Prevalence and resistance gene mutations of multi-drug resistant and extensively drug resistant *Mycobacterium tuberculosis* in the Eastern Cape

By

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DECLARATION

I, Cindy Hayes, 8520151, hereby declare that the dissertation for Master of Technology in Biomedical Technology is my own work and that it has not previously been submitted for assessment or completion of any postgraduate qualification to another University or for another qualification.

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ABSTRACT

The emergence and spread of multi-drug resistant (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) are a major medical and public problem threatening the global health. The objectives of this study were to (i) determine the prevalence of MDR-TB and XDR-TB in the Eastern Cape; (ii) analyze patterns of gene mutations in MDR-TB and (iii) identify gene mutations associated with resistance to second line injectable drugs in XDR-TB isolates. A total of 1520 routine sputum specimens sequentially received within a period of 12 months i.e. February 2012 to February 2013 from all MDR-TB and XDR-TB patients treated by Hospitals and clinics in the Eastern Cape were included in this study, of which 1004 had interpretable results. Samples were analyzed with the Genotype MTBDR*plus* VER 2.0 assay kit (Hain Lifescience) for detection of resistance to Rifampicin and Isoniazid while solid and liquid culture drug susceptibility tests were used for ethambutol, streptomycin, ethionamide, ofloxacin, capreomycin and amikacin. PCR and sequence analysis of short regions of target genes *gyrA*, (encode subunit of DNA topoisomerase gyrase), *rrs* (16S rRNA) and *tly*A (encodes a 2'-*O*-methyltransferase) were performed on 20 XDR-TB isolates. MTBDR*plus* kit results and drug susceptibility tests identified 462 MDR-TB, 284 pre-XDR and 258 XDR-TB isolates from 267 clinics and 25 hospitals in the Eastern Cape. There was a high frequency of resistance to streptomycin, ethionamide, amikacin, ofloxacin and capreomycin. Mutation patterns indicated differences between the health districts as well as differences between the facilities within the health districts. The most common mutation patterns observed were: (i) ΔWT3, ΔWT4, MUT1 [D516V+del515] (*rpo*B), ΔWT, MUT1 [S315T1] (*kat*G), ΔWT1 [C15T] (*inh*A) [39 MDR, 204 XDR-TB and 214 pre XDR-TB isolates], (ii) ΔWT8, MUT3 [L533P+S531L] (*rpo*B), ΔWT, MUT1 [S315T1] [145 MDR, 18 pre-XDR and 3 XDR-TB solates] and (iii) ΔWT3, WT4 [D516Y+del515] (*rpo*B), ΔWT, MUT1 [S315T1] (*kat*G) [75 MDR, 1 pre-XDR and 7 XDR-TB isolates]. Mutations in *inh*A promoter regions were strongly associated with XDR-TB isolates. Two thirds (66.6% (669/1004) of the isolates had *inh*A mutations present with 25.4% (170/669) found among the MDR isolates, 39.2% (262/669) among the pre-XDR isolates and 35.4% (237/669) among the XDR-TB isolates, which implies that these resistant isolates are being spread by transmission within the community and circulating in the province.

There was good correlation between XDR-TB drug susceptibility test results and sequence analyses of the *gyr*A and *rrs* genes. The majority of XDR-TB isolates contained mutations at positions C269T (6/20) and 1401G (18/20) in *gyr*A and *rrs* genes respectively. Sequence analysis of short regions of *gyr*A and *rrs* genes may be useful for detection of fluoroquinolone and amikacin/ kanamycin resistance in XDR-TB isolates but the *tly*A gene is not a sensitive genetic marker for capreomycin resistance. This study highlighted the urgent need for the development of rapid diagnostics for XDR-TB and raised serious concerns regarding ineffective patient management resulting in ongoing transmission of extremely resistant strains of XDR-TB in the Eastern Cape suggesting that the Eastern Cape could be fast becoming the epicenter for the development of Totally Drug-resistant Tuberculosis (TDR-TB) in South Africa.

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LIST OF ABBREVIATIONS

A	Adenine
A1232G	Substitution of Adenine by Guanine at nucleotide 1232
A1266G	Substitution of Adenine by Guanine at nucleotide 1266
A1278G	Substitution of Adenine by Guanine at nucleotide 1278
A1280G	Substitution of Adenine by Guanine at nucleotide 1280
A1281G	Substitution of Adenine by Guanine at nucleotide 1281
A1387T	Substitution of Adenine by Thymine at nucleotide 1387
A1400G	Adenine to Guanine mutation at position 1400
A1401G	Substitution of Adenine by guanine at nucleotide 1401
A1406C	Substitution of Adenine by Cytosine at nucleotide 1406
A1421G	Substitution of Adenine by Guanine at nucleotide 1421
A1427G	Substitution of Adenine by Guanine at nucleotide 1427
A1524C	Substitution of Adenine by Cytosine at nucleotide 1524
A281C	Substitution of a Adenine by Cytosine at nucleotide 387
A348D	Alanine to Aspartate amino acid change at codon 348
A355N	Alanine to Asparagines amino acid change at codon 355
A476T	Alanine to Threonine amino acid change at codon 476
A476T	Alanine to Threonine amino acid change at codon 476
A478E	Alanine to Glutamate amino acid change at codon 478
A480E	Alanine to Glutamate amino acid change at codon 480
A90V	Aspartate to Valine mutation at position 90
AC	Amplification control
ADC	Albumin Dextrose Catalase
AFB	Acid Fast Bacteria
AM	Amathole district
AMI	Amikacin
Asp94	Aspartate 94
BCM	Buffalo City Metropole
C	Cytosine
C1199-	Deletion of a Cytosine at nucleotide 1199
C1202T	Substitution of Cytosine by Thymine at nucleotide 1202
C1210-	Deletion of Cytosine at nucleotide 1210
C1211G	Substitution of Cytosine by Guanine at nucleotide 1211
C124-	Deletion of Cytosine at nucleotide 124
C1283G	Substitution of Cytosine by Guanine at nucleotide 1283
C1474T	Substitution of Cytosine by Thymine at nucleotide 1474
C15T	Cysteine to Threonine amino acid change at codon 15

