ABSTRACT

MOLECULAR EPIDEMIOLOGY OF *MYCOBACTERIUM TUBERCULOSIS* STRAINS FROM THE FREE STATE AND NORTHERN CAPE PROVINCES, SOUTH AFRICA.

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Background. Tuberculosis is increasing in the Free State and Northern Cape provinces of South Africa, but it is not clear how much of the disease is caused by reactivated latent infection and how much is attributed to interpersonal transmission. The discovery of the transposable DNA insertion sequence, IS*6110*, provided the desired polymorphism among different strains to track routes of transmission, study the degree of inter-person transmission versus reactivation, to detect laboratory contamination and disease outbreaks. Alternative methods include spoligotyping and the mycobacterial intergenic repetitive units or variable number of tandem repeats (MIRU-VNTR). Sustained studies performed on a small area in the Western Cape Province and some mines in the Gauteng Province of South Africa have found person-to-person transmission of tuberculosis to be high in these populations. In addition, resistance determinants to key antituberculosis drugs have remained unknown among tuberculosis causative organisms circulating in the Free State and Northern Cape. Thus, extensive DNA fingerprinting and gene mutation studies are needed to address these problems.

Methods. An area in the Free State suitable for long-term surveillance studies was defined using available information from the governmental database, the 1996 census statistics, and tuberculosis (TB) case loads and transfer data obtained from the National Tuberculosis Database. Each clinic's catchment information was provided by clinic managers and the population movement data from a 2002 student project. Sputum samples were collected and *Mycobacterium tuberculosis* isolated from tuberculosis positive patients from the defined area (Gamadi). Isoniazid resistant isolates received from a representative sample from the Free State and a few strains from the Northern

Cape Province were also included in the study. IS*6110*-directed restriction-fragment-length polymorphism (RFLP) analysis was performed on all isolates and drug susceptibility testing (indirect proportion method) done on the Gamadi isolates. Subtyping of identical strains (RFLP clusters) and some of the isolates with less than six IS*6110* bands was done using spoligotyping and the MIRU-VNTR typing. DNA sequencing analysis of the *katG* and *rpoB* genes was done in resistant isolates and a rapid PCR-based restriction enzyme *katG* gene mutation detecting method evaluated.

Results. An area characterised by extreme poverty (unemployment rate 69.0%), a relatively young population (69.0% below 35 years) of 61534 and with high incidence of tuberculosis (840/100 000) suitable for long-term surveillance studies was identified in the Free State. The area is served by three clinics and a hospital and is situated near the rural town of Thaba Nchu in the Free State province. Eighty eight *M. tuberculosis* isolates and a mycobacterium-other-than-tuberculosis (MOTT) were isolated from the 286 sputum specimens collected from the Gamadi area. Only two *M. tuberculosis* isolates tested isoniazid (INH) resistant and no rifampicin (RIF) resistant isolates were found. The MOTT was resistant to INH (0.2, 1 and 5 µg/ml) and to RIF.

Standard IS*6110*-based DNA fingerprinting of 84 of 88 (96.5%) isolates from the defined area was performed. Four of the isolates were cultured from duplicate sputum specimens provided by four patients. Two of these had identical fingerprint patterns to the first isolate of the patient and two had a different profile. The latter pair could be attributed to laboratory error. IS*6110* sequences were not detected in six isolates. Fourteen isolates had less than six IS*6110* hybridisation bands and four strains were in clusters. The remaining 57 (88.9%) strains had distinct RFLP profiles with more than six bands. The number of IS*6110* copies varied from seven to 21. A total of five strains was distributed in two clusters, one with two and the other with three members. Thirteen family groups, clustered at 65.0% on the similarity dendogram, each with two to eight strains, but no dominant groups were evident. A cluster of three isolates with five identical IS*6110* bands each was confirmed as one strain by MIRU-VNTR typing while two further isolates (both had three bands of different sizes) were confirmed as different strains by MIRU typing.

A total of 37 isoniazid-resistant *M. tuberculosis* was analysed. DNA fingerprint profiles showed nine isolates with less than six insertions (24.3%). Six of these isolates were from the Free State and three from the Northern Cape Province. Three of these isolates were multidrug resistant. The remaining 28 isolates (75.7%) contained between 9 and 18 copies of the IS*6110* insertion sequence. Twenty-six different IS*6110* RFLP types were identified. Only two clusters with two isolates, respectively, were found in each province. Eight clonally related groups (65.0% similarity) with two to four strains were present. Three clusters of two isolates (each with more than six bands) also exhibited identical spoligotype patterns. Spoligotyping of two of three isolates from a fourth cluster (4 RFLP bands each) showed two different banding patterns and all were shown to be different by MIRU-VNTR typing. The fifth cluster (2 bands) was made up of one isolate from each province. Spoligotyping of these strains was identical, but the MIRU was different. One isolate from Bloemfontein had identical IS*6110*-RFLP and spoligotyping patterns to a susceptible isolate from Gamadi.

Isoniazid resistance in 22/37 isolates was sequence linked to altered nucleotides of codon 315 of the *katG* gene. Twenty harboured the ACC variant at the codon. One strain carried the AAC mutation at this codon and the other GGC. The remaining 15 carried the wild type (AGC) genotype at this site. Two of the strains harbouring the AGC315ACC mutation belonged to the same *IS6110* cluster. Two mutations were found at codon 463 (CGG ® CTG; CGG ® CCG).

Thirteen MDR strains were investigated for *rpoB* gene alterations. Four of these isolates carried no mutations within the 157-bp amplified fragment while the others had various mutations.

Analysis of an 808bp fragment of the *kat*G gene from INH-resistant *M. tuberculosis* isolates after restriction with *Msp* I agreed with results obtained by sequencing. Thirteen isolates carried a pattern consisting of 228, 153, 146, 109, 79, 65 base pairs with the 153 bp fragment indicating the presence of the wild type AGC at codon 315 of the *katG* gene. Seventeen isolates demonstrated the 228, 146, 132, 109, 79, 65, 21 profile with the 132 bp fragments indicating the presence of an ACC mutation. Three isolates contained a mixed genotype and were digested into the fragments 228 bp, 153 bp, 146

bp, 132 bp, 109 bp, 79 bp, and 65 bp. Fragments with 146 bp and 65 bp are seen in strains with no mutation (bases CGG) at codon 463, while a 211 bp fragment shows a mutation at this spot. Four strains had the fragments 228, 211, 153, 109, and 79 bp. One strain was digested into six fragments of 228 bp, 211 bp, 132 bp, 109 bp 79 bp and 21 bp containing both a 315 (ACC) and 463 (CTG) codon mutation.

Discussion and conclusions. An area consisting of ten villages and characterised by a high incidence of tuberculosis was defined for long-term surveillance studies. Resistance in the area appears to be low and compares favourably to the situation in the Free State. Strains received from this area were highly diverse, but the presence of a cluster of five isolates indicated the need for continuous investigation. Recent transmission of INH resistance in the Free State province is not a significant factor, but since the isolates from the Northern Cape were not representative, no deduction could be made for this province. Resistance to INH is mostly associated with mutation AGC to ACC at codon 315 of the *katG* gene. The absence of alterations in a proportion of isolates is in agreement with published data implicating the involvement of more genes in causing INH resistance. Resistance to RIF was associated with various point mutations in the 81-bp core region of the *rpoB* gene. The high proportion of the ACC allele found among INH-resistant strains, cost effectiveness, ease to perform and rapid results, make PCR-RFLP an attractive option for detection of resistance especially in resource-poor countries.

Keywords: Tuberculosis; *Mycobacterium tuberculosis*; drug susceptibility testing; DNA fingerprinting; epidemiology; IS*6110* RFLP; transmission; resistance; DNA sequencing; PCR RFLP; restriction.

MOLECULAR EPIDEMIOLOGY OF *MYCOBACTERIUM TUBERCULOSIS* STRAINS FROM THE FREE STATE AND NORTHERN CAPE PROVINCES, SOUTH AFRICA.

by

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for the Degree Master of Medical Sciences (Microbiology)
in the Faculty of Health Sciences, School of Medicine
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DECLARATION

I declare that the dissertation hereby submitted by me for the M. Med. Sc. degree at the University of the Free State, Bloemfontein, is my own independent work and has not previously been submitted by me at another university/faculty. I furthermore cede copyright of the dissertation in favour of the University of the Free State.

Sehloho Zacharia Mokhethi

7th day of June 2004

DEDICATION

This work is dedicated to my family; your unwavering love is the wind beneath my wings. To Mr RA Codd for making matriculation possible, you were indeed my launching pad.

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TABLE OF CONTENTS

DECLARATION ii
DEDICATION iii
ACKNOWLEDGEMENTS iv
TABLE OF CONTENTS vi
LIST OF TABLES xi
LIST OF FIGURES xiii
ABBREVIATIONS xv

CHAPTER 1 1
INTRODUCTION 1

1.1 History 2
1.2 Prevalence 2
1.3 Drug-resistant tuberculosis 3
1.4 Interaction of TB and HIV/AIDS 5
1.5 Reservoirs of infection 5
1.6 Transmission of TB 6
1.7 The presentation and aetiology of tuberculosis 6
1.8 Genome 7
1.9 Chemotherapy and management of TB 8
1.10 Mechanisms of action of anti-tuberculosis drugs and resistance 9
    1.10.1 Streptomycin 10
    1.10.2 Isoniazid 10
    1.10.3 Rifampicin 11
    1.10.4 Pyrazinamide 11
    1.10.5 Ethambutol 11
    1.10.6 Cycloserine 12
    1.10.7 Fluoroquinolones 12
1.11 Laboratory diagnosis diagnosis of tuberculosis 12
    1.11.1 Microscopy 12

1.11.2 Culture 13
1.11.3 Diagnosis based on molecular techniques 13
1.11.4 Sensetivity testing 14
1.11.5 Strain typing 15
1.11.6 Gene mutation studies 17
1.12 Objectives 18

CHAPTER 2 19
MATERIALS AND METHODS 19

2.1 Defining of the study area 20
2.2 Collection and processing of samples 20
2.2.1 Sputum collection and processing 20
2.2.2 Isolation of mycobacteria 21
2.2.3 Identification of the isolates 21
2.2.3.1 Ziehl-Neelsen (ZN) staining 21
2.2.3.2 Catalase test 21
2.2.3.3 Nitrate test 22
2.2.4 Antimicrobial susceptibility testing 22
2.3. Strain collection from MRC 23
2.4 Extraction of DNA 23
2.5 DNA fingerprinting 24
2.5.1 Isolates tested 24
2.5.2 IS*6110* restriction fragment length polymorphism 25
2.5.2.1 Preparation of probes 25
2.5.2.2 Hybridisation, washing and detection 26
2.5.2.3 Analysis of IS6110 RFLP patterns 27
2.5.3 Spoligotyping 27
2.5.4 Mycobacterial interspersed repetitive units or variable number of tandem repeats (MIRU-VNTR) typing 28
2.6 *KatG* and *rpoB* genes analyses 29
2.6.1 Amplification of the *KatG* gene 29
2.6.2 Amplification of the *rpoB* gene fragment 30

2.6.3 Sequencing 30
2.6.3.1 katG gene 30
2.6.3.2 Sequencing of the rpoB gene 31
2.7 Restriction analysis of mutations in the *katG* gene 31

CHAPTER 3 33
RESULTS : SETTING IN THE FREE STATE PROVINCE OF SOUTH AFRICA 33

3.1 Introduction 34
3.2 Results 35
3.3 Discussion 38

CHAPTER 4 40
RESULTS: CULTURE AND IDENTIFICATION OF *M. TUBERCULOSIS* ISOLATES 40

4.1 Introduction 41
4.2 Results 42
4.2.1 Culture 42
4.2.2 Ziehl-Neelsen (ZN) staining 42
4.2.3 Identification of a selected number of isolates 45
4.3 Discussion 45

CHAPTER 5 48
RESULTS: DRUG SUSCEPTIBILITY TESTING 48

5.1 Introduction 49
5.2 Results 53
5.3 Discussion 54

CHAPTER 6 56
RESULTS: DNA FINGERPRINTING 56

6.1 Introduction 57

6.2 Results 60

6.2.1 INH resistant strains from the MRC 60

6.2.1.1 Patient data 60

6.2.1.2 IS6110-RFLP analysis of drug-resistant strains 62

6.2.1.3 Spoligotyping 65

6.2.2 Isolates from the defined area 66

6.2.2.1 Clinical characteristics of participants 66

6.2.2.2 IS6110 fingerprinting analysis 67

6.2.3 Subtyping by MIRU-VNTR 70

6.2.3.1 Evidence for recent transmission (Table 6.6) 71

6.3 Discussion 71

CHAPTER 7 79

RESULTS: MOLECULAR DETECTION OF ISONIAZID RESISTANCE 79

7.1 Introduction 80

7.2 Results 81

7.3 Discussion 84

CHAPTER 8 86

RESULTS: MUTATIONS IN THE *rpoB* GENE OF RIFAMPICIN-RESISTANT *M. TUBERCULOSIS* STRAINS 86

8.1 Introduction 87

8.2 Results 92

8.3 Discussion 93

CHAPTER 9 94

RESULTS: RAPID SCREENING FOR RESISTANCE 94

9.1 Introduction 95

9.2 Results 98

9.3 Discussion 99

CHAPTER 10 102
FINAL DISCUSSION 102
REFERENCES 107
SUMMARY 120
OPSOMMING 124

LIST OF TABLES

Table 2.1: Pairs of primers for amplifying different MIRUs. 28

Table 3.1: Number of tuberculosis patients reported during 2001 for the Free State province, the Motheo district and the Gamadi area. 38

Table 4.1: ZN staining results of randomly selected culture positive and culture negative sputum samples from Gamadi after decontamination to confirm quality of samples received for culturing. 44

Table 4.2: Identification of isolates from Gamadi using biological and biochemical characteristics. 46

Table 6.1: Data of strains received from MRC (location and resistance profiles). 61

Table 6.2: Demographic properties of fingerprinted INH-resistant patients from the Free State and Northern Cape. 62

Table 6.3: Strains numbers of sputum samples of six patients that gave more than one sputum sample from Gamadi. 66

Table 6.4: Characteristics of culture positive patients from the defined area: Gamadi. 67

Table 6.5: MIRU-VNTR patterns of twelve *M. tuberculosis* strains. Seven strains were from two RFLP clusters with less than six bands and two strains with unique RFLP patterns of three bands. The other five strains were isolates with less than six RFLP bands from the Gamadi area. MIRU-VNTR typing was performed using the twelve most polymorphic loci. 72

Table 6.6: Characteristics of groups of patients with identical IS6110 RFLP

patterns. Both INH resistant cases from MRC and susceptible cases from Gamadi. 73
Table 7.1: *KatG* gene mutations reported in the literature. 81

Table 7.2: Susceptibility data for INH, RIF, ETH, STR and mutations found by sequence analysis of the *katG* gene of 24 isoniazid resistant strains collected from the Free State and 13 strains from the Northern Cape. 83

Table 8.1: Common mutations in rpoB gene of rifampicin resistant *M. tuberculosis*. 88

Table 8.2: Other mutations reported in the rifampicin-resistant *M. tuberculosis* bp region of the *rpoB* gene. 89

Table 8.3: Missense mutation found in RIF-resistant strains from the FS and NC. 92

LIST OF FIGURES

Figure 3.1: Location of the study area in the Free State Province. 37

Figure 4.1: Summary of sputum samples received from patients in Gamadi area. 43

Figure 5.1: Summary of clinical Mycobacterial isolates from Gamadi subjected to isoniazid susceptibility testing. 51

Figure 5.2: Summary of Mycobacterial isolates from Gamadi subjected to rifampicin susceptibility testing. 52

Figure 6.1: INH-resistant *M. tuberculosis* from MRC and susceptible isolates and a MOTT from the Gamadi area selected for fingerprinting studies using IS6110-RFLP typing. 59

Figure 6.2: IS*6110* RFLP patterns of 37 INH-resistant *M. tuberculosis* isolates received from MRC, Pretoria. Strains were arranged according to similarities determined by the unweighted pair group method using arithmetical averages (UPGMA) and Dice coefficient with the GelCompar 4.3 program. 64

Figure 6.3: RFLP and spoligotyping patterns of nine INH-resistant isolates from MRC and one susceptible isolate from Gamadi that clustered with one of the INH-resistant strains. 65

Figure 6.4: IS*6110* RFLP fingerprinting patterns of 79 *M. tuberculosis* isolates from the Gamadi community. Strains were arranged according to similarities determined by the unweighted pair group method using arithmetical averages (UPGMA) and Dice coefficient with the GelCompar 4.3 program. 69

Figure 7.1: INH resistant isolates collected from the Free State and Northern Cape by the MRC. 81

Figure 8.1: Rifampicin resistant isolates from MRC, *rpoB* gene sequenced. 91

Figure 9.1: INH resistant *M. tuberculosis* isolates from the MRC and two mycobacterial strains from Gamadi tested for the presence of mutations using the *Msp* I RFLP assay. 97

Figure 9.2: *Msp* I restriction of control strain H37Rv and ten other isolates (MRC = 8, Gamadi = 20). 98

Figure 9.3: *Msp* I restriction of five TB isolates. 98

Figure 9.4: *Msp* I restriction of control strain H37Rv and 24 other TB isolates. 99

ABREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ATCC	American type collection culture
BCG	Bacillus of Calmette and Guerin
bp	Base pairs
CDC	Centres for Disease Control and Prevention
CFLP	Cleavase fragment length polymorphism
Da	Dalton
DOH	Department of health
DOTS	Directly Observed Treatment Short-course
E. coli	Escherichia coli
ETH	Ethambutol
HIV	Human Immunodeficiency Virus
FS	Free State
HPLC	High-performance liquid chromatography
INH	Isoniazid
Ipl	IS*6110* preferential locus
IS	Insertion sequence
Kb	Kilobases
LJ	Löwenstein-Jensen
MDRTB	Multidrug resistant TB
MIRU	Mycobacterial intergenic repetitive units
MMWR	Morbidity and mortality weekly report
MOTT	mycobacterium-other-than-tuberculosis
MPTR	Major polymorphic tandem repeat
MRC	Medical Research Council
MTB	*Mycobacterium tuberculosis*
MWM	Molecular weight marker
NAP	?-Nitro-a-acetylamino-ß-hydroxypropiophenane
NC	Northern Cape
NCCLS	National Committee for Clinical Laboratory Standards

ND	Not done
NHLS	National Health Laboratory Services
NTCP	National Tuberculosis Control Programme
PCR	Polymerase Chain Reaction
PGRS	Polymorphic GC-rich repetitive sequence
PTB	Pulmonary tuberculosis
PZA	Pyrazinamide
RFLP	Restriction fragment length polymorphism
RIF	Rifampicin
RRDR	Rifampicin-resistance-determining region
SA	South Africa
SCC	short course chemotherapy
SSCP	Single-strand conformation polymorphism
STR	Streptomycin
TB	Tuberculosis
UFS	University of the Free State
UK	United Kingdom
USA	United States of America
VNTR	Variable number of tandem repeats
WHO	World Health Organisation
ZN	Ziehl-Neelsen

CHAPTER 1

INTRODUCTION

1.1 History

Tuberculosis is an infectious disease of humans. Evidence of tuberculosis-compatible lesions dates back many thousands of years (Salo *et al*., 1994). Viewed from its historical and contemporary disease burden perspective, tuberculosis (TB) is one of the causes of human sufferings. In Europe the mortality ranged between 200 and 300 per 100 000 of the population at the beginning of the 19th century (Kato-Maeda M *et al*., 2001). The turn for the better came about in the 1880s when general living conditions improved and specific TB control and public health management measures came into place. Robert Koch discovered the causative agent of TB in 1882 (Zumla *et al*., 1999). However, treatment of TB with antibiotics had to wait for the discovery of streptomycin in 1944 (Chopra *et al*., 1998). Sir John Crofton and colleagues developed multidrug chemotherapy regimens in the 1950s. The subsequent discovery of rifampicin permitted the development of the present short course regimens (Zumla *et al*., 1999). Tuberculosis has since developed into an epidemic fuelled by the human immunodeficiency virus (HIV), non-compliance to treatment and poor management of the disease. This has resulted in an increase in reactivation rates, re-infection of cured patients, and the development of multidrug resistance.

1.2 Prevalence

One third of the world's population (about 2 billion people) is estimated to be infected with the tuberculosis bacillus, but a few (5-10%) will develop active tuberculosis. Globally, there are eight million new cases of tuberculosis per annum and three million people die from the disease annually (Raviglione *et al*., 1995). Predictions are quite grim. The incidence of TB varies between the developed and developing countries. For example, the incidence was estimated at 300 per 100 000 people in Ethiopia and 10 per 100 000 of the population in the Netherlands in 1995 (Hermans *et al*., 1995). Approximately two thirds of the world's cases occur in Asian countries, but the disease is also endemic in some countries in Africa and other regions (Dye *et al*., 1999). War and social upheaval have played a role in the spread of tuberculosis beyond endemic zones.

The increase in the global burden has resulted in the World Heath Organisation (WHO) in 1993 declaring tuberculosis a ‘Global emergency’ (Blumberg, 1995).

South Africa, with its cities being relatively attractive economic destinations in the Southern African region, but having poor and under-serviced rural areas, makes it a fertile ground for the disease to flourish. An estimated two thirds of the population in South Africa is infected by latent TB bacilli and 60.0% of TB patients are co-infected with HIV (DOH. Practical guidelines, 2000). Recent statistics revealed an increase (37.0%) in reported cases since the inception of the directly observed treatment strategy (DOTS) in 1996 to 2001. An incidence of 526/100 000 cases (in 2002) placed this country at number 9 in the world rankings. Nationally 144 910 new cases of pulmonary TB were reported in 2001. The Free State and Northern Cape contributed 9 978 (352/100 000) and 3 866 (438/100 000) cases respectively (Kironde *et al*., 2002).

1.3 Drug-resistant tuberculosis

Chemotherapy is the most potent weapon available in the fight against tuberculosis. When used properly, available anti-TB drugs are able to reach cure rates above the 85% target recommended by the World Health Organisation (WHO) (British Thoracic association, 1982). Early in the chemotherapy era, resistance associated with treatment failures emerged and has become a common occurrence worldwide. Of particular concern is the increasing prevalence organisms resistant to isoniazid and rifampicin, the two drugs that form the backbone of modern short-course therapy. Rifampicin (RIF) resistance occurs mostly in conjunction with INH resistance (90% of cases) and can be used as a surrogate marker for multidrug resistance.

Drug resistance in *M. tuberculosis* occurs as a result of random spontaneous chromosomal mutations during natural cell replication. These mutations are not drug-induced and are not linked. The probability of a drug-resistant mutant occurring is directly proportional to the size of the bacterial population. The frequency of primary resistant organisms varies for each drug; however, it is usually between 10^{-6} to 10^{-8}. Spontaneous resistance to isoniazid is estimated to occur once in every 10^{6} organisms, and to rifampicin once in every 10^{8} organisms. The probability of spontaneous mutants being simultaneously resistant to two or more drugs is the product of the individual mutants. The development of drug resistance is a man-made amplification of a naturally occurring phenomenon. Previous treatment for tuberculosis predisposes to the selection of multi-drug resistant organisms. Non-compliance is a major factor in allowing the resistant organisms to survive (Portaels *et al*., 1999). Multi-drug therapy is used to prevent the emergence of drug resistant mutants during the long duration of treatment. Resistance can be defined as single-drug, multi-drug, or poly-drug resistance depending on the number of drugs and/or which drugs are involved (Rieder, 2002).

Although an unequal global distribution of drug resistance exists between poor and rich countries, the problem is global. The regions where drug-resistant TB is more prevalent lack the resources to implement adequate measures to control even the susceptible types of the disease. Recent reviews have reported a high prevalence of primary multidrug resistant tuberculosis in Latvia (1998: 9.0%), Estonia (1998, 14.1%), The Dominican Republic (1994-1995: 6.6%), Ivory Coast (1995-1996: 5.3%), Argentina (1994: 4.6%), Russia (Ivanovo Oblast) (1998: 9%), Iran (1998, 5.0%) and Henan, China (1996, 10.8%). South Africa’s neighbours Botswana (1995-1996), Lesotho (1994-1995), and Swaziland (1994-1995) have reported encouraging results of 0.2%, 0.9%, and 0.9% respectively. Acquired multidrug resistance of higher than 20% was reported in Guinea (1998: 28.1), Latvia (1996: 54.4%), Mexico (1997: 22.4%) Italy (1999: 33.9%), Russia (Ivanovo Oblast) (1998: 25.9%) Tomsk Oblast (1999: 26.7%), Estonia (1998: 37.8%), Iran (1998: 48.2), Sierra Leone (1997: 23.1%), Argentina (1994: 22.2), and Spain (Barcelona) (1995-1996: 20.5%). Again acquired MDRTB was low in Botswana (1998: 9.0%), Mozambique (1999: 3.3%), Lesotho (1994-1995: 5.7%), and Swaziland (1994-1995: 9.1%) (Cohn *et al*., 1997, Espinal *et al*., 2001).

Previous treatment for tuberculosis predisposes to the selection of multi-drug resistant organisms and non-compliance is a major factor in allowing the resistant organisms to survive. In countries with a high incidence of MDRTB a DOTS plus system has been suggested. This approach is currently being tested in sentinel sites. It involves the use of specific treatment regimens together with sputum and susceptibility testing. Epidemiological surveillance of resistance and mutation monitoring are, however, still neglected in South Africa and should be the backbone of governmental continuous programmes as well as DOTS-plus programmes (Consensus statement, 2003).

1.4 Interaction of TB and HIV/AIDS

The advent of the HIV/AIDS pandemic has fuelled the spread of TB worldwide. Sub-Saharan Africa is the most devastated region with close to 70% of its inhabitants co-infected with HIV and TB (Harries, 1998). In South Africa, the rate of TB patients that were HIV positive reached 60% in 2002 (Kironde *et al*., 2002). Persons co-infected with HIV and TB have an increased risk of developing active tuberculosis. The result is an increase in TB cases among non-HIV infected persons due to a larger pool of source cases in the community (Harries, 1998). A disturbing issue is that this increase in the incidence that threatens to overwhelm TB control programmes will inevitably be accompanied by a rise in drug-resistant TB. The best approach to reduce the increasing TB caseload attributable to HIV infection will be to complement the DOTS with DOT-plus strategies that will rapidly identify MDR strains and their susceptibility patterns. The association between TB and the human immunodeficiency virus (HIV) infection is threatening to overwhelm control programmes globally. This has prompted the National Department of Health in South Africa to devise collaborative disease control and monitoring strategies (Kironde *et al*., 2002).

1.5 Reservoirs of infection

The incidence of TB varies between the developed and developing countries. The distribution of this disease is also uneven within countries. Certain groups within societies bear a disproportionately high burden. Groups such as AIDS patients, close contacts of TB sufferers, immigrants, medically under-serviced poor populations, alcoholics and intravenous drug users, people in long-term care facilities, correctional institutions, mental institutions, nursing homes/facilities, and other long-term residential facilities, mine workers and homeless people carry a higher TB burden (CDC, 1990).

1.6 Transmission of TB

Tuberculosis is primarily a disease of the lungs and infection is contracted by inhalation of aerosols. When a patient with active pulmonary TB coughs or spits, small droplets that contain TB bacilli will be produced. Anyone who inhales this air with droplets can then be infected and may subsequently develop TB disease. The infectiousness of a case of TB is dependent on the concentration of TB bacilli within the lungs and their spread into the air surrounding the patient with TB. The most infectious cases are those with a positive smear by microscopy (smear positive cases). Extra-pulmonary cases are almost never infectious, unless their lungs are infected as well (Metchock *et al*., 1995).

1.7 The presentation and aetiology of tuberculosis

Active pulmonary tuberculosis presents as fatigue, anorexia, lose of weight, low-grade fever, night sweats, chronic cough, and haemoptysis. Local symptoms depend on the parts affected. Active pulmonary tuberculosis is relentlessly chronic and, if untreated, leads to progressive destruction of lung tissue. Cavities form in the lungs and erosion into pulmonary blood vessels can result in life-threatening haemorrhage. Gradual deterioration of nutritional status and general health culminates in death due to wasting, infection, or multiple organ failure (Metchock *et al*., 1995).

Tuberculosis is characterized by the formation of tubercles and tissue necrosis, primarily because of host hypersensitivity and inflammation. Infection is usually by inhalation of airborne particles and the bacilli spread from the initial location in the lungs to other parts of the body via the blood stream, the lymphatic system, the airways or by direct extension to other organs. Primary tuberculosis is a mild or asymptomatic local infection. Regional lymph nodes may become involved, but in otherwise healthy persons, generalised disease does not immediately develop. Organisms in a primary lesion remain viable and can become reactivated months or years later to initiate secondary tuberculosis. Progression to the secondary stage eventually occurs in about 10% of people who have had primary tuberculosis (Metchock *et al*., 1995). Reactivated tuberculosis usually results in a chronic, spreading lung infection, most often involving the upper lobes. Tubercles develop in involved lung tissue, each consisting of a zone of caseation necrosis surrounded by chronic inflammatory cells. Pulmonary TB is the infectious and common form of the disease, occurring in over 80% of cases. Extra pulmonary tuberculosis is a result of the spread of tuberculosis to other organs, most commonly pleura, lymph nodes, spine, joints, genito-urinary tract, nervous system or abdomen. Tuberculosis may occur in any part of the body (Blumberg, 1995). Rarely, reactivation results in widespread dissemination of tubercles throughout the body (milliary tuberculosis). Variant syndromes are caused by organisms of the *Mycobacterium avium-intracellulare* complex, for example, tuberculous lymphadenitis in children and severe systemic disease in acquired immune deficiency syndrome (AIDS) sufferers (Metchock *et al*., 1995).

