosis, etc) (Table 3.1).

3.2.2 DNA fingerprinting

The technique of fingerprinting entails the growth of M. tuberculosis, extraction of DNA, restriction endonuclease digestion, Southern blotting of the digested fragments and probing with the IS element (van Soolingen *et al.*, 1994; Bendall *et al.*, 1995). These different stages in methodology are discussed below:

3.2.2.1 Growth

All strains were grown on Lowenstein-Jensen agar slants, then subcultured to 7H11 agar plates and, after three to four weeks of growth, the plate cultures were used to inoculate 50ml of Middlebrook 7H9 broth. These cultures were grown for three weeks.

3.2.2.2 Extraction of DNA

Colonies were scraped from L-J slopes and harvested into 0.5 ml of lysis buffer (15% sucrose, 0.05 M Tris pH 8.5, 0.05 M EDTA, 1mg/ml D-cycloserine). After SDS and proteinase K were added to a final concentration of 2% and 100 ug/ml respectively, the solution was incubated for 24 hours at 37°C and 30 minutes at 80°C. The DNA was purified by two extractions with lysate solution (1:1v/v) and once with chloroform alone.

The DNA was then precipitated by adding 0.1 volume of 3 M sodium acetate, pH 5.5 and 2 volumes of absolute ethanol to the aqueous phase. After overnight incubation at -20°C, precipitated DNA was recovered by centrifugation at 10 000 rpm for 15 minutes. The pellet was washed with 1 ml of cold 70% (v/v) ethanol to remove excess salt and recentrifuged. The supernatant was discarded and the pellet air-dried. The dried DNA was then resuspended in 40 ul of TE buffer, pH 8.0 (10 mM Tris-HCl pH 8.0; 1 mM EDTA, pH 8.0).

Table 3.1 Strains of *Mycobacterium tuberculosis* used in this study.

Lab ref. number	Age	Sex	Town/village
106	90	M	EAST.LONDON
145	N/A	F	MDANTSANE
149	N/A	M	N/A
153	49	M	MDANTSANE
159	19	M	MDANTSANE
164	29	M	NGQAMAKWE
487	23	M	MDANTSANE
488	28	M	BERLIN
491	25	F	MDANTSANE
500	24	M	MDANTSANE
502	28	M	K.W.T
577	35	F	MDANTSANE
578	23	F	K.W.T
580	24	F	ALICE
581	70	F	CHALUMNA
582	41	M	ALICE
583	59	M	MDANTSANE
586	45	M	MDANTSANE
588	37	M	K.W.T
590	48	M	MDANTSANE
591	37	M	NDEVANA
595	40	F	MDANTSANE
600	21	F	MDANTSANE
603	38	M	K.W.T
604	32	M	MDANTSANE
605	26	M	DIMBAZA
774	45	M	K.K.HOEK
780	30	M	MDANTSANE
782	44	M	K.W.T
792	50	F	N/A
793	66	F	KOMGA
916	43	M	DUNCAN VILLAGE
917	30	M	KWELERANA
919	54	M	ZWELITSHA
922	44	M	GINSBERG
933	31	M	MDANTSANE
934	28	M	N/A
936	38	M	MDANTSANE
5844	34	F	BUTTERWORTH
5948	32	F	MDANTSANE
5949	17	F	MDANTSANE
5950	19	F	K.W.T
5953	55	M	MOOIPLAAS
5954	42	M	K.W.T
5955	45	M	NGQAMAKWE
5961	22	F	ALICE

Definition of abbreviations: F=female, M=male, N/A=not available, CL=cluster, EL=East London, KWT=King Williams Town.

3.2.2.3 Purification of DNA

Dialysis membrane was immersed in boiling water with 5% sodium bicarbonate and then cooled down in cold water. To purify the DNA the membrane was filled with DNA, cut into appropriate sizes and tied at both ends. Dialysis was carried out overnight in 2 liters of water.

3.2.2.4. Restriction endonuclease digestion

Approximately 1 µg of DNA was digested overnight at 37 degrees with 10 units of *Pvu* II in a buffer supplied by the manufacturer (Boehringer Mannheim, Germany).

3.2.2.5 Separation of fragments by electrophoresis

The digested fragments were separated by electrophoresis through 1% agarose gel (20 cm gel) in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8). The gel was run at 1.5 V/cm for 16-24 hours at 4 degrees centigrade. Digoxigenin-labelled DNA marker III (Boehringer Mannheim, Germany), a mixture of 13 fragments obtained by digesting phage Lambda DNA with *Eco*RI and *Hind* III) was used to estimate the sizes of the separated fragments. These are 21.226, 5.148, 4.973, 4.268, 3.530, 2.027, 1.904, 1.584, 1.375, 0.947, 0.831, 0.564 and 0.125 kilobase pairs each in size (Boehringer Mannheim, Germany).

3.2.2.6 Southern blotting

Prior to transferring the DNA to the Hybond-N nylon membrane (Amersham Life Sciences, Germany), the gel was treated in depurination solution (0.25M HCl) for 15 minutes, denaturation solution (0.5 M NaOH, 1 M NaCl) for 30 minutes and finally in neutralising solution (0.5 M Tris-HCl, 1.5 M NaCl, pH 7.4) for 30 minutes. The Hybond-N membranes for Southern blotting were prepared according to the manufacturer's recommendation (Amersham Life Sciences, Germany) and the DNA was transferred overnight by capillary action using 20XSSC (3 M NaCl, 0.3 M Na citrate) as transfer buffer. After blotting, the nucleic acids were fixed to the membrane by baking at 80 ^{o}C for 30 minutes.

3.2.2.7 Synthesis of the probe

A 245bp probe was prepared by PCR from IS6110 using primer 1 (5'-CGTGAGGGCATCGAGGTGGC-3) and primer 2 (5'GCGTAGGCGTCGGTGACAAA-3) that corresponds to base-pair 631-650 and 856-875 of the 25 IS6110 insertion, respectively (van Soolingen *et al.*, 1994) as shown in Fig. 3.1. The amplification reaction was performed in a volume of 50 µl containing 5 µl of 10x stock solution of PCR buffer (20 mM Tris-HCl, 100 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA), 200 µM dNTP, 100 pmol of primer 1 and 2, 2.6 U of Taq polymerase enzyme (Roche Diagnostics, Germany) and 5 µl of template DNA.

The cycling parameters of denaturing-annealing-synthesizing cycles were as follows: a 10-min denaturation step at 95^0C (one cycle), followed by 25 cycles of denaturation at 94^0C for 1min, annealing at 68^0C for1.5-min, and extension at 72^0C for 1 min. This was followed by a final extension at 72^0C for 30 min. The probe was non-radioactively labelled using a digoxigenin labelling system as described by the manufacturer (Boehringer Mannheim, Germany).

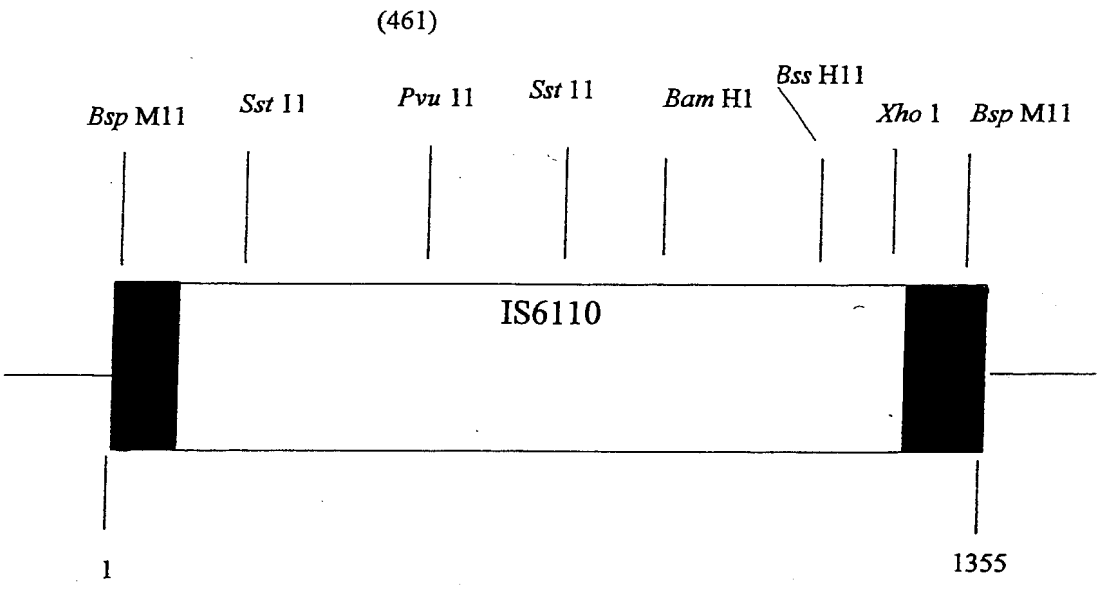

Figure 3.1: Restriction map of the.35-kb *M. tuberculosis* insertion element IS6110. The closed bars represent the 28bp inverted repeats bordering IS6110 DNA. The lines to the left and right denote chromosomal DNA (van Embden *et al.*, 1993).

The cleavage sites of several restriction enzymes on IS6110 are depicted in Fig 3.1. *Pvu* II cleaves the element at a base pair 461. Therefore, any chromosomal mycobacterial DNA fragment obtained by the recommended standard typing method is larger than 0.9kb because the probe used for this study is to the right of the *Pvu* II site on the physical map.

3.2.2.8 Recovery of Probe

The probe was run on a 2% agarose gel and recovered by removing and purifying DNA from the gel using the Geneclean 11 Kit manufactured by Bio 101, USA. A

volume of NaI stock solution, 3x the weight of the DNA containing gel slice, was added and incubated at 45°C to 55°C for 5 minutes to dissolve agarose. After adding 40 µl of glassmilk suspension, the solution was mixed and placed at room temperature for 5 minutes to allow binding of the DNA to the silica matrix. The glassmilk/DNA complex was pelleted for 5 seconds and the supernatant discarded. The DNA was washed 3 times with the New Wash solution, ensuring resuspension of the pellet by pipetting back and forth while digging into the pellet. After spinning the tube for 5 seconds, 40 µl of water was added to the DNA.

3.2.2.9 Probing with IS6110 element

The membrane was prehybridized for at least 2 hours at 68°C in a hybridisation solution containing 5X SSC; 1% w/v Blocking reagent (Amersham Life Sciences, Germany), 0.1% N-lauroylsarcosine, and 0.2% sodium dodecyl sulfate (SDS). After prehybridisation heat-denatured DNA probe was added and the membrane was incubated overnight at 68°C. Following hybridisation the membrane was washed twice for 5 minutes at room temperature with 2XSSC, 0.1% SDS and twice for 15 minutes at 68°C with 0.1x SSC, 0.1% SDS.

3.2.2.10 Detection

After post hybridisation washes the membrane was equilibrated in buffer1 (100 mM Tris-HCl,150 mM NaCl at pH 7.5) for one minute. The membrane was then blocked by agitating it in buffer 2 (0.5g blocking reagent, 100 ml buffer1) for 30 minutes.

Buffer 2 was removed and the membrane incubated in antibody solution (8 ul Anti DIG alkaline phosphatase and 40 ml buffer 2 for 30 minutes. The membrane was washed twice in buffer 1 to remove unbound antibodies. Thereafter the membrane was put in buffer 2 for two minutes and in colour substrate solution (NBT solution and X -phosphatase in buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$ at pH 9.5). The membrane was then blocked with buffer 4 (10 mM Tris-HCl and 1 mM at pH 8) for 5 minutes.