C269T	Cysteine to Threonine amino acid change at codon 269
C387-	Deletion of Cytosine at nucleotide 387
C387A	Substitution of a Cytosine by Adenine at nucleotide 387
CAC	Cacadu district
CAP	Capreomycin
CC	Conjugate control
CH	Chris Hani
CM	Capreomycin
D516V	Aspartate to Valine amino acid change at codon 516
D516Y	Aspartate to Tyrosine amino acid change at codon 516
D94A	Aspartate to Alanine amino acid change at codon 94
D94G	Aspartate to Glycine amino acid change at codon 94
D94H	Aspartate to Histidine amino acid change at codon 94
D94Y	Aspartate to Tyrosine amino acid change at codon 94
Del 129	Deletion at codon 129
Del 42	Deletion at codon 42
del406	Deletion at codon 406
del451	Deletion at codon 451
del458,	Deletion at codon 458
del459	Deletion at codon 459
del461	Deletion at codon 461
del462	Deletion at codon 462
del465	Deletion at codon 465
Del466	Deletion at codon 466
del467	Deletion at codon 491
del468	Deletion at codon 468
del478	Deletion at codon 478
del484,	Deletion at codon 484
del485	Deletion at codon 485
del487	Deletion at codon 487
del491	Deletion at codon 491
del494	Deletion at codon 494
Del495	Deletion at codon 495
del515	A deletion at codon 515
del589	Deletion at codon 589
del590	Deletion at codon 590
DEN	Denaturation buffer
DNA	Deoxyribonucleic Acid
DS	Drug susceptible
DST	Drug Susceptibility Testing

EC	Eastern Cape
EDTA	Ethylenediaminetetraacetic acid
EMB	Ethambutol
emB gene	Enzyme involved in synthesis of arabinogalactan
ETH	Ethionamide
F505L	Phenylalanine to Leucine amino acid change at codon 505
FIND	Foundation for innovative new diagnostics
FQs	Fluoroquinolones
G	Guanine
G262T	Substitution of a Guanine by Thymine at nucleotide 387
G280C	Glycine to Cysteine amino acid change at codon 280
G280T	Substitution of a Guanine by Thymine at nucleotide 280
G383A	Substitution of a Guanine by Adenine at nucleotide 383
G467C	Glycine to Cysteine amino acid change at codon 467
G491D,	Glycine to Aspartate amino acid change at codon 491
G494S	Glycine to Serine amino acid change at codon 494
G495V	Glycine to Valine amino acid change at codon 495
G536V	Glycine to Valine amino acid change at codon 536
G88A	Substitution of Guanine by Adenine at nucleotide 88
G88C	Glycine to Cysteine amino acid change at codon 88
gidB gene	Gene which encodes a conserved 7- methylguanosine methyltransferase
gyrA/B gene	Gene which encodes for a subunit of DNA topoisomerase gyrase
H417L,	Histidine to Leucine amino acid change at codon 417
H526C	Histidine to Cysteine amino acid change at codon 526
H526D	Histidine to Aspartate amino acid change at codon 526
H526L	Histidine to Leucine amino acid change at codon 526
H526N	Histidine to Asparagine amino acid change at codon 526
H526S	Histidine to Serine amino acid change at codon 526
HIV	Human Immunodeficiency Virus
HYB	Hybridization buffer
I462S	Isoleucine to Serine amino acid change at codon 462
I487L	Isoleucine to Leucine amino acid change at codon 487
I572M,	Isoleucine to Methionine amino acid change at codon 572
INH	Isoniazid
inhA gene	Encodes for the INH regulatory promoter region
IUATLD	International Union Against Tuberculosis and Lung Disease
JG	Joe Gqabi district
K310B	Lysine to Aspartate amino acid change at codon 310
K459Z	Lysine to Glutamine amino acid change at codon 459
K488R	Lysine to Arginine amino acid change at codon 488

kasA gene	Encodes β ketoacyl acyl carrier protein
katG gene	Encodes the catalase peroxide enzyme
KM	Kanamycin
KZN	Kwa-Zulu Natal
L343Z	Leucine to Glutamine amino acid change at 343
L384Y	Leucine to Tyrosine amino acid change at codon 384
L430R	Leucine to Arginine amino acid change at codon 430
L436E	Leucine to Glutamate amino acid change at codon 436
L533P	Leucine to Proline amino acid change at codon 533
LED	Light Emitting Diode
LiPA	Line probe assay MDR-TB
MDR	Multi- Drug Resistant Tuberculosis
MGIT	Mycobacteria growth indicator tube
MIC	Minimum Inhibitory Concentration
MOU	Memorandum of understanding
MP	Mutation Pattern
mRNA	messenger RNA
*msh*A	Encode an enzyme involved in mycothiol biosythesis
M.tb	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* Complex
MUT 1	Mutation 1
MUT2	Mutation 2
MUT3	Mutation 3
NALC-NaOH	N-acetyl-L-Cysteine sodium hydroxide
NHLS	National Health Laboratory Service
NMM	Nelson Mandela Metropole
OFL	Ofloxacin
OT	Oliver Tambo district
P347Z	Proline to amino acid change at codon 347
P375L	Proline to Leucine amino acid change at codon 375
P471R,	Proline to Arginine amino acid change at codon 471
P497S	Proline to Serine amino acid change at codon 497
PCR	Polymerase Chain Reaction
PE	Port Elizabeth
*pnc*A	Encodes for pyrazinamidase enzyme
POA	Pyrazinoic acid
PZA	Pyrazinamide
QRDR	Quinolone Resistance Determining Region
R128K	Arginine to Lysine amino acid change at codon 128
R395S	Arginine to Serine amino acid change at codon 395