The closely related subspecies that form the *M. tuberculosis* complex (MTBC) (*Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, and *Mycobacterium microti*) are the causative agents of tuberculosis (TB) in humans (Metchock BG *et al*., 1995). *M. tuberculosis* is the major cause of TB, but *M. africanum* represents up to 60% of cases of TB in certain regions in Africa (Zumla *et al*., 1999). Non-tuberculosis mycobacteria like *M. kansasii* and *M. avium-intracellulare* can also cause pulmonary diseases clinically indistinguishable from tuberculosis and are mainly found in patients infected with the human immunodeficiency virus (HIV) (Metchock *et al*., 1995).

M. tuberculosis is an aerobic, acid-fast, non-motile, non-sporeforming, non-capsulating thin bacillus. The cell envelope contains an additional layer beyond the peptidoglycan that is exceptionally rich in unusual lipids, glycolipids and polysaccharides. The high lipid content of their cell wall makes mycobacteria acid-fast. The cells are resistant to dehydration and are able to survive in dried, expectorated sputum. This characteristic may be important in its transmission by aerosol (Metchock *et al*., 1995).

1.8 Genome

The genome, made up of a single chromosome, consists of approximately 4.4 million base pairs, and contains around 4 000 genes. The DNA material is rich in repetitive

DNA, namely insertion sequences and short repetitive DNA, new multigene families and duplicated housekeeping genes. Most of the insertion sequences in *M. tuberculosis* appear to have inserted in intergenic or non-coding regions. Many are clustered, suggesting the existence of insertional hot spots that prevent genes from being inactivated. Genetic DNA elements called short repetitive DNA associated with some degree of diversity have been identified in the *M. tuberculosis* complex. Three of these, the polymorphic GC-rich tandem repeat sequence (PGRS), a repeat of the triplet GTG, and the major polymorphic tandem repeat (MPTR), are present at multiple chromosomal loci. The genome is also rich in G+C nucleotides. The presence of a high proportion of G+C-rich codons results in the increased use of amino acids Ala, Gly, Pro, Arg and Trp (Cole *et al*., 1998).

1.9 Chemotherapy and management of TB

Streptomycin was the first antibiotic to be used in the treatment of tuberculosis. Isoniazid was introduced in the early 1950s and rifampicin was included in tuberculosis treatment in 1971. Pyrazinamide was introduced as part of the short course chemotherapy regimen in the 1970s. Isoniazid, rifampin, ethambutol and pyrazinamide, administered as one combination tablet during the intensive phase (first two months) of tuberculosis treatment, form the DOTS strategy recommended by the WHO for the treatment of tuberculosis. Streptomycin is added in the treatment regimen in cases previously treated for tuberculosis. Other drugs, such as amikacin and ciprofloxacin, may be added or substituted. The success of treatment is dependent on two factors, that is, the sensitivity of the organisms to drugs in use and the risk of severe toxic effects produced by these agents. Unlike most infections treated with antibiotics, the period of tuberculosis treatment is measured in months and years. Long-term compliance therefore is one of the important challenges in control of the disease (Chopra *et al*., 1998).

According to the WHO, the principal reason for the spread of multidrug-resistant strains of *M. tuberculosis* is ineffectual management of tuberculosis control programmes, particularly in developing countries. Suboptimal administration of drugs not only leaves the patient still sick and still infectious, but also favours the selection of resistant bacteria. Currently the WHO urges that tuberculosis programs worldwide adopt the practice of directly observed therapy (DOT) (DOH: practical guidelines, 2000)

The objectives of the South African National Tuberculosis Control Programme (NTCP) are 1) to reduce mortality and morbidity attributable to TB, 2) to prevent the development of drug resistance, and 3) to ensure accurate measurement and evaluation of Programme performance. Short-term objectives of the NTCP are 1) to achieve smear conversion rates of at least 85% among new smear positive cases and 80% among retreatment cases at the end of the intensive phase of treatment, and 2) to cure at least 85% of new smear positive cases with short course chemotherapy. Prevention involves identification and subsequent treatment of sputum-positive patients, finding active cases of infection among their close contacts and the vaccination of all children with attenuated *M. bovis* BCG (bacillus Calmette-Guerin or BCG). All children under 5 years living in the household of the active index case receive prophylactic treatment with isoniazid. Drugs for TB are administered simultaneously as a single tablet (INH, rifampicin, pyrazinamide and ethambutol) for 2 months (induction phase) and for the next four months (continuous phase) isoniazid and rifampicin are administered as a combination tablet. Although therapy is usually given for months, the patient's sputum becomes non-infectious within a couple of weeks. Protracted therapy is attributed to (1) the intracellular location of the organism, (2) caseous material, which blocks penetration by the drug, (3) the slow growth of the organism, and (4) metabolically inactive organisms within a lesion. Antitubercular drugs may not kill metabolically inactive organisms, treatment may not eradicate the infection and reactivation of the disease may occur in the future (DOH: practical guidelines, 2000).

1.10 Mechanisms of action of anti-tuberculosis drugs and resistance

Mycobacterium tuberculosis is naturally resistant to many antibiotics. This resistance is due mainly to the cell envelope acting as a permeability barrier, but many potential resistance determinants are also encoded in the genome. These include hydrolytic or drug-modifying enzymes such as ß-lactamases and aminoglycoside acetyl transferases, and many potential drug-efflux systems (Hatfull, 1993).

1.10.1 Streptomycin

Streptomycin acts on ribosomes and causes misreading of the genetic code, inhibition of translation of mRNA and aberrant proofreading. Eventually synthesis of proteins is inhibited. Resistance results from mutations in components of the 30S ribosomal subunit, i.e. 16S RNA and the ribosomal protein S12. Mutations in genes encoding 16S ribosomal RNA (*rrs*) and ribosomal protein S12 (*rps*L) account for 64-68% of resistant strains (Chopra *et al*., 1998; Cockerill lll, 1999).

1.10.2 Isoniazid (INH)

Isoniazid interferes with the synthesis of mycolic acid, thus having a negative effect on the integrity of the cell wall. The catalase-peroxidase (*katG*) gene spans about 2 223 base pairs and encodes for an enzyme of 80 000 Da with substantial homology to hydroperoxidase I from *E. coli*, catalase-peroxidase from *M. intracellulare*, and other bacterial catalase-peroxidases. In most INH-resistant strains either simple base pair changes or small insertions or deletions in the *katG* gene result in catalase-peroxidase with less ability to activate INH (Musser, 1995). Missense mutations at codon 315 are found in a high proportion of INH-resistant strains. Mutations in the *katG* gene account for 50-70% of resistant strains (30-65% due to a single mutation S315T). Another mechanism for isoniazid resistance is the total deletion of the *katG* gene. This accounts for 10-24% of isoniazid-resistant isolates (Chopra *et al*., 1998; Cockerill lll, 1999).

Other causes of INH resistance have been found. A locus containing the *inh*A gene with a length of 744-bp encoding a 28.5-kDa protein has been found (Musser, 1995). Mutations in the *inh*A locus account for 5-10% of resistant strains. Mutations in the promoter region of *ahp*C encoding the alkylhydroperoxide reductase account for 6-13% of resistant strains (Cockerill lll, 1999). In recent years a gene called the *kas*A gene has joined the ranks as a strong culprit linked to INH resistance (Rieder, 2002).

1.10.3 Rifampicin (RIF)

Rifampicin is an inhibitor of DNA-dependent RNA polymerase. It acts by interfering with the synthesis of mRNA by binding to the RNA polymerase. Resistance to high levels of rifampicin occurs in a single step in *M. tuberculosis*, with mutants arising spontaneously in strains not exposed previously to the antibiotic at a frequency of one in 10^8. The molecular basis of *M. tuberculosis* resistance to rifampin has been shown to be associated with deletion, insertion, or missense mutations in the *rpoB* gene, which encodes the ß-subunit of the DNA-dependent RNA polymerase (Musser, 1995). This site referred to as the rifampicin resistance-determining region (RRDR) spans 81 bp coding for 27 amino acids. Published reports indicate that more than 95.0% of rifampicin-resistant *M. tuberculosis* isolates harbour specific mutations in this region ((Musser, 1995). Specific mutations in *rpoB* produce rifampicin resistance, apparently by diminishing its binding affinity to the enzyme.

1.10.4 Pyrazinamide (PZA)

The target of pyrazinamide in *M. tuberculosis* is not known, but the molecular basis of resistance is associated with the loss of pyrazinamidase activity. Multiple mutations, which span the entire coding region of the *pncA* gene have been identified as the cause for acquired pyrazinamide resistance in *M. tuberculosis* (Chopra *et al*., 1998). These mutations account for ~70% of resistant strains (Cockerill lll, 1999).

1.10.5 Ethambutol (ETH)

Ethambutol inhibits the synthesis of the mycobacterial cell wall by interfering with the polymerization of arabinogalactan. Mutations in the *embB* gene, which encodes polymerization of arabinose into arabinogalactan, account for ~70% of resistant strains (Chopral *et al*., 1998, Cockerill lll, 1999).

1.10.6 Cycloserine

Cycloserine inhibits the biosynthesis of the mycolylarabinogalactan-peptidoglycan complex by interfering with the activity of D-alanine racemase or D-alanine ligase enzymes. Resistance results from the overexpression of the AlrA enzyme – due to a single transversion (G F T) at the alrA promoter in cycloserine resistant strains (Chopra *et al*., 1998).

1.10.7 Fluoroquinolones

The principal target of fluoroquinolones is DNA gyrase, a type II topoisomerase that is composed of two A and two B subunits encoded by the genes *gyrA* and *gyrB*, respectively. Fluoroquinolone resistance in *M. tuberculosis* is associated with point mutations within a part of *gyrA* termed the quinolone-resistance-determining region (Chopra *et al*., 1998; Musser, 1995). This gene encodes the A subunit of DNA gyrase. These mutations account for ~100% of resistant strains.

1.11 Laboratory diagnosis detection of tuberculosis

1.11.1 Microscopy

Microscopic examination of sputum smears for acid-fast bacilli is used throughout the world as a diagnostic test for suspected pulmonary tuberculosis. Bacilli of mycobacteria can be demonstrated by Ziehl-Neelsen (ZN) or fluorochrome staining methods. The finding of acid-fast bacilli in sputum establishes a presumptive diagnosis of tuberculosis and indicates that the patient is capable of transmitting the infection and appropriate measures must be instituted to prevent infection. In all microscopic diagnostic methods the detection limit is between 10^4 and 10^5 bacilli per millilitre of specimen. This means those patients with fewer than 10^4 organisms per ml will be smear-negative and less infectious. Microscopy is rapid, cheap and relatively easy to perform. Sensitivity of microscopy approaches 60-70%. The sensitivity of acid-fast smears of sputum from HIV positive patients is even lower (i.e., 50% in adults). Organisms other than mycobacteria may also demonstrate various degrees of acid-fastness (*Nocardia asteroides)* leading to false smear-positive results (Blumberg, 1995).

1.11.2 Culture

Culture of sputum samples is more sensitive and can detect 10-100 organisms per ml. The increased sensitivity enables detection of cases earlier, often before they become highly infectious. However, *M. tuberculosis* grows slowly with a doubling time of approximately 18 hours. Because of slow growth, cultures of clinical specimens must be held for over a month before they can be recorded as negative. Media used for culture include among others Lowenstein-Jensen and the BACTEC liquid medium that contains radioactively marked carbon metabolites (e.g. palmitic acid). A definitive diagnosis of tuberculosis depends upon isolating the bacilli from the patient and identifying them as *M. tuberculosis* (Blumberg, 1995).

A patient who cannot produce sputum (in the case of pulmonary tuberculosis), gastric aspiration is a method of choice as a specimen for culture. Specimens used for the diagnosis of TB also include cerebrospinal fluid, bone marrow and liver biopsies. Routine urine cultures may be positive in only 7% to 10% of patients (Blumberg, 1995).

Species identification is dependent initially on growth rates and pigment production classifying *Mycobacterium* species into four groups. Species within these groups can be further identified by biochemical tests. In the BACTEC liquid-culture system, the NAP test is used to identify the growing organism as *M. tuberculosis*. Each *Mycobacterium* species can also be identified using high-performance liquid chromatography (HPLC) but this is expensive and seldomly used. Serodiagnostic test kits for anti-tuberculosis antibodies are available commercially, but serious concerns regarding interpretation of results from these tests in the South African setting have been voiced (Blumberg, 1995).

1.11.3 Diagnosis based on molecular techniques

Molecular biology-based methods have been proposed for use in the rapid diagnosis of tuberculosis. Several mycobacterial target genes have been investigated in assay systems, which allow the identification of a single or multiple mycobacterium species. Nucleic acid hybridization probes are commercially available that can hybridize specifically with a number of mycobacteria including *M. tuberculosis, M. avium*, *M. intracellulare*, *M. kansasii* and *M. gordonae*. A probe that exhibits 97.2% sensitivity and 96.1% specificity can identify *M. tuberculosis* within a few hours. Sensitive and rapid techniques based on detection of the DNA coding, highly conserved regions of the 16S ribosomal RNA (16S ribosomal DNA) of mycobacteria have been proposed. The only shortcoming of this technique is that at least 106 organisms need to be present. A polymerase chain reaction (PCR) assay amplifying a 123-bp sequence in IS*6110* may also be employed (Eisenach *et al*., 1993). *GyrB* genes, DNA sequencing, PCR assays targeting the internal transcribed spacer region (ITS) or PCR-RFLP analysis can also be employed to differentiate species of *Mycobacterium* (Park *et al*., 2000). However, the differentiation of *M. tuberculosis* and *M. africanum* type II cannot be achieved by analysis of molecular markers and remains based on phenotypic growth characteristics. PCR assays are most useful for *M. tuberculosis* detection in other sites where the number of cells are low, for example, bone, cerebrospinal fluid, pleural fluid, pericardial fluid or blood (Blumberg, 1995).

1.11.4 Sensitivity testing

The method that is recommended by the National Committee for Clinical Laboratory Standards (NCCLS) for susceptibility testing of *M. tuberculosis* is the modified agar proportion method. This method is inexpensive and relatively simple providing results in three to four weeks from a cultured isolate. The BACTEC system (Becton Dickinson) is also widely used. This system provides results in as few as five days, but requires expensive equipment and reagents and technical expertise. More recent innovations for *M. tuberculosis* antibiotic susceptibility testing are the Etest and the luciferase-based reporter mycobacteriophage that can also be used as a surrogate marker for drug susceptibility (Blumberg, 1995).

1.11.5 Strain typing

Different strains of *M. tuberculosis* form a very homogenous group at the phenotypic level. Classification of the disease evolved from differentiation of new cases of reactivated tuberculosis and acquired versus primary of drug resistant tuberculosis cases. Typing schemes that exploited phenotypic characteristics e.g. antigenic factors, resistance to defined antibiotics, susceptibility to phage infection and the production of certain chemical substances brought with them a glimpse of hope. The major drawback of these traditional methods was that markers used were often species-specific and relatively weak in identifying/confirming specific strains (Saunders, 1999 and Van Soolingen, 2001). Strain typing is, however, a powerful TB control tool that became more practical with the introduction of procedures based on DNA analysis. DNA typing differentiates organisms (even of the same species) on the bases of genetic variation at the level of chromosome or gene.

This approach was made possible by the discovery of the DNA insertion element, IS*6110*, 1355 bp long, present in different copy numbers (0-25 copies) and locations throughout the genome of *M. tuberculosis*. Although this insertion element can transpose to induce genetic recombination, rearrangements and insertion/deletion mutations, the frequency of change is relatively slow giving this method great molecular epidemiological power. A standard restriction fragment length polymophism (RFLP) method using the IS*6110* as a hybridisation target has been agreed upon internationally to enable comparison of results (Saunders, 1999 and Van Soolingen, 2001).

The discriminatory potential of this method is a function of the number IS*6110* copies present. The higher the IS*6110* copy number, the greater the likelihood that two or more identical RFLP patterns correspond to epidemiologically related strains. However, patterns containing few IS*6110* copies, even if identical, may correspond to epidemiologically unrelated cases of the disease (McHugh *et al.,* 1998). Thus, other genetic markers must be used for confirming relatedness between strains exhibiting identical RFLP patterns. More than 16 different insertion sequences (IS) belonging to five IS families have been documented in the TB bacillus (*e.g.* IS*1081*, IS*1547 etc*). However, little polymorphism is observed when using these sequences as epidemiological markers. Many other DNA fingerprinting techniques have since been discovered and are used in cases where the IS*6110* based method is found to be insufficient. The most commonly used alternatives include the polymorphic GC-rich tandem repeat sequence (PGRS), a repeat of the triplet GTG, the direct repeat sequence typing (Spoligotyping), and the mycobacterial interspersed repeat units (MIRU) method. For spoligotyping each of the 43 spacer sequences found in the direct repeat domain of the genomic DNA is detected by a specific probe. The presence or absence spacer or sequences is used in the differentiation of strains. MIRUs are repeat sequences found in tandem and are of variable numbers in intergenic regions. Estimation of the length in base pairs of each provides an indication of the number of repeats in that locus. The 12 most polymorphic MIRU-VNTR give a 12-number configuration that can be used to differentiate strains (Saunders, 1999, Kamerbeek *et al*., 1997, Supply *et al*., 1997).

Initially, the IS*6110*-directed RFLPs were used to confirm suspected cases of transmission, to detect laboratory contamination and to study disease outbreaks. Most studies have focused on tracing tuberculosis among contacts, for example, outbreaks among HIV-positive instutionalised people, bar patrons and churchgoers. Presently this tool is used to characterize the clones that are circulating in a particular restricted geographic region. Analyses of DNA fingerprints have also shed light on the routes of transmission and the degree of recent transmission versus reactivation (Warren *et al*., 1996). This method is used worldwide to monitor transmission of TB strains, even across geographical borders (Mazurek *et al*., 1991, Casper *et al*., 1996). Studies on the molecular epidemiology of tuberculosis have almost invariably shown the global dissemination strains belonging to the Asian lineage, designated W-Beijing clonal family of strains. This family of strains is associated with drug resistance in Asian countries. Subsequent investigations in Europe, United States of America, South Africa (Western Cape and KwaZulu-Natal), and other parts of the world discovered strains that are genetically related to the Beijing strain (Bifani *et al*., 2002). The prevalence of this family of strains in the Free State and Northern Cape provinces has remained unknown. Analysis of data obtained has proven valuable in improving national/regional TB control programmes.

A governmental database established in South Africa aimed at monitoring the national incidence and outcome of this disease is often not very accurate. Questions concerning transmission routes within or across our borders, the degree of exogenous re-infection versus endogenous reactivation, risk factors, spread of drug-resistant strains and development of resistance to anti-TB drugs within communities remain unanswered. Sustained studies performed on a small area in the Western Cape Province and some mines sought to clarify transmission, reactivation, and resistance development perspectives of this epidemic (Warren *et al*. 1996, Godfrey-Faussett *et al*., 2000, Churchyard *et al*., 2000, Sonnenberg *et al*., 2001). DNA fingerprinting has shown that person-to-person transmission of MDRTB is proving to be a public health menace (Warren *et al*., 1996). This data, though informative, cannot be used to address problems unique to other areas, but will remain relevant to the geographical area studied. A recent DNA fingerprinting study by the Department of Medical Microbiology, University of the Free State, found that in a convenience sample of MDRTB cases in Bloemfontein, large clusters were not evident (Van der Spoel van Dijk *et al*., 1996). A limitation of this study was that the sample was not necessarily representative of MDRTB in the Free State. The extent of drug resistance as well as the determination of reactivation and transmission patterns in the Free State and the Northern Cape is incomplete and an extensive DNA fingerprinting and gene mutation study is needed to address this problem.

1.11.6 Gene mutation studies

Mycobacterium tuberculosis is a slow growing microorganism. Therefore, an antimicrobial susceptibility test based on traditional methods may be time consuming. The need to minimize the transmission of drug-resistant TB strains resulted in the development of DNA amplification assays that greatly shortens antimicrobial resistance detection time. These assays are used as rapid additional tools assisting TB control programmes in the screening of resistant strains from clinical samples. The genetics of antimicrobial resistance have been elucidated in part for *M. tuberculosis*, enabling certain genes to be associated with resistance to anti-TB drugs: *katG*, *inhA*, *aphC*, *kasA* for isoniazid resistance; *rpoB* for rifampin resistance; *rpsL* and *rrs* for streptomycin resistance; *embB* for ethambutol resistance; *pncA* for pyrazinamide resistance; and *gyrA* for fluoroquinolones. Resistance to multiple drugs is the consequence of an accumulation of mutations. PCR-RFLP, PCR-SSCP, PCR-CFLP, PCR-ddF, PCR-LiPA, PCR-molecular beacon sequence analysis, PCR-HDP analysis, PCR-dot blot, rifoligotyping (rifampicin oligonucleotide typing) and PCR-DNA sequencing have all been employed to detect mutations associated with specific drug resistance (Brow *et al*., 1996, Felmlee *et al*., 1995, Marttila *et al*., 1996, Rossau *et al*., 1997).

1.12 Objectives

The objectives of the study were to;

(1) define an area in the Free State where long-term molecular epidemiological surveillance and resistance developmental studies can be done.

(2) describe strain diversity and transmission rates in two sample sets collected in the Free State and the Northern Cape:

  (a) INH-resistant strains collected during a survey of the National Tuberculosis Research Program of the MRC in the Free State and the Northern Cape;

  (b) Samples from all consecutive smear positive patients in a defined population in the Free State over a period of 18 months.

(3) determine susceptibility to INH and RIF of strains circulating among patients attending three clinics in Thaba ‘Nchu.

(4) investigate missense and mutation development in the *katG* and *rpoB* genes of INH resistant strains in the two sample sets.

CHAPTER 2

MATERIALS AND METHODS

2.1. Defining of the study area

Population data were retrieved from the 1996 Census. Tuberculosis (TB) case loads and transfer data were obtained from the National Tuberculosis Database. Each clinic's catchment information was provided by managers and movement data from a 2002 student project to define an area with a relatively stable population with a high TB caseload.

2.2. Collection and processing of samples

2.2.1. Sputum collection and processing

Sputum samples were collected from all consecutive sputum smear positive (according to the local NHLS laboratory) TB patients attending clinics in the defined population. Recommendations published by Small and colleagues in 1993 were followed to minimise the risk of cross-contamination. Sputum specimens were processed in batches of 16. Only one tube at a time was uncapped for addition of solutions. Buffer solutions were prepared as individual aliquots in single use tubes. Tubes were only opened 5 min after centrifugation.

Sputum specimens were collected from 286 microscopy confirmed TB patients from three primary health care clinics (Dinaane, Mafane, and Gaongalelwe) and a hospital in the Gamadi area near the rural town of Thaba Nchu. A detailed explanation concerning the ethics of the study was provided to each participant. Patients older than 12 years of age were enrolled only after giving a written consent. The samples were collected from all patients after TB confirmation, but before initiation of treatment, during the period June 2001 through to April 2003. The samples underwent digestion by means of the N-acetyl-L-cysteine sodium hydroxide method in order to decontaminate the sample before TB culture (Metchock *et al*., 1995). Equal volumes of specimen and decontamination reagent (1N NaOH, 0.1N sodium citrate, N-acetyl-L-cysteine) were added to a 15-ml plastic centrifuge tube. The reagent and specimen were placed on a shaker for 20 minutes before the tube was filled with phosphate buffer (pH 6.8). The mixture was then centrifuged (3000 xg, 15min), the supernatant discarded and the pellet resuspended with a further 10 ml of phosphate buffer. Centrifugation and addition of buffer was repeated until the pH of the mixture was neutral.

2.2.2. Isolation of mycobacteria

The decontaminated specimens (10 µl) were inoculated onto a 1.2% (v/v) glycerol containing Löwenstein-Jensen (LJ) medium. The LJ slopes were incubated in a slanted position for at least 24 h at 37ºC. All cultures were examined after 5-7 days of incubation and weekly thereafter for 4-6 weeks. Caps were opened once a week for a short interval to aerate the cultures and to examine bottles for positive growth. Cultures showing no growth after 6 weeks of incubation were excluded from the study. ZN stain, nitrate and catalase tests were performed on cultures positive for purposes of species identification. Cultures identified as mycobacterium-other-than-tuberculosis (MOTT) were excluded from the study. All positive cultures were stored at -20°C for later use.

2.2.3. Identification of the isolates

2.2.3.1. Ziehl-Neelsen (ZN) staining

A Ziehl-Neelsen (ZN) stain was performed on the decontaminated sputum specimen or growth from a solid media slant. Carbol Fuchsin stained the *M. tuberculosis* red, while 5% acid alcohol was used to decolorize and Löffler's methylene blue was used to counterstain. The slides were examined under the 100x oil immersion objective of a microscope for acid fast organisms.

2.2.3.2. Catalase test

Two loopfuls of *M. tuberculosis* were suspended in 0.5 ml Sorenson's buffer in screw-cap tubes (16 mm by 125mm). The second tube was the blank control. The tubes were placed in a 68°C water bath for 20 min and then cooled to room temperature. Half a millilitre each of a 10% Tween 80 and 30% H_2O_2 were added and the appearance of bubbles indicated a positive catalase test. Bottles were held for 20 min before being discarded as negative. *M. tuberculosis* is catalase negative. The blank also had to be negative (Metchock *et al*., 1995).

2.2.3.3. Nitrate test

Two millilitres of nitrate buffer in screw-capped tubes were inoculated with 2 loopfuls of each *M. tuberculosis*, and one tube being a negative control. The contents of the tubes were mixed and incubated in a 37°C incubator for 4 h. After incubation, 1 drop HCl, 2 drops of 0.2% sulfanilamide, and 2 drops 0.1% N-naphthylethylene-diamine were added. The solutions were examined for the development of a pink/red colour contrasting with the control. A pinch of powdered zinc was added to all the negative tubes to reduce nitrate to nitrite. The formation of a red color only after the addition of the zinc, confirmed a negative nitrate test.

2.2.4. Antimicrobial susceptibility testing

Susceptibility to isoniazid (INH) and rifampicin (RIF) was determined by the proportion method on Lowenstein-Jensen egg-based slopes containing critical concentrations of INH and RIF (0.2 µg/ml, 1 µg/ml and 5 µg/ml, 40 µg/ml respectively) (Kleeberg *et al*., 1980). Standard antibiotic powders (INH and RIF) were obtained from Sigma-Aldrich (South Africa).

The inoculum was prepared by directly suspending colonies grown for approximately three weeks on Lowenstein-Jensen drug-free slopes to a turbidity equivalent to a 1.0 MacFarland standard. The 1.0 MacFarland standardised suspension was further diluted 10^{-1} and 10^{-3}. The 10^{-1} suspension was subsequently inoculated on the drug-containing medium. Two drug-free LJ slopes were inoculated with 1:10 and 1:1000 diluted suspensions of a 1.0 MacFarland standardised inoculum. This was done for each sample tested. The drug-susceptible MTB reference strain ATCC 27294 (H37Rv) was used as a susceptible control and a known resistant strain (FS956/01) collected by the Medical Research Council was used as a resistant control. The slopes were incubated at 37°C and read after 3 and 4 weeks. An isolate was considered resistant if the proportion of bacilli resistant to the critical concentration of a drug exceeded 1%.

2.3. Strain collection from MRC

The second component of this study comprised 40 INH-resistant isolates cultured by the National Tuberculosis Research Programme, Medical Research Council (MRC), Pretoria. The isolates were collected from selected Primary Health Care clinics in the Free State and Northern Cape provinces as part of a nation-wide study: "National Survey of Tuberculosis Drug Resistance in South Africa" during 1999 – 2001 and were representative of all INH resistant cases in each province. Cultures received included isoniazid and multidrug resistant *M. tuberculosis* (MTB) isolates. The susceptibility data of the isolates were provided by the MRC.

These cultures were subcultured only on LJ media for DNA extraction.

2.4. Extraction of DNA

The LJ slant cultures were heat-inactivated at 80°C for 1 hour before DNA extraction was performed in a P2 laminar flow cabinet. The growth from a slant culture was suspended in 6 ml of DNA extraction buffer (5% monosodium glutamate, 50mM Tris-HCl, pH 7.0 and 25mM EDTA) in a sterile 50ml polypropylene tube which contained approximately thirty 5 mm glass balls. The bacterial clumps were disrupted by vigorous shaking and vortexing of the tube. Five hundred microlitres of Lysozyme (Amersham Biosciences, Greece)(50 mg per ml) and 10µl of RNAseA (Amersham Biosciences, Greece) (10 mg per ml) were added to the tube. The contents of the tube were mixed by gentle inversion and then incubated at 37°C for 2 hours. After incubation, 600 µl of 10x Proteinase K buffer and 150µl of Proteinase K (Amersham Biosciences, Greece) (10 mg per ml) were added. The sample was gently mixed (inverting the tube a few times) and then incubated overnight at 45°C. Proteins were removed by Phenol/Chloroform (5 ml) and the chloroform/isoamyl-alcohol (5 ml) extraction. DNA was then precipitated with the addition of 600 µl 3M Sodium-Acetate (pH 5.5) and 7 ml of cold (-20°C) isopropanol. The precipitated DNA was collected on a glass loop and washed with 1ml of 70% ethanol for approximately 1 minute. The DNA was air – dried and dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

2.5. DNA fingerprinting

2.5.1. Isolates tested

Eighty-nine isolates (including one MOTT) were collected from selected clinics (Dinaane, Gaongalelwe, and Mafane) and Dr JS Moroka hospital to use for restriction fragment length polymorphism (RFLP) typing. Patients diagnosed at Dr JS Moroka hospital but residing in the Gamadi area were transferred to their nearest clinic in order to start with treatment. Patients from whom sputa were received from the Dr JS Moroka hospital were then followed to their respective clinics in the Gamadi area.