3.2.2.11 Computer- assisted analysis of DNA fingerprints

Blot images were first scanned into a computer using Agfa T1200 scanner. Digital images were normalized by alignment of RFLP patterns to a reference marker included in the first and last lane of the gel. The bands were automatically assigned to each track by GelCompar 4.0 software (Applied Maths, Kortrijk, Belgium). The fingerprint patterns were analyzed for similarity by using the Dice coefficient, and the unweighted-pair group method using average linkages (UPGMA) was used to cluster the patterns. Band positions were determined by using the peak finder of the GelCompar software and were controlled manually by comparison with the original IS6110 fingerprint blots.

Table 3.2 Results of drug susceptibility tests and IS6110 DNA fingerprinting of *M. tuberculosis* isolates from Nkqubela Hospital in East London.

Lab ref. number	Age	Sex	Town/village	Susceptibility tests	TB History	IS6110 Copy Number	Cluster	Family
106	90	M	EAST.LONDON	SUSCEPTIBLE	NEW	4	CL C	F5
145	N/A	F	MDANTSANE	SUSCEPTIBLE	NEW	9		F3
149	N/A	M	N/A	N/A	N/A	16		F4
153	49	M	MDANTSANE	SUSCEPTIBLE	R	14		F4
159	19	M	MDANTSANE	N/A	NEW	5	CL D	F5
164	29	M	NGQAMAKWE	N/A	NEW	13		F4
487	23	M	MDANTSANE	SUSCEPTIBLE	R	10		
488	28	M	BERLIN	N/A	NEW	9		F2
491	25	F	MDANTSANE	SUSCEPTIBLE	NEW	9		
500	24	M	MDANTSANE	N/A	NEW	4	CL C	F5
502	28	M	K.W.T	SUSCEPTIBLE	R	11		F4
577	35	F	MDANTSANE	SUSCEPTIBLE	NEW	12		F3
578	23	F	K.W.T	SUSCEPTIBLE	NEW	8		F3
580	24	F	ALICE	INH+, RIF-, ETH	NEW	16	CL A	F4
581	70	F	CHALUMNA	SUSCEPTIBLE	R	12		F4
582	41	M	ALICE	SUSCEPTIBLE	NEW	13	CL B	F4
583	59	M	MDANTSANE	SUSCEPTIBLE	NEW	11		F1
586	45	M	MDANTSANE	SUSCEPTIBLE	NEW	19		F4
588	37	M	K.W.T	SUSCEPTIBLE	R	13		F4
590	48	M	MDANTSANE	SUSCEPTIBLE	NEW	14		F4
591	37	M	NDEVANA	SUSCEPTIBLE	R	8		F3
595	40	F	MDANTSANE	SUSCEPTIBLE	NEW	10		F3
600	21	F	MDANTSANE	ALL RESISTANT	NEW	11		
603	38	M	K.W.T	INH+, RIF-, ETH	NEW	2		F4
604	32	M	MDANTSANE	N/A	NEW	12		F4
605	26	M	DIMBAZA	SUSCEPTIBLE	R	11		F2
774	45	M	K.K.HOEK	ALL RESISTANT	R	14		F4
780	30	M	MDANTSANE	N/A	NEW			
782	44	M	K.W.T	CULTURE+I	NEW	13		F4
792	50	F	N/A	SUSCEPTIBLE	NEW			
793	66	F	KOMGA	N/A	R	16		
916	43	M	DUNCAN VILLAGE	SUSCEPTIBLE	NEW	1		
917	30	M	KWELERANA	SUSCEPTIBLE	NEW	16	CL A	F4
919	54	M	ZWELITSHA	INH+, ETH-, RIF	NEW	14		F4
922	44	M	GINSBERG	SUSCEPTIBLE	R	14		F4
932	29	M	DIMBAZA	INH+, RIF-, ETH-	R	4	CL C	F5
933	31	M	MDANTSANE	SUSCEPTIBLE	NEW	11		F4
934	28	M	N/A	N/A	N/A	10		
936	38	M	MDANTSANE	N/A	NEW	10		F3
5844	34	F	BUTTERWORTH	N/A	NEW	5	CL D	F5
5948	32	F	MDANTSANE	INH+, RIF-, ETH-	NEW	4		
5949	17	F	MDANTSANE	SUSCEPTIBLE	NEW	14	CL B	F4
5950	19	F	K.W.T	INH+, RIF-, ETH-	NEW	14		F4
5953	55	M	MOOIPLAAS	SUSCEPTIBLE	R	5	CL D	F5
5954	42	M	K.W.T	N/A	NEW	6		F1
5955	45	M	NGQAMAKWE	SUSCEPTIBLE	NEW	4	CL C	F5
5961	22	F	ALICE	SUSCEPTIBLE	NEW	9		F1

Definition of abbreviations: F=female, M=male, N/A=not available, CL=cluster, EL=East London, KWT=King Williams Town, R=retreatment and - = negative and + =positive

3.3 RESULTS

The DNA fingerprints of 47 *M. tuberculosis* isolates from tuberculosis patients available for testing were analyzed by RFLP using IS6110 as a probe. The copy number of IS6110 in each of the isolates was determined from the number of bands hybridizing with the probe. It was found that most isolates possessed more than four copies of IS6110 genome and only one isolate carried a single copy of the insertion sequence. The occurrence of IS6110 varied from 1 to 19 with most isolates containing 14 copies (Table 3.2).

The similarities of all DNA patterns were calculated by computer analysis. Dendograms were constructed to show the degree of relatedness among strains (Fig 3.2). In Table 3.2 different isolates displayed identical RFLP patterns. About 72.3% (34 isolates) of strains with non-identical RFLP patterns showed common band patterns, including the position and grouping of bands (Table 3.3). These strains were therefore grouped into families F1-5 (Table 3.2), such that all the members of each family were identical or differed by only one to three bands while still maintaining the general band structure of the fingerprint. Grouping at the family level was based on band pattern identity greater than 70%, based on band sharing between strains (Warren *et al.*, 1999). Only two isolates (934 and 916) were not attached to any family. F1 had four isolates, the smallest family (F2) contained two isolates, F3 and F5 both had seven isolates each and the largest family (F4) comprised 21 isolates.

A cluster of *M. tuberculosis* isolates was defined as two or more isolates which exhibited the same number of copies of IS6110 fragments of identical molecular weight (i.e 100% identical) (Wilkinson *et al.*, 1997 and Warren *et al.*, 1999). Four such clusters were identified and numbered (CL A-D). Eleven (23.4%) isolates were in clusters (Table 3.3), the largest cluster (CL C) contained four isolates; CL D contained three isolates; and the other two clusters (CL A and CL B) each contained two isolates. Cluster A and Cluster B fell into Family 4, Cluster C and Cluster D fell into Family 5 (Fig. 3.2). Of the 11 isolates in clusters, 3 (27.3%) had unknown susceptibility to the four drugs tested, 6 isolates were susceptible and 2 were resistant to INH alone.

Among the 36 nonclustered isolates, 9 had unknown susceptibility, 5 exhibited drug resistance, with 2 showing multiple drug resistance. Twenty-one isolates were susceptible to the four drugs tested.

3.3.1 IS6110 RFLP clustering

3.3.1.1 Correlation by gender and age (Table 3.2)

Cases in male patients (72.7%) far exceeded those in female patients (27.3%). Younger patients (0-44 years) were strongly associated with clustering. Isolates from patients within this group belonged to a cluster more frequently than older patients (63.6% vs 36.4%).

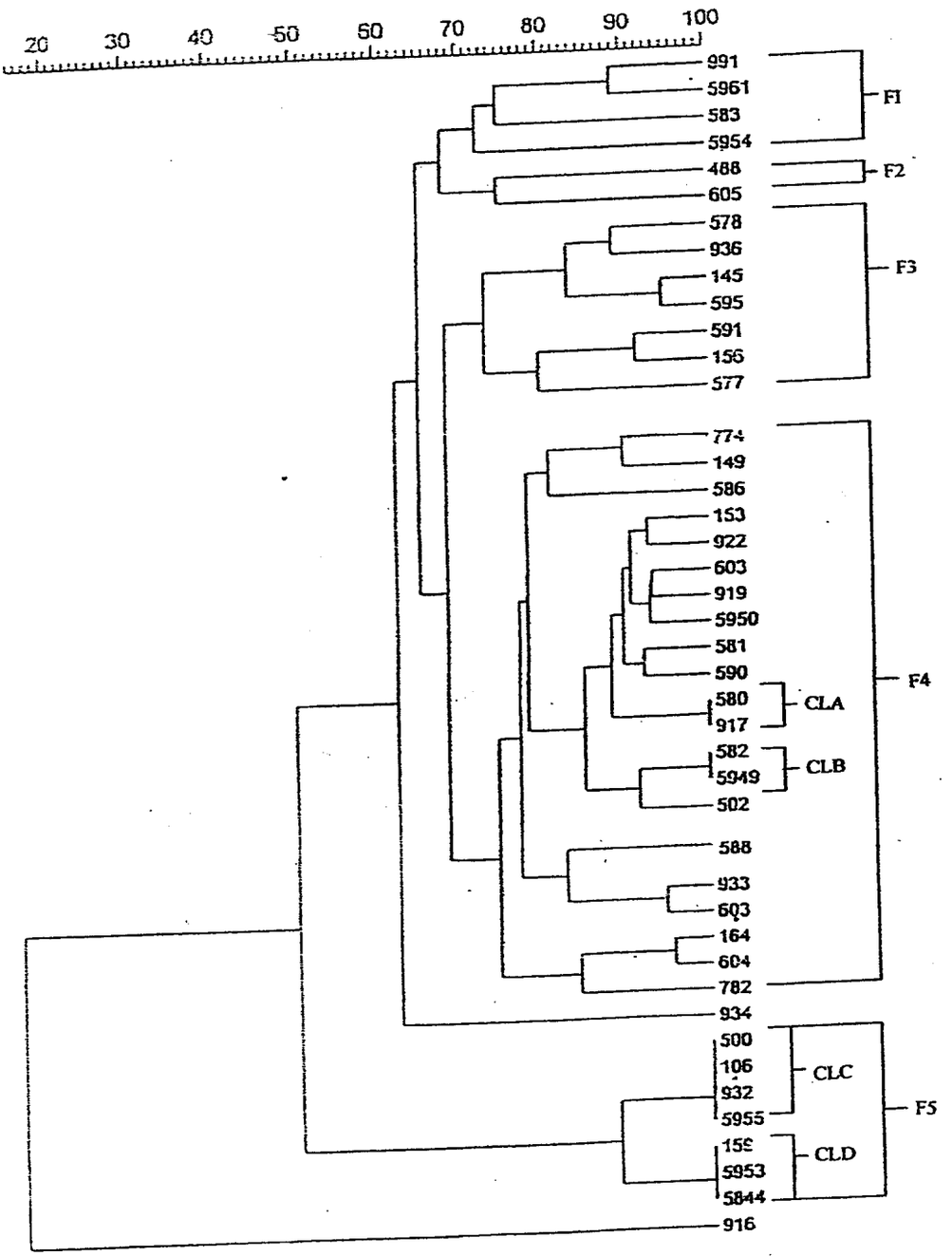

Figure 3.2: Dendogram based on computer comparison of DNA fingerprints of 47 isolates obtained from Nkqubela Hospital. Banding patterns are ordered by similarity. The position of each IS6110 band is normalized so that banding patterns of all strains are mutually comparable.

3.3.1.2 Epidemiological investigation of the RFLP clusters

There was no apparent connection detected in Cluster C (CL C) which consisted of 4 cases, except that they were all male patients and that the relevant patients lived in separate areas. Cluster D (CL D) consisted of three patients which were not connected to each other. There was no correlation between the two patients in Cluster A (CL A) and the two cases in Cluster B (CL B) were new TB cases and both susceptible to the drugs.