R463L	Arginine to Leucine amino acid change at codon 463
R484P	Arginine to Proline amino acid change at codon 484
RIN	Rinse solution
RMP	Rifampicin
*rpo*B gene	Encodes for a portion of the RNA polymerase B subunit
*rps*L gene	Encodes for the S12 ribosomal protein
rRNA	ribosomal Ribonucleic Acid
rrs	Encodes a 16S rRNA
S315T	Serine to Threonine mutation at position 315
S315T1	Serine to Threonine amino acid change at codon 315 INH mono-resistant
S315T2	Serine to threonine amino acid change at codon 315
S446R,	Serine to Arginine amino acid change at codon 446
S460K	Serine to Lysine amino acid change at codon 460
S474Z	Serine to Glutamine amino acid change at codon 474
S481L	Serine to Leucine amino acid change at codon 481
S509T	Serine to Threonine amino acid change at codon 509
S531W	Serine to Tryptophan amino acid change at codon 531
SAMRC	South African Medical Research Council
SM	Streptomycin
STI	Sexually transmitted infection
STR	Streptomycin
STR*	Stringent wash solution
T	Thymine
T1197C	Substitution of a Thymine by Cytosine at nucleotide 1197
T1264G	Substitution of Thymine by Guanine at nucleotide 1264
T1276-	Deletion of Thymine at nucleotide 1276
T1386A	Substitution of a Thymine by Adenine at nucleotide 1386
T1399C	Substitution of a Thymine by Cytosine at nucleotide 1399
T1525A	Substitution of Thymine by Adenine at nucleotide 1525
T394I	Threonine to Isoleucine amino acid change at codon 394
T488I	Threonine to Isoleucine amino acid change at codon 488
T508A	Threonine to Alanine amino acid change at codon 508
T589P	Threonine to Proline amino acid change at codon 589
T8C	Threonine to Cysteine amino acid change at codon 8
TB	Tuberculosis
*tly*A	Encodes a 2'-O- methyltransferase
TUB	*M. tuberculosis* control
UV	Ultra Violet
V465L	Valine to Leucine amino acid change at codon 465
V466L	Valine to Leucine amino acid change at codon 466

VER	Version
VM	Viomycin
W412R	Tryptophan to Arginine amino acid change at codon 412
WHO	World Health Organisation
WT1	Wild type 1
WT2	Wild type 2
WT3	Wild type 3
WT4	Wild type 4
WT7	Wild type 7
WT8	Wild type 8
XDR-TB	Extensively Drug Resistant Tuberculosis
Y129 stop	Tyrosine to Stop codon amino acid change at codon 129
Y390D	Tyrosine to Aspartate amino acid change at codon 390
Y390H	Tyrosine to Histidine amino acid change at codon 390
Y470L	Tyrosine to Leucine amino acid change at codon 470
14C	Carbon
14CO	Carbon monoxide

CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

Development of drug resistance poses a serious challenge in the control of tuberculosis (TB). Potential factors contributing to development of drug resistance include inadequate treatment regimens prescribed by health staff, poor case management of TB patients, poor drug supply, poor drug quality, patient error in following prescribed regimens and misuse of TB drugs, including non-compliance (Crofton *et al.* 1997; Green *et al.* 2008; Mlambo *et al*. 2008). This is exacerbated by prolonged diagnostic delay and failure to ensure optimal treatment (Warren *et al*. 2009).

Multi-drug resistant tuberculosis (MDR-TB) is defined as TB that is resistant to the two first line anti-TB drugs rifampicin (RMP) and isoniazid (INH). MDR-TB is a challenge to TB control due to its complex diagnostic and treatment obstacles. Extensively drug resistant tuberculosis (XDR-TB) is currently defined as multi-drug resistant TB to at least the two most potent anti-TB drugs, rifampicin and isoniazid, in addition to resistance to any one of the fluoroquinolones and to at least one of the injectable second-line drugs capreomycin, kanamycin and amikacin. XDR-TB emerges through mismanagement of MDR-TB treatment. XDR-TB is already spread throughout all regions of the world with 9.4 million new cases and 1.7 million deaths (WHO, 2010b).

The global HIV (Human Immunodeficiency Virus) infection epidemic has caused explosive increases in TB incidence and is especially severe in South Africa (USAID, 2009). By 2011, more than 50% of new TB cases in South Africa were estimated to be co-infected with HIV (WHO, 2012). Analysis of the host population in the Eastern Cape region showed HIV co-infection to be a risk factor for the spread of the atypical Beijing strains (Strauss *et al.* 2008) with other reports indicating that 92% of isolates from the Eastern Cape were of the atypical Beijing strain (Chihota *et al.* 2011) and

the emergence of “total drug resistance” (Klopper *et al.* 2013). This raises concern for the spread of all drug-resistant strains in vulnerable populations.

The diagnosis of MDR and XDR-TB is based on mycobacterial culture and drug susceptibility testing (DST) on liquid or solid media, with results available in weeks to months. During this time patients may be inappropriately treated, drug resistant strains may continue to spread, and amplification of resistance may occur. Therefore rapid diagnosis and identification of MDR-TB or XDR-TB are imperative.

The World Health Organization (WHO) approved the use of the HAIN Genotype MTBDR*plus* test for the rapid diagnosis of INH and RMP resistance, which also allows for the simultaneous identification of *M. tuberculosis* complex (MTBC) strains in clinical isolates (WHO, 2008b; Warren *et al*. 2009). However, there are no reports of the performance of HAIN Genotype MTBDR*plus* assay kit, for the screening of MDR-TB in the Eastern Cape region as this kit was only implemented in December 2009 at the NHLS, Port Elizabeth.

Prevalence studies of MDR-TB and XDR-TB isolates, detection of the common as well as rare resistance gene mutations (distribution, frequency) in these isolates and the epidemiology of such mutants may reflect the extent of MDR and XDR-TB transmission in this region. XDR-TB is only identified up to phenotypic level at NHLS as a molecular assay has not yet been validated; hence the analysis of mutations in target genes in XDR-TB provided new information on this aspect of XDR-TB.