A further 37 isolates received from the MRC resistance survey were also subjected to RFLP typing. The MRC study was a population-based, cross-sectional study designed according to the international WHO protocol for drug resistance surveillance (WHO/TB/96.216, 1997). Province-specific calculations of appropriate sample sizes using a cluster design approach; particularly to avoid the risk of missing large diagnostic centers were done. At least 30 diagnostic centers per province were included. The total number of patients registered in the preceding year was divided by the number of clusters to obtain the sampling interval. Referral centers for MDR tuberculosis were excluded to avoid over-estimation of resistance prevalence. Patients included were all newly registered patients with culture-confirmed tuberculosis at the selected diagnostic centers. These specimens were sent to the MRC laboratories in Pretoria. Specimens from all persons suspected of having tuberculosis were investigated, i.e. not only those from patients with positive sputum smears (Weyer, personal communication).

One isolates from the clinic study and nine from the MRC study were sent for spoligotyping to the Gelre Hospital in Apeldoorn, The Netherlands.

Twelve isolates were analysed using the mycobacterial intergenic repeat units (MIRU) typing method with the assistance of colleagues at the Institute of Tropical Medicine in Antwerp, Belgium.

2.5.2. IS*6110* restriction fragment length polymorphism

RFLP was performed using the standardised IS*6110* technique (Van Embden *et al*., 1993). The extracted genomic DNA was restricted with *Pvu*II in reaction mix (final volume 30 µl) consisting: 3 µg of genomic DNA, 15 units *Pvu*II in 3 µl of the prescribed restriction buffer (Amersham biosciences, Greece). The restriction mix was incubated overnight (± 16 h) at 37°C. At the end of digestion, the reaction was incubated at 65°C for 10 min to inactivate any remaining enzyme activity. The restricted products were run on a 0.8% (w/v) Seakem® ME agarose (BioWhittaker molecular applications, USA) using 1 × TBE (g/l 21.6 g Tris, 11 g Boric acid, 1.5 g EDTA, pH 8.3) buffer for 1h to test for completion of digestion.

A 0.8% SeaKem® ME agarose gel (200 cm^2) was prepared and loaded with 20µl of *Pvu*II restricted genomic DNA, 2 µl loading mix, and 2 µl of molecular weight marker X (Roche, Germany). Fragments of DNA were separated by gel electrophoresis in 1 × TBE buffer at 40 V for 18 h. Fragmentation of DNA molecules involved irradiation of the gel with ultraviolet light for 5 min and rinsing it in 0.25 M HCl for 10 min. Two denaturation steps of 20 min each in 0.4 M NaOH followed treatment with 0.25 M HCl.

The DNA in the gel was transferred onto a Hybond–N^+ membrane (Amersham Life Science) using a vacuum blotter (BioRad Laboratories, Hercules, CA, USA) according to the manufacture's instructions. Transfer of the DNA was carried out using 10 × SSC buffer (1.5 M sodium chloride, 0.5 M sodium citrate, pH 7) for 2.5 h.

2.5.2.1 Preparation of probes

Four microlitres (3 ng) of DNA probe was used as template for amplification of a 245-bp fragment. The reaction was carried out in 100 µl consisting of 1.5 mM of $MgCl_2$; 200 µM each of the dNTP's; 0.2 µM each of the primers (INS-1: 5'-CGT GAG GGC ATC GAG GTG GC-3') and INS-2: 5'-GCG TAG GCG TCG GTG ACA AA-3') (Van Embden *et al*., 1993) and 0.5U of Super-Therm Taq polymerase (Southern Cross, South Africa). Incubation cycles in the thermocycler (Perkin Elmer, 9600, USA) were as follows: 96°C for 5 min (denaturing) followed by 30 cycles at 94°C for 1 min, annealing at 67.5°C for 30 sec and a final extension step at 72°C for 10 min. The resultant PCR product was fractionated electrophoretically in 1.5% NuSieve 3:1 agarose (BioWhittaker molecular applications, USA) gel at 80V for 2h. Purification of the PCR product was carried out following the Qiaquick gel extraction kit protocol.

The Gene Images CDP star labelling module (Amersham biosciences, Greece) was used to label the IS*6110* probe. The amplified 245-bp DNA fragment was diluted with sterile distilled water to a concentration of 15 ng/µl. This was then denatured in the thermocycler at 96°C for 5 min and immediately cooled on ice. The following reagents were added; nucleotide mix (10 µl), primer (5 µl), denatured DNA (20 µl), Klenow enzyme solution (1 µl), and sterile distilled water (14 µl). Mixing was achieved by pipetting up and down a few times. The contents were then centrifuged briefly and incubated at 37°C for 1h.

2.5.2.2 Hybridisation, washing and detection

Hybridisation was performed in tubes in a rotating hybridisation oven (Techne, Cambridge, England). The membrane was covered with 25 ml of hybridisation buffer and prehybridised for one hour at 60°C. The labelled IS*6110* was then added and hybridisation continued for 18 h at 60°C.

Hybridisation was followed by stringency washes: a brief rinse in 2 × SSC, 0.1% (w/v) SDS; two incubations, 5 min each in 200ml 2 × SSC, 0.1% (w/v) SDS; two rinses, 10 min each in 200ml 1 × SSC, 0.1% (w/v) SDS; and four washes, 5 min each in 200ml 0.1 × SSC, 0.1% (w/v) SDS.

Signal generation and detection according to Gene Images CDP-Star detection module (Amersham bioscience, Greece) followed the stringency washes. An exposure time of 8 min was usually sufficient to produce a good signal on the autoradiograph.

2.5.2.3 Analysis of IS6110 RFLP patterns

Comparison of RFLP profiles was achieved by using the computer software Gelcompar II, version 2.5 (Applied Maths, Kortrijk, Belgium). The DNA fingerprints were analysed using the Dice coefficient unweighted pair-group method with arithmetic averages (UPGMA).

2.5.3. Spoligotyping

Spoligotyping to detect 43 known spacer sequences in the direct repeat (DR) section of *M. tuberculosis* was performed using a modification of the method described by Kamerbeek *et al* (1997). The changes in the PCR mixture consisted of 3.0 mM $MgCl_2$, 50 pmol of each primer, 15 mM Tris, pH 9.0.

The extracted DNA was added to 50 µl of PCR mixture. The mixture was heated for 60 minutes at 37°C for uracil DNA glycosylase incubation, three minutes at 95°C for uracil DNA glycosylase inactivation and DNA denaturation, one minute at 57°C for primer annealing and one minute at 72°C for primer extension. Twenty-five cycles consisting of one minute at 95°C, one minute at 57°C, and 30 seconds at 72°C were performed.

The presence of these spacer sequences in the *M. tuberculosis* strain was detected by reverse line blotting using an oligonucleotide-impregnated membrane. Hybridised DNA with peroxidase labelled streptavidin was detected using the ECL detection reagent (Amersham International, Amersham, Buckinghamshire, UK).

2.5.4. Mycobacterial interspersed repetitive units or variable number of tandem repeats (MIRU-VNTR) typing

Polymerase chain reaction was carried out for *in vitro* amplification of 12 known loci containing MIRUs using the HotStart Taq DNA polymerase kit (Qiagen, Hilden, Germany). The twelve pairs of primers used are listed in Table 2.1. The $MgCl_2$ concentrations varied depending on the locus. A 1.5 mM $MgCl_2$ solution was used in reaction mixtures for MIRUs 20, 24, 26, and 27; 2 mM $MgCl_2$ for MIRUs 2, 4, 10,16,31 and 40; and 2.5 mM $MgCl_2$ for MIRUs 23 and 39. Incubation cycles in the thermocycler were as follows: 95°C for 15 min (denaturing) followed by 40 cycles at 94°C for 1 min, annealing at 59°C for 1 min, extension at 72°C for 1 min 30 sec, and a final extension step at 72°C for 10 min. The resultant PCR product was fractionated electrophoretically in 3% NuSieve 3:1 agarose (BioWhittaker molecular applications, USA) gels prepared in 1 × TBE buffer. Electrophoresis was performed at 120 V for 5 h using a 100 bp ladder as the size marker. The gel was stained with 0.7 µg/ml ethidium bromide, observed under ultraviolet light and photographed (Supply *et al*., 1997).

Table 2.1 Pairs of primers for amplifying different MIRUs

Locus	Primer (5' – 3')
MIRU 2	2F - TGGACTTGCAGCAATGGACCAACT 2R - TACTCGGACGCCGGCTCAAAAT
MIRU 4	4F – GCGCGAGAGCCCGAACTGC 4R - GCGCAGCAGAAACGTCAGC
MIRU 10	10F – GTTCTTGACCAACTGCAGTCGTCC 10R - GCCACCTTGGTGATCAGCTACCT
MIRU 16	16F – TCGGTGATCGGGTCCAGTCCAAGTA 16R - CCCGTCGTGCAGCCCTGGTAC
MIRU 20	20F – TCGGAGAGATGCCCTTCGAGTTAG 20R - GGAGACCGCGACCAGGTACTTGTA
MIRU 23	23F – CTGTCGATGGCCGCAACAAAACG 23R - AGCTCAACGGGTTCGCCCTTTTGTC
MIRU 24	24F – CGACCAAGATGTGCAGGAATACAT

	24R - GGCGAGTTGAGCTCACAGAA
MIRU 26	26F – TAGGTCTACCGTCGAAATCTGTGAC 26R - CATAGGCGACCAGGCGAATAG
MIRU 27	27F – TCGAAAGCCTCTGCGTGCCAGTAA 27R - GCGATGTGAGCGTGCCACTCAA
MIRU 31	31F – ACTGATTGGCTTCATACGGCTTTA 31R - GTGCCGACGTGGTCTTGAT
MIRU 39	39F – CGCATCGACAAACTGGAGCCAAAC 39R - CGGAAACGTCTACGCCCCACACAT
MIRU 40	40F – GGGTTGCTGGATGACAACGTGT 40R - GGGTGATCTCGGCGAAATCAGATA

2.6. *KatG* and *rpoB* genes analyses

2.6.1 Amplification of the *katG* gene

The 808-bp fragment of the *katG* gene was amplified employing oligonucleotides RTB 59 (5’-TGG CCG CGG CGG TCG ACA TT-3’) and RTB 36 (5’-TCG GGG TCG TTG ACC TCC CA-3’) (Victor T *et al*., 1999). One hundred and fifty nanogrammes of genomic DNA were used as a template for amplification in a 50 µl reaction mixture consisting of magnesium chloride, 1.5 mM; dNTP's (dATP, dGTP, dCTP, and dTTP), 200 µM each; primers 0.2 µM each and 0.5 U of Super-Therm Taq Polymerase (Southern Cross, South Africa). Amplification was performed in a thermal cycler (Perkin Elmer 9600, USA) as follows: 93°C for 3 min followed by 35 cycles at 93°C for 1 min, annealing at 66°C for 1 min and extension step at 72°C for 2 min. Final extension was performed at 72°C for 10 min. Amplification of the desired fragment was confirmed by gel electrophoresis on 2% agarose gels (LM SIEVE, Whitehead Scientific, South Africa).

2.6.2 Amplification of the *rpoB* gene fragment

In vitro amplification of the *rpoB* gene was achieved by PCR using the following synthetic oligonucleotide primers TR8 (5'-TGC ACG TCG CGG ACC TCC A-3') and TR9 (5'-TCG CCG CGA TCA AGG AGT-3') (Pretorius *et al*., 1996). One microlitre (5 ng) of DNA was used as template for amplification in a 100 µl reaction mixture made up of $MgCl_2$, 200 µg dNTPs, 0.25 µM each primer, and 0.5 U of Super-Therm Taq polymerase (Southern Cross, South Africa). Amplification was performed in a thermocycler (Perkin Elmer, 9600, USA) using the following steps: 95°C for 2 min followed by 35 cycles of 1 min denaturation at 93°C and 2 min annealing at 58°C and 2 min extension at 72°C. The PCR product (157-bp) was then excised from the ethidium bromide stained 3% LM sieve agarose gel (Whitehead Scientific, South Africa).

2.6.3. Sequencing

2.6.3.1 katG gene

One microlitre of the PCR product was used for sequencing. Both the sense and antisense strands were sequenced using the primers RTB 59 and RTB 36 respectively. The BigDye Ready Reaction Terminator Cycle Sequencing Kit (Applied Biosystems, USA) was used and performed as follows: 25 cycles of 96°C for 10 sec, 50°C for 5 sec, and 60°C and 4 min with a ramp of 2 sec/1°C. The unincorporated dye terminators and primers were separated from the extension products by spin column purification (Centri-Sep; Princeton Separations Inc., Adelphia, NJ) and dried. The reaction mixtures for sequencing were analysed by the Department of Microbiology and Biochemistry, University of the Free State, on a Sequence Navigator gel. Computer-assisted analysis was performed with ABI PRISM, Model 377 (Perkin Elmer, USA). The products were analysed by the ABI Prism TM 377 system (Applied Biosystems, USA).

2.6.3.2 Sequencing of rpoB gene

DNA was eluted by incubating the gel block in 150 µl of TE buffer (pH 8). For direct sequencing the primer was the same as that used for PCR. Both sense and antisense strands were sequenced to cross check results. Approximately 1.2 µl of each eluted PCR product was used for sequencing. Both the sense and antisense strands were sequenced using the primers TR8 and TR9 respectively and the BigDye Ready Reaction Terminator Cycle Sequencing kit (Applied Biosystems, USA). Sequencing conditions were as follows: 25 cycles of 96°C for 10 sec, 50°C for 5 sec, and 60°C and 4 min with a ramp of 2 sec/1°C. Unincorporated dye terminators and primers were separated from the extension products by spin column purification (Centri-Sep; Princeton Separations Inc., Adelphia, NJ) and dried. The reaction mixtures for sequencing were analysed by the Department of Microbiology and Biochemistry, University of the Free State, on a Sequence Navigator gel. Computer-assisted analysis was performed with ABI PRISM, Model 377 (Perkin Elmer, USA). The products were analysed by the ABI Prism TM 377 system (Applied Biosystems, USA). Codon numbers were designated according to the E. coli *rpoB* sequence with a portion of the translated *M. tuberculosis* sequence.

2.7. Restriction analysis of mutations in the *katG* gene

The 808-bp fragment of the *katG* gene was amplified employing oligonucleotides RTB 59 (5'-TGG CCG CGG CGG TCG ACA TT-3') and RTB 36 (5'-TCG GGG TCG TTG ACC TCC CA-3') (Victor et al.,1999). One hundred and fifty nanogrammes of genomic DNA were used as a template for amplification in a 50 µl reaction mixture consisting of magnesium chloride, 1.5 mM; dNTP's (dATP, dGTP, dCTP, and dTTP), 200 µM each; primers 0.2 µM each and 0.5 U of Super-Therm Taq Polymerase (Southern Cross, South Africa). Amplification was performed in a thermal cycler (Perkin Elmer 9600) as follows: 93°C for 3 min followed by 35 cycles at 93°C for 1 min, annealing at 66°C for 1 min and extension step at 72°C for 2 min. Final extension was performed at 72°C for 10 min. Amplification of the desired fragment was confirmed by gel electrophoresis on 2% agarose gels. Seventeen microlitres of the PCR product was restricted with *Msp 1* restriction enzyme (Roche, Germany) and the fragments separated by electrophoresis in a 4% LM-SIEVE agarose (Whitehead Scientific, South Africa) prepared using 1 × TBE (pH 8.3). The hundred base-pair ladder was used in each gel as a size marker. The presence of a 211 base pair fragment indicated the presence of a mutation in codon 463. The absence of a 153 base pair fragment indicated the presence a wildtype allele at codon 315, whereas the presence of 132 base pair fragment indicated an AGC to ACC mutation at that codon.

CHAPTER 3

RESULTS:

SETTING IN THE FREE STATE PROVINCE OF SOUTH AFRICA

3.1 Introduction

The global annual incidence of tuberculosis is growing at a rate of 0.4% per year. The growth rate is reported to be much higher in Southern African countries (5% per year) (WHO report 2003). An increase in the annual incidence was noted in South Africa from 1996 to 2001 despite the implementation of the WHO recommended strategies such as the DOTS. An increase in the incidence of tuberculosis was also observed in the Free State province in the period between 1996 and 2001 (Kironde *et al*., 2002). The upsurge of tuberculosis in recent years is a strong indication that this disease needs fresh approaches that will lead to a better understanding of its transmission.

The last decade has seen the increased application of molecular epidemiological methods that has led to a better understanding of the disease dynamics. Earlier studies found that in high incidence countries strains showed less DNA polymorphism than in low incidence countries (Hermans *et.al.,* 1995). Recent transmission was therefore expected to be a more significant force in these countries than reactivation of previous infection. This was in contrast to the findings of a study in the high incidence area of Western Cape, South Africa, where strain diversity was reported as high, indicating that reactivation was a problem. Since the Free State and Western Cape do not share a common border, isolates in the Free State cannot be assumed to have similar genotypic characteristics as was found in a small study in the Bloemfontein area (Van der Spoel van Dijk *et al*., 1996).

Furthermore, the extent of drug resistance was largely unknown in the Free State province until the Medical Research Council conducted a surveillance study between 2001 and 2002. This study registered some concerns regarding the equal distribution of resistance between new and relapse patients (Weyer *et al*., 2002).

In order to thoroughly study the disease dynamics in the Free State, long-term surveillance will be needed. This is however, not feasible in an area as large as the Free

State and the cost would be astronomical. Long-term studies in poor countries are made possible by limiting the study area to a subset that carries all the characteristics of the area of interest. The value of such an endeavor is evident in the data generated by investigators from a small area in the Western Cape Province (Beyers *et al*., 1996, Van Rie *et al*,. 1999, Van Rie *et al*,. 2000, Warren *et al*,. 1996).

The aim of this part of the study was to define an area in the Free State representative of the province where long-term molecular epidemiological surveillance (determination of the spread of strains) and monitoring of resistance development in a stable population could be done.

3.2 Results

The population of Thaba ’Nchu a rural town in the Free State was estimated at 77 208 in 1996, with 99.0% of the residents belonging to the black race. The female gender makes up 52.0% of the population’s gender distribution. Sixty nine percent of residents are younger than 35. About half of the residents (49.0%) live on tribal authority administered lands, 48.0% in semi-urban areas. The remaining 3.0% live on other non-urban areas (commercial farms and small holdings) (Van Rensburg *et al*., 2001).

Sixty nine percent of the people are jobless, 20.0% of the working force earn between R1 and R1000, while 10.0% earn more than R1000 per month. The area has 28 primary, five secondary, nine intermediate, one special school, and a technical school. Only 3.0% percent of the residents have had education beyond matriculation. The residents mostly live in either mud houses or western style dwellings (87.0%). Taps supply water to 95.0% of the people, 4.0% receive it from carriers, tankers, boreholes, rainwater and wells, while 0.2 % uses dams, rivers, streams and springs. A total of 73.0% of households uses electrical energy for lighting purposes; while 64.0% have access to a telephone (they either have their own or live near one). The majority of households (74.0%) use conventional toilets (pit or bucket latrines) for sanitation purposes, while 21.0% use sewage-linked appliances. Seventy four percent (74.0%) of women in their childbearing years have between one and more children. The Department of Welfare reports that of the total grants paid out per month 45.0% goes towards child support (Van Rensburg *et al*., 2001).

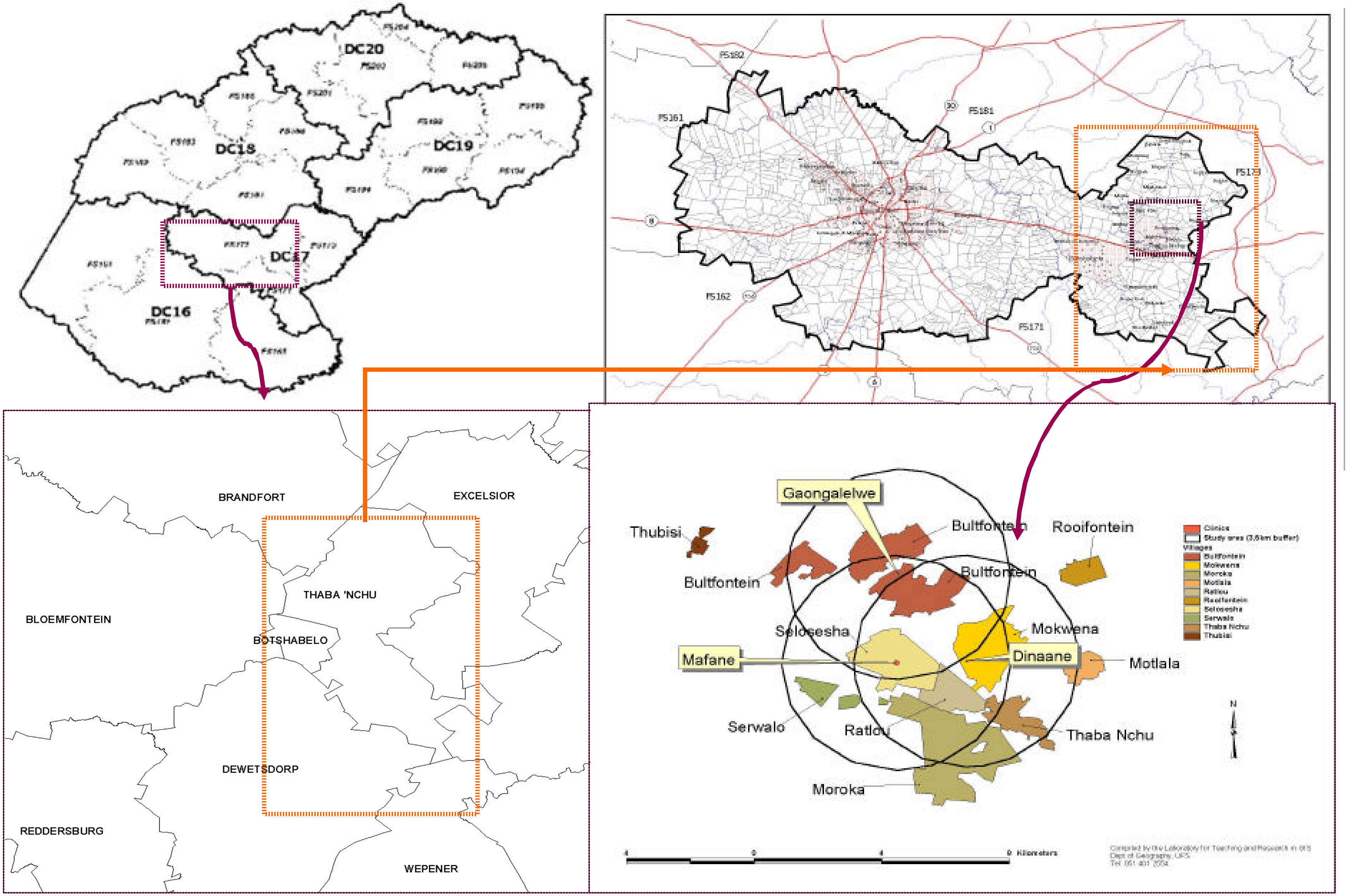
The main causes of mortality in the area are infectious and parasitic diseases, respiratory diseases, and what are described as ill-defined conditions. These together accounted for 61.0% of deaths. In 2000 there were 590 cases of tuberculosis reported, and Dinaane, Gaongalelwe, and Mafane clinics together contributed 374 cases to this pool (Van Rensburg *et al*., 2001).

A semi-urban part of the Motheo district in the Free State referred to as Gamadi from henceforth, consists of ten neighboring villages between the small rural town of Thaba 'Nchu and surrounding non-urban areas (Figure 3.1). The Gamadi area is served by three clinics Gaongalelwe (24 hours), Mafane and Dinaane (24 hours) and a hospital (Moroka hospital). The hospital also houses an MDR unit that provides health care to patients from other parts of the province and beyond. The three clinics mentioned previously serve a population of 61524 people and together they received 111091 visits from the public in 2000. Immigrants and the homeless do not form a common feature but the majority inhabitants live under abject poverty.

Five hundred and seventeen persons (517) were diagnosed with TB in the area in 2001. This figure translates into 840/100 000 population per annum. Pulmonary tuberculosis (PTB) accounted for 441 (85.3%) of the affected patients. New PTB made up 315 (71.4%) of the cases with 284 (90.2%) of the patients confirmed by microscopy. Hundred and fifty five cases (54.0%) were females. The new smear positive patients consisted of 148 (52.0%) cases that are between 20 – 39 years of age. Retreatment cases accounted for 126 (28.6%) of the PTB patients. Taking of medicines is supervised by nurses in 44.0% of the cases, DOTS supporters in 24.6% and relatives in 31.4% of the cases. A survey on HIV infection among antenatal woman in 2000 reported a prevalence of 27.9%.

A survey conducted in 2003 among TB patients in the Gamadi area found that two-thirds (66.6%) of adult TB patients were single, divorced, or widowed. Slightly over 90.0% of the cases were unemployed. More than four-fifths (86.9%) of the interviewees had remained at the stated address for a period longer than two years. More than half (59.7%) of the patients spend short periods away from home. The remaining 40.3% of

Figure 3.1: Location of study area in the Free State Province



the patients rarely travel away from home. Of those that stayed away from home 48.1% were away for less than two months at a time.

Fifty-six percent of the patients are of sober and drug-free habits. Forty-two percent of patients use legalised substances such as alcohol and tobacco and 2% use marijuana.

The National Tuberculosis database was used to determine the patient caseload of the area in 2001.

Table 3.1: Number of tuberculosis patients reported during 2001 for the Free State Province, the Motheo district and the Gamadi area.

Area	PTB	%	Other TB	%	Total
Free State	9975	76.61	3046	23.39	13021
Motheo	2851	73.67	1019	26.33	3870
Gamadi	441	85.30	76	14.70	517

3.3 Discussion

The rural town of Thaba ’Nchu is situated approximately 12 km from a large settlement (Botshabelo) that forms part of the metropolis of Mangaung. The Gamadi area is a well defined and relatively stagnant area influenced by distant geographical areas such as the Goldfields, towns, the provincial capital (Bloemfontein) and surrounding towns. It is a poverty-stricken semi-urban area made up of 10 villages that form part of the poorly industrialised, rural town of Thaba ’Nchu. This town has a young population as 69% of the residents are younger than 35 years (Van Rensburg et al., 2001). Tuberculosis mostly affects individuals in the age between 20 and 39 years followed by the 40 – 59 age group. A review of the status of TB in the defined area has revealed that the annual incidence was extremely high per 100 000 population (840/100 000). Young persons in the peak of their productive years are mostly affected by tuberculosis (20 – 39 age group) (http://healthweb.ofs.gov.za). The majority of TB patients in the area are unemployed. Investigations have revealed that the residents do not travel much out of the area. However, dire poverty may encourage intracommunity migration as most people try to look for employment in limited industries in the town. More people may travel around in order to be near the support of relatives (the working minority or welfare grants receivers.

Comparing the status quo in Thaba ’Nchu to the Free State in general we observed striking similarities. A reasonably high percentage of the population is black (Free State 85.0%, Thaba ’Nchu 99.0%). The gender distribution in the Free State is fairly even with 51% males and in Thaba ’Nchu the ratio is 52/48 in favour of males. The provincial age distribution shows that 70.0% of the population is younger than 35 years. The Free State has a higher urbanisation than Thaba ’Nchu, but the setting of this study is semi urban. The degree of poverty is similar since 69.0% in Thaba ’Nchu and 64.0 % in the province are unemployed (Van Rensburg et al., 2001).

In conclusion, the population and health service structures of the Gamadi area and its unique resemblance to the larger Free State make it suitable for long-term molecular epidemiology studies and the second part of the current study concentrated on TB strains from this area.

CHAPTER 4

RESULTS:

CULTURE AND IDENTIFICATION OF M. TUBERCULOSIS ISOLATES

4.1 Introduction

Clinical suspicion of tuberculosis is based on clinical signs and symptoms. Suspicion of tuberculosis can be confirmed to some degree by the tuberculin skin test and radiographic patterns but bacteriologic examinations (microscopy and culture) are the cornerstones of the DOTS strategy (DOH, practical guidelines, 2000). Of the two, bacteriologic tests, culture have the advantage of making definite identification of the bacillus possible. Drawbacks stem from its cost and results that are available after a protracted period (Blumberg, 1995).

In developing countries microscopy forms the basis of correctly identifying cases which are infectious and therefore having the highest priority for care. In South Africa, a member of the 22 countries that together carry 80% of the global TB burden, culture is requested only under special conditions (Kironde *et al*., 2002). Diagnostic and follow-up smear microscopy for acid-fast bacilli is performed at the local general bacteriologic laboratories. Patients are started on the tuberculosis treatment programme once a presumptive mycobacterial infection has been established. Culture, identification and drug susceptibility tests (DST) are requested only on patients failing to convert (become smear negative for TB after two months of treatment). Drug resistance statistics (in particular single drug resistance) determined by referral bacteriological laboratories may present an under estimation of the extent of the problem, because of the strict selection process of cases (Blumberg, 1995).