Table 3.3 Number of clinical isolates and patterns obtained by RFLP analysis using IS6110 probe.

	Total	**Percentage**
Isolates with IS6110 based fingerprint.	47	100%
Isolates with identical patterns grouped in different clusters.	11	23.4%
Isolates in groups of families	34	72.3.%
Isolates not in groups of families	2	4.3%

Cluster analysis was based on 100% identity.

3.4 DISCUSSION

In order to differentiate *M. tuberculosis* isolates, RFLP patterns of 47 randomly selected isolates of *M. tuberculosis* were analyzed and clustering was considered indicative of recent transmission tuberculosis in this area. Of the 47 cases studied, 11 fell into 4 clusters that consisted of 2 to 4 patients. Clusters may represent recent transmission, previous outbreaks with reactivation of cases, or unrelated organisms with similar fingerprint patterns. Two of the clustered cases were retreatment patients which resulted from reactivation of infection and 9 resulted from recently transmitted tuberculosis. The total amount of clustering, defined by Small *et al.*, (1994) as [total no. of strains in clusters]-[no. of clusters]/total no. of strains, was 11-4/47=14.9%.

It has been reported by Glynn and colleagues (1999) that as the proportion of TB cases in the population which is included in the study decreases, the proportion of isolates identified as clustered decreases. The extent of underestimation of clustering was also found to be inversely related to cluster size, i.e it is large if underlying cluster sizes are small.

Samples that grouped in clusters were from patients that lived in the same district but not at the same address. Male patients were mostly affected and more likely to belong to clusters than female patients. More than one drug susceptibility pattern was observed among the individual isolates belonging to the same cluster. One cluster contained only drug susceptible isolates and three clusters included either isolates

resistant to one drug or susceptible to all of them. In three isolates belonging to two clusters there was no information available about their drug susceptibility profile. These findings suggest a relatively low transmission of drug resistant strains in the study area.

It is possible that the patients with "unique" isolates of *M. tuberculosis* were infected by sources outside the geographical or temporal catchment of the study. It is likely that some strains more easily establish themselves by virtue of their transmissibility, virulence and resistance to treatment. In this chapter it was shown that the percentage of isolates that contained less than 5 copies of the IS6110 element, was 20%. For these, a secondary typing method was used to further differentiate the different isolates (Chapter 4).

CHAPTER 4. COMPARISON OF THE TYPING EFFICIENCIES OF AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP) AND RFLP (CHAPTER 3).

4.1 Introduction

In recent years the range of molecular techniques available for epidemiological fingerprinting has expanded and there are now many genotypic methods that allow high levels of discrimination between bacterial strains. Restriction Fragment Length Polymorphism (RFLP) as described in chapter 3 is based on IS6110 insertion element and has become the standard method for fingerprinting of *M. tuberculosis*. RFLP disadvantages are that it requires large amounts of genomic DNA, defined nucleic acid probes and laborious hybridization procedures. Since the discriminatory power of IS6110 fingerprinting for strains harboring only one to five copies of IS6110 is poor, it has been proposed that all low copy-number strains should be confirmed by an alternative typing method, e.g AFLP (Yang *et al.*, 1996).

A novel PCR-based DNA fingerprinting technique, AFLP, which was originally developed for plant breeding purposes (Vos *et al.*, 1995), overcomes many of the problems of various other typing methods, including discriminatory power, flexibility and reproducibility. AFLP has recently been used in the genotyping of different bacterial species and strains such as *Acinetobacter, Aeromonas, Xanthomonas, Helicobacter pylori* and *Pseudomonas*. In addition, AFLP has proved to be a highly useful taxonomic tool for the differences of genomic groups in *Aeromonas* (Janssen *et*

al., 1997). Recently, Goulding and co-workers (Goulding *et al.*, 2000) determined the value of fluorescent AFLP for genetic analysis of *M. tuberculosis* and concluded that their methodology can be used in conjunction with IS6110 RFLP typing to further unravel the epidemiology and evolution of this species.

The AFLP technique is based on the principles of restriction fragment length polymorphism analysis and PCR amplification. AFLP involves ligation of the adapter molecules to restriction enzyme fragments which subsequently serve as primer binding sites for PCR amplification. The amplification process is selective since the AFLP primers contain one or more nucleotides on their 3′-end; thus only a subset of the restriction fragments will be amplified. The subset of amplified fragments are then analyzed by denatured polyacrylamide gel electrophoresis to generate the fingerprint (Gibco BRL: Life Technologies, USA). The combination of different restriction enzymes and the choice of selective nucleotides in the primers for PCR make AFLP a useful new system for typing of microorganisms (Lin *et al.*, 1996).

Since relatively small amounts of DNA are digested and detection of AFLP fragments does not depend on hybridization, partial digestion and faint patterns which are sources of irreproducibility with RFLP, can easily be avoided with AFLP (Vaneechoutte 1996). Furthermore, the possibility of using stringent PCR annealing temperatures renders the AFLP analysis method more reproducible and robust than other genotyping methods (Blears *et al.*, 1998). However, AFLP also exploits the variation in the nucleotides that

match the selective 3' ends. Most important is that AFLP analysis displays more fragments than other fingerprinting techniques because it determines whole-genome polymorphisms of the organism (Vaneechoutte 1996).

Typing and identification using AFLP can be standardised by defining windows of similarity. Patterns with 90 to 100% identity are considered to be derived from identical strains, patterns with 60 to 90% identity indicate different strains of the same species, while 40 to 60% identity is obtained with isolates of the same genus but of different species. Less than 40% identity denotes isolates from different genera (Vaneechoutte 1996).

In this study we have investigated the efficacy of AFLP in confirming the validity of fingerprinting patterns which have been generated through IS6110 RFLP. When a total of 21 *M. tuberculosis* clinical strains (previously typed using IS6110 probe) (Table 4.1), *M. tuberculosis* H37Rv and *M. vaccae* were analyzed with AFLP method, two different groups could be identified based on the banding patterns observed.

4.2 Material and Methods

M. tuberculosis H37Rv was used as a model to identify primers and to optimize PCR conditions. A primer kit (GIBCO BRL Life Technologies) which contained *Eco*RI and *Mse*I restriction enzyme adapters, PCR primers and primer mixture was used in performing AFLP reactions and the reactions were performed following the protocol

in the GIBCO BRL AFLP System II manual. The entire reaction was electrophoresed on a 1.5% agarose gel in 1X TAE buffer. Gels were stained with ethidium bromide and visualized with UV illumination.

4.2.1 Bacterial cultures

All bacterial cultures used in this study (Table 4.1) were grown on Lowenstein Jensen media at 37°C. Culture stocks were stored in 15% glycerol at -70°C.

4.2.2 Restriction endonuclease digestion, and ligation of adapters

M. tuberculosis H37Rv DNA was isolated as described in section 4.2.4.1. The DNA was digested at 37°C for 2 h with *Eco*RI/*Mse*I enzyme in the 1x reaction buffer provided with the enzyme (final concentration of 50 mM Tris-HCl pH 7, 50 mM Mg-acetate, 250 mM K-acetate) added in a final volume of 25 µl. The enzyme was inactivated by incubating the mixture at 70°C for 15 min. Digested DNA was used in a ligation reaction containing 24 µl adapter ligation solution (*Eco*RI/*Mse*I adapters, 0.4 mM ATP, 10 mM Tris-HCl pH 7.5, 10 mM Mg-acetate, 50 mM K-acetate), 1 µl T4 DNA ligase (1 U/µl in 10 mM Tris-HCl pH 7.5, 1 mM DDT, 50 mM KCl, 50% glycerol v/v) in a final volume of 50 µl held at 20°C for 2 h. The complementary oligonucleotide sequences used for the adapter are shown in Table 4.2.

4.2.3 PCR primers and Optimization of PCR for AFLP analysis

The two primers used in the PCR are shown in Table 4.2. Pre-amplification reactions were performed in a total volume of 51 µl containing 5 µl of DNA, 2.7 µl of 10µM stock solution of primer *Eco*RI, 12 µl of 10µM stock solution of primer *Mse*I, 5 µl of 10x PCR buffer with 5 mM $MgCl_2$, and 1 µl (1 U/µl) of Taq polymerase (Roche Diagnostics, Germany). The amplification cycles were 20 cycles of 94° C for 30 s, 56° C for 60 s and 72° C for 60 s. A 1:50 dilution was performed and both unused diluted and undiluted reactions stored at -20°C.

Selective amplification was performed by preparing "Mix 1" and "Mix 2". Mix 1 contained 5 µl of selective *Eco*RI (O, A, C, T and G,) and 45 µl *Mse*I (O, A, C, T and G) of different primer combinations. Mix 2 contained 20 µl of 10x PCR buffer plus Mg and 1ul (5 U/µl) of Taq DNA polymerase added to a total volume of 100 µl. The AFLP amplification was assembled by combining 5 µl of Mix 1, 10 µl of Mix 2 and 5 µl of DNA template to a total volume of 20 µl. The PCR conditions were an initial 12 cycles of 94° C for 30 s, 65° C (decreasing to 60° C after 6 cycles) for 30 s, and 72° C for 60 s followed by 23 cycles of 94° C for 30 s, 56° C for 30 s and 72° C for 60 s and a final extension for 5 min at 72°C. Amplified fragments were separated by electrophoresis in a 1.5% agarose gel in TAE buffer overnight at 40V and were stained with ethidium bromide.

Table 4.1 A total of 21 *M. tuberculosis* clinical isolates which were previously typed using IS6110 probe in Chapter 3 were analyzed by AFLP technique.

Lab ref. number	Age	Sex	Town/village	Susceptibility tests	TB History
149	N/A	M	N/A	N/A	N/A
487	23	M	MDANTSANE	SUSCEPTIBLE	R
491	25	F	MDANTSANE	SUSCEPTIBLE	NEW
500	24	M	MDANTSANE	N/A	NEW
502	28	M	K.W.T	SUSCEPTIBLE	R
577	35	F	MDANTSANE	SUSCEPTIBLE	NEW
578	23	F	K.W.T	SUSCEPTIBLE	NEW
582	41	M	ALICE	SUSCEPTIBLE	NEW
588	37	M	K.W.T	SUSCEPTIBLE	R
591	37	M	NDEVANA	SUSCEPTIBLE	R
595	40	F	MDANTSANE	SUSCEPTIBLE	NEW
780	30	M	MDANTSANE	N/A	NEW
782	44	M	K.W.T	CULTURE+1	NEW
792	50	F	N/A	SUSCEPTIBLE	NEW
793	66	F	KOMGA	N/A	R
916	43	M	DUNCAN VILLAGE	SUSCEPTIBLE	NEW
919	54	M	ZWELITSHA	INH+, ETH-, RIF	NEW
932	29	M	DIMBAZA	INH+, RIF-, ETH-	R
5954	42	M	K.W.T	N/A	NEW
5949	17	F	MDANTSANE	SUSCEPTIBLE	NEW
5961	22	F	ALICE	SUSCEPTIBLE	NEW

Definition of abbreviations: F=female, M=male, N/A=not available, KWT=King Williams Town, R=retreatment and - = negative and + =positive

4.2.4 Typing of clinical isolates

4.2.4.1 Extraction of DNA

Colonies were scraped from L-J slopes and harvested into 0.5ml of sterile distilled water. After SDS was added to a final concentration of 2% the solution was incubated at 37°C for 24 h. Thereafter the bacteria was killed for 30 min at 80° C. The lysate was purified by two extractions with phenol:chloroform solution (1:1 v/v) and once with chloroform alone. Dialysis was carried out overnight in 2 liters of water at 4°C.