1.2 *MYCOBACTERIUM TUBERCULOSIS*

1.2.1 Taxonomy

Mycobacterium tuberculosis belongs to the genus *Mycobacterium* which, belongs to the family *Mycobacteriaceae,* order *Actinomycetales* and the class *Actinomycetes.* Mycobacteria are straight/ slightly curved rods which have mycolic acids in their cell walls which contribute to their environmental survival and protect them from host defenses. They have a long generation time of 22 - 24 hours (Madiraju *et al*. 1999)

and can take 8 weeks to grow on solid media such as Lowenstein Jensen slants. *M. tuberculosis* cells are difficult to stain because of the high lipid content, as well as the presence of N-glycolylmuramic acid in the cell wall. Their resistance to decolorization with acid alcohol after being stained with basic fuchsin dye reveals their acid-fast characteristic which distinguishes these organisms from other members of the genus (Forbes *et al*. 2007).

1.2.2 Epidemiology

The Stats SA 2009 report on "Mortality and causes of death in South Africa" states that TB is the fourth highest cause of death in children between 1 and 4 years of age. The prevalence of XDR-TB among MDR-TB patients is 9% - in line with the global trend, but the numbers are among the highest in the world. The four provinces with the highest burden of MDR and XDR-TB are Eastern Cape, KwaZulu-Natal, Western Cape and Gauteng (Bateman, 2010).

A study conducted in 2008, in Khayelitsha, a township in the Western Cape, involving patients suspected of having pulmonary tuberculosis, showed that the incidence of MDR-TB was estimated to be at 51/100,000 cases per year as opposed to the national estimate of 26/100,000 cases for South Africa (Cox *et al*. 2010). This township, like many others in South Africa, is characterized by poverty, overcrowding, and a high prevalence of HIV and Tuberculosis. The data presented in this study revealed that the MDR-TB epidemic appeared to be largely driven by primary transmission amongst new cases as opposed to the usual risk factors, such as, non-compliance, poor TB control programmes and HIV infection (Cox *et al*. 2010). New cases did not benefit from DST in South Africa at this time, resulting in probable treatment failure due to ineffective anti-tuberculosis therapy, increased transmission and ultimately increased mortality (Cox *et al*. 2010).

A retrospective study conducted between 2005 and 2007, in Tugela Ferry, Kwa-Zulu Natal showed that the majority of hospital based MDR-TB patients were resistant to isoniazid, rifampicin and streptomycin (80/123, 65%) and that most XDR-TB patients were resistant to isoniazid, rifampicin, streptomycin, ethambutol, ciprofloxacin and kanamycin (77/139, 55%). The study also highlighted a higher frequency of women

among the XDR-TB isolates, compared with MDR and drug susceptible (DS) patients. The data in this study also indicated a higher prevalence of HIV among XDR-TB patients (98%) than MDR-TB patients (92%, p=0.06) or DS TB patients (87%, p<0.01) (Andrews *et al.* 2010).

The World Health Organization estimates that there were approximately 9 million global TB incident cases in 2011 with around 1.4 million TB deaths in HIV negative patients and approximately 0.43 million HIV-associated TB deaths. The global incidence of TB was 26% and occurred in Africa with South Africa ranking third with an incidence of 0.40 million–0.6 million cases in 2011. Seventy nine percent of global TB cases co-infected with HIV were from the African continent which has the highest incidence (39%) of TB cases co-infected with HIV (WHO, 2012).

According to the National Strategic Plan on HIV, STIs (Sexually Transmitted Infections) and TB report of 2012 - 2016, 80% of South Africa's population is infected with TB, with health care workers, mine workers, prisoners, prison officers and household contacts of confirmed TB cases being among the high risk group of the population to develop active TB disease. Included in the high risk group are also children, people infected with HIV, diabetics, smokers, people with silicosis, alcohol and substance abusers and malnourished individuals (National Strategic Plan on HIV**,** STIs and TB 2012 **-** 2016 report). It is also estimated that approximately 1% of South Africa's population develops TB disease each year (National Strategic Plan on HIV**,** STIs and TB 2012 **-** 2016 report) with the number of cases increasing from 148,164 in 2004 to a total of 389 974 cases notified in 2011 (WHO, 2012).

MDR TB is further aggravating the global TB epidemic with an estimated 3.7% (2.1 – 5.2%) of new cases and 20% (13 – 26%) previously treated cases having MDR TB. 9.0% (6.7–11.2%) of these MDR-TB cases are estimated to have XDR-TB (WHO, 2012). Studies on the population structure of MDR and XDR strains of TB in four provinces in South Africa revealed that 92% of Beijing isolates from the Eastern Cape belonged to the atypical Beijing group (Chihota *et al.* 2011). In addition a strong association was found between the atypical Beijing *M. tuberculosis* strain and pre-XDR and XDR isolates from the Eastern Cape (Klopper *et al.* 2013). Alarmingly, this study also revealed that 93% of the XDR isolates had mutations conferring

resistance to at least 10 anti-TB drugs, with some strains also exhibiting resistance to *para*-aminosalicylic acid. This suggests the emergence of totally drug-resistant tuberculosis in the Eastern Cape. However these strains were geographically widely distributed throughout the province and likely to have been in circulation for a long period of time (Klopper *et al.* 2013).

1.3 TREATMENT OF *MYCOBACTERIUM TUBERCULOSIS*

Managing MDR and XDR TB is extremely challenging. Several factors such as the cost of treatment, length of treatment, patient compliance, drug availability, drug management, side effects, as well as limited drug choice with the added complication of cross-resistance between drugs, drug toxicity and poor efficacy of these drugs, all contribute to the challenges that are faced.

Several treatment strategies are followed in South Africa, in accordance with the Department of Health policy guidelines (Department of Health, 2012). The standardized treatment regimen is designed around national drug resistance survey data and consists of at least five drugs being administered over an intensive phase of six months. MDR patients qualify for standardized treatment on the grounds that they have only been treated with regimen 1 or 2 of the National TB control programme. The choice of drugs include kanamycin/amikacin (injectable), moxifloxacin (fluoroquinolone), ethionamide, terizidone and pyrazinamide, to be taken daily, at least six days a week during the intensive phase followed by the continuation phase which continues for at least 18 months from the time of culture conversion (Department of Health, 2012). Moxifloxacin may be substituted with levofloxacin where intolerance is noted and ethambutol may be used as an extra drug in the standardized regimen in areas with a low prevalence of ethambutol resistance.