Information about resistance in the Free State has recently been investigated with encouraging results (Weyer *et al*., 2002). This province is characterised by enormous contrasts between economically rich cities (the Goldfields and the provincial capital, Bloemfontein), towns and the dire poverty of the rural communities. The incidence of TB and anti-mycobacterial drugs in poverty-stricken peripheral populations has hitherto remained unclear.

The aim of this section was to isolate *M. tuberculosis* strains from sputum specimens by culture. In addition, to identify randomly selected isolates by the ZN stain, heat-stable catalase test, and nitrate reduction test.

4.2 Results

4.2.1 Culture

Sputum samples received are summarised in figure 4.1. In the analysis period 286 sputum samples were cultured on LJ slopes from 219 smear confirmed pulmonary tuberculosis patients and 35 suspects. The first sputa from 67 of the smear-confirmed cases were collected some time after tuberculosis treatment was initiated. Ninety-six specimens (96/184) were negative after a period of culture. *M. tuberculosis* was isolated from 88 samples collected from 82 consecutive patients. Six of these patients also gave 7 follow-up sputa which subsequently tested culture negative. Five patients (5/82) were asked to give the second sputa within 2 days to three weeks of the first sputum specimen. The sixth patient provided the additional sputum at six months. Culture positive results comprised 30.77% of all tested specimens. A rapidly growing mycobacterium species was also isolated from the third sputum specimen provided by a patient with a prior history of tuberculosis treatment. The first two sputum specimens from this patient yielded no positive result.

4.2.2 Ziehl-Neelsen (ZN) staining

Since 95/184 sputa from patients having been diagnosed previously by the national laboratory as smear positive was found to be culture negative, forty digested and concentrated sputum specimens were randomly selected (20 culture negative and 20 culture positive) and subjected to smear microscopy. Five of 20 and 16/20 respectively were smear positive (Table 4.1).

Figure 4.1: Summary of sputum samples received from patients in Gamadi area.

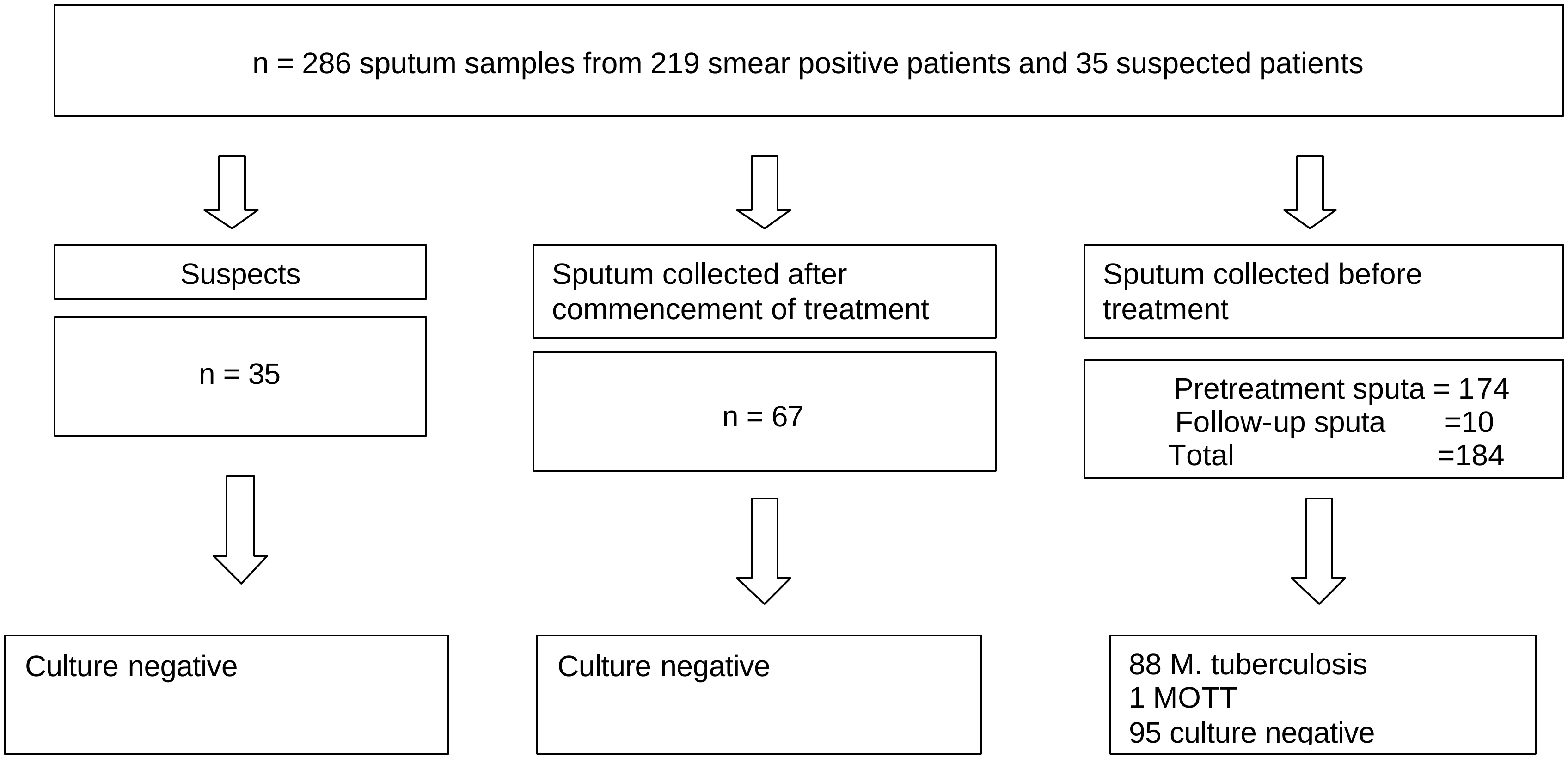


Table 4.1 ZN staining results of randomly selected culture positive and culture negative sputum samples from Gamadi after decontamination to confirm quality of samples received for culturing.

Culture positive	**Result +/-**	**Culture negative**	**Results +/-**
Dinaane		**Dinaane**	
02020051	3+	02020019	-
02020026	-	01020014	-
03020076	3+	01020012	-
03020102	Scanty	02020028	-
01020013	2+	02020053	-
Gaongalelwe		**Gaongalelwe**	
01010020	3+	02010057	-
02010073	1+	02010070	1+
02160023	2+	01010014	-
02010123	-	02010111	-
02010026	2+	03010152	-
Mafane		**Mafane**	
01030003	2+	02030009	Scanty
02030002	3+	02030027	2+
03030043	1+	02030030	-
03030097	-	03030035	-
03030071	3+	03030046	-
Moroka		**Moroka**	
03110016	2+	03110037	-
03110034	3+	03110031	Scanty
03110064	-	03110035	3+
03160025	2+	03110020	-
03110036	3+	03110019	-

4.2.3 Identification of a selected number of isolates

Twenty cultures with inconclusive biological characteristics were subjected to ZN staining, catalase, and the nitrate test. Table 4.2 presents the results of the tests performed.

Ten of the isolates were ZN positive, catalase negative, and reduced nitrate during testing. One isolate that exhibited rapid growth had positive ZN, catalase test, and nitrate reduction results. This strain will be identified later using further tests. The remaining nine strains proved to be smear negative and, thus, further tests were not necessary.

4.3 Discussion

According to our knowledge this is the first study to be conducted over a protracted period in the Gamadi area. A significant misunderstanding resulted in the collection of sputum specimens from patients already on treatment (22.6%). These sputa turned out to be culture negative as expected. This was evident in the early phase of sample collection when most of the health care workers were still unsure of the collection criteria to be followed. It could be advantageous to ensure training of several staff members when doing research in places with limited staff to minimize the effect of staff vacations and rotation.

Although high frequency of culture positivity was expected, a large proportion of specimens collected turned out to be negative upon culture. Twenty concentrated samples were randomly selected from the resultant negative cultures and subjected to ZN staining. Three quarters of these cases were smear negative. Comparing these results to 20 randomly selected concentrated sputa of culture positive isolates a high proportion was found to be smear positive (80%). The high number of culture negative samples raises some serious concerns about the quality of the specimens collected for this study. The fact that the quality of samples received was so poor seriously affected the study as a whole since it can be concluded that the samples received were in fact not representative of the Gamadi area, allowing for very little conclusions to be drawn from the data. Studies elsewhere have confirmed the necessity of establishing quality control systems when doing research projects of this nature, as well as within fully functional DOTS programmes. It is, therefore, imperative for the province to implement quality control programmes focusing on continuos-professional-development related workshops, proficiency training and regular quarterly slide assessments and feedback (Lan *et al*., 1999, Addo *et al*., 2002).

Biochemical tests confirmed 10 isolates as *M. tuberculosis* and one isolate as *Mycobacterium*-other-than-tuberculosis (MOTT) species. Definite identification of this strain will be performed at a later date. A MOTT strain was isolated from a study in Namibia and identified as *M. kansasii* (Haas *et al*., 1999). In the United States of America increased isolation of *M. avium-intracellulare* complex strains is often associated with HIV infection (Metchock *et al*., 1995). The proportion of TB burden caused by different species of mycobacteria in the Gamadi area is however not known.

Table 4.2 Identification of isolates from Gamadi using biological and biochemical characteristics.

Sample no	**Biological characteristics**				**Biochemical characteristics**			**Result**
	Morphology	**Growth rate**	**Temperature (celsius)**	**Pigment production**	**ZN**	**Catalase test**	**Nitrate test**	
02020022	Butterous	Rapid	37	Red	Negative	ND	ND	ND
03020059	Butterous	Rapid	37	Yellow	Negative	ND	ND	ND
02020026	Rough	Rapid	37	Buff	Positive	Positive	Positive	MOTT
02020068	Rough	Slow	37	Buff	Positive	Negative	Positive	MTB
03020082	Rough	Slow	37	Buff	Positive	Negative	Positive	MTB
01010004	Rough	Slow	37	Buff	Positive	Negative	Positive	MTB
02010056	Rough	Slow	37	Buff	Negative	ND	ND	ND
02010057	Rough	Rapid	37	Yellowish-green	Negative	ND	ND	ND
02010077	Butterous	Rapid	37	White	Negative	ND	ND	ND
03010164	Butterous	Rapid	37	Buff	Negative	ND	ND	ND
03010151	Smooth	Rapid	37	Red	Negative	ND	ND	ND
02010074	Rough	Slow	37	Buff	Positive	Negative	Positive	MTB
03010155	Rough	Slow	37	Buff	Positive	Negative	Positive	MTB
03010147	Rough	Slow	37	Buff	Positive	Negative	Positive	MTB
02030022	Butterous	Rapid	37	Buff	Negative	ND	ND	ND
02030031	Butterous	Rapid	37	Buff	Negative	ND	ND	ND
03030071	Rough	Slow	37	Buff	Positive	Negative	Positive	MTB
03030036	Rough	Slow	37	Buff	Positive	Negative	Positive	MTB
03110016	Rough	Slow	37	Buff	Positive	Negative	Positive	MTB
03160024	Rough	Slow	37	Buff	Positive	Negative	Positive	MTB

ND = Not determined, MTB = M. tuberculosis

CHAPTER 4

RESULTS:

CULTURE AND IDENTIFICATION OF M. TUBERCULOSIS ISOLATES

4.1 Introduction

Clinical suspicion of tuberculosis is based on clinical signs and symptoms. Suspicion of tuberculosis can be confirmed to some degree by the tuberculin skin test and radiographic patterns but bacteriologic examinations (microscopy and culture) are the cornerstones of the DOTS strategy (DOH, practical guidelines, 2000). Of the two, bacteriologic tests, culture have the advantage of making definite identification of the bacillus possible. Drawbacks stem from its cost and results that are available after a protracted period (Blumberg, 1995).

In developing countries microscopy forms the basis of correctly identifying cases which are infectious and therefore having the highest priority for care. In South Africa, a member of the 22 countries that together carry 80% of the global TB burden, culture is requested only under special conditions (Kironde *et al*., 2002). Diagnostic and follow-up smear microscopy for acid-fast bacilli is performed at the local general bacteriologic laboratories. Patients are started on the tuberculosis treatment programme once a presumptive mycobacterial infection has been established. Culture, identification and drug susceptibility tests (DST) are requested only on patients failing to convert (become smear negative for TB after two months of treatment). Drug resistance statistics (in particular single drug resistance) determined by referral bacteriological laboratories may present an under estimation of the extent of the problem, because of the strict selection process of cases (Blumberg, 1995).

Information about resistance in the Free State has recently been investigated with encouraging results (Weyer *et al*., 2002). This province is characterised by enormous contrasts between economically rich cities (the Goldfields and the provincial capital, Bloemfontein), towns and the dire poverty of the rural communities. The incidence of TB and anti-mycobacterial drugs in poverty-stricken peripheral populations has hitherto remained unclear.

The aim of this section was to isolate *M. tuberculosis* strains from sputum specimens by culture. In addition, to identify randomly selected isolates by the ZN stain, heat-stable catalase test, and nitrate reduction test.

4.2 Results

4.2.1 Culture

Sputum samples received are summarised in figure 4.1. In the analysis period 286 sputum samples were cultured on LJ slopes from 219 smear confirmed pulmonary tuberculosis patients and 35 suspects. The first sputa from 67 of the smear-confirmed cases were collected some time after tuberculosis treatment was initiated. Ninety-six specimens (96/184) were negative after a period of culture. *M. tuberculosis* was isolated from 88 samples collected from 82 consecutive patients. Six of these patients also gave 7 follow-up sputa which subsequently tested culture negative. Five patients (5/82) were asked to give the second sputa within 2 days to three weeks of the first sputum specimen. The sixth patient provided the additional sputum at six months. Culture positive results comprised 30.77% of all tested specimens. A rapidly growing mycobacterium species was also isolated from the third sputum specimen provided by a patient with a prior history of tuberculosis treatment. The first two sputum specimens from this patient yielded no positive result.

4.2.2 Ziehl-Neelsen (ZN) staining

Since 95/184 sputa from patients having been diagnosed previously by the national laboratory as smear positive was found to be culture negative, forty digested and concentrated sputum specimens were randomly selected (20 culture negative and 20 culture positive) and subjected to smear microscopy. Five of 20 and 16/20 respectively were smear positive (Table 4.1).

Figure 4.1: Summary of sputum samples received from patients in Gamadi area.

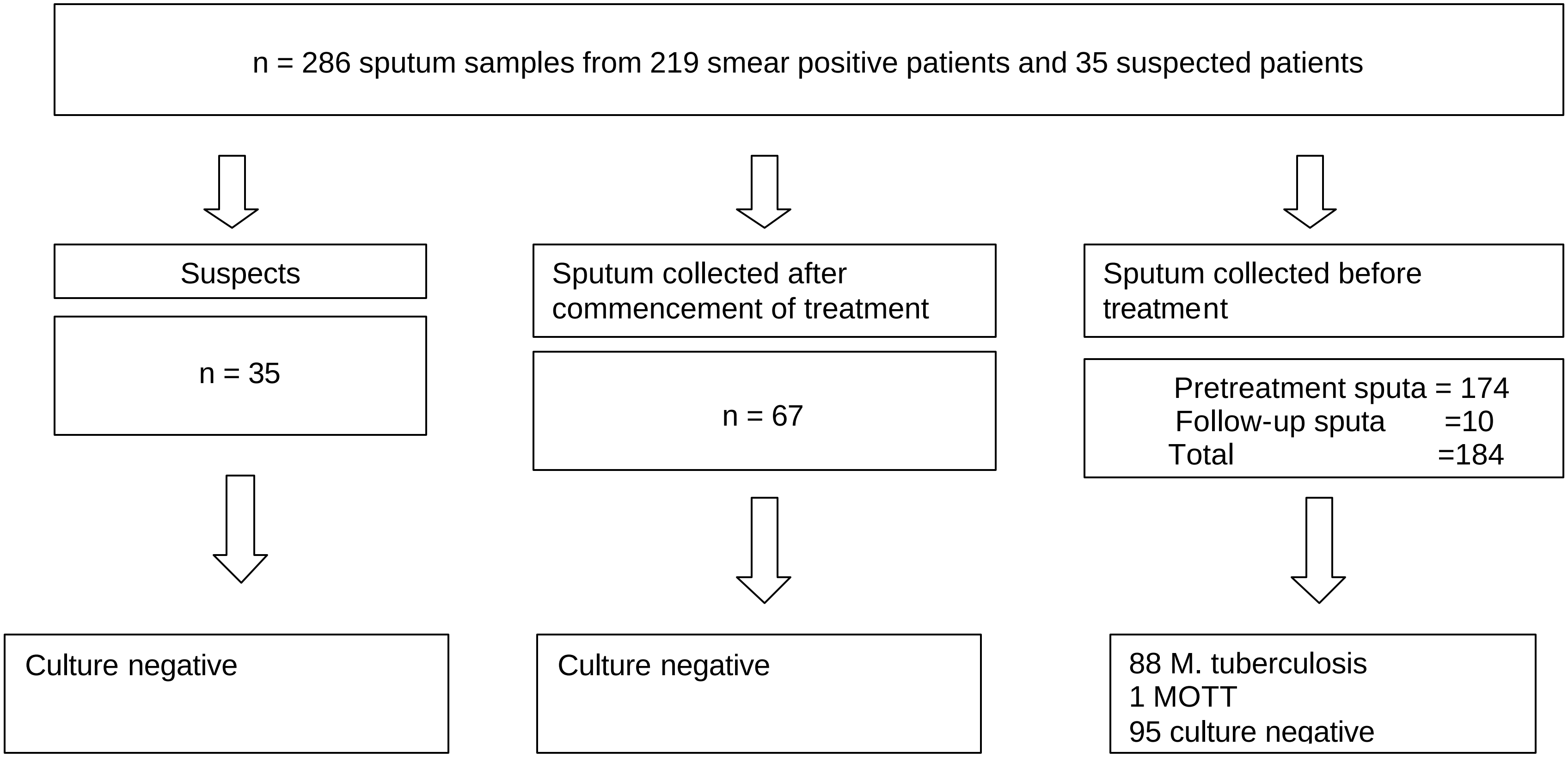


Table 4.1 ZN staining results of randomly selected culture positive and culture negative sputum samples from Gamadi after decontamination to confirm quality of samples received for culturing.

Culture positive	**Result +/-**	**Culture negative**	**Results +/-**
Dinaane		**Dinaane**	
02020051	3+	02020019	-
02020026	-	01020014	-
03020076	3+	01020012	-
03020102	Scanty	02020028	-
01020013	2+	02020053	-
Gaongalelwe		**Gaongalelwe**	
01010020	3+	02010057	-
02010073	1+	02010070	1+
02160023	2+	01010014	-
02010123	-	02010111	-
02010026	2+	03010152	-
Mafane		**Mafane**	
01030003	2+	02030009	Scanty
02030002	3+	02030027	2+
03030043	1+	02030030	-
03030097	-	03030035	-
03030071	3+	03030046	-
Moroka		**Moroka**	
03110016	2+	03110037	-
03110034	3+	03110031	Scanty
03110064	-	03110035	3+
03160025	2+	03110020	-
03110036	3+	03110019	-

4.2.3 Identification of a selected number of isolates

Twenty cultures with inconclusive biological characteristics were subjected to ZN staining, catalase, and the nitrate test. Table 4.2 presents the results of the tests performed.

Ten of the isolates were ZN positive, catalase negative, and reduced nitrate during testing. One isolate that exhibited rapid growth had positive ZN, catalase test, and nitrate reduction results. This strain will be identified later using further tests. The remaining nine strains proved to be smear negative and, thus, further tests were not necessary.

4.3 Discussion

According to our knowledge this is the first study to be conducted over a protracted period in the Gamadi area. A significant misunderstanding resulted in the collection of sputum specimens from patients already on treatment (22.6%). These sputa turned out to be culture negative as expected. This was evident in the early phase of sample collection when most of the health care workers were still unsure of the collection criteria to be followed. It could be advantageous to ensure training of several staff members when doing research in places with limited staff to minimize the effect of staff vacations and rotation.

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01010004	Rough	Slow	37	Buff	Positive	Negative	Positive	MTB
02010056	Rough	Slow	37	Buff	Negative	ND	ND	ND
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03160024	Rough	Slow	37	Buff	Positive	Negative	Positive	MTB

ND = Not determined, MTB = M. tuberculosis

CHAPTER 5

RESULTS:
DRUG SUSCEPTIBILITY TESTING

5.1 Introduction

Tuberculosis is an ancient disease causing great concern in resource rich and resource poor countries alike. In recent years infection with *M. tuberculosis* has been complicated by the development of multidrug resistance (Espinal *et al*., 2001). It can be predicted that poor case-holding, non-adherence, combined with overstrained and inadequately funded TB control programmes in the advent of AIDS, may result in the emergence of increased numbers of resistant strains. If these spread in a community, TB may become progressively uncontrollable using the current efficient, cheap, and cost effective drugs.

In South Africa, data of *Mycobacterium tuberculosis* (TB) resistance comes from microbiologic studies in a few provinces. Primary INH resistance (in new cases) of 3.9% versus 10.8% of acquired resistance (in patients treated for tuberculosis before) was reported in the Western Cape in 1993 (Weyer *et al*., 1995). Primary and acquired multidrug resistance was reported as 1.5% and 8.0% in the Mpumalanga province of South Africa 1997 (Espinal *et al*., 2001). INH resistance in South Africa from 1980 to 1988 was reported as 14.2% and in Bloemfontein in 1996 as 20%, with isoniazid/rifampicin resistance 14% (Van der Spoel van Dijk *et al*., 1996). Over the years it became difficult to classify resistance into primary and acquired resistance and recent research tends to refer to resistance in new cases versus resistance in previously treated cases.

Preliminary data of a national study during 2001/2002 reported a rate of isoniazid resistance in new cases as 6.3% and that of previously treated patients as 6.9% for the Free State. MDRTB was detected in 1.6% of new cases and in 1.7% of retreatment patients (Weyer *et al*., 2002). The prognosis of MDRTB is very poor, and cure rates of less than 50% have been reported in South Africa (DOH: practical guidelines, 2000). Few studies have been conducted in the Free State to properly assess the extent of drug resistance.

Drug-resistant mutants that occur in TB are a man-made amplification of natural selective pressure. Resistance to any anti-tuberculosis chemotherapy in *M. tuberculosis* is mutation mediated and not plasmid borne. The development of mutations in bacteria is a natural phenomenon that occurs randomly at a defined frequency during replication. Existing drug-resistant mutants do not have a natural selective advantage, but are able to survive in a setting of monotherapy while the drug susceptible bacilli are killed. Treatment with two drugs implies that the other drug kills mutants resistant to one drug. The probability of resistance to multiple drugs is multiplicative. This means that the chance of resistance development in regimens consisting of INH, RIF, ETH, and PZA amounts to 1 in 10^{26} bacilli. A cavity in the lung normally has between 10^{7} and 10^{9} TB bacilli. Thus, it is theoretically improbable that resistant mutants can arise to all four drugs in most patients treated with the four-drug combination tablet (CDC, 1993; Blumberg, 1995)). In a drug free environment, resistant mutants do not have any selective advantage, unless they are exposed to an erratic TB control regimen.

In the current era, characterised by treatment of diseases by antibiotics, inadequate exposure of an organism to the correct antimicrobial agent is the leading cause of drug resistance. Previous inadequate treatment with or without compliance of patients to treatment, are key risk factors that allow resistant organisms to multiply. Subsequent transmission of such organisms to contacts infected may lead towards the creation of a pool of infectious patients harbouring drug-resistant organisms and unintentionally propagating primary drug resistance.

The threat of drug resistance in TB, particularly multidrug resistance (MDRTB), can be averted by strict adherence to the central tenets of the directly observed treatment (DOTS) strategy and also effective treatment of known drug-resistant infectious cases with potent second line drugs. This approach should be supported by consistent laboratory resistance surveillance.

The aim of the present study was to assess the activity of INH and RIF against MTB strains collected from consecutive smear positive patients attending selected primary health care clinics in Gamadi near Thaba ’Nchu.

Figure 5.1: A summary of clinical *Mycobacterial* isolates from Gamadi subjected to isoniazid susceptibility testing.

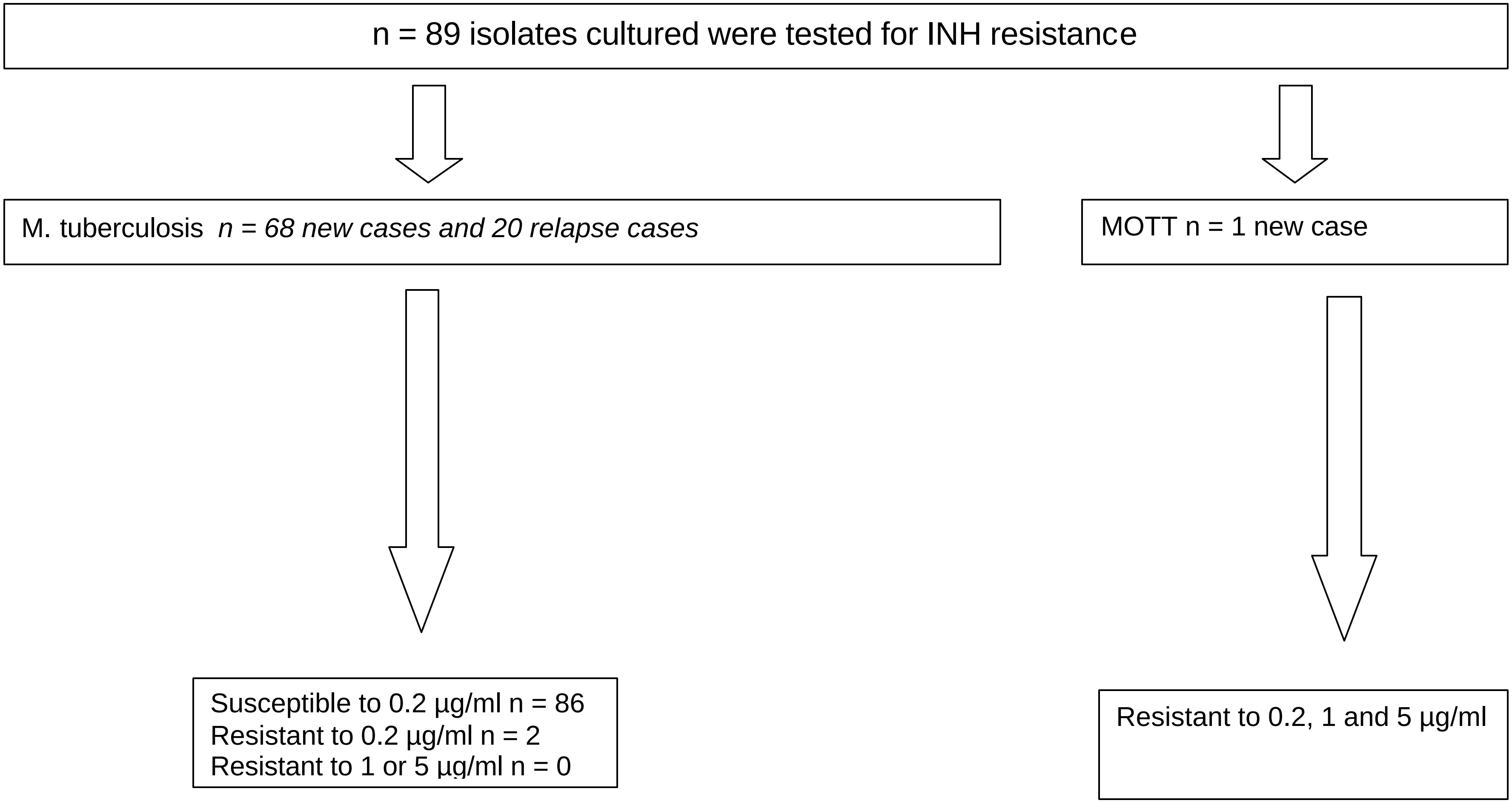
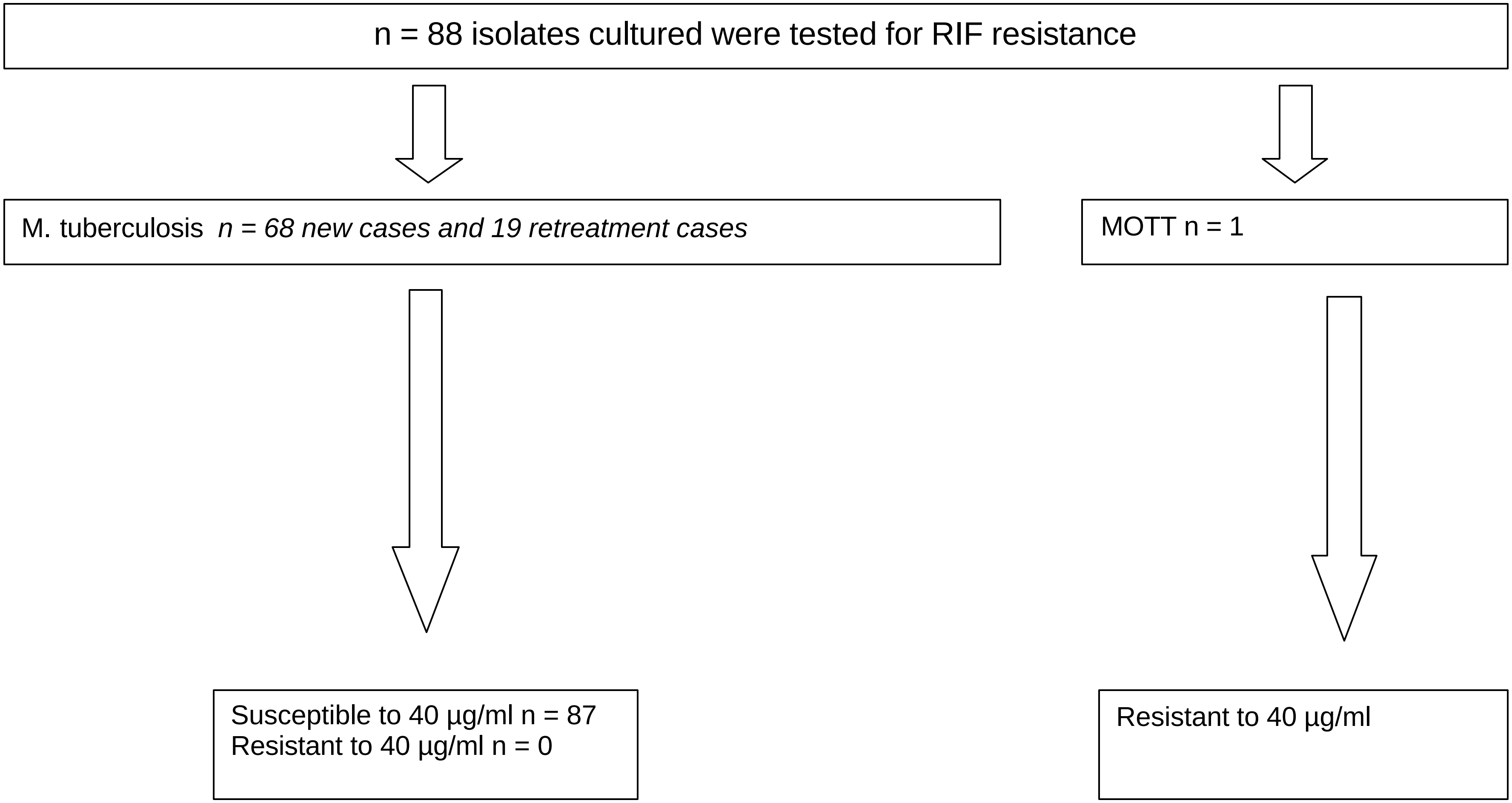


Figure 5.2: A summary of *Mycobacterial* isolates from Gamadi subjected to Rifampicin susceptibility testing.