4.2.4.2 Restriction enzyme digestion of DNA

Five microliters of DNA was digested for 1h at 37°C in a total volume of 20 µl. The reaction mixture contained 5U of *Mse*I, 10x *Mse*I buffer, 0.2 µl of 10x bovine serum albumin (BSA) for 1hr at 37°C. To this digest was added 5U (1 µl) of *Eco*RI, 2 µl of *Eco*RI buffer and 2 µl of dH_2O (total volume, 25 µl), and the reaction mixtures were incubated at 37°C for a further 1hr. Endonucleases were inactivated at 65°C for 10 min prior to ligation.

4.2.4.4 Ligation of adapters

To the double-digested DNA was added 25 µl of a solution containing 40U (4 µl) of T4 DNA ligase, 10 pmol of *Eco*RI adapter, 100 pmol of *Mse*I adapter and 5 µl of 10x T4 ligase buffer. The reaction mixture was incubated at 12°C for 17 h, heated at 65°C for 10 min to inactivate the ligase, and stored at -20°C.

Table 4.2 Adaptors and PCR primers used in this study. Primers for AFLP consist of a core region and a 3' extension (E) of nucleotides (Janssen *et al.*, 1996).

***Eco*RI-adaptor:**	5' -CTCGTAGACTGCGTACC - 3'
*Eco*RI (G/AATTC)	3'- CTGACGCATGGTTAA - 5'
E-primer core sequence:	5' -GACTGCGTACCAATTCE - 3'
***Mse*I-adaptor:**	5' -GACGATGAGTCCTGAG - 3'
*Mse*I (T/TAA)	3'- CTACTCAGGACTCAT- 5'
M-primer core sequence:	5' -GATGAGTCCTGAGTAAE - 3'

4.2.4.5 PCR Amplification

PCRs were performed in 20 µl volumes containing 2 µl of ligated DNA, 0.1 µM *Eco*RI primer with an extension of A, 0.25 µM *Mse*I primer with no extension, 2 µl of 10x Taq polymerase buffer, 0.4 µl of dNTP mixture and 1.5U of Taq DNA polymerase (Roche Diagnostics, Germany). A negative control which contained all reagents but no template DNA was included.

(i) Selective amplification

PCR amplifications were performed with a Perkin-Elmer model 3600 by using the touchdown PCR cycling conditions as follows: a 2 min denaturation step at 94°C, followed by 30 cycles of denaturation at 94°C for 20 s, a 30s annealing step, and a 2

min extension step at 72°C. The annealing temperature for the first cycle was 66°C; for the next nine cycles, the temperature was decreased by 1^0C at each cycle. The annealing temperature for the remaining 20 cycles was 56°C. This was followed by a final extension at 60°C for 30 min. The amplification products were stored at -20°C (Arnold *et al.*, 1999).

4.2.4.6 Electrophoresis of PCR products

The amplification products were separated on a 4% denaturing polyacrylamide gel on a Mini VE electrophoresis system (Hoeffer). The gel was prepared by using 4.75% (w/v) acrylamide , 0.25% (w/v) methylene bis-acrylamide, 7.5 M urea, 0.5X TBE buffer (100 mM Tris,100 mM boric acid, 2 mM EDTA, pH 8), 40 µl ammonium persulphate and 20 µl TEMED. Gels were allowed to polymerize overnight at room temperature. The sample (10 µl) was added to 3 µl of loading buffer which was a mixture containing 98% formamide, 10 mM EDTA, bromophenol blue and xylene cyanol (Goulding *et al.*, 2000). The sample mix was heated at 90°C for 3 min, cooled on ice and immediately loaded onto the gel. Gels were pre-electrophoresed at constant power 1W for 20 min to maintain optimal gel temperature during electrophoresis. Two gels were run separately at 1W using 0.5X TBE as an electrophoresis buffer for about 10 h and 15 h. Standard references, i.e AFLP template of *M. vaccae* and *M. tuberculosis* strain H37Rv, were included in the second and third lane respectively. A standard Marker X (Roche Biochemicals) was also included in the first and the last lane.

4.2.4.7 Detection

Gels were treated with both ethidium bromide and visualized by UV transillumination and/or silver stained. Results were observed immediately after electrophoresis. For silver staining, the gel was fixed in 1% nitric acid until the bromophenol blue turned yellow. The fixing solution was discarded and distilled water added to rinse the gel. After rinsing, the gel was stained in 0.125 % w/v silver nitrate. Again the gel was rinsed in distilled water and then reduced in 0.28 M sodium carbonate and 100 µl of 37% formaldehyde per 200 ml. The gel was developed until the DNA bands were visible. After developing the gel it was rinsed with distilled water and then soaked in 10% glacial acetic acid for 10 minutes. After rinsing in distilled water the gel was fixed in 5% glycerol for 5 minutes.

4.3 RESULTS

The current investigation was initiated to evaluate the potential of the whole-genome coverage DNA fingerprinting technique, AFLP, to discriminate between different strains of *M. tuberculosis*. The choice of primers to amplify the DNA was tested on H37Rv to evaluate efficacy. Among the 23 primer combinations that were tested only 12 pairs successfully amplified the DNA yielding fragments of which the molecular weight ranged between 517 and 2036 base pairs. The PAGE gel produced a good degree of band resolution compared to agarose gel.

A total of 21 *M. tuberculosis* clinical strains, *M. tuberculosis* H37Rv and *M. vaccae* were analyzed with the AFLP method, using *Eco*R1 (A) and *Mse*1 (O), as described in Materials and Methods. The clinical isolates used in this study were previously typed by RFLP and grouped into families and clusters. When AFLP products were analyzed, two different groups could be identified based on the banding patterns observed (Fig 4.1). The first group of clinical isolates including the isolates which were previously grouped into clusters by RFLP and H37Rv laboratory reference strain had a similar pattern and the second group which differed from the first group a with few fragments was a non-tuberculous strain *M. vaccae.* AFLP showed a better resolution of bands with the *M. tuberculosis* isolates which harboured one IS6110 copy.

The use of primer combination *Eco*RI+A/*Mse*I+O did not increase the resolving power within the *M. tuberculosis* complex (*M. tuberculosis* and *M. tuberculosis H37Rv*), but resulted in a better separation of bands (Fig. 4.1 and 4.2). Because of the relatively low degree of genotypic variation among the AFLP band patterns of the clinical isolates, no correlation could be found with previously reported molecular typing method RFLP using IS6110 (Chapter 3) and the 27 remaining of the 47 isolates were excluded. As a result of the low degree of genetic variation throughout their respective genomes, the non radioactive AFLP methodology seems less promising for individual strain differentiation between *M. tuberculosis* strains.

4.4 DISCUSSION

AFLP is a technique in which adapter molecules are ligated to restriction enzyme fragments and are subsequently used as a target sites for primers in a PCR amplification process (Vos *et al.*, 1995). AFLP analysis provides a means of examining DNA segments distributed over the entire genome of an organism, and it offers this advantage over methods that examine restriction site changes in single genes (Gibson *et al.*, 1998).

AFLP patterns obtained in this study comprised 9 to 31 bands, whereas the simplified method used by Picardeau *et al.*, (1997) generally yielded three to eight bands. In this regard these authors concluded that AFLP was very suitable as a pre-screening technique, but recommended combined use with Pulse Field Gel Electrophoresis (PFGE) analysis for high-level strain characterization.

In our study described here, the enzymes *Eco*R1 and *Mse*1 were chosen for AFLP template preparation based on the fact that they have been used extensively in the analysis of other microorganisms. Visual inspection of the band patterns between 506 base pairs and 220 base pairs revealed species-specific bands, differentiating *M. tuberculosis* from *M. vaccae.* Occasionally, AFLP fingerprints of *M. tuberculosis* exhibited strain specific band differences. However, no correlation could be found between the observed AFLP polymorphisms and IS6110 RFLP. There was no correlation found between AFLP fingerprint and drug resistance patterns of isolates.

The increase of running time of the gel from 10 hours to 15 hours did not improve discrimination among *M. tuberculosis* isolates but did allow for improved separation of bigger fragments (Fig. 4.1 and Fig. 4.2). Due to lack of clear separation of bands, GelCompar programme was not used in this chapter to analyze data because not all bands could be assigned in lanes. In conclusion, the present investigation has demonstrated the usefulness of the AFLP technique as a reliable taxonomic tool for the differentiation of *Mycobacterium vaccae* and *Mycobacterium tuberculosis* and can be used in future for differentiation of *Mycobacterial species*. These findings correlates with recently published work by Goulding *et al.*, 2000. In the next chapter restriction fragment length polymorphism technique was used to differentiate *M. tuberculosis* clinical isolates.

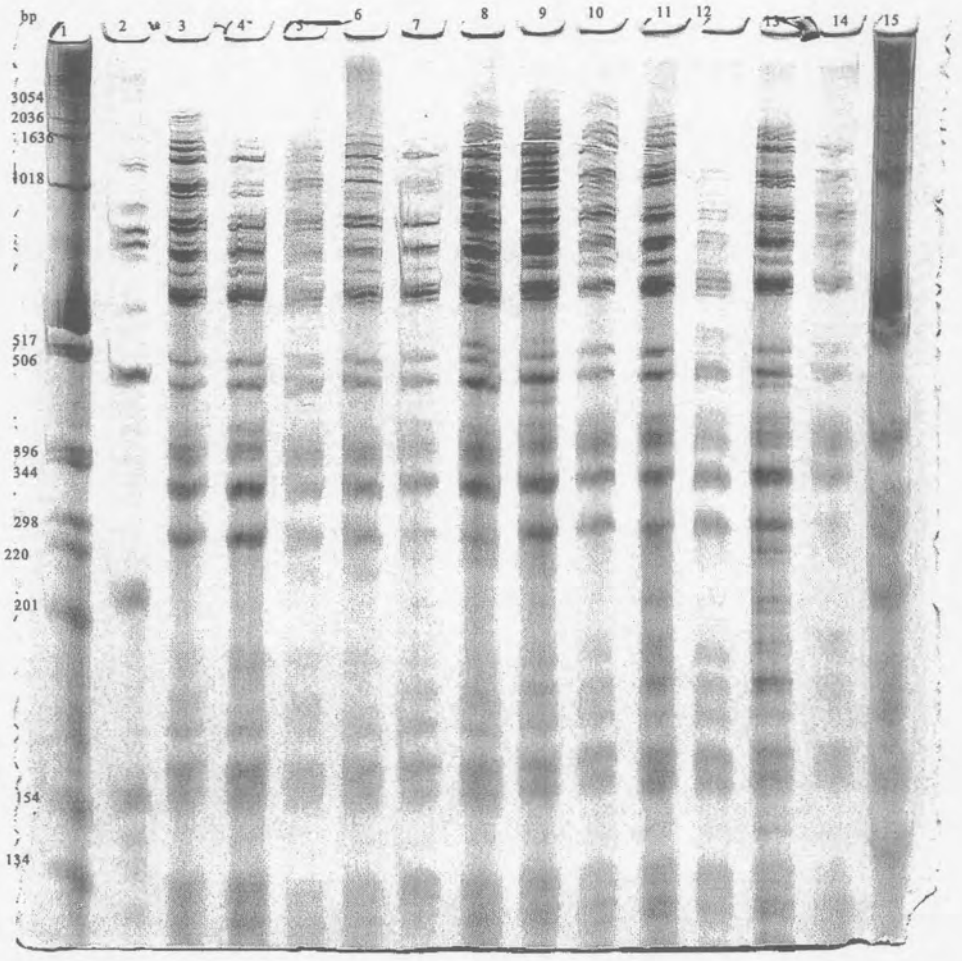

Figure 4.1: AFLP band patterns of *M. vaccae* (lane 2), *M. tuberculosis* H37Rv (lane 3) and 11 of the 21 clinical isolates analyzed (lane 4-14), generated by primer EA-MO. Standard marker X was loaded in lane 1 and 15. The gel was electrophoresed for 10 hours.