The current regimen for TB recommended by WHO is two months with a combination of four first line drugs (isoniazid, rifampicin, pyrazinamide and ethambutol) followed by four months with two drugs (isoniazid and rifampicin) (Manzano *et al.* 2008). However a first time case is defined as a patient who has

never been treated (new case) or has only received prior treatment for less than one month. Unfortunately the first line treatment can fail due to non-compliance leading to the emergence of MDR-TB. For the treatment of MDR-TB, the standardized treatment recommended by WHO consists of a four month intensive phase with five anti-TB drugs aminoglycosides (kanamycin, amikacin), capreomycin, cycloserin, para-aminosalicylic acid, thiomides (ethionamide, prothionamide) and fluoroquinolones (ciprofloxacin, ofloxacin, levofloxacin), followed by a twelve months continuation phase with three drugs (ethionamide, ofloxacin and cycloserin or ethambutol) (Villemagne *et al.* 2012). It was recommended that these anti-TB drugs should be administered five times per week in outpatient clinics and seven times per week in hospitals. However, the continuation period could be shortened provided that twelve months of treatment had been given after sputum culture was negative for three consecutive months (Villemagne *et al.* 2012). Capreomycin and para-aminosalicylic acid were made available for treatment of XDR-TB (Streicher *et al.* 2011).

1.4 DETECTION OF *MYCOBACTERIUM TUBERCULOSIS*

The diagnosis of MDR and XDR-TB is based on sputum smear acid fast staining and microscopy and culture techniques with Lowenstein-Jensen and Middlebrook 7H9 media (Isenberg *et al.* 2007; Woods *et al*. 1997; Caviedes *et al.* 2000). Lowenstein Jensen media are more susceptible to contamination, while Middlebrook 7H10 allows for easier and earlier bacterial visibility and are less prone to contamination. However results are available in weeks to months. During this time, patients may be inappropriately treated, drug resistant strains may continue to spread, and amplification of resistance may occur.

Other detection methods include: Mantoux (tuberculin) skin test, BACTEC MGIT, commercially available DNA probes and Gene Expert PCR kit. The gold standard is the BACTEC MGIT 960 automated system which makes use of a mycobacteria growth indicator tube (MGIT tube) that is designed to detect mycobacteria in all types of clinical specimens (blood and urine excluded). The tube contains a modified Middlebrook 7H9 broth, a growth supplement as well as an antimicrobial mixture that

acts to suppress the growth of contaminating organisms (BD BBL™ MGIT™ package insert). The tube also contains a silicone button at the bottom of the tube which is embedded with a fluorescent compound. The principle of the MGIT 960 method is that the fluorescent emissions by the compound at the bottom of the tube are initially suppressed by the large amounts of dissolved oxygen in the tube. Once microorganisms begin to grow and oxygen is consumed, the level of oxygen in the MGIT tube begins to drop, allowing the fluorescent compound to fluoresce which is then detected by the instrument. The use of a solid medium in conjunction with the liquid culture (MGIT 960) is recommended for optimal recovery of mycobacteria in clinical specimens.

Of the many proteins that *M. tuberculosis* has been known to produce, MPT64 is the most predominant. This has led to the production of a commercial rapid test, namely, an immunochromatographic assay for rapid identification of *M. tuberculosis* complex (ICA) (BIO-LINE SD Ag MPT64 TB) using solid or liquid based cultures (Marzouk *et al.* 2011).The test involves using a cassette device that contains a T (test) line as well as a C (control) line on the surface of the pad. These lines are not visible prior to the addition of the sample. The control line is used as a procedural control and must develop for the test result to be accepted. The test is based on the principle of immobilizing mouse monoclonal anti-MPT64 on a nitrocellulose membrane (test line). A second antibody that is able to recognize another epitope of MPT64 is conjugated with colloidal gold particles and used for antigen capture and detection in a sandwich type assay. The test sample is added to the sample well where it spreads laterally through the membrane. If MPT64 antigen is present in the test sample, it binds to the colloidal gold conjugate. This complex then flows further until it binds with the mouse monoclonal anti-MPT64 on the test line (solid phase). A red to purple coloured band develops on the test line in the presence of MPT64 antigen. No band is detected in the absence of MPT64 antigen (Marzouk *et al*. 2011). This test is routinely used in most South African NHLS TB laboratories.

1.5 METHODS FOR ANTIMICROBIAL SUSCEPTIBILITY TESTING

A number of techniques are available to perform antimicrobial susceptibility tests on *M. tuberculosis*. Phenotypic techniques involve culturing of *M. tuberculosis* in the presence of anti-TB drugs and observing growth, which indicates resistance to the drug or the inhibition of growth which indicated susceptibility to the drug. Poor standardization and reproducibility of phenotypic methods due to differences in media being used, inoculum concentration, minimum drug concentrations and resistance criteria, resulted in the WHO and the International Union Against Tuberculosis and Lung Disease (IUATLD) meeting and agreeing on the definitions for drug resistance and three categories of acceptable methods for phenotypic drug susceptibility testing (i) Absolute concentration method (MIC) (ii) Resistance ratio method and (iii) Proportion method (Drabiewski *et al*. 2007).

1.5.1 BACTEC MGIT (mycobacteria growth indicator tube) 960

The gold standard for phenotypic testing for first and second line drugs is the BACTEC™ MGIT 960™ TB System (WHO, 2010b). The MGIT 960 mycobacteria detection system is an automated system for the growth and detection of mycobacteria with a capacity to incubate and continuously monitor the 960 MGIT every 60 minutes for increase in fluorescence. Growth detection is based on the AFB metabolic oxygen utilization and subsequent identification of an oxygen quenched fluorescent dye contained in a tube of modified MGIT (Venkataraman *et al.*1998). If growth is inhibited in the presence of anti-TB drugs, the oxygen levels remain high and fluorescence is quenched. If growth is observed in the presence of anti-TB drugs, the oxygen levels drop and fluorescence occurs (Becton Dickenson BD BBL™ MGIT™ package insert). The inoculated MGIT tubes are loaded into susceptibility “Set carriers”, depending on different drug combinations and incubated in the MGIT 960 instrument at 37°C for 5 – 13 days. Once the test is complete, the instrument will indicate that the results are ready (Siddiqi *et al*. 2006).