5.2 Results

Eighty-eight *M. tuberculosis* isolates (Figure 5.1 and 5.2) and one MOTT were cultivated from sputa received from smear-positive patients. Sixty-nine isolates were collected from 67 new cases while 20 came from 16 relapse cases. All isolates were tested for resistance to 0.2 µg/ml of INH. Eighty-seven (97.8%) were susceptible to INH while 2 (2.2%) were resistant. Resistant isolates were found to be susceptible to 1 µg/ml and 5 µg/ml of INH. The eighty-nineth isolate was a MOTT resistant to higher concentrations of INH. Patients harbouring the resistant *M. tuberculosis* isolates were two males of 43 and 62 years old respectively, who had no previous history of the disease.

Eighty-eight (98.9%) of 89 isolates (*M. tuberculosis* = 87, MOTT = 1) were tested for resistance to 40 µg/ml RIF and all *M. tuberculosis* were susceptible to this concentration. The isolate from one patient was no longer viable when sub-cultured. *M. tuberculosis* isolates resistant to both INH and RIF (i.e. multidrug resistant strains) were not detected during this period.

5.3 Discussion

This was the first time research of this nature was conducted in the area. Due to unknown reasons much fewer samples were sent to our laboratory during the study period than could have according to the provincial TB database. The primary INH resistance prevalence rate of 2.3% appears to be significantly lower than the provincial rate of 6.3% as reported by the findings of the MRC. A long-term study that will ensure a representative collection of specimens needs to be undertaken to remove discrepancies detected above. Such a study will also give a true reflection of the state of resistance in the area that boasts to have one of the best DOTS support systems in the province. The Free State is essentially a rural province and the Goldfields are the economic centers populated by migratory male labourers from Southern African states and former self-governing territories like Qwaqwa, Thaba ´Nchu (Gamadi) and others. Previous investigations to determine the state of TB among gold miners in the province revealed high rates of acquired resistance for isoniazid, but primary rates were comparable to those of general population (Churchyard *et al*., 2000). The proportion of primary MDRTB was significantly higher in the inhabitants of the province than miners. Miners generally stay in congregated male gender accommodations, a known TB transmission factor. The aim of this study was to determine the extent of resistance to both isoniazid and rifampicin among the residents of a defined rural area in the Free State, taking into consideration the influences of commercial hubs like the Goldfields. In the defined area, isoniazid and rifampicin can still be used as first line initial empiric regimens with careful monitoring of clinical response and antibiotic sensitivities.

Primary INH resistance, is regarded as a sensitive measure of the effectiveness of a treatment programme as this drug has been used in almost all tuberculosis control programmes. In resource rich nations the prevalence of primary isoniazid resistance is below 10%, but in developing countries where control programmes are not fully functional, rates in excess of 20% have being reported. In this survey the prevalence of isoniazid resistance was relatively low at 2.3% in new patients and no acquired INH resistance was observed. These observations should, however, not lead to a false sense of security, given the study limitations presented above. A serious concern arises from a high percent of treatment interruptions (31.25%) observed, a known risk factor for the development of drug resistance. Since no MDR strains were observed in this study, it is difficult to comment on this type of resistance in the area.

In conclusion, more effort should be directed towards stressing the importance of adherence using all available and easy to understand methods of communication. Modern technologies such as easy to interpret posters, the print and electronic media in the local languages may be vehicles of choice to reach the general public. Most importantly, regular resistance surveillance studies using recent molecular techniques should be conducted to provide information regarding the temporal spread and transmission of antituberculosis drug resistance. In addition, the *in vitro* activity of second-line antituberculosis drugs among strains in the Free State remains unknown. It is also imperative to undertake a study that will unravel the extent of resistance to these drugs in the province. However, testing of first-line anti-TB remains a priority given the currently low prevalence of MDRTB.

CHAPTER 6

RESULTS:

DNA FINGERPRINTING

6.1 Introduction

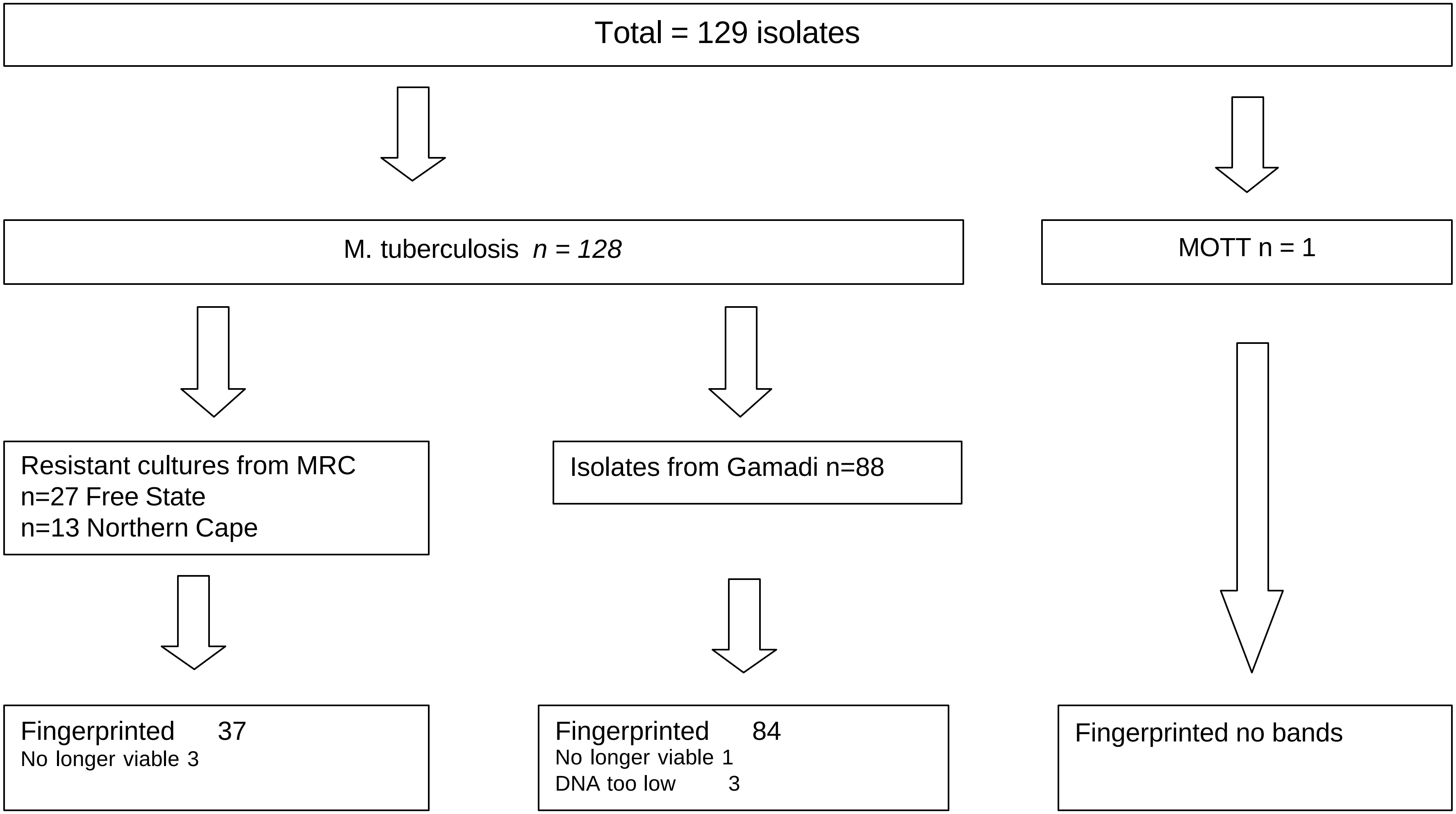
The control of tuberculosis requires a multi-faceted approach consisting of case finding, chemotherapeutic treatment with a regimen that includes isoniazid (INH) and rifampicin (RIF), evaluation of the performance of treatment and vaccination with the attenuated bacillus of Calmette-Guérin (BCG) (DOH, 2000). The disease is however continued in its ravaging path despite the worldwide reported successes of the directly observed treatment (DOTS) (Feng-Zeng *et al*,. 1996, WHO report 2003). Central to the DOTS strategy is the pivotal role of microscopy in the detection of active pulmonary TB cases (DOH, 2000). The major disadvantage of the acid-fast stain is that the stain cannot be used to distinguish between the various species of *Mycobacterium* or for assessing drug resistance.

The threat posed by the increased TB prevalence aroused interest in studying its disease dynamics. Active TB may be caused by reactivation of previously acquired infection or recent infection. In developed countries, where the annual incidence is low and most cases occur among senior citizens, more than 90.0% of cases are thought to result from reactivation of earlier infection. The high prevalence of the human immunodeficiency virus (HIV) in developing nations has resulted in the increase of reactivated TB among infected cases (CDC, 1990). Studies of outbreaks in the USA among patients in congregated settings have all suggested that recent transmission is a significant force. Recent transmission has also been observed in community based studies. Thus, in studies conducted in New York City, St Louis (Missouri) and San Franscisco as many as 37.5% to 40.0% cases were due to recent transmission (Alland *et al*., 1994 and Bifani *et al*., 1996).

In South Africa DNA fingerprinting does not form part of the national TB control programme, but a group of investigators in the Western Cape province have been generating data on the transmission dynamics of TB in communities around Cape Town (Warren *et al*., 1996 and Van Rie *et al*., 1999). Strains with the genotype characteristic of the Beijing strain and resistant to both INH and RIF have been isolated frequently. This genotype is also a recent transmission driving force among prison inmates in

KwaZulu-Natal (Pillay *et al*., 2003). Other information available comes from periodic studies conducted among employees in gold mines in the country. These studies show that recent transmission is significant, estimated at between 30.0 – 40.0% (Godfrey-Faussett *et al*., 2000). To date, information on molecular mechanisms of drug resistance, the rate of recent transmission and genetic heterogeneity of strains circulating in the Free State and Northern Cape is almost non-existent. The little that is known comes from sporadic strain typing and gene studies conducted in communities around Mangaung by the Department of Medical Microbiology at the University of the Free State since 1996. These studies performed with non-representative but convenience samples revealed extremely diverse fingerprint patterns with small clusters (Van der Spoel van Dijk *et al*., 1996). Non-representative studies have resulted in the failure to identify the presence of the Beijing strains or any strain belonging to these provinces. In this study fingerprinting by IS*6110*, spoligotyping and mycobacterial intergenic repetitive units or variable number of tandem repeats (MIRU-VNTR) were used to determine whether INH resistant strains of *M. tuberculosis* isolated from patients with pulmonary tuberculosis in the Free State and Northern Cape provinces were genetically related clones or diverse organisms. In addition, the investigation aimed to establish the routes of TB transmission in a relatively stable, well – defined community that is representative of the Free State.

Figure 6.1: INH-resistant *M. tuberculosis* isolates from MRC and susceptible isolates and a MOTT from the Gamadi area selected for fingerprinting studies using IS*6110*-RFLP typing.



6.2 Results

6.2.1 INH resistant strains from the MRC

6.2.1.1 Patient data

From May 2001 through April 2002 a total of 40 isoniazid-resistant or isoniazid and/or any other drug resistant *M. tuberculosis* isolates from pulmonary TB patients were collected by the Medical Research Council (MRC) in the Free State (n=27) and Northern Cape (n=13) provinces of South Africa. Eight of the isolates from the Free State and 5 from Northern Cape were multidrug resistant (MDR). The origins of the isolates and susceptibility profiles (MRC, Pretoria) are shown in Table 6.1. Demographic characteristics of fingerprinted patients are shown in Table 6.2. Of the 40 patients enrolled in this study, 25 (62.5%) were males between 17 to 70 years of age and 24 (60%) were below 39 years of age. Eleven patients (27.5%) had a previous history of TB treatment. Three of the 40 isolates (FS1140/01, FS884/01, and FS2260/01) were no longer viable for further tests. The number of isolates received from the Northern Cape area was far less than expected and will probably not yield any information about the molecular epidemiology of the area. Since five of these isolates were however multidrug resistant it was envisaged that the isolates could contribute essential information about the *katG* and rpoB mutations present in this area and therefore were not excluded from the study.

Table 6.1: Data of drug resistant isolates from MRC (location and resistance profiles).

		Resistance pattern							
No	Strain	INH	RIF	ETH	STR	District	Sub-district	Town	Clinic
MRC01	FS230	R	S	S	S	DC 17	FS 173	Thaba'Nchu	Gaongalelwe
MRC02	FS824	R	S	S	S	DC 17	FS 172	BFN	Opkoms
MRC03	FS1136	R	S	S	S	DC 17	FS 172	BFN	Batho
MRC04	FS2701	R	S	S	S	DC 17	FS 172	BFN	Opkoms
MRC05	FS2788	R	S	S	S	DC 17	FS 172	BFN	Opkoms
MRC06	FS2972	R	S	S	S	DC 17	FS 172	BFN	Opkoms
MRC07	FS475	R	R	R	R	DC 17	FS 172	BFN	Mangaung
MRC08	FS1212	R	S	S	R	DC 17	FS 172	BFN	Opkoms
MRC09	FS956	R	S	S	S	DC 18	FS 184	Welkom	Welkom
MRC10	FS1156	R	S	S	S	DC 18	FS 184	Welkom	Welkom
MRC11	FS1283	R	S	S	S	DC 18	Nala	Wesselsbron	Albert Luthuli
MRC12	FS2012	R	S	S	S	DC 18	Nala	Wesselsbron	Albert Luthuli
MRC13	FS2295	R	S	S	S	DC 18	FS 184	Welkom	Welkom
MRC14	FS975	R	R	S	S	DC 18	FS 184	Welkom	Welkom
MRC15	FS2189	R	R	S	R	DC 18	Nala	Wesselsbron	Albert Luthuli
MRC16	FS2771	R	R	R	S	DC 18	FS 184	Welkom	Pedisanang
MRC17	FS1773	R	S	S	S	DC 19	Dihlabeng	Bethlehem	Bethlehem
MRC18	FS264	R	S	S	S	DC 19	Nketoana	Qwa Qwa	Bolata
MRC19	FS266	R	S	S	S	DC 19	Nketoana	Qwa Qwa	Marakong
MRC20	FS1591	R	R	S	R	DC 20	FS 184	Welkom	Welkom
MRC21	FS2516	R	R	R	R	DC 20	FS 184	Welkom	Welkom
MRC22	FS1794	R	R	S	S	DC 20	FS 184	Sasolburg	Zamdela
MRC23	FS117	R	R	R	S	DC 17	FS 173	Botshabelo	U/W
MRC24	FS637	R	S	S	S	DC 17	FS 173	BFN	Opkoms
MRC25	FS884	R	S	S	S	DC 18	FS 184	Welkom	Boithusong
MRC26	FS1140	R	S	S	S	DC 17	FS 173	BFN	Opkoms
MRC27	FS2260	R	R	S	S	DC 17	FS 173	BFN	Thusong
NC01	NC 1243	R	R	S	S	F. Baardt	F. Baardt	NA	Betty Gaetsewe
NC02	NC 928	R	S	S	S	Hantam	Hantam	NA	Calvinia
NC03	NC 1300	R	R	S	S	Kgalagadi	Kgalagadi	NA	kuruman
NC04	NC 802	R	S	S	S	DC 8	Lower Orange	NA	Keimoes eilande
NC05	NC 646	R	S	S	S	DC 7	Upper-Karoo	NA	Montana
NC06	NC 931	R	S	S	S	DC 7	Upper-Karoo	De Aar	De Aar
NC07	NC 948	R	S	S	S	DC 7	Upper-Karoo	De Aar	Nonzwakazi
NC08	NC 984	R	S	S	S	DC 7	Upper-Karoo	Colesberg	Colesberg
NC09	NC 910	R	R	S	S	DC 7	Upper-Karoo	K/ Ville	Montana
NC10	NC 19	R	R	R	S	D field	D field	Valspan	Valspan
NC11	NC1094	R	S	S	S	DC 7	Upper-Karoo	H/ Valley	Montana
NC12	NC1087	R	R	S	R	DC 8	Lower Orange	NA	Progress
NC13	NC1047	R	S	S	S	DC 7	Upper-Karoo	NA	Montana

Table 6.2: Demographic properties of fingerprinted INH-resistant patients from the Free State and Northern Cape.

Gender	
Female	12
Male	25
New cases	
Female	10
Male	16
Retreatment	
Female	2
Male	9
Age	
0 - 14	0
15 - 19	1
20 - 39	23
40 - 59	9
=60	4

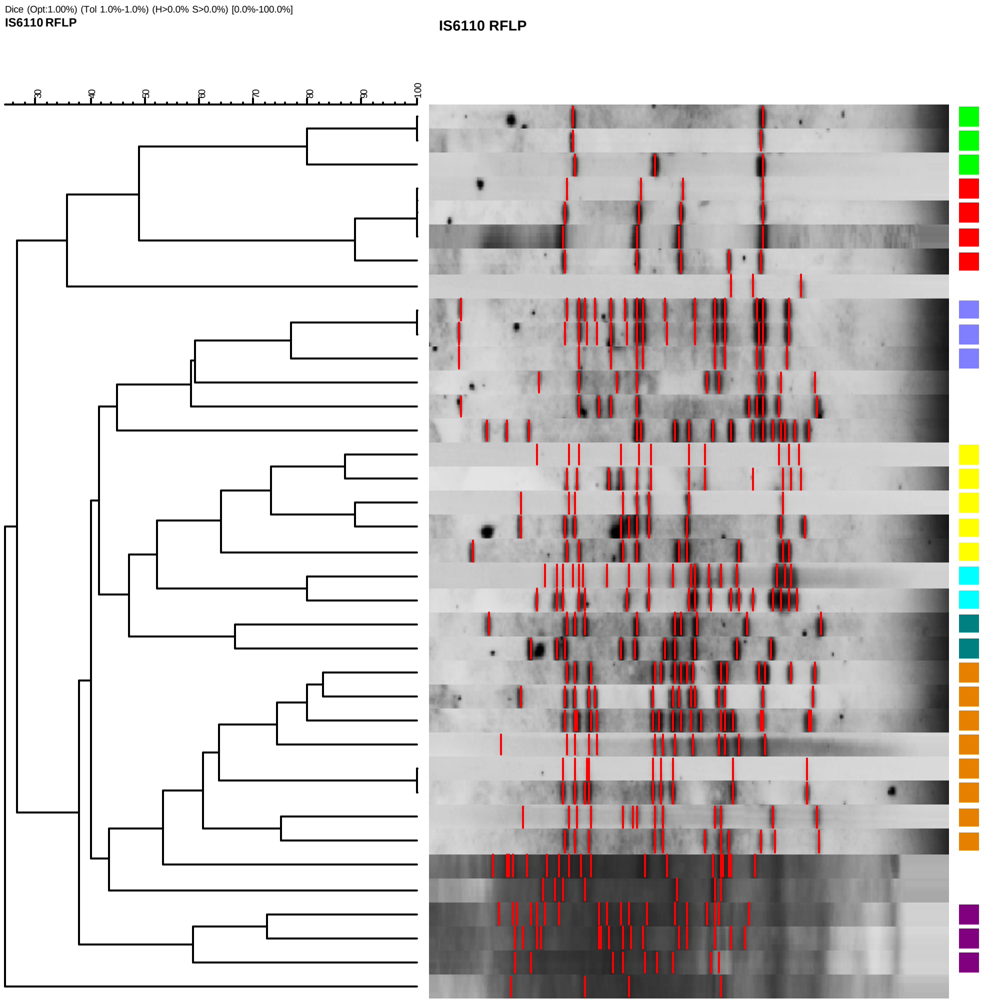
6.2.1.2 *IS*6110-RFLP analysis of drug-resistant strains

A total of 37 isoniazid-resistant *M. tuberculosis* were analysed using the standard IS*6110* based DNA fingerprint method (Figure 6.1). The number of IS*6110* copies per isolate varied from two to 18. DNA fingerprint profiles showed nine isolates from nine patients with less than six insertions (24.3%). Six of these isolates were from the Free State and three from the Northern Cape Province. The remaining 28 isolates (75.7%) contained between 9 and 18 copies of the IS*6110* insertion sequence. Of these four (14.3%) isolates were found in clusters and the remaining 24 (85.7%) each gave a unique pattern. One cluster (each two patients) was observed in the Free State and the other in the Northern Cape Province.

Eight clonally related groups (65.0% similarity) with two to four strains were present (Figure 6.2). Of the 13 MDR strains (Table 6.1) three (MRC16, NC03, and NC09) had six and less insertions.

These strains need to be sub typed using different methods to provide clarity.

Figure 6.2: IS*6110* RFLP patterns of 37 INH resistant *M. tuberculosis* isolates received from MRC, Pretoria. Strains were arranged according to similarities determined by the unweighted pair group method using arithmetical averages (UPGMA) and Dice coefficient with the GelCompar II version 2.5 program.



MRC16	M. tuberculosis	FS2771/0	Pedisanang	Welkom	Goldfield FS
NC03	M. tuberculosis	NC1300/0	Kuruman	Kuruman	N.Cape
MRC08	M. tuberculosis	FS1212/0	Opkoms	Bloemfontein	Mangaung FS
MRC10	M. tuberculosis	FS1156/0	Welkom	Welkom	Goldfield FS
MRC11	M. tuberculosis	FS1283/0	Albert Luthuli	Wesselsbron	Free State
MRC24	M. tuberculosis	FS637/01	Opkoms	Bloemfontein	Mangaung FS
NC09	M. tuberculosis	NC910/01	Montana	K/Ville	N.Cape
MRC09	M. tuberculosis	FS956/01	Welkom	Welkom	Goldfield FS
NC08	M. tuberculosis	NC984/01	Upper-Karoo	Colesberg	N.Cape
NC07	M. tuberculosis	NC948/01	Nonzwakazi	De Aar	N.Cape
NC02	M. tuberculosis	NC928/01	Calvinia	Calvinia	N.Cape
MRC12	M. tuberculosis	FS2012/0	Albert Luthuli	Wesselsbron	Free State
NC04	M. tuberculosis	NC802/01	Keimoes eilande	Keimoes	N.Cape
MRC21	M. tuberculosis	FS2516/0	Welkom	Welkom	Goldfield FS
MRC14	M. tuberculosis	FS975/01	Welkom	Welkom	Goldfield FS
MRC18	M. tuberculosis	FS264/01	Bolata	Qwa Qwa	Qwaqwa FS
MRC07	M. tuberculosis	FS475/01	Mangaung	Bloemfontein	Mangaung FS
NC06	M. tuberculosis	NC931/01	De Aar	De Aar	N. Cape
MRC20	M. tuberculosis	FS1591/0	Welkom	Welkom	Free State
MRC03	M. tuberculosis	FS1136/0	Batho	Bloemfontein	Mangaung FS
MRC19	M. tuberculosis	FS266/01	Marakong	Qwa Qwa	Qwaqwa FS
MRC06	M. tuberculosis	FS2972/0	Opkoms	Bloemfontein	Mangaung FS
NC05	M. tuberculosis	NC646/01	Montana	Upper-Karoo	N.Cape
MRC13	M. tuberculosis	FS2295/0	Welkom	Welkom	Goldfield FS
NC01	M. tuberculosis	NC1243/0	Betty Gaetsewe	N.Cape	N.Cape
MRC15	M. tuberculosis	FS2189/0	Albert Luthuli	Wesselsbron	Free State
ZT39	M. tuberculosis	FS230/01	Gaongalelwe	Thaba Nchu	Mangaung FS
MRC02	M. tuberculosis	FS824/01	Opkoms	Bloemfontein	Mangaung FS
MRC04	M. tuberculosis	FS2701/0	Opkoms	Bloemfontein	Mangaung FS
MRC17	M. tuberculosis	FS1773/0	Bethlehem	Bethlehem	Free State
MRC05	M. tuberculosis	FS2788/0	Opkoms	Bloemfontein	Mangaung FS
MRC22	M. tuberculosis	FS1794/0	Zamdela	Sasolburg	Free State
NC10	M. tuberculosis	NC19/01	Valspan	Valspan	N.Cape
NC12	M. tuberculosis	NC1087/0	Progress	Lower Orange	N.Cape
NC13	M. tuberculosis	NC1047/0	Montana	Montana	N.Cape
MRC23	M. tuberculosis	FS117/01	U/W	Botshabelo	Free State
NC11	M. tuberculosis	NC1094/0	Montana	H/Valley	N.Cape

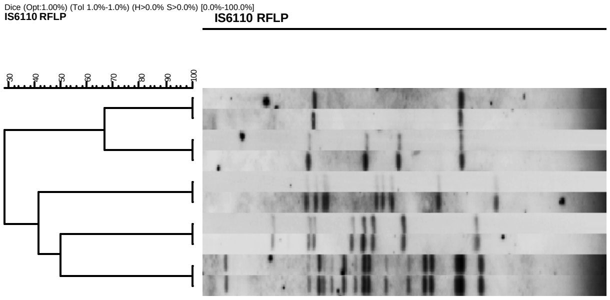
Five clusters with identical RFLP patterns were present and were confirmed by spoligotyping. Interviews could not be conducted with patients from clusters. Only 3 (MRC07, MRC14, and MRC20) of the remaining 10 MDR strains were in the same family group (65.0% similarity).

6.2.1.3 Spoligotyping

Spoligotyping was used as an additional tool in order to confirm results of clustered isolates (Figure 6.3).

Figure 6.3: RFLP and spoligotyping patterns of 9 INH-resistant isolates from MRC and one susceptible isolate from Gamadi that clustered with one of the INH-resistant strains.

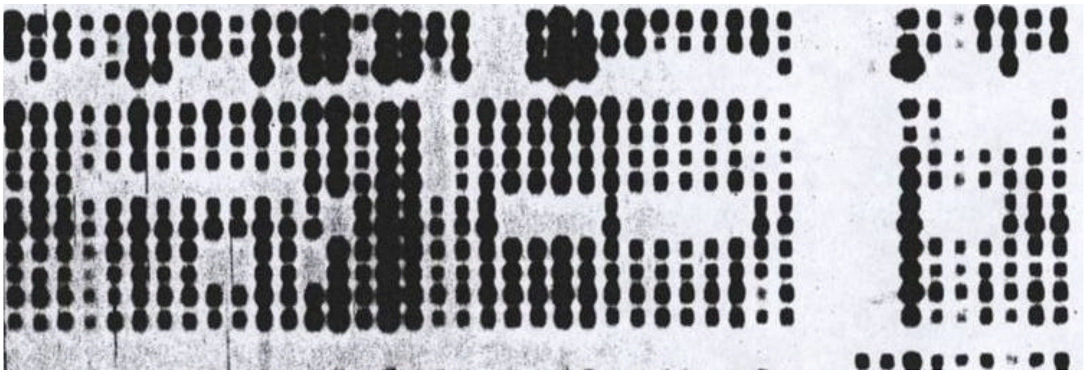
RFLP patterns



MRC16	.FS2771/02	.Pedisanang	Welkom	Goldfield FS	.
NC03	.NC1300/02	.Kuruman	Kuruman	N.Cape	.
MRC10	.FS1156/01	.Welkom	Welkom	Goldfield FS	.
MRC11	.FS1283/01	.Albert Luthuli	Wesselsbron	Free State	.
MRC02	.FS824/01	.Opkoms	Bloemfontein	Mangaung FS	.
MRC04	.FS2701/01	.Opkoms	Bloemfontein	Mangaung FS	.
MRC07	.FS475/01	.Mangaung	Bloemfontein	Mangaung FS	.
ZT59	.FS2879/02	.Gaongalelwe	Thaba Nchu	Mangaung FS	.
NC07	.NC948/01	.Nonzwakazi	De Aar	N.Cape	.
NC08	.NC984/01	.Upper-Karoo	Colesberg	N.Cape	.