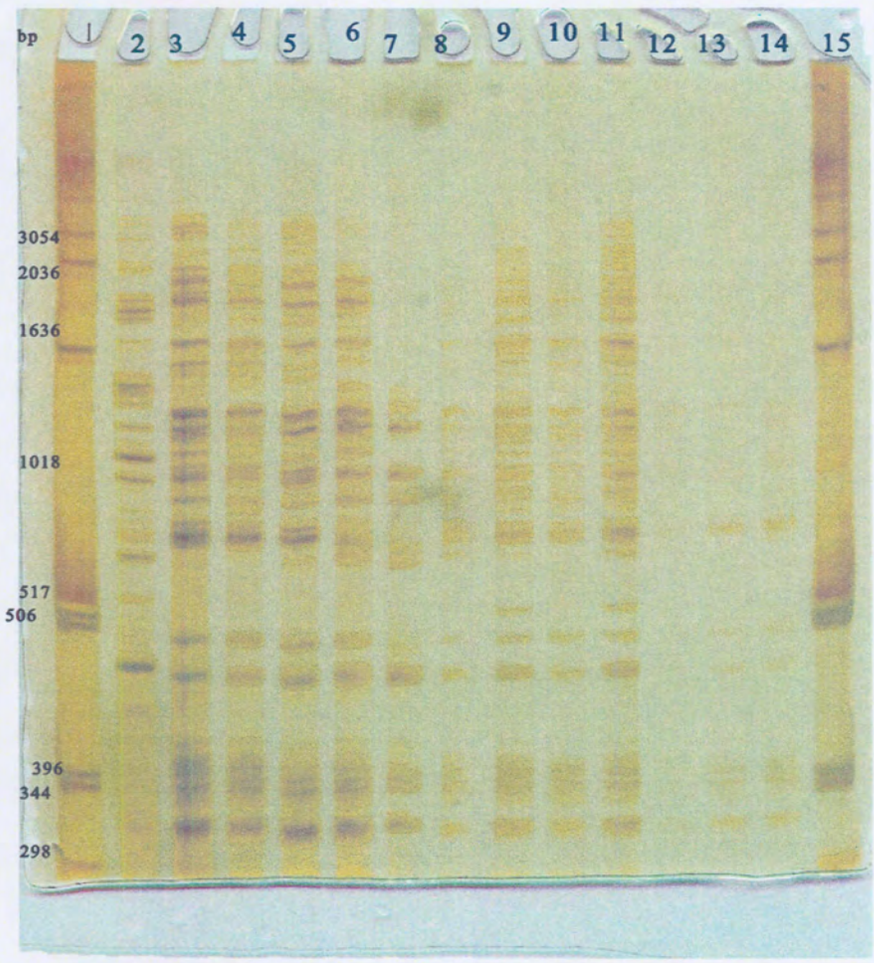

Figure 4.2: AFLP band patterns of *M. vaccae* (lane 2), *M. tuberculosis* H37Rv (lane 3) and 11of the 21 clinical isolates analyzed (lane 4-14), generated by primer EA-MO. Standard marker X was loaded in lane 1 and 15. The gel was electrophoresed for 15 hours.

CHAPTER 5. MOLECULAR CHARACTERIZATION OF ISONIAZID (INH) RESISTANT ISOLATES FROM THE EASTERN-CAPE (EC) PROVINCE AND NORTH -WEST (NW) PROVINCE.

5.1 INTRODUCTION

Tuberculosis and HIV commonly appear together in developing countries. No matter how much we try to separate them, these diseases are closely linked as dangerously inseparable diseases. Worse still, they have a third partner and progeny: multi-drug resistant tuberculosis (MDR-TB) (Anonymous 2000). HIV infection of itself does not increase the risk of an individual with tuberculosis developing drug resistance but should an HIV infected individual acquire infection with drug resistant organism as with drug sensitive organism it is more likely to progress to active disease than it is in an immunocompetent person. Therefore targeting TB for eradication is essential in the era of HIV (Anonymous 2000).

Drug resistance in tuberculosis emerges by means of two processes, firstly, acquired drug resistance occurs as a result of poor adherence to treatment, or incorrect prescription of drugs for treatment programmes. This allows for the emergence of naturally occurring drug resistant mutants, causing clinical disease (Lipsitch and Levin 1998; Mitchison 1998). Therefore a high level of this type of resistance is a mark of a poorly functioning TB control program (Bloom 1994). The second process involves resistant organisms from infected patients who subsequently infect and cause disease

in other individuals who may or may not have been previously infected with *M. tuberculosis*. Primary drug resistance, i.e the strains infecting patients are already drug-resistant at the time of infection is also an indicator of efficacy of previous TB control efforts (Weyer and Kleeberg 1992). A recent study, employing RFLP analysis of *M. tuberculosis* isolates, demonstrated that drug resistance can develop through exogenous reinfection with a new multidrug resistant strain of *M. tuberculosis* during or after therapy for an episode of drug resistant tuberculosis (Small *et al.*, 1993). If infection with multiple drug resistant strains involves a patient already under treatment for TB caused by another strain, the process is referred to as superinfection.

The relationship between clustering, strain diversity and drug susceptibility patterns may be used to examine whether recently transmitted drug resistant strains between patients took place or if drug resistance was acquired during treatment. Recently transmitted resistance caused by clonal dissemination of resistant strains, will exhibit identical or near identical RFLP profiles and limited RFLP diversity among drug resistant strains in a population (Moss *et al.*, 1997; Niemann *et al.*, 1997). Matching fingerprints may however also demonstrate transmission in the past between patients who acquired primary tuberculosis, followed by dormancy and subsequent reactivation, often years after primary infection. This situation has been shown to occur in populations where tuberculosis is highly prevalent e.g in Ethiopia and Tunisia (Hermans *et al.*, 1995) and French Polynesia (Torrea *et al.*, 1995). In a recent review article on the use of RFLP based DNA fingerprinting in developing countries, Cohn

and O'Brien (1998) discussed differences in the transmission of tuberculosis between such poor countries and industrialized countries. Based on the comparison between African countries and the Netherlands (van Soolingen *et al.*, 1991), it was concluded that more recent transmission occurs in areas of high incidence while disease reactivation and importation of cases are reflected in DNA fingerprinting-based studies in areas of low incidence. In the context of poor countries where tuberculosis is common, Godfrey-Fausset in a commentary in The Lancet in 1999, emphasized that "clusters will be formed by repeated generation of transmission limited by emigration and stability of the DNA fingerprint itself. Unless there is much mobility of population, clusters may have little to do with recent infection" (Godfrey-Fausset 1999). Wilkinson *et al.* (1997) reported results of a study of DNA fingerprinting and conventional contact tracing on 246 TB patients in rural South Africa, showing that approximately 40% of isolates were in clusters and that epidemiologic links could be established for 27% of clustered patients. A second South African study demonstrated that recent *M. tuberculosis* transmission rather than relapse was responsible for most cases of recurrent TB (van Rie *et al.*, 1999).

This study, differed from Chapter 3 because it was population-based. The study was conducted at the Medical Research Council and designed to (i) determine the prevalence of isoniazid (INH) resistance and (ii) study and estimate the incidence of tuberculosis that results from recent transmission of the disease in the Eastern-Cape (EC) and North-West province (NW). All isoniazid resistant cultures from both

provinces collected and isolated between August 2000 and October 2001, were cultured on LJ media and typed for fingerprinting using IS6110 as a probe.

Results for resistance to isoniazid were available in 27/40 patients from the EC province and 17/31 from NW province, all with INH positive *M. tuberculosis* culture. Taking into account study limitations, the incidence of INH resistance was 6.7% in EC and 8.4% in the NW province.

5.2 MATERIAL AND METHODS

5.2.1 Study population

During the period from August 1999- October 2001, 2168 cases of tuberculosis from 18 districts in the Eastern Cape Province were collected. A total of 598 patients were identified as positive, and 40 cases were found to be resistant to isoniazid. Twenty seven of these cultures were viable and ready to be used for RFLP fingerprinting. The remaining isolates were resistant to one or more drug other than isoniazid. Of the 1409 patients for which samples were collected in the NW Province 370 were positive and 31 patients were resistant to isoniazid. Only 17 had cultures available for RFLP fingerprinting. All the patients whose isolates were not typed were excluded because their culture results were thought to represent possible laboratory contamination or mislabeling. Listing of patients on admission regarding their treatment history as 'Yes' or 'No' cases is given in Table 5.1 and 5.3.

5.2.2 Bacterial strains

TB patients investigated during the study submitted one acid fast bacillus (AFB) positive sputum smear and resided in the Eastern Cape and North West Province. Each hospital in those provinces was requested to submit sputum samples of TB cases to the Medical Research Council-TB laboratory. The patients included treatment failures, relapse and default patients (retreatment cases). In patients who had never received anti-tuberculous drugs (listed in Table 5.1 and 5.3 as No) bacterial resistance was assumed to be primary. In patients with a record of previous treatment (listed in Table

5.1 and 5.3 as YES), the bacterial resistance was classified as acquired.

5.2.3 Bacterial growth

Smear positive samples were cultured on Lowenstein-Jensen medium, only patients with culture positive TB and strains resistant to any resistance to INH, were ultimately included in the study.

5.2.4 DNA fingerprinting and analysis

Characterization of strains by IS6110 DNA fingerprinting was done using the same methodology described in Chapter 2. Evaluation of laboratory cross contamination was performed by comparing the DNA fingerprints of all culture-positive cases that were processed on the same day. In the EC twenty six isolates were found to share eight common processing dates (two to six cultures per day). In the NW province 16 isolates were found to share 6 common processing dates. The remaining isolates were culture positive specimens which were processed on different dates. Comparison of the restriction fragment length polymorphism patterns of the two to five isolates that were processed on the same day demonstrated that no isolates processed on the same day shared a common RFLP pattern. Therefore, the data indicate that laboratory cross contamination during specimen processing was not likely to be a factor contributing to RFLP clustering of isolates.