1.5.2 Molecular methods

There are two commercially available hybridisation kits for detection of drug resistant tuberculosis: the line probe assay (INNO-LiPARif.TB; Innogenetics) and the Genotype MTBDR*plus* assay (HAIN Lifescience) for simultaneous detection of

rifampicin (RMP) + isoniazid (INH) resistance respectively. The Genotype MTBDR*plus* assay (HAIN Lifescience) is a PCR based amplification and reverse blotting assay that employs specific probes hybridized to nitrocellulose strips to detect RMP and INH resistance (Tukvadze *et al.* 2012).

The assay detects mutation in the *rpo*B gene for RMP, in the *katG* gene for high level INH resistance and in the *inh*A regulatory region gene for low level INH resistance (Tukvadze *et al.* 2012). The test procedure is divided into different steps which include DNA extraction from decontaminated specimen, amplification of the mycobacterial DNA by PCR, hybridization of amplicons with specific probes and the detection of amplicon probe – complex on a lateral flow dipstick (Zhang *et al.* 2010). After DNA isolation the nucleic acids are amplified by PCR and amplicons are denatured and hybridized with specific probes. These specific probes have a gold binding site and the amplicon probe hybrid is marked with gold on the test strip (Tukvadze *et al.* 2012). However, by using the lateral flow buffer system the marked complex reaches a specific binding site on the test strip and attaches itself there and the reaction causes a visible band on the strip (Huang *et al.* 2009). Evaluation of the results arise when a specific band appears, this shows that a pathogen or genetic marker being tested is present in the specimen (Zhang *et al*. 2010). Recently, the WHO approved the use of the HAIN Genotype MTBDR*plus* test for the rapid diagnosis of INH and RMP resistance, which also allows for the simultaneous identification of *M. tuberculosis* complex (MTBC) strains in clinical isolates (WHO, 2010b; Warren *et al*. 2009).

In addition further investigations demonstrated the feasibility of the HAIN MDRTB*plus* assay as an effective tool for MDR-TB screening in a high TB, and high MDR-TB incidence, region and good concordance with phenotypic DST results (Hillemann *et al*. 2007; Miotto *et al*. 2008; Barnard *et al*. 2008; Nikolayevskyy *et al.* 2009). Reports demonstrated that application of this test reduces diagnostic delay to less than 48 hours, with high sensitivity and specificity to identify RMP resistance which is an important marker of MDR and XDR-TB (Barnard *et al.* 2008). Specificity was good for INH although sensitivity estimates were lower and variable because several genes are involved in conferring INH resistance and probes for these genes are not included in the HAIN Genotype MTBDR*plus* test kit (Warren *et al.* 2009).

However, molecular tests should not be applied alone and therefore cannot totally replace culture methods for several reasons: (i) apart from rifampicin and isoniazid susceptibility testing, culture is needed for all other drug susceptibility tests; (ii) rifampicin and isoniazid susceptibility must be confirmed, since the possibility that a strain is resistant cannot be excluded for a strain with a wild-type pattern by the HAIN Genotype MTBDR*plus* assay and (iii) in the case of a mixed infection with an MTBC strain and a non-tuberculosis *Mycobacterium*, interpretation may be difficult (Hillemann *et al*. 2007). Furthermore, the HAIN Genotype MTBDR*plus* assay kit does not detect the full spectrum of mutations conferring resistance in *M. tuberculosis*. Amplification and sequencing of target genes can be restricted to the investigation of less frequent mutations or when the interpretation of the hybridization pattern is not straightforward (Hauck *et al.* 2009).

An evaluation of the HAIN Genotype MTBDR*plus* test in the Western Cape Province demonstrated the presence of an *inhA* gene or promoter mutation in 53.6% of INH mono-resistant strains and 38.2% of cases with MDR-TB strains (Barnard *et al.* 2008). Analysis of drug resistant TB cases collected during 2000-2006 from the Boland, Overberg, Karoo and Southern Cape regions of the Western Cape also showed high proportions of isolates with *inhA* promoter mutations: 24% among cases with INH mono-resistant TB and 29% among cases with MDR-TB (Warren *et al.* 2009). However, there are no reports of the performance of HAIN Genotype MTBDR*plus* assay kit, for the screening of MDR-TB in the Eastern Cape region as this kit was only implemented in December 2009 at the NHLS, PE.

1.6 MECHANISMS OF ANTIMICROBIAL DRUG RESISTANCE

Genetic resistance to an anti-tuberculosis drug is due to the spontaneous chromosomal mutations at a frequency of 10^6 to 10^8 mycobacterial replication. The accumulation of these mutations leads to multi drug resistance. Mutations are enhanced through patient compliance such as monotherapy due to irregular drug supply, inappropriate doctor prescription and most importantly, poor patient adherence to treatment (Vareldzis *et al.* 1994). The MDR/XDR phenotype is caused by the sequential accumulation of mutations in different genes involved in individual

drug resistance. INH resistance is the most common form of anti-tuberculosis drug resistance encountered whether in isolation or in combination with other drugs (WHO, 2010c).

1.6.1 Rifampicin (RMP)

Rifampicin is an important first-line drug considered to be the backbone for the treatment of TB and its resistance is often associated with MDR-TB (Laurenzo and Mousa, 2011). RMP is bactericidal for *M.tb* with a MIC ranging from 0.05-1μg/ml on solid or liquid media. RMP interferes with RNA synthesis by binding to the β subunit of RNA polymerase (*rpoB*). The *rpo*B gene encodes the β subunit of the RNA polymerase and mutations in a portion of the RNA polymerase B subunit gene (*rpoB*), hotspot region encompassing codons 507-533 are responsible for 97% rifampicin resistant strains (Telenti *et al.* 1993; Ahmad and Mokaddas, 2010).