Spoligotypes:

C
C
C
-
01 – FS2771
02 – NC1300
03 – FS1156
04 – FS1283
05 – FS 824
06 – FS2701
07 – FS 475
08 – FS2879
09 – NC 948
10 – NC 984
-
C



Two clusters, one consisting of strains from the Free State (MRC02 and MRC04, 9 bands each) and a second from the Northern Cape (NC07 and NC08, 16 bands each) gave matching spoligotypes. Two of three isolates from a third clone (MRC10, MRC11 and MRC24, 4 bands each) gave no match with this typing method. The fourth cluster (2 bands) was made up of one isolate from each province (Free State, MRC16; Northern Cape, NC03). Spoligotyping of these strains was identical. One isolate from Bloemfontein (MRC07) had identical IS6110-RFLP and spoligotyping patterns to a susceptible isolate (ZT59) from Gamadi (Figure 6.3).

6.2.2 Isolates from the defined area

6.2.2.1 Clinical characteristics of participants

A total of 84 isolates was analysed. Six of the patients provided two sputa each (Table 6.3). For five of these patients the interval between the first and the second sputum varied from one to three weeks. The sixth patient provided the additional sputum after five months.

Table 6.3: Strain numbers of sputum samples of six patients that gave more than one sputum sample from Gamadi.

Patient	1st sputum (date collected)	2nd sputum (date collected)
1	FS2239/01 (02.11.2001)	01010007 (23.11.2001)
2	01010009 (03.11.2001)	FS2241/01 (13.11.2001)
3	FS2401/01 (05.12.2001)	01010017 (18.12.2001)
4	01010052 (05.12.2001)	FS2513/01 (28.12.2001)
5	03110064 (04.11.2002)	02010133 (29.11.2002
6	FS2588/02 (22.01.2002)	02010074 (07.06.2002)

Demographic data of 83 of the patients were available and are given in Table 6.4.

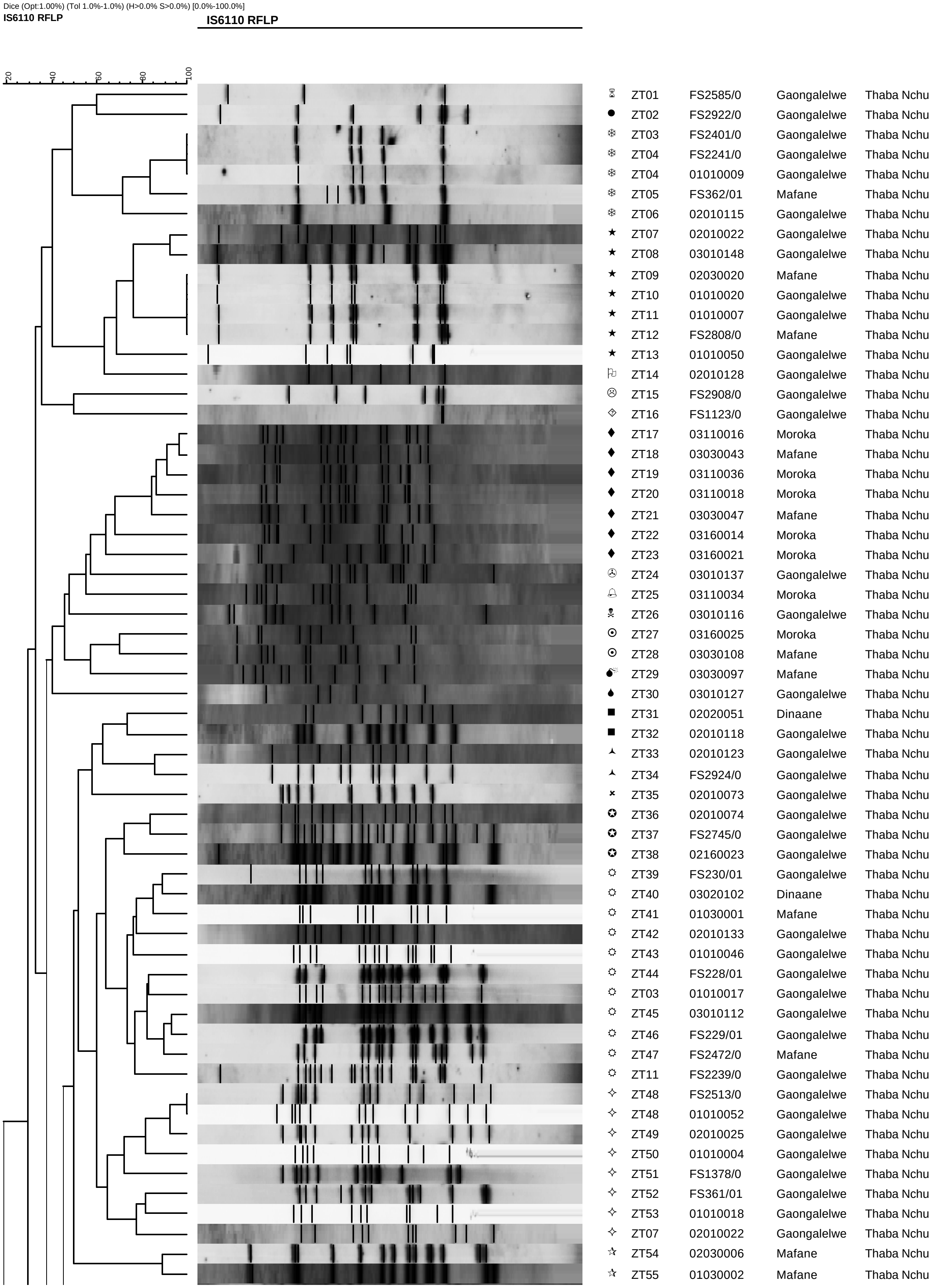
Table 6.4: Characteristics of culture positive patients from the defined area: Gamadi.

Total patients = 83	**Isolates = 88**	**INH resistant = 2**
Gender		
Female	40	
Male	43	
New cases		
Female	36	
Male	35	
Retreatment cases		
Female	7	
Male	9	
Age		
0 - 14	0	
15 - 19	4	
20 - 39	45	
40 - 59	30	
=60	3	

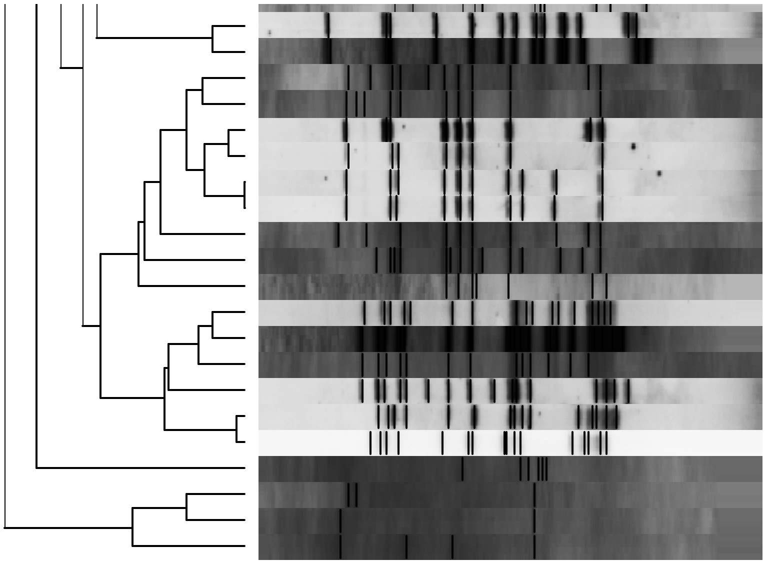
6.2.2.2 IS6110 fingerprint analysis

DNA fingerprint analysis could not be performed on 4 of the 88 isolates (01020006 no longer viable, 02010124, 03110064, and FS2588/02 had DNA concentrations that were too low to allow IS*6110*-based fingerprinting). DNA fingerprinting was performed on 84/88 (95.5%) isolates (Figure 6.3). Of the 84 isolates fingerprinted, eight were from patients that provided two specimens each. For these four patients both strains were fingerprinted as a means of checking laboratory contamination. For two (ZT04 and ZT48) of the four patients the two strains had identical patterns. These duplicate isolates were cultured on different days and fingerprinted on different gels. For the other two patients (ZT03 and ZT11) the two sputa had different fingerprints. One of the sputa of patient ZT03 had the same fingerprinting pattern as patient ZT04 and was fingerprinted on the same gel as this strain. The same was true for the two strains of patient ZT11. Both these strains were cultured in the same batches as the strains with identical patterns and laboratory contamination, accidental labeling or switching errors are assumed.

Figure 6.4: IS*6110* RFLP fingerprinting patterns of 79 *M. tuberculosis* isolates from the Gamadi community. Strains were arranged according to similarities determined by the unweighted pair group method using arithmetical averages (UPGMA) and Dice coefficient with the GelCompar II version 2.5 program.



⌛	ZT01	FS2585/0	Gaongalelwe	Thaba Nchu
●	ZT02	FS2922/0	Gaongalelwe	Thaba Nchu
❄	ZT03	FS2401/0	Gaongalelwe	Thaba Nchu
❄	ZT04	FS2241/0	Gaongalelwe	Thaba Nchu
❄	ZT04	01010009	Gaongalelwe	Thaba Nchu
❄	ZT05	FS362/01	Mafane	Thaba Nchu
❄	ZT06	02010115	Gaongalelwe	Thaba Nchu
★	ZT07	02010022	Gaongalelwe	Thaba Nchu
★	ZT08	03010148	Gaongalelwe	Thaba Nchu
★	ZT09	02030020	Mafane	Thaba Nchu
★	ZT10	01010020	Gaongalelwe	Thaba Nchu
★	ZT11	01010007	Gaongalelwe	Thaba Nchu
★	ZT12	FS2808/0	Mafane	Thaba Nchu
★	ZT13	01010050	Gaongalelwe	Thaba Nchu
⚐	ZT14	02010128	Gaongalelwe	Thaba Nchu
☹	ZT15	FS2908/0	Gaongalelwe	Thaba Nchu
◇	ZT16	FS1123/0	Gaongalelwe	Thaba Nchu
♦	ZT17	03110016	Moroka	Thaba Nchu
♦	ZT18	03030043	Mafane	Thaba Nchu
♦	ZT19	03110036	Moroka	Thaba Nchu
♦	ZT20	03110018	Moroka	Thaba Nchu
♦	ZT21	03030047	Mafane	Thaba Nchu
♦	ZT22	03160014	Moroka	Thaba Nchu
♦	ZT23	03160021	Moroka	Thaba Nchu
✇	ZT24	03010137	Gaongalelwe	Thaba Nchu
🔔	ZT25	03110034	Moroka	Thaba Nchu
☠	ZT26	03010116	Gaongalelwe	Thaba Nchu
⊙	ZT27	03160025	Moroka	Thaba Nchu
⊙	ZT28	03030108	Mafane	Thaba Nchu
💣	ZT29	03030097	Mafane	Thaba Nchu
💧	ZT30	03010127	Gaongalelwe	Thaba Nchu
■	ZT31	02020051	Dinaane	Thaba Nchu
■	ZT32	02010118	Gaongalelwe	Thaba Nchu
⅄	ZT33	02010123	Gaongalelwe	Thaba Nchu
⅄	ZT34	FS2924/0	Gaongalelwe	Thaba Nchu
×	ZT35	02010073	Gaongalelwe	Thaba Nchu
✪	ZT36	02010074	Gaongalelwe	Thaba Nchu
✪	ZT37	FS2745/0	Gaongalelwe	Thaba Nchu
✪	ZT38	02160023	Gaongalelwe	Thaba Nchu
☼	ZT39	FS230/01	Gaongalelwe	Thaba Nchu
☼	ZT40	03020102	Dinaane	Thaba Nchu
☼	ZT41	01030001	Mafane	Thaba Nchu
☼	ZT42	02010133	Gaongalelwe	Thaba Nchu
☼	ZT43	01010046	Gaongalelwe	Thaba Nchu
☼	ZT44	FS228/01	Gaongalelwe	Thaba Nchu
☼	ZT03	01010017	Gaongalelwe	Thaba Nchu
☼	ZT45	03010112	Gaongalelwe	Thaba Nchu
☼	ZT46	FS229/01	Gaongalelwe	Thaba Nchu
☼	ZT47	FS2472/0	Mafane	Thaba Nchu
☼	ZT11	FS2239/0	Gaongalelwe	Thaba Nchu
✧	ZT48	FS2513/0	Gaongalelwe	Thaba Nchu
✧	ZT48	01010052	Gaongalelwe	Thaba Nchu
✧	ZT49	02010025	Gaongalelwe	Thaba Nchu
✧	ZT50	01010004	Gaongalelwe	Thaba Nchu
✧	ZT51	FS1378/0	Gaongalelwe	Thaba Nchu
✧	ZT52	FS361/01	Gaongalelwe	Thaba Nchu
✧	ZT53	01010018	Gaongalelwe	Thaba Nchu
✧	ZT07	02010022	Gaongalelwe	Thaba Nchu
✰	ZT54	02030006	Mafane	Thaba Nchu
✰	ZT55	01030002	Mafane	Thaba Nchu



☆	ZT54	02030006	Mafane	Thaba Nchu
☆	ZT55	01030002	Mafane	Thaba Nchu
♊	ZT56	03010114	Gaongalelwe	Thaba Nchu
♊	ZT57	02020058	Dinaane	Thaba Nchu
♊	ZT58	02010060	Gaongalelwe	Thaba Nchu
♊	ZT59	FS2879/0	Gaongalelwe	Thaba Nchu
♊	ZT60	02010026	Gaongalelwe	Thaba Nchu
♊	ZT61	FS2909/0	Gaongalelwe	Thaba Nchu
♊	ZT62	03010155	Gaongalelwe	Thaba Nchu
♊	ZT63	03020064	Dinaane	Thaba Nchu
➔	ZT64	02020068	Dinaane	Thaba Nchu
☺	ZT65	01020013	Dinaane	Thaba Nchu
☺	ZT66	02020066	Dinaane	Thaba Nchu
☺	ZT67	02020067	Dinaane	Thaba Nchu
☺	ZT68	02030007	Mafane	Thaba Nchu
☺	ZT69	FS2894/0	Gaongalelwe	Thaba Nchu
☺	ZT70	01030003	Mafane	Thaba Nchu
➲	ZT71	03030044	Mafane	Thaba Nchu
☞	ZT72	03160024	Gaongalelwe	Thaba Nchu
☞	ZT73	03030071	Mafane	Thaba Nchu
⌦	ZT74	03030048	Mafane	Thaba Nchu

One isolate from this patient had a pattern that correlated with a pattern of a cluster consisting of four isolates from patients ZT09, ZT10, and ZT12. These discrepancies were the only evidence of laboratory contamination found.

IS*6110* sequences were not detected in six isolates. Fourteen isolates had less than IS*6110* hybridisation bands and four strains were in clusters. The remaining 57 (88.9%) strains had distinct RFLP profiles with more than six bands. The number of IS*6110* copies varied from seven to 21. A total of five strains distributed in two clusters, one with two and the other three members were evident.

Thirteen family groups, clustering at 65.0% on the similarity dendogram, each with two to eight strains were evident but, no dominant groups were obvious (Figure 6.3 first column with symbols).

6.2.3 Subtyping by MIRU-VNTR

Twelve strains were sub-typed using MIRU-VNTR typing (Table 6.5).

A cluster made up of one isolate from each province, MRC16 and NC03 (each with two bands), gave differing MIRU results. Three isolates (MRC10, MRC11, MRC24) with identical IS*6110* profiles gave unrelated MIRU and spoligotyping patterns (MRC10, MRC11). Strains MRCO8 and MRC09 (both INH-resistant) and ZTO1 had different RFLP and MIRU permutations. A cluster of three isolates (ZT03 and two x ZT04, five identical IS*6110* bands, figure 6.3) had identical MIRU profiles while one, (ZT06) with a unique three-band RFLP pattern, also gave a distinct MIRU pattern.

6.2.3 Evidence for recent transmission (Table 6.6)

Medical files of patients in clusters were reviewed and interviews conducted to establish possible evidence of transmission and index cases. Patients in clusters A and D came from the same village and three in cluster E (ZT10, ZT11 and ZT13) were from the same village as those of clusters A and D. Two of these patients were friends (ZT10, ZT11). The remaining two members of cluster E (ZT09, ZT12) lived in another village.

6.3 Discussion

The isolates used in this study come from the Free State, Northern Cape, and a defined area (Gamadi) that models the Free State province. The provinces are similar in being of the poorest in South Africa. Nevertheless, the DOTS strategy has been successfully implemented in the two provinces. A resistance profile of an isolate is requested in instances of failure to convert to sputum smear negative result (DOH, practical guidelines, 2000). Since resistance phenotypes are poor indicators of transmission patterns, the use of molecular typing is essential for epidemiological studies.

Although isolates from the Northern Cape were apparently not representative of the province due to difficulties that the MRC encountered with collection of the samples (Weyer, personal communication), the fingerprint patterns of the Northern Cape isolates were as diverse as those obtained for the Free State area. For this reason the Northern Cape samples were included in the study. DNA fingerprint profiles with less than six IS*6110* insertions were observed from 24.3% INH resistant strains (Figure 6.2). The percentage of strains with few IS*6110* sequences was as high as 33.3% among strains in the Western Cape in 1996 (Warren *et al*., 1996). Isolates with few copies appear to have a global distribution. They have been reported in many countries including

Botswana, Ethiopia, United Kingdom and the United States among others (Haas *et al*., 1999, Hermans *et al*., 1995, Kumar *et al*., 2000, Frieden *et al*., 1996)

Two clusters were present among INH resistant strains of the Free State and the Northern Cape, with less than six bands. The cluster containing strains MRC16 and NC03 were confirmed by spoligotyping, but had different MIRU patterns. The cluster containing MRC10 and MRC11 as analysed by spoligotyping had in strain MRC 11 a whole block of spacers absent from the pattern as defined by Kamerbeek *et al.,* 1997 while the MIRU identification differed in the sizes of several of the alleles (Supply *et.al*., 1997). This finding confirms the need for secondary typing in strains with less than six IS*6110* insertions. The majority (75.7%) of the strains had seven to eighteen bands. Only four of the 28 strains from the seven to 18 bands group (14.3%) were found in clusters and the rest (85.7%) had distinct patterns. This is in sharp contrast to the results of Van Rie *et al.* (2000) who reported that 76.0% of MDR strains (in the Western Cape Province) were found in clusters (Van Rie *et al*., 2000).

The high degree of diversity observed suggests that reactivation of a latent isoniazid-resistant infection is a significant factor in a population of drug-resistant cases in the Free State and Northern Cape. It is also probable that these results may underestimate the importance of recent transmission, since some patients may have contracted the disease while outside the provinces. These patients then formed part of a recent transmission group, the extent of which lies outside the catchment area of this study. This premise may hold true since two-thirds of cases (67.6%) are males who might have been migrant workers in other provinces at certain times of their lives. Since contact could not be established, one can only speculate on loose casual contact or the presence of a common index case beyond the geographic and temporal confines of this study.

Observation of clusters is also associated with patients presenting at clinics with advanced TB. Decrease in the risk of infection for the community may only be obtained

Table 6.5: MIRU-VNTR patterns of twelve *M. tuberculosis* strains. Seven strains were from two RFLP clusters with less than six bands and two strains with unique RFLP patterns of three bands. The other five strains were isolates with less than six RFLP bands from the Gamadi area. MIRU-VNTR typing was performed using the twelve most polymorphic loci.

No.	Strain No.	MIRU 02	MIRU 04	MIRU 10	MIRU 16	MIRU 20	MIRU 23	MIRU 24	MIRU 26	MIRU 27	MIRU 31	MIRU 39	MIRU 40
01 - MRC16	FS2771/02	2	2	4	3	2	5	1	4	3	3	2	4
02 - N03	NC1300/01	2	2	4	3	2	5	1	4	3	3	2	3
03 - MRC08	FS1212/01	2	2	4	3	2	5	1	5	3	3	2	3
04 - MRC10	FS1156/01	2	7	4	3	2	6	2	2	3	4	1	4
05 - MRC11	FS1283/01	2	2	4	3	2	5	1	5	3	3	2	4
06 - MRC24	FS637/01	2	2	4	3	2	5	1	4	3	2/3	2	2
07 - MRC9	FS956/01	2	5	4	3	2	6	2	2	3	4	1	3
08 - ZT01	FS2585/02	2	2	4	3	2	5	1	5	2	3	2	3
09 - ZT03	FS2401/01	2	2	4	3	2	5	1	4	3	3	2	2
10 - ZT04	FS2241/01	2	2	4	3	2	5	1	4	3	3	2	2
11 - ZT04	01010009	2	2	4	3	2	5	1	4	3	3	2	2
12 - ZT06	02010115	2	2	5	3	2	5	0	3	3	3	2	4
CT	H37Rv	2	3	3	2	2	6	1	3	3	3	2	1

Table 6.6 Characteristics of groups of patients with identical IS*6110* RFLP patterns. Both INH resistant from MRC and susceptible cases from Gamadi.

Cluster	IS copy no.	Strain no.	Gender	Age	Link	Secondary typing results	
						Spoligotyping	MIRU
A	4	ZT03 ZT04	F M	36 56	Not interviewed	ND	Match
B	9	MRC02 MRC04	F M	17 34	No link Same township	Match	ND
C	8	MRC07 ZT63	M F	35 34	Cannot trace Different antibiotic pattern	ND	ND
D	10	ZT64 ZT65	F M	40 26	Not interviewed	ND	ND
E	8	ZT12 ZT09 ZT11 ZT10 ZT13	M M M F F	37 41 25 UNK UNK	Not interviewed Not interviewed Friends Not interviewed	ND	ND
F	4	MRC16 NC03	F M	49 31	Not interviewed Not interviewed	Match	No match
G	4	MRC24 MRC11 MRC10	M M M	38 35 46		Not identical	Not identical All strains different
H	3 (2 = Identical, 1 different size)	MRC08 ZT01	M F	29 27		ND	Identical
I	16	NC948/01 NC984/01	M M	43 70	Not interviewed	Identical	ND

by preventing the patient's and health care worker's delay. The efforts to educate of the community on the nature of tuberculosis should be an ongoing process. In addition, activities aimed at elevating the index of suspicion among health care workers should continue. These efforts coupled with the amelioration of the socio-economical situation, will reduce the delays and retard the spread of the disease. Studies in the Western Cape and KwaZulu-Natal have identified strains of the Beijing genotype lineage as a significant force (Pillay *et al.*, 2003). The Beijing genotype are often associated with multidrug-resistant TB in Asian countries, USA (W strain), and the Western Cape Province (U strain) of South Africa (Van Rie A *et al.*, 1999). Contrary to that found in certain regions of the world no strains with an *IS6110* pattern characteristic of the Beijing-W type were observed in the samples collected from the Free State and Northern Cape. Furthermore, the existence of recently discovered multidrug and poly-drug-resistant strain (designated DRF150) has remained unclear in the two areas (www.promedmail.org).

These results indicate low transmission of INH-resistant strains. In addition, the absence of clusters among MDRTB strains is a heartening observation, since the spread of such strains may be difficult to contain. However, continuous monitoring of drug resistance and disease dynamics in the general population is imperative for the situation to be reversed or remain constant, at the least.

The second arm of the study evaluated the population structure and transmission routes of *M. tuberculosis* strains from Gamadi in the Free State. This part of the study was seriously hampered by poor quality of samples and thus the collected sputa did not result in consecutive cultures for use in DNA typing. This factor caused the isolates collected to be less representative thus limiting the scope of conclusions that could be drawn about the diversity, transmission and reactivation of the tuberculosis disease. Eighty-four isolates were fingerprinted. Six strains without IS*6110* bands will be re-analysed at a later date. A further 14 strains with less than six IS*6110* bands were observed and left for later analysis. Of the remaining 64 isolates with more than six bands, three were suspected to result from laboratory contamination and therefore omitted. Of the remaining 61 isolates six were in two clusters. The different banding patterns were calculated to be 61 minus six in clusters, plus the two cluster patterns. It was calculated that 57 (93.4%) different patterns were present. This high degree of strain variability suggests that reactivated TB is a significant problem in the area. This is in accordance with previous reports from the Western Cape. Diversity may be high at strain level, but greater relatedness was deduced from the few clonal families (65.0% on the similarity dendogram) observed. Altogether, nine clonal families each with two to eight strains were evident. Comparing strain families in Gamadi to others in the database (from other regions in the province) revealed similarity differences. This is in keeping with limited migration of the residents in this area.

The IS*6110* fingerprint patterns of strains isolated in succession from one patient (ZT11) revealed some discrepancies consistent with laboratory contamination. The strains were isolated by different laboratories, fingerprinted on different gels and had different RFLP patterns (one fingerprint pattern belonged to a cluster of strains run in the same gel). Laboratory contamination was also suspected in three other cases. One was from a fingerprint of a strain ZT03 that formed a cluster with two identical strains (ZT04) from one patient. The second strain (ZT03) had a unique DNA fingerprint on another gel. Therefore the ZT03 (01010017) strain that did not cluster with the ZT04 strain is most likely the correct isolate. Different profiles were observed for a single strain (ZT07, the same DNA) run on different gels and dates. The results of a cluster consisting of ZT03 and ZT04 were confirmed by MIRU typing.

Subtyping by spoligotyping was supportive in confirming clustering of a susceptible strain from Gamadi (ZT59) and an INH resistant strain (MRC07) from Bloemfontein (Free State). It could not be established if the resistant strain was from a patient that was a new case or that the patient was previously treated for tuberculosis since insufficient information was supplied.

A significant number (16.7%) of strains with less than six IS*6110* were detected. Strains with such patterns have been previously reported from Tunisia, Ethiopia, and the Western Cape Province. The high number of these strains makes it imperative to utilise additional genetic markers with every epidemiological surveillance studies undertaken. Such endeavours will assist in informing the public about the arrival or spread of unique strains, such as the multiple drug resistant DRF150. The DRF150 strain may be establishing itself in a different DNA fingerprint configuration, hence the need for secondary typing of every strain with less than six IS*6110* bands. The DRF150 strain has so far been detected in the Western Cape, Limpopo, Mpumalanga provinces of South Africa and Kenya (www.promedmail.org), but not in the Free State and therefore RFLP surveillance with subsequent further typing is imperative.

In conclusion, fingerprint patterns arousing suspicion of multiple or super-infection were not evident in this study. Studies in the Western Cape have revealed multiple infections of individuals as a significant problem in an area of high TB incidence (Van Pittius *et al*, 2003). Since Gamadi has a heavy burden of TB, we urgently need to investigate the role of such infections against TB control efforts in the province. This study has highlighted the role of reactivated TB in the Free State province. Perhaps poverty and its subsequent problems plays a leading role in the reactivation past infections. Finally, the observation of a cluster of five patients (ZT09, ZT10, ZT11, ZT12 and ZT13) in this community calls for more investigations in order to determine the spread and persistence of this strain in the population.

CHAPTER 7

RESULT:
MOLECULAR DETECTION OF ISONIAZID RESISTANCE

7.1 Introduction

Production of toxic by-products is an inevitable consequence of cellular metabolism. To survive the deleterious effects of these harmful substances, bacteria have developed some damage control mechanisms. In particular, most possess the capacity to convert oxygen radicals to harmless compounds such as oxygen and water. In *M. tuberculosis* a bi-functional protein called catalase-peroxidase, encoded by the *katG* gene detoxifies oxygen radicals.

Isoniazid (isonicotinic acid hydrazide) is the cornerstone of the first-line drugs included in the DOTS antituberculosis regimen. It has the most potent early bactericidal activity of all known drugs against drug-susceptible *M. tuberculosis*. The major mechanism of acquisition of resistance to INH in *M. tuberculosis* has been shown to be associated with the point mutations in the *katG* gene encoding the catalase-peroxidase enzyme of this organism. The resulting defective katG protein fails to convert INH (thought to be a prodrug) to hydrazine derivatives needed to inhibit the formation of mycolic acids. (Musser, 1995). Table 7.1 lists *katG* mutations reported in the literature. Although the importance of codon 315 mutations in INH resistance has been established, the involvement of other mutations (for example, codons 275 and 328) is unclear. It has also been demonstrated that other mutations, for example, CGG to CTG (Arg to Leu) at codon 463, do not seem to confer any degree of resistance to INH. However, it has been suggested that different alleles of codon 463 of the *katG* gene link *M. tuberculosis* strains to specific geographic regions (Haas *et al*., 1997).

Table 7.1 *KatG* gene mutations reported in the literature

Codon	Base changes	Amino acid change	References
315	AGC→ACC AGC→ACA AGC→AAC AGC→ATC AGC→CGC	Ser→Thr Ser→Thr Ser→Asn Ser→Ile Ser→Arg	Musser *et al.,* 1996
463	CGG→CTG	Arg→Leu	Musser *et al.,* 1996
710	GTC→GCC	Val→Ala	Musser *et al.,* 1996
94	GAC→GCC	Asp→Ala	Musser *et al.,* 1996
328	TGG→TTG TGG→TGC	Trp→Leu Trp→Cys	Haas *et al.,* 1997

Asp = asparagine, Arg = arginine, Ser = serine, Cys = cysteine, Leu = leucine, Thr = Threonine, Val = valine, Trp = tryptophan, Ala = alanine, Ile = isoleucine, Asn = asparagine.