Table 5.1 Characteristics of patients with drug resistant TB isolates from the Eastern Cape Province

Lab ref.no	Age	Sex	District	Town/village	TB history	Date specimen received	Date processed	Drug sensitivity	IS6110 copies	cluster
26	32	M	UITNHAGE	KWANOBUHLE	NO	08.08.2000	08.09.2000	H+,	12	
106	40	M	FORT BEAUFORT	BEDFORD	YES	08.10.2000	08.11.2000	H+, R+, S+		
134	27	M	UMTATA	LIBODE	N/A	08.14.2000	08.15.2000	H+	11	
136	43	M	UMTATA	LIBODE	N/A	08.14.2000	08.15.2000	H+	11	CL3
137	17	F	UMTATA	JIXINI	NO	08.14.2000	08.15.2000	H+, S+	9	
143	34	M	UMTATA	TABASE	NO	08.14.2000	08.15.2000	H+	12	CL2
146	20	F	LIBODE	NKWILINI	YES	08.14.2000	08.15.2000	H+, R+		
156	29	F	NGQELENI	NTLAZA	N/A	08.14.2000	08.15.2000	H+, R+	14	
214	46	M	PE	PE	NO	0815.2000	08.16.2000	H+, S+	12	
242	24	M	QUEENSTOWN	SADA	YES	08.15.2000	08.16.2000	H+	9	
281	30	F	PE	NOXOLO	NO	08.17.2000	08.18.2000	H+, S+	14	
286	32	F	PE	GELVANDALE	YES	08.17.2000	08.18.2000	H+, R+, E+	12	
374	27	M	PE	GELVANDALE	N/A	08.22.2000	08.23.2000	H+, S+	14	
379	35	M	PE	N/A	YES	08.22.2000	08.23.2000	H+, S+	13	
396	21	M	ALIWAL NORTH	STERKSPRUIT	YES	08.22.2000	08.23.2000	H+, S+	13	CL 1
497	34	M	UITENHAGE	UITENHAGE	NO	08.24.2000	08.25.2000	H+	2	
521	20	F	PE	PE	NO	08.24.2000	08.25.2000	H+, R+, S+, E+	11	
583	31	M	UMTATA	NGANGELIZWE	NO	08.28.2000	08.29.2000	H+, R+	15	
591	21	F	LIBODE	MHLANGANISWENI	YES	08.28.2000	08.29.2000	H+, R+	15	
594	36	M	LIBODE	MGWENYANE	YES	08.28.2000	08.29.2000	H+, S+, R+	12	CL2
682	34	M	QUEENSTOWN	QUEENSTOWN	NO	08.29.2000	08.30.2000	H+		
875	65	M	BUTTERWORTH	KENTANE	YES	09.07.2000	09.07.2000	H+, S+, R+	10	
715	20	F	BUTTERWORTH	BUTTERWORTH	NO	08.29.200	08.31.2000	H+, S+	1	
933	60	F	UMTATA	UMTATA	YES	09.11.2000	09.11.2000	H+, R+	15	
958	44	M	PORT ST JOHNS	LIBODE	YES	09.11.2000	09.11.2000	H+	9	
1278	44	M	MOUNT FLETCHER	DODRECHT	YES	10.02.2000	10.12.2000	H+	11	
1365	52	M	BUTTERWORTH	WILLOWVALE	NO	10.06.2000	10.12.2000	H+, S+		
1391	48	M	PE	N/A	YES	09.10.2000	10.12.2000	H+, R+	5	
1392	23	F	PE	N/A	NO	10.09.2000	10.12.2000	H+, R+		
1550	46	M	FLAGSTAFF	MANTLWENI	YES	12.10.2000	10.14.2000	H+	8	
1838	17	F	PORT ELIZABETH	PORT ELIZABETH	NO	11.14.2000	11.15.2000	H+	8	
1905	26	F	PORT ELIZABETH	N/A	NO	11.21.2000	11.22.2000	H+	9	
1912	28	M	GRAFF REINET	N/A	NO	11.23.2000	11.27.2000	H+, S+, E+		
1930	N/A	M	BUTTERWORTH	WILLOWVALE	NO	11.23.2000	11.27.2000	H+	15	
2031	33	M	UMTATA	ROSEDALE	NO	01.02.2001	01.03.2001	H+	11	CL3
2039	N/A	F	UMTATA	LIBODE	NO	01.02.2001	01.03.2001	H+	3	
2053	45	M	BUTTERWORTH	KENTANE	NO	12.14.1999	09.01.2001	H		
2090	52	F	GRAFF REINETE	LOTUSVILLE	NO	01.23.2001	01.25.2001	H+, R+, S+, E+		
2121	32	M	BUTTERWORTH	WILLOWVALE	NO	02.14.2001	02.15.2001	H		
2146	55	M	KENTANE	NQAMAKWE	YES	02.28.2001	03.01.2001	H+, R+, E+	14	CL1

Definitions of abbreviations: F=Female, M=Male, PE=Port Elizabeth, N/A=Not Available, CL=Cluster, H+=Isoniazid positive, S= Streptomycin, R= Rifampicin, E=Ethambutol.

5.3 RESULTS

5.3.1 Geographical distribution of patients from EC province

Geographical data of the patients with drug resistant tuberculosis isolated are shown in Table 5.1. Eight patients were from Umtata district, 10 from Port Elizabeth, 6 Butterworth, 3 Libode, two from each of the following districts, Uitenhage, Queenstown and Graaff Reinet and one patient from Aliwal North, Port St Johns, Mount Fletcher, Flagstaff, Kentane, Ngqeleni, and Fort Beaufort.

5.3.2 RFLP patterns in the EC province

Only 27 IHN resistant *M. tuberculosis* clinical strains from EC province were viable and examined for their IS6110-associated RFLP patterns. Of the 27 EC clinical isolates subjected to RFLP analysis, six were identified in three clusters of two isolates each. Only 24 different patterns were observed and 21of these were found only once. Dendograms were constructed to show the degree of relatedness among strains (Fig 5.1). About 66.7% (18 isolates) of isolates with non-identical RFLP patterns showed common band patterns, including the position and grouping of bands (Fig 5.1). These strains were grouped into families (F1-4), such that all the members of each family were identical or differed by only one to three bands. The band pattern identity was so high, that individual strains could only be resolved at identity levels of 79% and above. Nine isolates were not attached to any family. F1 had 8 isolates, F2 contained 5 isolates, F3 had 3 isolates, F4 consisted of 2 isolates. F1 consisted of 50% (4 isolates) MDR isolates and 4 of these were previously treated for tuberculosis. The remaining

3 isolates were resistant to isoniazid and streptomycin only, and the treatment history of one in this family isolate was not available. In F3 60% of isolates were singly resistant to isoniazid, 20% were MDR and another 20% were resistant to isoniazid and streptomycin only. The relatedness between F1 and F3 was 88%.

A cluster was defined as one or two patients whose isolates share identical (100%) IS6110-DNA fingerprint patterns (Warren *et al.*, 1999). Three clusters (CL1-3) were identified in this province (Fig 5.1), and they all consisted of two isolates in each cluster. All three clusters consisted of male patients between the age 21-55 and CL1 and CL2 grouped into F3. CL1 composed patients who were both previously treated for tuberculosis and lived in different districts far from another. Both patients in CL 2 were living in districts not far from another. CL 3 consisted of patients from the same district and both resistant to isoniazid only. About 50% of clustered patients have worked in the mines. Clustering of these isolates from male patients was associated with working in the mines.

The minimum percentage of tuberculosis cases due to recently transmitted tuberculosis in the EC province (calculated according to the formula proposed by Small *et al.*, 1994, as [Total number of isolates in clusters]-[no. of clusters]/[Total No. of strains] was 11.1 % based on isolates.

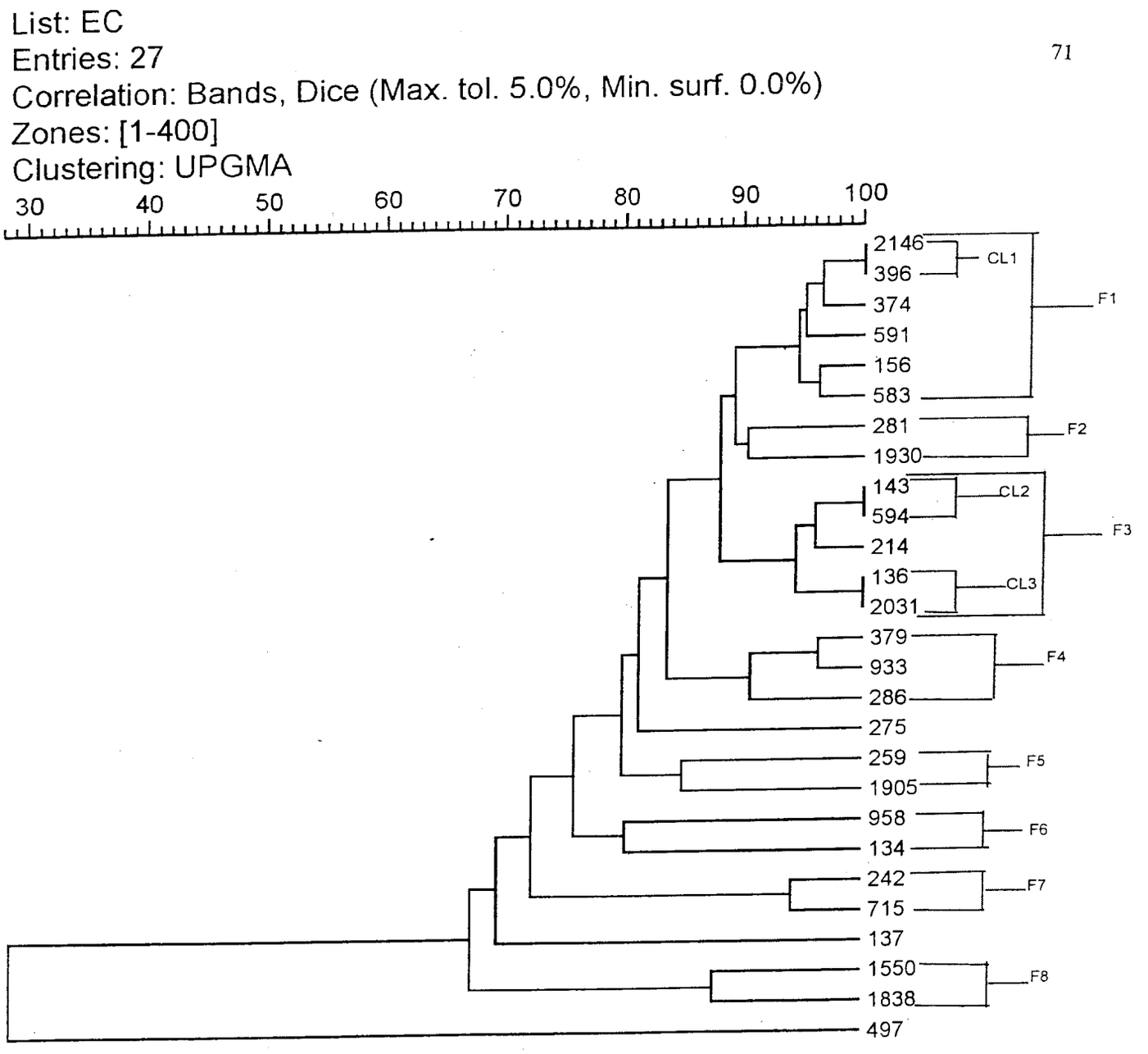

Figure 5.1 Dendogram of DNA fingerprints of 27 *M. tuberculosis* clinical isolates obtained from the Eastern Cape province. The percentages of similarity are shown above the dendogram and the strain Numbers, clusters (CL1-3) and families (F1-4) are shown on the right.

5.3.3 Drug resistance profile of isolates from EC

All 40 isolates were resistant to INH and of these 17 were mono-resistant. Of the 23 remaining isolates resistant to more than one drug, 14 complied with the definition of MDR (resistant to both INH and RIF) (Table5.2). The other 9 isolates, apart from being resistant to INH, were in addition resistant to streptomycin and ethambutol. Patients who reported previous tuberculosis treatment were more likely to harbor multidrug resistant isolates than were patients who did not report a history of treatment (Table 5.2). The total percentage of INH resistance was calculated using the following formula: resistant isolates/total number of positive isolates x 100 = total percentage of INH resistance (40/598 x 100 = 6.6%).

5.3.3.1 Initial resistance in EC

Of the 40 INH resistant tuberculosis cases, 21 were defined as 'new' patients (i.e no TB history) and resistance in this group was termed 'initial'. Initial resistance to the four major drugs is presented in Table 5.2. Among the 598 positive isolates identified in this province, primary resistance to isoniazid only was 1.67%. Initial resistance to isoniazid and streptomycin was 1.0%. Since almost all tubercle strains showing resistance to rifampin had associated isoniazid resistance, the estimate for multidrug resistance in new patients was 0.67%. Resistance to both isoniazid and ethambutol was not found.