The mechanism of resistance to rifampicin involves missense mutations in a well characterized region of the *rpoB* gene. Resistance to rifampicin is an indicator of possible multi-resistance as 90% of rifampicin resistant strains are also isoniazid resistant (Somoskovi *et al.* 2001). However, mutations in the regions 526 and 531 confer high resistance while some specific mutations in codons 511, 516, 518 and 522 are associated with low level resistance to RMP. Most of the developed molecular diagnostic tools are centered on detecting these mutations in the amino acid regions 507-533 (Laurenzo and Mousa, 2011).

1.6.2 Isoniazid (INH)

INH is a first line drug that is only active against growing tubercle bacilli and is not active against non-replicating bacilli or under anaerobic conditions. INH is a prodrug activated by the catalase peroxide enzyme (KatG) encoded by the *katG* gene (Zhang *et al*. 1992) to produce a range of highly reactive oxygen and nitrogen species, which then attack multiple targets in *M*. *tuberculosis* (Zhang *et al.* 2000). Its mechanism of resistance by organisms is complex involving one or more gene mutations. INH resistant clinical isolates of *M.tb* often lose catalase and peroxidase enzyme encoded by *katG* and *inhA* genes (Laurenzo and Mousa, 2011). Mutations in these

two genes are the main mechanism of INH resistance. The *kat*G S315T mutation is the most common mutation in INH-resistant strains accounting for 50-95% of INH resistant clinical isolates (Ando *et al*. 2010). A line probe was developed for the detection of *kat*G mutations associated with high level INH resistance. Mutations in the regulatory promoter region of *inhA* (8-24nucleotides) have been observed (Laurenzo and Mousa, 2011). There are a number of missense mutations detected in the *inhA* structural gene; these mutations decrease the NADH binding affinity of *inhA,* thus protecting the enzyme from isoniazid inactivation (Morlock *et al*. 2003).

The *inhA* promoter mutations are more frequently present at codons 24(G-T) and 16 (A-G), or 8 (T-G/A) and 15 (C-T) (Johnson *et al.* 2006b). It has been reported that mutations in the *katG* gene tend to confer high level INH resistance while mutations in the *inhA* gene or its promoter region confer low level resistance (Springer *et al.* 2008; Warren *et al.* 2009). A positive correlation between *inhA* promoter mutations and high level ethionamide resistance has been observed (Morlock *et al*. 2003; Baulard *et al.* 2000). Another mutation in the *kasA* gene, which encodes a β-ketoacyl-ACP synthase, is also thought to be associated with isoniazid resistance (Sun *et al*. 2007). Mutation in *mshA* encoding an enzyme involved in mycothiol biosynthesis, have recently been shown to confer INH and ETH resistance in MTB strains *in vitro* but its role in clinical resistance remains to be demonstrated (Ahmad and Mokaddas, 2010).

The presence of the *inh*A mutation has been shown to be strongly associated with XDR TB in the Eastern Cape where 91.9% of XDR isolates had an *inh*A promoter mutation while only 62.4% of MDR isolates had mutations (Müller *et al*. 2011). The predominant *inh*A mutation was at nucleotide position -17 (Müller *et al*. 2011) which is contrary to previous reports that *inh*A mutations were more frequently present at codons 24(G-T) and 16 (A-G), or 8 (T-G/A) and 15 (C-T) (Johnson *et al.* 2006b).

1.6.3 Fluoroquinolones (FQs)

Fluoroquinolones (FQs) inhibit DNA gyrase in bacteria (topoisomerase II) and topoisomerase IV, resulting in microbial death. They act by inhibiting DNA supercoiling (topoisomerase II /DNA gyrase), thus preventing replication and cell division, The DNA gyrase is encoded by *gyrA* and *gyr*B (Laurenzo and Mousa,

2011). A conserved region the quinolone resistance determining region(QRDR) of *gyrA* (320 bp) and *gyrB* (375 bp) has been found to be a most important area involved in the inhibition of FQ resistance in MTB (Ginsburg *et al.* 2003).

Resistance to fluoroquinolones used to treat MDR-TB is thought to be mediated by mutations in target genes *gyr*A and less frequently *gyr*B which encode the respective subunits of the DNA topoisomerase gyrase. Amikacin and capreomycin resistance are associated with mutations in the 16S rRNA gene (*rrs*) and additionally for capreomycin mutations in *tly*A gene (encodes 2'-*O*-methyltransferase) (Takiff *et al.* 1994; Suzuki *et al*. 1998; Feuerriegel *et al*. 2009; Zhang and Yew, 2009). Mutation within the QRDR of *gyr*A have been identified in clinical and laboratory –selected isolates of MTB, largely clustered at codons 90, 91, 94 with Asp 94 being relatively frequent (Takiff *et al.* 1994). Mutation at codon 94 is considered a common position followed by substitution mutation at codon A90V.

1.6.4 Aminoglycosides

Streptomycin (SM) is an aminoglycoside antibiotic that is active against a variety of bacterial species including *M.tb*. It kills actively growing tubercle bacilli with MICs of 2-4µg/ml (Heifets *et al.* 2005). It is inactive against non-growing or intracellular bacilli. SM inhibits protein synthesis by binding to the 30S subunit of bacterial ribosome causing misreading of the mRNA during translation (Johnson *et al.* 2006b). Two mutations A1400G and A1401G have been identified as the initial cause of resistance to amikacin/kanamycin while resistance to SM is caused by mutation in the S12 protein encoded by *rpsL g*ene and 16S rRNA encoded by *rrs* gene (Laurenzo and Mousa, 2011). Mutation in *rpsL* and *rrs* are the major mechanism of SM resistance accounting for about 50% and 20% of SM-resistant strains respectively (Honore *et al.* 1995). However A1400G mutation appears to be the major mechanism of resistance to amikacin/kanamycin, Recently mutation in *gid*B encoding a conserved 7-methylguanosine (m(7)G) methyltransferase specific for 16S rRNA has been found to cause low level SM resistance in 33% of resistant MTB isolates (Okamoto *et al*. 2007).