Clinical INH-resistant *M. tuberculosis* isolates collected in the Free State and Northern Cape are characterised by being genetically diverse, with only small clusters and lacking strains of the Beijing clonal family. The aim of this study was to investigate mutations in the *katG* gene in INH resistant strains by DNA sequencing and to assess the spread and relative frequency of mutations associated with resistance in the Free State and Northern Cape.

Figure 7.1: INH resistant isolates collected from the Free State and Northern Cape by the MRC.

The *KatG* gene of all viable cultures from MRC were sequenced 37
Free State n=24; Northern Cape n=13

7.2 Results

A fragment of 808 bp (between bases 2743 and 3510) was amplified from the *katG* gene of 37 INH-resistant isolates. Codons were numbered according to the Genbank accession no. x68081. Mutations were demonstrated at codon 315 of 22/37 isolates (Table 7.2). The AGC315ACC exchange was observed in 20/37 (54.1%) of these strains. The previously reported AGC → AAC change was present in one strain and an AGC → GGC exchange in strain NC06 that has not been reported before.

Table 7.2 Susceptibility data for INH, RIF, ETH, STR and mutations found by sequence analysis of the *katG* gene of 24 isoniazid resistant strains collected from the Free State and 13 strains from the Northern Cape.

NO.	**Strain**	**Resistance pattern**				**Codon 315**		**Codon 463**	
		INH	RIF	ETH	STR	Mutation	Amino acid	Mutation	Amino acid
MRC01	FS230/01	R	S	S	S	ACC	Threonine	None	Arginine
MRC02	FS824/01	R	S	S	S	ACC	Threonine	None	Arginine
MRC03	FS1136/01	R	S	S	S	None	Serine	None	Arginine
MRC04	FS2701/02	R	S	S	S	ACC	Threonine	None	Arginine
MRC05	FS2788/02	R	S	S	S	ACC	Threonine	None	Arginine
MRC06	FS2972/01	R	S	S	S	ACC	Threonine	None	Arginine
MRC07	FS475/01	R	R	R	R	ACC	Threonine	None	Arginine
MRC08	FS1212/01	R	S	S	R	None	Serine	None	Arginine
MRC09	FS956/01	R	S	S	S	ACC	Threonine	None	Arginine
MRC10	FS1156/01	R	S	S	S	None	Serine	None	Arginine
MRC11	FS1283/01	R	S	S	S	None	Serine	None	Arginine
MRC12	FS2012/01	R	S	S	S	ACC	Threonine	None	Arginine
MRC13	FS2295/01	R	S	S	S	ACC	Threonine	None	Arginine
MRC14	FS975/01	R	R	S	S	ACC	Threonine	None	Arginine
MRC15	FS2189/01	R	R	S	R	None	Serine	None	Arginine
MRC16	FS2771/02	R	R	R	S	ACC	Threonine	None	Arginine
MRC17	FS1773/01	R	S	S	S	None	Serine	None	Arginine
MRC18	FS264/01	R	S	S	S	None	Serine	None	Arginine
MRC19	FS266/01	R	S	S	S	None	Serine	None	Arginine
MRC20	FS1591/01	R	R	S	R	None	Serine	None	Arginine
MRC21	FS2516/02	R	R	R	R	None	Serine	CTG	Leucine
MRC22	FS1794/02	R	R	S	S	ACC	Threonine	None	Arginine
MRC23	FS117/01	R	R	S	S	ACC	Threonine	None	Arginine
MRC24	FS637/01	R	S	S	S	ACC	Threonine	None	Arginine
NC01	NC1243/01	R	R	S	S	None	Serine	None	Arginine
NC02	NC928/01	R	S	S	S	ACC	Threonine	None	Arginine
NC03	NC1300/01	R	R	S	S	ACC	Threonine	None	Arginine
NC04	NC802/01	R	S	S	S	AAC	Asparagine	None	Arginine
NC05	NC646/01	R	S	S	S	None	Threonine	None	Arginine
NC06	NC931/01	R	S	S	S	GGC	Glycine	None	Arginine
NC07	NC948/01	R	S	S	S	ACC	Threonine	None	Arginine
NC08	NC984/01	R	S	S	S	ACC	Threonine	CCG	Proline
NC09	NC910/01	R	R	S	S	ACC	Threonine	None	Arginine
NC10	NC19/01	R	R	R	S	None	Serine	None	Arginine
NC11	NC1094/01	R	S	S	S	ACC	Threonine	None	Arginine
NC12	NC1087/01	R	R	S	R	None	Serine	None	Arginine
NC13	NC1047/01	R	S	S	S	None	Serine	None	Arginine

Two strains (MRC21 and NC08) had mutations at codon 463 (CGG → CTG; CGG → CCG) and strain NC08 also had the ACC change at codon 315. The remaining 14 carried no mutations at either codon 315 or 463. Two of the strains (MRC02, MRC04) harbouring the Ser315Thr mutation belonged to the same RFLP-*IS6110* cluster suggesting normal transmissibility of the strain.

7.3 Discussion

Investigations into the genetics of isoniazid resistance have linked several genes to the phenotype. This study looked at the nature and spread of mutations that occur within an 808-bp fragment of the *katG* gene spanning susceptible codons (315 and 463). In addition, the fragment included codons 275 and 328 where mutations speculated as determinants of resistance were described previously (Haas *et al*., 1997).

Missense mutations were observed at codon 315 in slightly more than half (54.08%) of the strains evaluated. The AGC to ACC mutation resulting in the Ser to Thr amino acid exchange was encountered in 20 of the 37 (54.05%) clinical isolates analysed. Two other mutations at this site (AAC and GGC) pointed to the vulnerability of this codon to variants associated with resistance. Furthermore, these results confirm the findings of Musser *et al* (1996) implicating a few mutations in isoniazid resistant *M. tuberculosis*. Thus far only three types of codon 315 mutations (AGC to ACC, AAC or ACA) have been documented, including the two observed in this report (Haas *et al*., 1997, Tracevska *et al*., 2002, Van Soolingen *et al*., 2000). The ACA variant associated with the resistant W strain in the USA (Bifani *et al*., 1996), a close relative of the Beijing type, was not observed in this study, confirming the findings that this strain is absent in the Free State and Northern Cape provinces. The role of codons 275 and 328, however, remained unclear as mutations at these locations remained elusive. A significant proportion (40.5%) of isolates carried the wild-type genotype suggesting the probable involvement of other genes alluded to above.

Focusing on codon 463, only 2 strains had mutations at this site. Both carried the previously described substitutions, CGG → CCG (Arg to Pro) and the common CGG→

CTG (Arg to Leu) (Haas *et al*., 1997, Van Doorn *et al*., 2001). The existing large body of evidence in literature seems to exclude this codon from INH resistance (Dobner *et al*., 1997, Van Doorn *et al*., 2001, Lee *et al*., 1997).

Studies by groups in The Netherlands (Van Soolingen *et al*., 2000) and Russia (Toungoussova *et al*., 2002) correlated mutations at codon 315 of the *katG* gene of INH resistant strains and other drug resistance. This study has confirmed these observations in that the ACC genotype occurred in 8/14 (57.1%) of the strains with resistance to INH and one or more other drugs.

AGC to ACC (Ser to Thr) (Cockerill III., 1999). This mutation has been associated with acquisition of resistance to other drugs (especially rifampicin) in Russia (Tracevska *et al*., 2002), the Netherlands (Van Soolingen *et al*., 2000), and South Africa (Haas *et al*., 1997). Furthermore, isolates with mutations at codon 315 of the *katG* gene are just as virulent and are capable of being transmitted (Toungoussova *et al*., 2002, Haas *et al*., 1997).

CHAPTER 8

RESULTS:

MUTATIONS IN THE rpoB GENE OF RIFAMPICIN-RESISTANT M. TUBERCULOSIS STRAINS.

8.1 Introduction

Drug-resistant tuberculosis is threatening the efficacy of hitherto effective and affordable therapeutic agents. Currently, the most sterilizing agent used in the short course chemotherapy against drug-susceptible *M. tuberculosis* is rifampicin (RIF). Although RIF is the central component of many protocols drug resistant tubercle bacilli have been isolated with increasing frequency in recent years. The major mechanism of resistance to RIF in *M. tuberculosis* is associated with the development of genetic variations in the defined region of the *rpoB* gene encoding the ß-subunit of the RNA polymerase. Observation of mutations in this region is an indicator of the resistant phenotype. Research into the development of rapid resistance identification procedures has thus been stimulated and genetic mechanisms are offering a glimpse of hope. Resistance to rifampicin (RIF) in *M. tuberculosis* cannot be emphasized enough as it is often associated with resistance to INH in clinical isolates (CDC, 1992).

Investigations have revealed that mutations in codons 516, 526, and 531 of the *rpoB* gene are often implicated (Table 8.1). Mutations at codon 531 occur at a relatively higher frequency, but different groups have reported disproportionate frequencies of mutations at these codons. Groups working in Greece (Matsiota-Bernard *et al*., 1998), Germany, Sierra Leone (Rinder *et al*., 1997) and South Africa (KwaZulu-Natal) (Kiepiela *et al*., 1998) have reported differences in the relative abundance of these mutations among rifampicin-resistant *M. tuberculosis*. In addition, the frequency of Ser531Leu remains the most common in isolates with a resistant phenotype. Other mutations often detected are shown in Table 8.2.

Table 8.1 Common mutations in *rpoB* gene of rifampicin resistant *M. tuberculosis*

Codon	Base changes	Amino acid change	References
516	GAC→GTC	Asp→Val	Pozzi *et al.,* 1999
	GAC→AAC	Asp→Asn	Schilke *et al.,* 1999
	GAC→TAC	Asp→Tyr	Schilke *et al.,* 1999
	GAC→GGC	Asp→Gly	Schilke *et al.,* 1999
	GAC→AAA	Asp→Lys	Mani *et al.,* 2001
526	CAC→GAC	His→Asp	Pozzi *et al.,* 1999
	CAC→TAC	His→Tyr	Pozzi *et al.,* 1999
	CAC→CGC	His→Arg	Pozzi *et al.,* 1999
	CAC→TGC	His→Cys	Schilke *et al.,* 1999
	CAC→CTC	His→Leu	Schilke *et al.,* 1999
	CAC→CTG	His→ Leu	Valim *et al.,* 2000
	CAC→ACC	His→Thr	Mani *et al.,* 2001
531	TCG→TTG	Ser→Leu	Pozzi *et al.,* 1999
	TCG→TGG	Ser→Trp	Schilke *et al.,* 1999

Asp = asparagine, Lys = lysine, His = histidine, Arg = arginine, Ser = serine, Cys = cysteine, Leu = leucine, Tyr = tyrosine, Thr = Threonine, Val = valine, Gly = glycine, Asn = asparagine, Trp = tryptophan.

DNA fingerprint analysis of INH-resistant isolates from the Free State province in 1996 indicated that a few small clusters were features of the *M. tuberculosis* population structure. Based on this report, the spread and the nature of mutations associated with resistant strains in the two provinces need to be established. In the present study DNA sequencing of the *rpoB* gene was performed on rifampicin-strains isolated from the Free State and Northern Cape of South Africa.

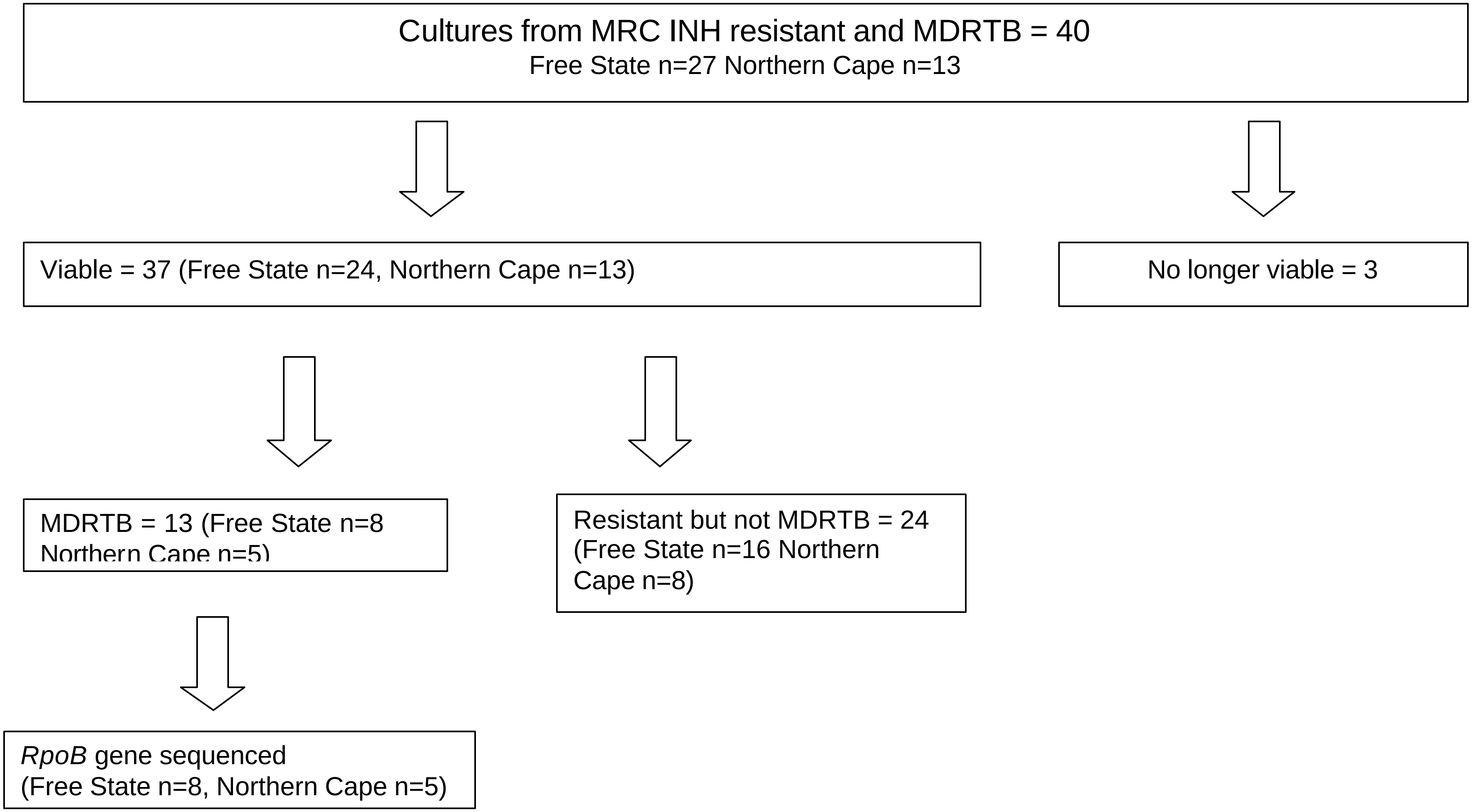
Table 8.2: Other mutations reported in the rifampicin-resistant *M. tuberculosis* 81 bp region of the *rpoB* gene.

Codon	Base changes	Amino acid change	References
507	GGC→GAC	Gly→Asp	Kim *et al.*, 1997
508	ACC→AGC	Thr→Ser	Mani *et al.*, 2001
511	CTG→CCG CTG→ATG CTG→CTA CTG→CGG	Leu→Pro Leu→Met Leu→Leu (silent) Leu→Arg	Rinder *et al.*, 1997 Mani *et al.*, 2001 Kim *et al.*, 1997 Valim *et al.*, 2000
512	AGC→AGG AGC→ACC	Ser→Arg Ser→Thr	Mani *et al.*, 2001 Pozzi *et al.*, 1999
513	CAA→AAA CAA→CCA CAA→CAC CAA→CTA	Gln→Lys Gln→Pro Gln→His Gln→Leu	Mani *et al.*, 2001 Kim *et al.*, 1997 Valim *et al.*, 2000 Schilke *et al.*, 1999
514	TTC→GTC	Phe→Val	Valim *et al.*, 2000
515	ATG→GTG	Met→Val	Kim *et al.*, 1997
518	AAC→CAC	Asn→His	Mani *et al.*, 2001
519	GCG→GCAAla		
522	TCG→TTG TCG→TCC	Ser→Leu Ser→Ser (silent)	Kim *et al.*, 1997 Schilke *et al.*, 1999
524	TTG→TGG	Leu→Trp	Valim *et al.*, 2000
525	ACC→CCC	Thr→Pro	Valim *et al.*, 2000
532 (silent)	GCG→GCA	Ala→Ala	Mani *et al.*, 2001
533 (silent)	CTG→CTT CTG→CCG	Leu→Leu Leu→Pro	Mani *et al.*, 2001 Schilke *et al.*, 1999
Insertions			
513? 514	TTC	Phe	Rinder *et al.*, 1997
Deletions			

514	TTC	Phe	Valim *et al.,* 2000
515	ATG	Met	Valim *et al.,* 2000
516	GAC	Asp	Valim *et al.,* 2000
517	CAG	Gln	Mani *et al.,* 2001
518	AAC	Asn	Valim *et al.,* 2000
527	AAG	Lys	Valim *et al.,* 2000

Asp = asparagine, Lys = lysine, His = histidine, Arg = arginine, Ser = serine, Cys = cysteine, Leu = leucine, Tyr = tyrosine, Thr = Threonine, Val = valine, Gly = glycine, Asn = asparagine, Trp = tryptophan, Gln = glutamine, Met = methionine, Phe = phenylalanine, Pro = proline.

Figure 8.1: Rifampicin resistant isolates from MRC, *rpoB* gene sequenced.



8.2 Results

Analysis of 13 *M. tuberculosis* rifampicin resistant strains from the Free State (n=8) and Northern Cape (n=5) provinces (Table 6.1), by DNA sequencing identified 12 missense mutations within an 81-bp rifampicin-resistance-determining region (RRDR) of the *rpoB* gene (Table 8.3).

Table 8.3: Missense mutation found in RIF - resistant strains from the FS and NC.

Strains	Gene	Codon no.	Mutation	Amino Acid exchanges
MRC07-FS475	*Rpo*B	516	GAC-GTC	Asp - Val
MRC14-FS975	*Rpo*B	No Mut		
MRC15-FS2189	*Rpo*B	No Mut		
MRC16-FS2771	*Rpo*B	523	GGG-TGG	Gly - Trp
		524	TTG-TTC	Leu - Phe
MRC20-FS1591	*Rpo*B	529	CGA-AGA	Arg - Arg
		531	TCG-TTG	Ser - Leu
MRC21-FS2516	*Rpo*B	No Mut		
MRC23-FS1794	*Rpo*B	526	CAC-TAC	His - Tyr
		534	GGG-GGA	Gly - Gly
		535	CCC-ACC	Pro - Ser
MRC24-FS117	*Rpo*B	531	TCG-TTG	Ser - Leu
		544	GGG-TTG	Gly - Leu
NC01-NC1243	*Rpo*B	531	TCG-TTG	Ser - Leu
		533	CTG-GTG	Leu - Val
NC03-NC1300	*Rpo*B	526	CAC-CTC	His - Leu
NC09-NC910	*Rpo*B	526	CAC-CTC	His - Leu
		527	AAG-GAG	Lys - Glu
		531	TCG-TTG	Ser - Leu
NC10-NC19	*Rpo*B	511	CTG-ATG	Leu - Met
		529	CGA-AGA	Arg- Arg
		533	CTG-GTG	Leu - Val
NC11-NC1087	*Rpo*B	No Mut		

Asp = asparagine, Lys = lysine, His = histidine, Arg = arginine, Ser = serine, Cys = cysteine, Leu = leucine, Tyr = tyrosine, Thr = Threonine, Val = valine, Gly = glycine, Asn = asparagine, Trp = tryptophan, Met = methionine, Phe = phenylalanine, Pro = proline.

Mutations in nine isolates affected seven amino acids. Four of the 13 isolates carried no mutation. One synonymous mutation was observed in two strains (MRC20, NC10) at codon 529 and the other in one strain (MRC23) at codon 534. Of the widely reported point mutations codon 516 (GAC→GTC, Asp→Val) was affected in one isolate

(MRC07) (7.7%), codon 526 in three strains (MRC23, NC03, NC09) (23.1%), and codon 531 in four isolates (30.8%) (MRC20, MRC24, NC01, NC09). One strain with CAC→CTC at codon 526 carried the His→Leu mutation. Three isolates (MRC23, NC09, and NC10) had three point mutations at three separate sites of the gene, all but two previously reported. Three strains (MRC16, MRC20, and NC01) each harboured two mutations each at two separate codons. Two of the mutations were not previously reported (529, CGA→AGA; 544, GGG→TGG).

8.3 Discussion

Point mutations were observed in an 81-nucleotide core region of the *rpoB* gene among two-thirds of rifampicin resistant *M. tuberculosis* strains. A significant proportion (30.77%) of isolates carried no mutations within the amplified region. This point to the possible existence of other mechanisms of resistance or functional mutations outside the analysed region as previously suggested. Two new (529 and 534) mutations observed in this study were silent, but a new double base substitution (codon 544) in strain MRC24 was observed, exchanging Gly for Leu. Codons often affected (516, 526, and 531) had mutations in more than half (61%) of the cases. Strains with multiple mutations were a common feature in this study. Fifty four percent (54%) of the isolates carried two to three mutations at separate codons. Other research teams have also reported multiple mutations (Kiepiela *et al*., 1998, Mani *et al*., 2001). Nucleotide substitutions observed at codons 531 and 526 (except for codon 526 in strain N03) were in association with point mutations at other codons. The involvement of codons 516, 526, and 531, occurred at a frequency of 7.7%, 23.1%, and 30.8%, respectively. Studies conducted between 1996 and 1997 within communities in and around Mangaung have shown the presence of the Ser531Leu, His526Tyr, His526Asp, and Asp516Val mutations in 44.0, 10.0, and 10.0% of RIF-resistant isolates, respectively (Van der Spoel van Dijk *et al*., 2001). The results of this study showed that the widely reported mutations predominate in the two provinces (53.9% of isolates), but the frequency of each determinant seems to be in concordance with the disproportionate geographical distribution as reported by Rinder et al (1997).

CHAPTER 9

RESULTS:
RAPID SCREENING FOR RESISTANCE

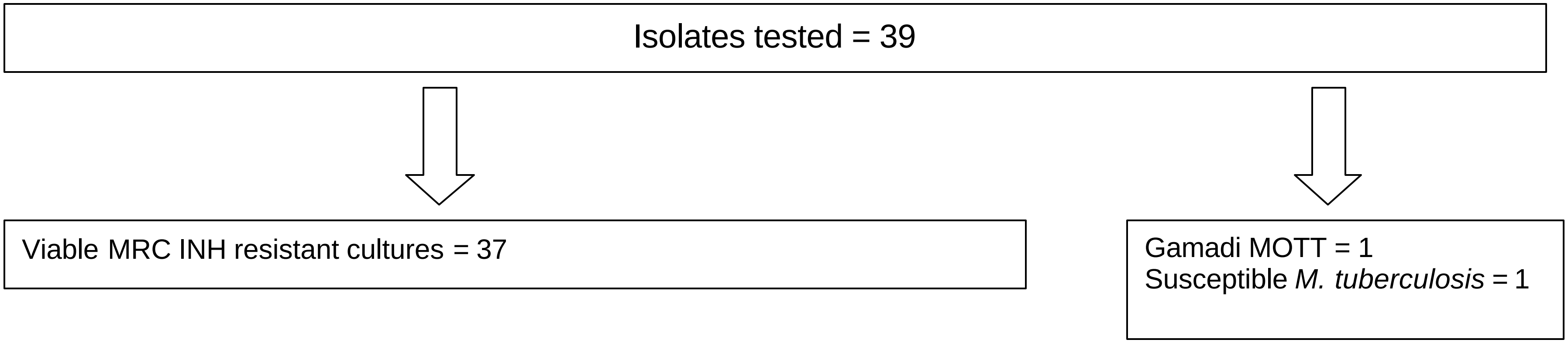
9.1 Introduction

The current in vitro methods of anti-tuberculosis drug susceptibility methods are either time-consuming or very expensive to be applied fully within National Tuberculosis Control Programmes of poor countries (Blumberg, 1995). The greatest cause for concern is that a patient infected with a resistant strain may unintentionally be spreading the disease while waiting for DST results. Early detection of drug resistant tuberculosis is crucial in order to stop the spread of drug resistant organisms within communities. Recent reviews have indicated that the extent of drug resistant tuberculosis varies between the developed and poor countries, but the problem is global (Espinal *et al*., 2001). Tuberculosis is treated with a combination of drugs, which are cheap and effective. The dilemma facing humanity at this point is that there is a limited armamentarium against tuberculosis. It has also been shown that alternative drugs are more expensive (Gupta *et al*., 2001), less effective, more toxic to the host and have higher resistance rates. The widespread use of mainstay drugs (Rifampicin and isoniazid) has led to the emergence of multidrug-resistant strains, threatening to make both drugs useless (Espinal *et al*., 2001). On the other hand simple yet rapid techniques of identifying resistant cases may help in breaking the chain of TB transmission, including MDRTB.

The major mechanism of acquisition of resistance to INH in *M. tuberculosis* has been shown to be associated with the point mutations in the *katG* gene. In particular, amino acid substitutions at codon 315 of *katG* have been shown to be present in INH-resistant, but not INH-susceptible bacilli. (CDC, 1993). Although a more complex genetic system that involves several genes either singly or jointly control INH resistance, the significant volume of data available seems to single out the ACC variant of position 315 in the *katG* gene as the greater culprit (Musser *et* al., 1996 and Soolingen *et al*., 2000). The frequency of mutant variants differs from one region to another, but in some regions (e.g. Russia) the AGC→ACC variant has been observed in 100% of MDR Beijing genotype strains. A double mutation AGC315ACA was observed among MDRTB strains, genetically similar to Beijing strains (designated W strains) and their progenies, unique to the United States of America (Musser *et al*., 1996). Strains genetically related to the W-Beijing genotype have been reported in the Western Cape and KwaZulu-Natal provinces of South Africa (Van Rie *et al*., 1999, Pillay *et al*., 2003). The influence of these strains on the spread of resistance determinants in the country is unknown. Haas and others have reported a *kat315* mutation frequency of 68.0% among INH-resistant *M. tuberculosis* complex strains in 1997 (Haas *et al*., 1997), but the frequency of these mutations per province remained unknown.

Since the Free State and Northern Cape provinces have not as yet reported W-Beijing strains, the nature, frequency and spread of 315 (*katG*) and 463 alleles unique to these provinces is of great interest. The genetic basis of resistance was elucidated using the modified restriction enzyme *Msp* I (Abal *et al* 2002). This ingenious PCR-based technique was used as a rapid screening assay for INH-resistant phenotypes isolated from the two provinces. Briefly, the mutation often associated with isoniazid resistance involves the AGC→ACC substitution at codon 315 of the *katG* gene. This substitution results in the acquisition of the recognition site for restriction endonuclease *Msp* I (GCGG to CCGG). The next common mutation occurs at codon 463 changing the bases from CCGG to a configuration not recognisable by *Msp* I. The described mutations result in predictable restriction patterns of PCR-amplified fragments encompassing these codons. The presence and nature of mutations were confirmed by nucleotide sequencing using the BigDye terminator ready reaction kit (Applied Biosystems, USA).

Figure 9.1: INH resistant *M. tuberculosis* isolates from the MRC and two mycobacterial strains from Gamadi tested for the presence of mutations using the *Msp* 1 RFLP assay.



9.2 Results

Amplification of DNA extracted from *M. tuberculosis* strain H37Rv and 39 INH-resistant isolates yielded an 808 bp PCR product (Figures 9.1, 9.2 and 9.3). Restricting the known susceptible H37Rv with *Msp* I resulted in fragments of predicted sizes 228, 153, 146, 109, 79, 65 base pairs (Figure 9.1).

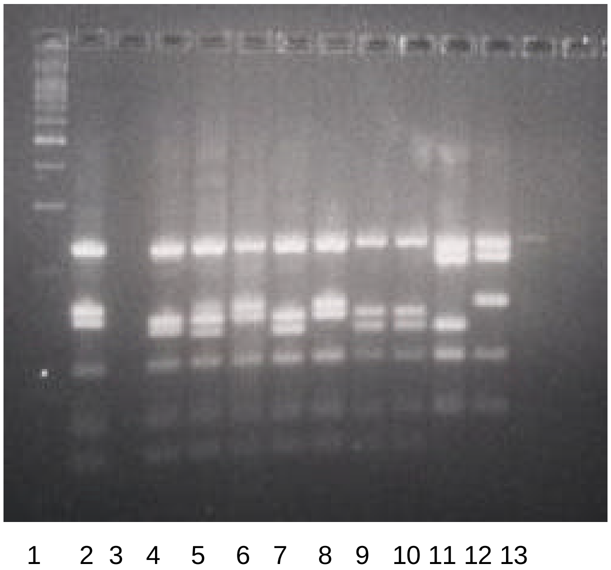


Figure 9.2: *Msp* I restriction of control strain H37Rv and ten other isolates (MRC = 8, Gamadi = 2) strains. Lane 1, 100bp marker, lane 2, H37Rv control strain, lane 3 empty and lanes 4 to 13: NC06, NC07, NC12, NC09, NC01, MRC23, MRC24, MRC22, 01030007 and 02020026 (MOTT).