5.3.3.2 Acquired resistance in EC

A total of 15 patients gave a history of tuberculosis treatment and bacterial resistance occurring in this group was termed 'acquired'. Acquired resistance to the four first line drugs is presented in Table 5.2. Acquired resistance to isoniazid only was 0.67% among the TB positive isolates identified. Acquired resistance of MDR isolates among the TB positive isolates was 1.5%.

Table 5.2: Initial and acquired *M. tuberculosis* resistance of patients from Eastern Cape province.

Drug	**Initial** ($N = 21$)	**Acquired** ($N = 15$)	**Unknown** ($N = 4$)	**Overall** ($N = 40$)
Isoniazid	11	4	2	17
Isoniazid + Streptomycin	6	2	1	9
Isoniazid + Ethambutol	0	0	0	0
MDR(Isoniazid + Rifampin+ Streptomycin/ Ethambutol)	4	9	1	14

Table 5.3: Characteristics of patients with Ioniazid (INH) resistant TB isolates from the North West Province.

Lab ref. number	Age	Sex	District	Town/village	TB history	Date specimen received	Date processed	Drug sensitivity	IS6110 copies	cluster
20	51	M	RUSTENBURG	TLHABANE	YES	07.20.2000	24 .07.2000	H+	10	
25	29	F	ZEERUST	BORAKALO	NO	20.07.2000	24.07.2000	H+	13	
39	50	M	KLERKSDORP	STILFONTEIN	NO	07.20.2000	07.24.200	H+	13	
169	41	M	KLERKSDORP	STILFONTEIN	YES	07.20.2000	07.25.2000	H +, S+, R+	4	
180	49	M	KLERKSDORP	STILFONTEIN	N/A	07.25.2000	07.26.2000	INH+		
214	22	F	VRYBURG	HUHUDI	YES	07.25.2000	07.26.2000	H+, R+		
215	48	F	VRYBURG	COLRAGE	YES	07.25.2000	07.26.2000	H+, R+	4	
219	N/A	M	ZEERUST	N/A	NO	07.25.2000	07.26.2000	H+, S+		
239	17	M	POTCHEFSTROOM	POTCHEFSTROOM	NO	07.27.2000	07.28.2000	H+	14	
295	29	F	KLERKSDORP	N/A	NO	08.03.2000	08.04.2000	H+	11	
339	68	M	MOGWASE	MOKOSHONG	YES	08.08.2000	08.09.2000	H+, S+		
474	36	F	GANYESA	BEESPLAAS FARM	YES	08.22.2000	08.23.2000	H+	10	
494	33	M	LICHTENBURG	BODIBE	NO	08.22.2000	08.23.2000	H+	12	
513	64	M	MOGWASE	MAGONG	YES	08.23.2000	08.25.2000	H+, S+		
530	46	F	DELAREVILLE	WELEVREDE	NO	29.03.2000	31.08.2000	H+,R+,E+,S+		
557	39	M	KLERKSDORP	STILFONTEIN	NO	08.29.2000	08.31.2000	H+, R+, S+		
606	37	M	KURUMAN	BOTHITONG	YES	31.08.2000	09.09.2000	H+,R+,E+,S+		
668	48	M	POTCHEFSTROOM	PRIMOSA	NO	09.05.2000	09.06.2000	H+		
674	47	F	S/REINECKE	N/A	NO	09.05.2000	09.07.2000	H+		
738	46	M	KLERKSDORP	STILFONTEIN	YES	09.12.2000	09.13.2000	H+,S+		
741	43	M	KLERKSDORP	STILFONTEIN	NO	09.12.2000	09.13.2000	H+, S+	4	
750	37	M	KLERKSDORP	KHUMA LOCATION	NO	09.12.2000	09.13.2000	H+,S+		
816	24	F	GANYESA	PIET PLESSIS	NO	09.19.2000	09.21.2000	H+,		
830	35	M	KLERKSDORP	N/A	YES	09.19.2000	09.21.2000	H+,S+		
1033	35	M	KLERKSDORP	STILFONTEIN	NO	10.12.2000	10.23.2000	H+, R+,E-, S-		
1063	51	F	KLERKSDORP	RUSTENBURG	YES	10.17.2000	10.23.2000	H+, R+	6	
1111	53	M	KLERKSDORP	STILFONTEIN	NO	10.26.2000	10.30.2000	H+, S+, R+	5	
1131	29	M	KLERKSDORP	JOUBERTON	NO	10.31.2000	11.02.2000	H+		
1263	27	M	KLERKSDORP	JOUBERTON	NO	12.07.2000	12.09.2000	H+	8	
1294	32	M	KLERKSDORP	STILFONTEIN	NO	01.11.2001	01.12.2001	H+,S+		
1331	47	M	KLERKSDORP	KLERKSDORP	NO	02.06.2001	02.07.2001	H+,S+		

Definitions of abbreviations: F=Female, M=Male, PE=Port Elizabeth, N/A=Not Available, CL=Cluster, H+=Isoniazid positive, S= Streptomycin, R= Rifampicin, E=Ethambutol

5.3.4 Geographical distribution of patients from NW province

Data of 31 patients collected from 10 districts of NW province is shown in Table 5.3. Sixteen patients were from Klerksdorp district, 2 from Mogwase, one from Delareyville, Kuruman, Rustenburg, Lichtenburg and Schweizer Reneke, two patients from each of the following districts Zeerust, Vryburg, Potchefstroom and Ganyesa. All the 17 typed isolates were resistant to INH and of these, 13 were mono-resistant. Of all the remaining 18 isolates resistant to more than one drug, four complied with the definition of MDR (resistant to both INH and RIF). The other two isolates apart from being resistant to INH were in addition resistant to streptomycin (Table 5.3 and 5.4).

5.3.5 RFLP patterns in NW province

Among 31 isolates only 17 were viable and subjected to RFLP analysis. Eleven patients had a history of tuberculosis and nineteen were never treated for tuberculosis. One patient had no information on the history of tuberculosis. Seventeen patterns were observed with 4 to 15 IS6110 copies. There were four different families (F 1-4) with two isolates each, identified at 79% relatedness. About 50% of the patients in these families are previous miners. In F1, the isolates in this group were both resistant to isoniazid only. These patients had previously been treated for TB. In F2 both patients were new patients. The isolates in this family were resistant to isoniazid only. The 2 cases identified in F3 were both males from different district, with multidrug resistant TB isolates.

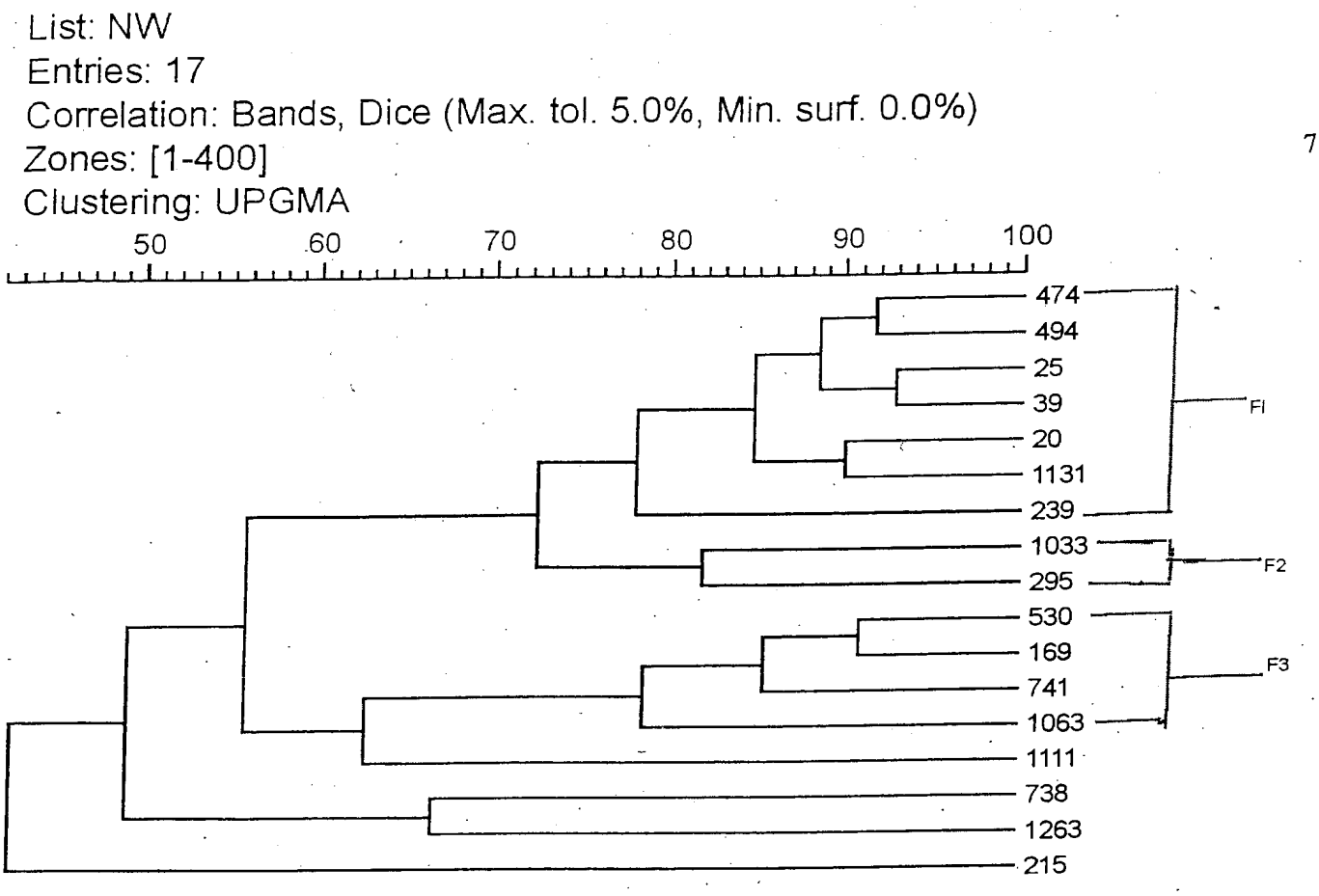

Figure 5.2 Dendogram of DNA fingerprints of 17 *M. tuberculosis* clinical isolates obtained from the North West province. The percentages of similarity are shown above the dendogram and the strain numbers and families (F1-3) are shown on the right.

5.3.6 Drug resistance profile of isolates from NW

Among the 31 collected isolates which were resistant to INH, 13 isolates were resistant to isoniazid only, 9 were multidrug resistant and another 9 resistant to isoniazid and streptomycin (Table 5.4).

5.3.6.1 Initial resistance in NW

Of the 31 INH resistant tuberculosis cases, 11 were registered as new patients (i.e no TB history). Among the 370 positive isolates identified, initial resistance to isoniazid only was 2.7%, 0.9% higher than the EC province (Table5.4). The estimate for multidrug resistance in new patients was 1.08%, 0.59% higher than the EC province. Resistance to streptomycin in addition to isoniazid only was found in 9 isolates.

Table 5.4: Initial and acquired *M. tuberculosis* resistance of patients from North West province.

Drug	**Initial** ($N = 19$)	**Acquired** ($N = 11$)	**Unknown** ($N = 1$)	**Overall** ($N = 31$)
Isoniazid	10	2	1	13
Isoniazid + Streptomycin	5	4	0	9
MDR (Isoniazid + Rifampin+ Streptomycin/ Ethambuthol	4	5	0	9

5.3.6.2 Acquired resistance in NW

Twelve isolates were identified to have acquired resistance (Table 5.4). Among 370 isolates identified, acquired resistance to isoniazid only was 0.5% and multidrug resistance to those patients who reported history of tuberculosis was 1.4%. This indicates that non-compliance of people to treatment in this area resulted in a high level of multidrug resistance.

5.3.7 Grouping of RFLP DNA fingerprinting patterns from both provinces (EC and NW).

All the isolates from both provinces were grouped in 6 families (F1-6) at 79% relatedness. In F1 22% of these isolates were from NW and 78% from EC. Both isolates from NW were resistant isoniazid only, 44% were MDR isolates. In this group two patients (one from EC and the other from NW) were previous miners and there is a possibility that they could have worked in the same mine.

Family F2 consisted of 7 isolates 86% of them resistant to isoniazid only. Five of these isolates were from EC and 2 isolates were from NW. Three of the 7 isolates were previous miners 2 from EC and 1 from NW. The previous miner from NW might have been in contact with the other patient from NW. Family F4-F6 consisted of 2 isolates each and were grouped by provinces.

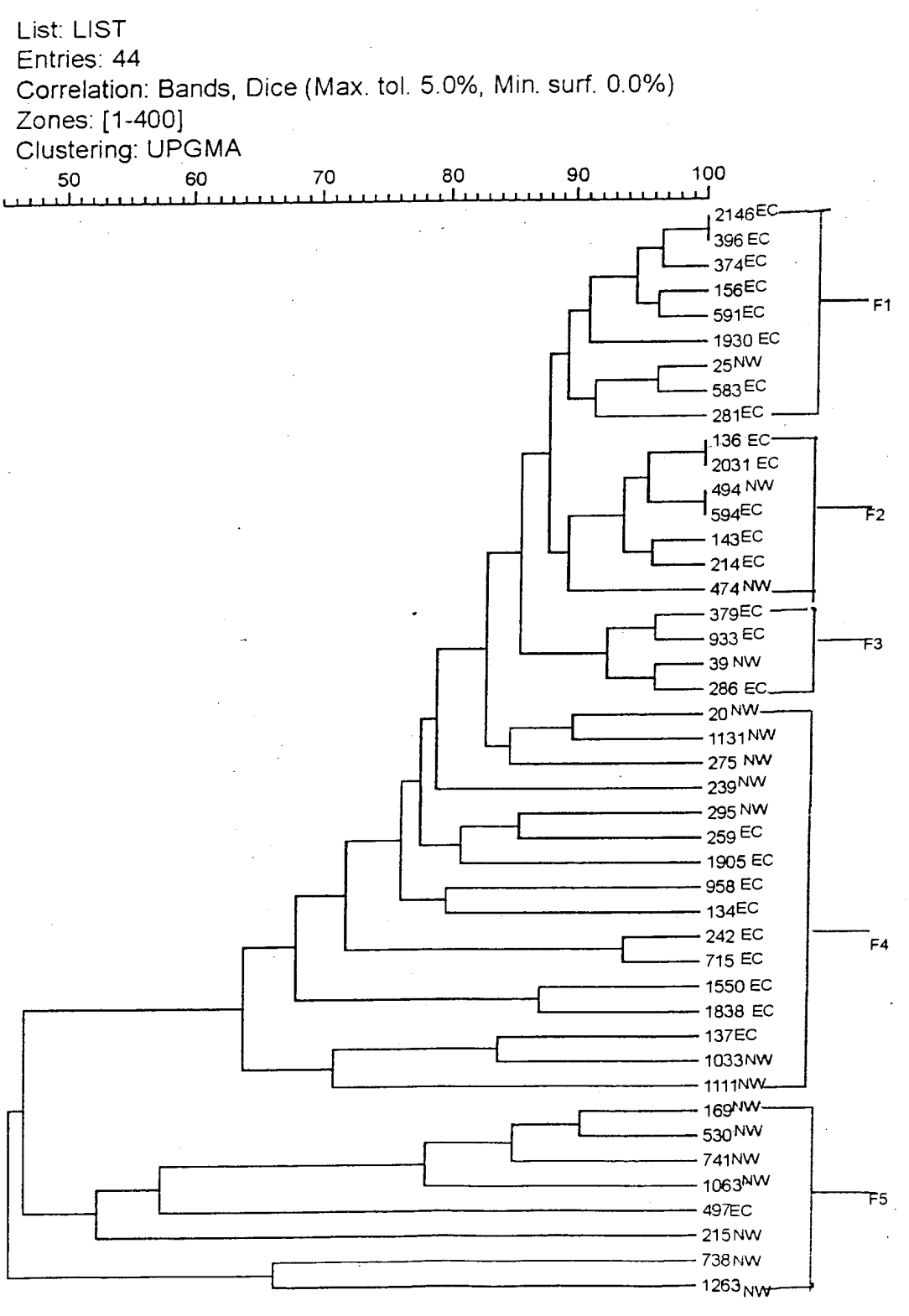

Figure 5.3 Dendogram of DNA fingerprints of 44 *M. tuberculosis* clinical isolates obtained from the Eastern Cape (EC) and North West (NW) province. The percentages of similarity are shown above the dendogram and the strain numbers and families (F1-5) are shown on the right.

5. 4 DISCUSSION

Molecular typing methods have proved to be powerful tools in the investigation of important aspects of the activity of *M. tuberculosis* in a population. The use of RFLP analysis to identify patterns of tuberculosis transmission within a community is based on the premise that epidemiologically unrelated cases will have occurred as a result of reactivation of latent infection and thus have unique RFLP patterns, whereas cases that are linked as a consequence of recent infection will have the same patterns (appear in defined clusters) (Small *et al.*, 1994). In this study, the first statement is supported by the diversity of RFLP patterns of all the isolates typed in the NW province. The second is supported by the 6 isolates in 3 clusters from the EC province. This means recent transmission of TB is very low at 22% of the isolates studied.

The prevalence of drug resistant *M. tuberculosis* in this study has been found to be low (6.7% and 8.4%) and is similar to earlier reports on drug resistance among workers in the South African gold mines (Cowie 1990) and in children patients of the Western Cape (Weyer *et al.*, 1995). The NW province had a higher proportion (8.4%) of drug resistant tuberculosis than EC province (6.7%). Drug resistance rates in patients who had previously reported a prior history of tuberculosis treatment were (3.2%) in NW but it was not possible to categorize the reasons for retreatment such as retreatment failure, relapse or defaulting. High levels of acquired resistance, however, indicate poor programme performance in that area. Initial resistance to INH was at 3.5% EC and 5.1% NW and is generally accepted as a sensitive indicator of the overall success of a

treatment programme in a country since this drug is widely used in treatment regimens (Weyer *et al.*, 1995).

When MDR rates in TB positive cases from EC (0.67 % new + 1.5% previously treated) and NW (1% new + 1.4% previously treated) province are compared with figures of multi drug resistance from other countries, it is clear that MDR in these provinces is relatively low. In China, India, Iran, Mozambique and Russia high levels of MDR-TB of over 3% in new TB cases has been reported (WHO 2000). Additionally Israel, Italy, Mexico reported MDR-TB in over 6% of both new and previously treated cases combined (WHO 2000).

It is worth noting however that the number of positive TB cases on the records obtained thus far was as low as between 26% and 27.5% in these provinces. The possible reason for this low percentage could be that the tuberculosis screening procedure for a positive smear result before treatment, as outlined in the National Tuberculosis Control Program Practical guidelines might have been applied too leniently at clinic level and many of the sputum smear negative patients were missed. In other studies that exceed positive TB cases of 60% it means that either many of the tuberculosis patients are presenting late to the health services for diagnosis and hence infecting many of their contacts before they start treatment (South African Health Review (SAHR) 2000).

In accordance with previous reports in the literature, there was no correlation found between antibiotic resistance profile and RFLP patterns. For this reason, drug resistant strains were shown to belong to the same cluster/s with strain/s that were susceptible to that particular drug. These data are consistent with those obtained in various other research programmes, that demonstrate a lack of change in DNA fingerprinting pattern after *in vivo* selection of drug resistance by *M. tuberculosis* of (van Soolingen *et al.*, 1991, Godfrey-Fausset *et al.*, 1993). It is likely that some drug resistant strains, including MDR strains, may have acquired part of, or their full complement of resistance in the past in the mines and then appeared in the villages following reactivation of their initial drug resistant infection. Most men in the villages of these provinces are migrant labourers who are recruited from areas where high frequencies of initial drug resistance have been reported (Fourie *et al.*, 1980). These men could thus be expected to develop reactivated pulmonary tuberculosis with urban acquired drug resistant, particularly isoniazid resistant *M. tuberculosis* while in the communities.

CHAPTER 6. CONCLUDING REMARKS

The incidence of tuberculosis is rising throughout the world, prompting detailed investigation of the epidemiology of this disease. Typing techniques based on the polymorphism of insertion sequence position in the genome (IS6110) has provided molecular methods to distinguish strains of *M. tuberculosis* for outbreak investigations (Rodriguez *et al.*, 2000), detect transmission (Aznar *et al.*, 1995), and confirm lab cross-contamination (Small *et al.*, 1993).

The objectives of this study were 1) to determine the extent of transmission of tuberculosis in a referral hospital (Nkqubela Hospital) situated in the East London district of the Eastern Cape Province by differentiating *M. tuberculosis* isolates using the RFLP technique discussed in chapter 3, 2) to investigate the efficacy of AFLP in confirming the validity of fingerprinting patterns which have been generated through IS6110 RFLP in chapter 3, and 3) to study and estimate the incidence of tuberculosis that result from recent transmission of the disease in the Eastern-Cape (EC) and North-West province (NW).

In chapter 3 of this study the extent of recent transmission of tuberculosis was determined by estimating the levels of clustering in that area. It was shown that the amount of clustering in this area was as low as 14.9% compared to several studies which have identified very high levels of clustering (as high as 40%) (Small *et al.*,

1994). Two of the clustered cases were retreatment patients in which reactivation of infection had occurred. Nine were from patients with recently transmitted tuberculosis. Samples from younger patients (63%) were most likely to group in clusters than those from the elderly (36.4%) as was found in previous studies (Small *et al.*, 1994, Yang *et al.*, 1995). This was expected as elderly patients have had many more years of life during which they could have become infected and are likely to have reactivation of the disease.

In chapter 4, AFLP technique generated two different groups that could be identified based on the banding patterns observed (Fig 3.1). The first group of clinical isolates and the H37Rv laboratory reference strain displayed a similar AFLP pattern. The second group was represented by non-tuberculous strain *M. vaccae* differed from the first group with at least fragments. AFLP showed its potential use in differentiating *Mycobacterial species* and gives a better resolution of bands.

The prevalence of drug resistant *M. tuberculosis* studied in Chapter 5 has been found to be low (6.7% for the Eastern Cape and 8.4% for North West Province) and is similar to earlier reports on drug resistance among workers in the South African gold mines (Cowie 1990) and in paediatric patients of the Western Cape (Weyer *et al.*, 1995). *M. tuberculosis* isolates in these provinces exhibited a high degree of strain diversity, with some tendency to group according to their provinces (Fig.5.3)

Initial resistance to INH +/- other drugs was at 3.5% in the EC and 5.1% in the NW province. The low percentages indicate the overall success treatment programme in the province since this drug is widely used in treatment regimens. According to the WHO, an effective TB control programme is defined by a high detection rate, high cure rate and low level of drug resistance (WHO, 1997). The low prevalence of MDR detected among newly diagnosed TB cases suggests that the ongoing transmission of drug resistance within both provinces is still low.

Finally we conclude that IS6110 DNA fingerprinting of *Mycobacterium tuberculosis* in this study helped to estimate the magnitude of recent transmission in the Eastern Cape (22%) and North West province. In order to increase the knowledge of active transmission of the disease in these two provinces and to identify the risk factors associated with transmission, this study can be extended to all the provinces of South Africa by studying the prevalence of other drugs used for the treatment of tuberculosis. A bank of *M. tuberculosis* RFLP patterns can also be established for future studies.

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