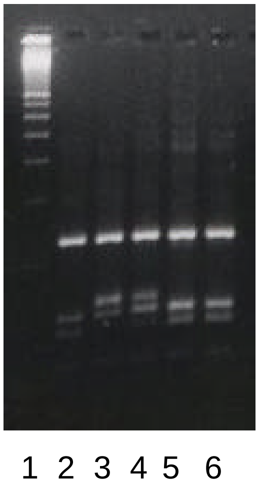


Figure 9.3: *Msp* I restriction of five TB strains. Lane 1 the 100bp marker and lanes 2 to 6: MRC5, NC05, NC08, NC03 and MRC16 (All INH resistant from MRC).

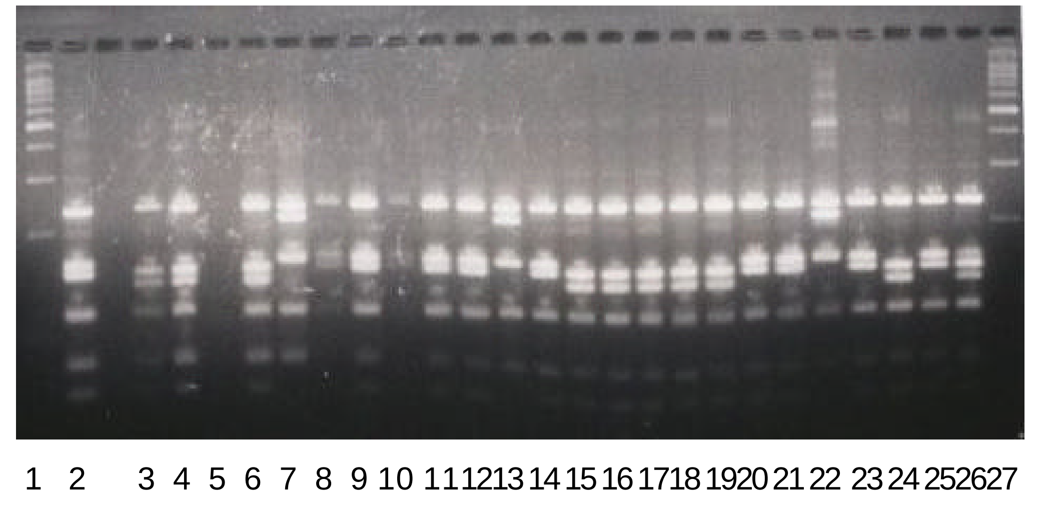


Figure 9.4: *Msp* I restriction of control strain H37Rv and 24 other TB strains. Lane 1 the 100 bp marker, Lane 2 H37Rv control strain, lanes 3 to 27 (Resistant isolates from MRC), MRC01, MRC02, MRC09, MRC14, MRC03, MRC10, MRC08, MRC17, NC13, MRC18, MRC19, MRC11, MRC12, MRC13, MRC04, MRC07, MRC06, MRC20, MRC15, MRC21, NC10, NC11, NC04, NC02.

PCR-RFLP of 16 INH-resistant strains (41.0%) demonstrated the 228, 146, 132, 109, 79, 65, 21 (invisible) profile. Thirteen (33.3%) other strains carried a pattern similar to that of the susceptible strain. Four strains (10.26%) had bands with bp sizes of 228, 211, 153, 109, and 79 while the PCR product from 1 strain (2.6%) was digested into 6 fragments of 228 bp, 211 bp, 132 bp, 109 bp, 79, and 21 bp. The PCR products from three isolates (7.7%) were digested into 228 bp, 153 bp, 146 bp, 132 bp, 109 bp, 79 bp, and 65 bp fragments. The presence of the 153 bp fragment indicates the existence of AGC at codon 315, while the 132 bp fragment results from the ACC allele. Fragments with 146 bp and 65 bp are seen in strains with bases CGG at codon 463, while a 211 bp fragment shows a mutation at codon 463. Direct DNA sequencing of the PCR product confirmed the existence of mutations.

9.3 Discussion

The key in the control of drug resistant tuberculosis lies in the prevention of its transmission in the community. The magnitude of the problem becomes apparent when cornerstone drugs (isoniazid and rifampicin) are involved, thus reducing treatment success to 60.0% (Espinal *et al*., 2000). During this time a patient with an MDRTB strain is an unknown source of infection. The global increase in reported cases of drug resistant strains has sparked an interest in molecular genetics research. This promising avenue seemed a far-fetched idea because of the number of genes involved. The involvement of codon 315 of the *katG* gene has been established in recent years, with the AGC→ACC (Ser→Thr) nucleotide change occurring at a higher frequency. A previous study has shown the presence of the Ser315Thr mutations in 60.0% of MDR isolates from South Africa (Haas *et al*., 1997). Comparison of these results with the results of the current study shows that the Ser315Thr mutation also predominates in the Free State and Northern Cape provinces of South Africa. Using the PCR-*Msp* I assay a high prevalence of this mutation has also been observed in Kuwait (Abal *et al*., 2002).

The *Msp* I endonuclease restriction of the 808-bp PCR product resulting in a fragment of 132 bp suggests a codon exchange to ACC (threonine), while a 153-bp fragment indicates the wildtype (AGC) genotype. The 51.4% occurrence rate of the 132 bp indicates a high prevalence of the ACC genotype at codon 315. Results of this study are in agreement with published data (Musser *et* al., 1996, Soolingen *et al*., 2000, Haas *et al*., 1997). In addition, 3/20 isolates exhibited the 228 bp, 153 bp, 146 bp, 132 bp, 109 bp, 79 bp, and 65 bp profile. This pattern suggests the presence of a mixed phenotype. Also of importance, a substantial number of INH resistant isolates gave a pattern with a 153-bp fragment (43.7%). This observation supports the role of other genes, for example, the *ahpC*, *kasA*, and *inhA* genes in INH resistance. Furthermore, the PCR-*Msp* I assay could not detect the presence of other mutations as confirmed by direct DNA sequencing. This observation emphasises the importance of DNA sequencing as the ultimate genotypic method. Among the resistant isolates, 5/39 (12.8%) strains carried a mutation at codon 463, thus substituting leucine with another amino acid. Two of the strains came from the Gamadi area. Studies elsewhere could not correlate this mutation to a resistant phenotype (Lee *et al*., 1997, Van Doorn *et al*., 2001). The prevalence of Ser315Thr mutations within the majority of isoniazid-resistant clinical *M. tuberculosis* isolates strengthens the potential this PCR based test has as a rapid screening method for monitoring the spread of resistance determinants. However, the reduced sensitivity of this method suggests that traditional methods of susceptibility testing are still required.

The use of the *Msp* I-RFLP technique in detecting nucleotide mutations provides a rapid, accurate and relatively inexpensive method for predicting the resistance profile of the isolated organism. The methods used in detecting these genes could be adopted to assist in monitoring the spread of INH resistance in our setting.

CHAPTER 10

FINAL DISCUSSION

The aim of this study was to investigate the molecular epidemiology and the spread of resistance determinants of tuberculosis, including multidrug-resistant tuberculosis in Gamadi, the Free State and Northern Cape provinces using conventional and genetic methods. Traditional laboratory methods used in the control of tuberculosis have inherent limitations, but molecular techniques can provide useful hidden data.

The first phase of the study involved the definition of an area within the Free State where surveillance of the disease can be conducted at regular and long-term intervals. An area called Gamadi near the rural town of Thaba 'Nchu in the Free State has characteristics suitable for such surveillance studies. The area is poverty-stricken and 99.0% of the population is black. It has a high unemployment rate (69.0%) and a high incidence of tuberculosis (840/100 000 population). Movement within the community is high, but migration in and out of the area is minimal.

Many problems were encountered in the initial stages of the project. Few sputum specimens were collected since information regarding the procedures to follow in collecting samples were not adequately implemented by health workers. Many of the samples turned out to be culture negative as they were obviously collected after treatment was initiated. The number of samples increased towards the end of the study, but many were still negative (overall 68.9%). Ziehl-Neelsen staining of decontaminated sputum specimens, 20 culture negative and 20 culture positive, showed the positivity of 5/20 and 16/20 respectively. Of the 20 cultures identified by biological characteristics, ZN staining, the catalase and the nitrate tests, nine were confirmed to be *M. tuberculosis* and one a MOTT. Since confirmation of TB by microscopy is central to the control of this disease in South Africa, there is a need to implement quality control systems within a fully functional DOTS programmes.

A total of 88 *M. tuberculosis* isolates and a MOTT were cultured from 286 sputum specimens. Drug susceptibility testing showed primary INH resistance of 2.3% to 0.2 µg/ml of this drug. The eighty seven strains tested against rifampicin were found to be susceptible. The MOTT was resistant to rifampicin and to higher concentrations of INH (1 µg/ml and 5 µg/ml).

The RFLP profiles indicated that a high proportion of INH-resistant TB in the two provinces was as a result of reactivation and not recent transmission. Two contrasting conclusions can be drawn from the high genetic diversity observed. Firstly, reactivation of previously acquired infection may indeed be a true reflection of the state of affairs in the two provinces. This may well be so since the two provinces have limited job opportunities and the consequences of poverty impacts negatively on the immune system of healthy individuals. As the immune system is weakened, the chances of latent TB infection to progress to active disease also increase. The alternative is that recent transmission may have been underestimated, since the study was conducted in a period of less than two years. Most individuals who become infected with TB experience clinical symptoms in a period longer than two years (>90%). A small proportion has a much faster progression to active disease after infection. Therefore, the different strains observed may be part of transmission chains, of which the index cases of which lie outside the temporal boundaries of this study.

As for the Gamadi area, the high intracommunity movement observed might have resulted in the loss of TB patients from the clinics participating in the study. This might explain the high strain diversity observed, contrasting reports from high TB incidence areas. In addition, the few clonal families (65% similarity index) observed suggest that recent transmission of strains able to survive within the TB control programme may have established themselves in the two provinces. The spread of the INH-resistant strain (ZT39) seems to have been contained, since strains with the related genotype were not observed in the area. This finding emphasises the need to incorporate molecular techniques in the National TB control programmes as performance evaluating tools. The use of PCR-based methods (spoligotyping and MIRU-VNTR typing) was of value in differentiating strains with difficult patterns to interpret e.g. those with identical RFLP patterns but with less than six bands. Strain MRC16 from the Free State and strain NC03 from the Northern Cape with identical RFLP and spoligotyping were differentiated by the MIRU-VNTR typing. Similarly, the relationship between a multiple drug resistant strain isolated from a patient in the city of Bloemfontein with identical RFLP and spoligotype patterns to a susceptible strain from the Gamadi area could be clarified by MIRU-VNTR typing. Since TB was not cultured from a large proportion of sputa collected from Gamadi region, either spoligotyping or MIRU-VNTR typing can investigate transmission of TB in this area better. These methods do not require DNA extracted from live TB cultures, can be performed on inactivated sputum samples or stained smear slides used in the initial diagnoses of TB. Since less exposure to live tuberculosis is needed with these methods, risk of infection of overworked clinic staff is reduced and this may eliminate the possible apathy towards research.

Direct sequencing of 37 INH-resistant isolates indicated that most (54.1%) of the resistance in the two provinces was mediated by the ACC mutation at codon 315 of the *katG* gene. This mutation was also associated with resistance to other anti-tuberculosis drugs. The clusters observed among INH-resistant isolates also carried mutation ACC at codon 315. This finding suggests that INH-resistant isolates, including MDRTB isolates, can be as infectious as susceptible isolates. This calls for intensified efforts aimed at active tracing of immediate contacts of the patient. Since DNA sequencing is an expensive technique that cannot be performed regularly in developing countries, cheaper alternatives able to detect the ACC mutation can be of value. The enzyme *Msp* 1 was used successfully in this study to detect this mutation among INH-resistant strains with known alleles at codon 315. *Msp* 1 restriction endonuclease assay is a PCR-assisted method that can be used in conjunction with spoligotyping or MIRU-VNTR typing procedures. DNA sequencing of the rifampicin-resistance-determining region of the *rpoB* gene indicated that multiple mutations were common (53.8%) in MDRTB strains isolated from these provinces. Mutations at codon 516, 526 and 531 were also observed in more than half (53.9%) of the strains. Since resistance to rifampicin and the ACC mutation are associated with resistance to other drugs, characterisation of the *rpoB* mutations and the PCR-RFLP of the *katG* gene by the *Msp* 1 restriction enzyme can be used together as rapid MDRTB screening methods in high TB incidence areas such as Gamadi.

In conclusion, DNA fingerprinting of isolates from the two provinces has excluded the presence of strains belonging to the Beijing genotype. Furthermore the DRF150 strain was also not observed in this study. Limitations, such as a too small a sample received from a high incidence area (Gamadi) may give a false reflection of the dynamics of TB in that setting. As a consequence, other concerns, such as the extent of multiple infections and re-infections with new strains during treatment, have remained unclear. The true extent of TB transmission and the efficiency of the DOTS strategy can only be assessed satisfactorily when long-term genotypic studies are conducted in the area. The presence of the Beijing and the DRF150 strains in other provinces calls for continuous or regular monitoring of strains circulating in the two provinces. The data so obtained can assist the provincial TB control programme in putting sound strategic plans in place.

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SUMMARY

The objective of the study was to investigate the molecular epidemiology and resistance development of tuberculosis (TB), including drug resistant tuberculosis in the Gamadi community, the Free State and Northern Cape provinces using both traditional and genetic methods. Traditional methods involved microscopy, culture and drug susceptibility testing of sputum specimens collected from patients diagnosed with pulmonary tuberculosis. The standardised IS*6110*, spoligotyping and the mycobacterial interspersed repeat units (MIRU) methods were used to gain insight into the transmission dynamics of different strains of M. *tuberculosis*. DNA sequencing and the polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) procedures were used to investigate genetic changes associated with drug resistance.

Chapter 1 is a literature review emphasising the magnitude of TB in the world, South Africa, the Free State and Northern Cape provinces. The routes of transmission of tuberculosis are better understood in the developed countries because molecular technologies have gained widespread use. The literature review focused on the traditional and genetic methods used in the control of TB and the advantages and limitations of each approach. Information such as the rate of recent transmission versus reactivation and the spread of resistance determinants in TB cannot be evaluated by Ziehl-Neelsen staining and culture methods. Characterisation of *M. tuberculosis* strains by genotypic methods provides data that can be used by local TB control programmes in the management of this age old problem.

Chapter 2 describes the different methods used in the various sections.

In chapter 3 the Gamadi region of the Free State with a high incidence of TB, is defined. The area is characterised by poverty, a relatively young population with low migration. The Gamadi area compares well with the Free State at large and was found to be suitable for TB surveillance studies. Genotypic data obtained will provide insight into the

rate of recent transmission versus reactivated TB and resistance mechanisms involved in drug resistance.

In Chapter 4 a sample of decontaminated sputum specimens was investigated by Ziehl-Neelsen (ZN) staining and positive cultures identified by biological characteristics (growth rate, growth temperature and colony morphology) and biochemical properties (ZN staining, heat-stable catalase test and the nitrate test). Ziehl-Neelsen staining of decontaminated sputum specimens, 20 culture negative and 20 culture positive, was positive in 5/20 and 16/20 respectively. Of the 20 cultures identified by biological characteristics, ZN staining, the catalase and the nitrate tests, nine were confirmed to be *M. tuberculosis* and one a *M. tuberculosis* other than TB (MOTT).

Resistance to isoniazid (INH) and rifampicin (RIF) in the Gamadi area was described in chapter 5. A variant of the indirect proportion method was employed to determine the susceptibility profiles of 87 tuberculosis isolates. An organism was resistant to the drug if growth on the medium, containing the critical concentration of the drug, exceeded 1%. Drug susceptibility testing showed primary INH resistance of 2.3% to 0.2 µg/ml of this drug. The 87 strains tested against rifampicin were found to be susceptible. The MOTT was resistant to rifampicin and also to higher concentrations of INH (1 µg/ml and 5 µg/ml).

The restriction fragment length polymorphism (RFLP) patterns of INH-resistant isolates from patients in the Free State, the Northern Cape and the Gamadi area were discussed in chapter 6. A high proportion of cases were due to reactivation of old tuberculosis. Thirty-two diverse fingerprint patterns from 37 persons with INH resistant TB were found. Evidence of recent transmission could be deduced from two clusters (identical RFLP patterns) of two patients and two putative (strains with 6 and less bands) clusters. One of the putative clusters had a matching spoligotype, but a different MIRU type and the second had non-matching spoligotype and MIRU patterns. Only 8 clonal families (65% similarity index) of two to four members were evident among INH resistant strains.

DNA fingerprinting of 84 isolates from the Gamadi area using the IS*6110*-based method produced 14 patterns with six or less bands. Two isolates that belonged to a cluster of three (five bands) came from the same patient. The other eleven RFLP patterns were all different. Sixty fingerprint patterns with more than six bands from 70 isolates were evident, but three resulted from laboratory contamination. Two clusters were observed (one with two and the other with four identical isolates). An epidemiological link could be established with two patients in the four isolate cluster, but loose casual contact could be speculated between other members of the cluster. Nine clonal groups, clustering at 65% on the similarity dendogram, each with two to eight strains were evident.

MIRU confirmed the results of a cluster consisting of strains ZT03 and ZT04. Two isolates having different resistance and IS*6110*-RFLP patterns of three bands (the susceptible ZTO1 and INH resistant MRCO8 strains) gave identical MIRU patterns. A cluster made up of one isolate from each province, MRC16 and NC03 (each with two bands), had identical spoligotypes, but different MIRU results. Three isolates with identical IS*6110* profile gave unrelated MIRU (MRC10, MRC11, MRC12) and spoligotyping patterns (MRC10, MRC11).

The proportion of isolates resistant to INH due to mutations in the *katG* gene was discussed in Chapter 7. Direct sequencing of the *katG* gene indicated that a high percent (54.0%) of mutations occur at codon 315 changing AGC to ACC. Two strains had other mutations at this site. The previously reported AGC to AAC (NC04) change was present in one strain and the other had an AGC to GGC (NC06) exchange, which has not been reported before. Thirteen strains carried no mutations at either codon 315 or 463. One strain had mutations at both codons 315 and 463 and another strain carried a mutation at codon 463. Two of the strains harbouring the AGC to ACC mutation belonged to the same *IS6110* cluster, suggesting normal transmissibility of strains having that mutation.

The involvement of mutations in the 81 bp rifampicin-resistance-determining region of the rpoB gene was described in chapter 8. Analysis of 13 rifampicin resistant *M. tuberculosis* strains by sequencing identified 12 missense mutations. Four of the 13 isolates carried no mutation within the 81 bp core region. More than half of the strains (53.9%) carried mutations at known sites (codons 516, 526 and 531). Seven strains (53.8%) carried multiple missense mutations.

In chapter 9 the validity of a rapid and cheaper alternative for sequencing, the assessing of the predominant ACC allele at codon 315 of the katG gene was investigated. The PCR-based *Msp* I restriction endonuclease assay was used for screening for the prevalent mutation (ACC) at codon 315 of the *katG* gene. Of the 37 INH resistant strains tested, 20 were digested into fragments that indicated the presence of the ACC mutation at codon 315. The results of this assay were in agreement with the findings of DNA sequencing. The advantage of the *Msp* I restriction endonuclease assay is that it is cheap, quick and easy to perform.

In chapter 10 the findings of the various sections of the study are discussed. The use of genetic methods in this study has revealed that reactivation of TB, including drug resistant TB, is a significant problem in the Free State province. The same holds true for INH and multidrug resistant TB in the Northern Cape Province. Genotypic typing of isolates from the two provinces has excluded the presence of strains belonging to the Beijing genotype. Furthermore the DRF150 strain was also not observed in this study. Limitations, such as a too small a sample received from a high incidence area (Gamadi) may give a false reflection of the dynamics of TB in that setting. As a consequence, other concerns, such as the simultaneous infection with more than one strain (multiple infections) and re-infections with new strains during treatment have remained unclear. The true extent of TB transmission and the efficiency of the DOTS strategy can only be assessed satisfactorily when long-term genotypic studies are conducted in the area. The presence of the Beijing and the DRF150 strains in other provinces calls for continuous or regular monitoring of strains circulating in the two provinces. The data so obtained can assist the provincial TB control programme in putting sound strategic plans in place.

OPSOMMING

Die doel van die studie was om die molekulêre epidemiologie en ontwikkeling van weerstand in tuberkulose (TB) in die Gamadi, Vrystaat en Noord Kaap provinsies van Suid-Afrika met behulp van tradisionele en genetiese metodes te ondersoek. Tradisionele metodes soos mikroskopie, kweking en sensitiwiteits bepalings van sputum monsters wat van pasiente met pulmonale tuberkulose versamel is, is gebruik. Die gestandardiseerde IS*6110* restriksie fragment stuk polimorfisme (RFLP) tipering, spoligotipering en invoegings herhalings eenhede (MIRU) tipering is gebruik om insig te verkry in die transmissie dinamika van verskillende stamme van *M. tuberculosis.* DNA volgorde bepaling (sequencing) en die PKR-RFLP metode is gebruik om genetiese veranderinge wat met tuberkulose-middel weerstand geassosieer is, te bestudeer.

Hoofstuk een is ’n literatuuroorsig wat die omvang van tuberkulose in die wêreld, die Vrystaat en Noord Kaap beklemtoon. Molekulêre tegnieke word tans wydverspeid in ontwikkelde lande gebruik wat daartoe gelei het dat die roetes van oordrag van tuberkulose tussen mense in die lande beter verstaan word. Die literatuuroorsig fokus op die tradisionele en genetiese metodes wat gebruik word in die beheer van tuberkulose, asook hulle voordele en beperkinge. Die onlangse oordragingsyfer teenoor heraktivering van die siekte en die verspreiding van weerstandsbepalers kan nie deur middel van Ziehl-Neelsen (ZN) kleuring en kweking bepaal word nie. Karakterisering van stamme met behulp van genotipiese metodes lewer data wat deur die bestuurders van die TB beheer programme gebruik kan word.

In hoostuk twee is die verskillende metodes wat in die onderskeie seksies gebruik is, beskryf.

In hoofstuk drie word Gamadi, ’n gebied waarvan die tuberkulose insidensie hoog is en wat soortgelyk aan die Vrystaat is, gedefinieer. Gamadi, het ‘n geweldige arm populasie met min migrasie uit die gebied en vergelyk goed met die groter Vrystaat. Gamadi is uiters geskik vir tuberkulose studies. Data van genetiese studies kan inligting verskaf oor

onlangse oordragingsyfers van TB teenoor reaktiewe TB, asook weerstandsmeganismes betrokke by weerstand in die Gamadi gebied, die Vrystaat en Noord Kaap Provinsies.

In hoofstuk vier word ’n aantal gedekontamoneerde sputum monsters ondersoek met behulp van ZN kleuring. Gekweekte TB stamme word deur middel van biologiese (groei tempo en kolonie morfologie) en biochemiese eienskappe (ZN kleuring, hitte stabiele katalase en nitraat toetse) geklassifiseer. ZN kleuring van gedekontamineerde sputum monsters, 20 kweking negatief en 20 kweking positiewe isolate, het onderskeidelik vir 5/20 en 16/20 *M. tuberculosis* positiewe resultate gesorg. Die 20 kulture wat op grond van biologiese eienskappe, ZN kleuring, katalase toets en nitraat toets geklassifiseer is, het nege as *M.tuberculosis* en een as ’n *M.tuberculosis* anders as TB (MOTT) geidentifiseer.

Weerstand teen isoniasied (INH) en rifampisien (RIF) in die gemeenskap van Gamadi word in hoofstuk 5 bespreek. Die veranderde indirekte proporsie metode is gebruik en ’n organisme is as weerstandig teen ’n antibiotikum beskou as groei op die medium, bevattende ’n kritiese konsentrasie van ’n antibiotika, een persent oorskry het. Primêre weerstandigheid teen INH was 2.3% vir 0.2 µg/ml van die antibiotikum. Die 87 stamme wat vir RIF weerstand getoets is, was almal sensitief. Die MOTT was egter weerstandig teen rifampicin van 1 µg/ml, asook 5 µg/ml.

RFLP patrone van INH weerstandige stamme van pasiente in die Vrystaat, die Noord Kaap en die Gamadi gebied word in hoofstuk ses bespreek. ’n Hoë proporsie van gevalle was te wyte aan reaktiewe tuberkulose. Twee en dertig vingerafdrukke van 37 pasiente met INH weerstand teen tuberkulose is gevind. Interpasient oordrag van tuberkulose is afgelei van twee klone met twee pasiente elk en twee moontlike klone (minder as ses RFLP bande). Een van die moontlike klone het ook eenderse spoligotiperings patrone gehad, maar verskillende MIRU patrone is aangetoon. Slegs agt families met ‘n 65% ooreenkoms tussen die stamme, elk bestaande uit twee tot vier stamme, is gevind onder INH weerstandige isolate.

IS*6110*-RFLP analise van 84 isolate uit die Gamadi gebied het 14 isolate met minder as ses bande opgelewer. Twee isolate in ’n kloon van drie (minder as vyf bande) was van dieselfde pasient en elf onverwante patrone is gevind. Sestig RFLP patrone met meer as ses bande is van 70 isolate gevind en drie isolate het foutiewe patrone gehad as gevolg van laboratorium kontaminasie. Twee klone met identiese patrone is gevind, die een met twee isolate en die een met vier isolate. Verwantskappe tussen twee pasiente van die kloon met vier isolate kon bevestig word, maar daar kan slegs oor informele kontak tussen die ander pasiente met identiese vingerafdrukpatrone gespekuleer word. Nege familie groepe (65% ooreenkoms), elk met twee tot agt stamme is gevind.

MIRU tipering het die 100% ooreenkoms van een kloon met stamme ZT03 en ZT04 bevestig asook twee isolate met drie RFLP bande (een was vatbaar en een weerstandig teen INH). ’n RFLP kloon met een stam uit elke provinsie, MRC16 en NC03 (elk met twee bande) het identiese spoligotiperings patrone gehad, maar verskillende MIRU tiperings. Drie verdere isolate met eenderse RFLP bande het verskillende MIRU en spoligotiperings patrone getoon.

Die proporsie van isolate met weerstandig teen INH as gevolg van mutasies in die *katG* geen is in hoofstuk sewe bespreek. Direkte basisvolgorde bepalings van die *katG* geen het getoon dat 54% van die isolate ’n verandering in kodon 315 van AGC na ACC gehad het. Twee stamme het ander mutasies in dieselfde posisie gehad. Die AGC na ACC (NC04) verandering was teenwoordig in een isolaat en ’n AGC na GGC (NC06) verandering is nie tevore gepubliseer nie. Dertien stamme het geen mutasies in of kodon 315 of 463 gehad nie. Een isolaat het mutasies in beide kodons 315 en 463 gehad en een isolaat slegs ’n mutasie in kodon 463. Twee van die stamme het die AGC na ACC mutasie getoon asook dieselfde RFLP patroon wat ’n oordrag tussen pasiente bevestig.

Die invloed van mutasies in die rifampisien weerstandsbepalings gedeelte van die *rpoB* geen is in hoofstuk agt geevalueer. Analise van 13 RIF weerstandige *M. tuberculosis* isolate deur gebruik van basisvolgorde bepalings, het basis vervangings in twaalf van die stamme getoon. Vier van die 13 isolate het geen mutasie in die 81bp kern gedeelte van die geen getoon nie. Meer as die helfte (53.9%) van die isolate het basis vervangings mutasies in die bekende gedeeltes (kodons 516, 526 en 531) gehad. Sewe stamme (53.8%) het meer as een basis vervangings mutasie gehad.

In hoofstuk nege is die geldigheid van die mees prominente ACC allele van kodon 315 van die *katG* geen as ’n vinnige en goedkoper alternatief tot basisvolgorde bepaling, ondersoek. Die PKR gebasseerde *Msp* I beperkings ensiem analise is gebruik om die ACC mutasie in kodon 315 van die *katG* geen op te spoor. Van die 37 INH weerstandige stamme wat getoets is, was 20 gesny in fragmente wat die teenwoordigheid van die ACC mutasie van kodon 315 aangedui het. Die resultate van hierdie analise was in ooreenstemming met die resultate van die basisvolgorde bepaling. Die voordeel van die *Msp* I beperkings endonuklease ensiem analise is dat dit ’n goedkoop en maklike metode is.

In hoofstuk tien word die bevindinge van die verskillende studies bespreek. Die gebruik van die genetiese metodes in die studie het aangedui dat heraktivering van vorige TB asook weerstandige TB ’n ernstige probleem in die Vrystaat is. Dieselfde is waar vir INH en multiweerstandige tuberkulose in die Noord Kaap. Genetiese tipering van isolate uit die twee provinsies het die afwesigheid van stamme wat tot die Beijing genotipe behoort, aangedui. Verder is die bekende multiweerstandige DRF150 stam ook nie gevind nie. Beperkinge van die studie, bv. dat te min monsters van die hoë insidensie gebied (Gamadi) ontvang is, kan moontlik ’n effense skewe beeld van die dinamika van TB in hierdie gebied gee. As gevolg hiervan is die omvang van veelvuldige infeksies en herinfeksies met nuwe stamme gedurende behandeling, steeds onduidelik. Die ware omvang van TB oordrag en die effektiwiteit van die DOTS strategie kan alleen bevredigend bepaal word as langtermyn genotipiese studies in die gebied uitgevoer word. Die teenwoordigheid van die Beijing en DRF150 stamme in ander provinsies vereis gereelde monitering van stamme wat in die Vrystaat en die Noord Kaap sirkuleer. Die data wat so verkry word kan die bestuur van die TB beheer program behulpsaam wees om doeltreffende strategiese beplanning te doen.