Some low-level streptomycin resistance seems to be caused by an increased efflux. Capreomycin is a polypeptide antibiotic and a gene called *tlyA* encoding

rRNAmethyltransferase was shown to be involved in resistance to capreomycin (Maus *et al.* 2005). Variable cross resistance may be observed between KM, AMK, CM or viomycin (VM). Multiple mutations may occur in the *rrs* gene in one strain, conferring cross-resistance among these agents. SM resistant strains are usually still susceptible to KM and AMK.

1.6.5 Ethambutol (EMB)

Ethambutol interferes with cell wall biosynthesis of arabinogalactan by inhibiting the polymerization of cell wall arabinan (arabinogalactan) as well as lipoarabinomannan. It also induces the accumulation of D-arabinofuranosyl-P-decaprenol, an intermediate of arabinan biosynthesis. The enzyme arabinoyltransferase, involved in the synthesis of arabinogalactan, is encoded by *emb*B and is thought to be the target of EMB in *M. tuberculosis* and *M. avium* (Zhang and Yew, 2009). It is a bacteriostatic agent active for growing bacilli and has no effect on non-replicating bacilli. It also interferes with the biosynthesis of cell wall arabinogalactan. The inhibition prevents the formation of a mycolyl-arabinogalactan peptidoglycan complex which increases cell wall permeability. The main mechanism likely to cause resistance is the over-expression or structural change of the Emb proteins particularly EmbB (Laurenza and Mousa, 2011). Phenotypic resistance is difficult to standardize in part due to the instability of EMB in both solid and liquid culture media. A specific mutation at codon 306 of *embB* gene has been found to be responsible for most of the ethambutol resistance. A low percentage of resistant strains contain gene mutation thus conferring high level of resistance. The other mutations that have been found to confer significant levels of resistance are at codons 330 and 630 (Laurenzo and Mousa, 2011).

1.6.6 Ethionamide (ETH) / Prothionamide and Thioamides

ETH is a derivative of isonicotinic acid as well as a thioamide antibiotic used as a second line drug for MDR-TB. It is bactericidal only against MTB, *M*. *avium*-intracellulase and *M*. *leprae.* The MICs of ETH are 0.5-2 µg/ml in liquid. The exact mechanism by which ethionamide exerts its effect is unclear. Ethionamide is a pro-drug, activated by *EtaA/ EthA* (mono-oxygenase). It inhibits the same target as INH (*inhA* in mycolic acid synthesis pathway). Mutations in *EtaA/EthA* cause resistance

to Ethionamide. In addition, mutations in the *InhA* target results in resistance to both INH and ETH (Zhang and Yew, 2009).

The activation of ethionamide causes conversion to 4-pyridylmethanol which is a proxy for activating isoniazid via *kat*G. This link illustrates a relationship between isoniazid and ethionamide on *inhA*, therefore resistance to one overlaps with the others. Strains of isoniazid resistance with a *kat*G mutation also exhibit susceptibility to ethionamide (Laurenzo and Mousa, 2011). The main mechanism of ethionamide resistance is not through gene mutation but rather by an overproduction of EtaR (Rv3855 a number given to a protein that negatively regulates the production of ETHA) which is a regulatory protein conferring ethionamide resistance. However EtaR negatively controls the hypersensitivity of the enzyme adjacent to Eta operon (EtaA) therefore the overproduction of EtaR results in the lowering of EtaA and thus reducing the sensitivity to ethionamide (Laurenzo and Mousa, 2011).

1.7 SCOPE AND OBJECTIVES

The emergence and spread of multi-drug resistant (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) are a major medical and public problem threatening the global health. Prevalence studies of MDR-TB and XDR-TB isolates, detection of the common as well as rare resistance gene mutations (distribution, frequency) in these isolates and the epidemiology of such mutants may reflect the extent of MDR and XDR-TB transmission in the Eastern Cape region. There are few publications addressing the status of MDR-TB and XDR-TB in the Eastern Cape while most publications have focused on results from laboratories in the larger cities like Gauteng, Cape Town and Durban. This study would provide information pertaining to *M. tuberculosis* in the Eastern Cape region and assist in identifying common genotype groups and molecular epidemiological markers in the isolates. Furthermore this project would impact on identification of high risk groups, infection control, rapid case detection and appropriate treatment.

1.7.1 Hypotheses tested

It was hypothesised that there was a high prevalence of multi-drug resistant and extensively drug resistant *M. tuberculosis* in the Eastern Cape. It was further hypothesised that there were specific genotype groups present with common as well as rare resistance gene mutations.

1.7.2 Objectives

The following objectives have been established to test the above hypotheses:

- To determine the prevalence of MDR-TB and XDR-TB in the Eastern Cape and to collate demographic data on patient population to establish epidemiology status,
- To establish the pattern of gene mutations in MDR-TB isolates using HAIN Genotype MTBDR*plus* assay kit,
- To perform sequence analysis of short regions of target genes in XDR-TB isolates: *gyrA*, (encode subunit of DNA topoisomerase gyrase), *rrs* (16S rRNA) and *tlyA* (encodes a 2'-O-methyltransferase) for detection of second line TB drug resistance among isolates.

CHAPTER TWO

MATERIALS AND METHODS

2.1 STUDY SETTING

This study took place at the National Health Laboratory Services (NHLS) Tuberculosis laboratory in Port Elizabeth. Data request approval from NHLS and authorization from the Department of Health was granted. Ethics approval for this project was obtained from the NMMU Research Ethics Committee (Human) [Ref.: H11-SCI-BCM-006]. Demographic information was recorded however clinical information concerning patient history could not be obtained.

2.2 SPUTUM SPECIMENS

Routine sputum specimens sent to the NHLS TB laboratory from all MDR-TB and XDR-TB patients treated by Hospitals and clinics in the Eastern Cape were included in this study. Specimens were sequentially received within a period of 12 months i.e. February 2012 to February 2013. A total of 1 520 specimens were used in this investigation of which 1 004 had interpretable results and were therefore included in the analysis.

2.3 SPECIMEN PROCESSING/ CULTURE/ PHENOTYPIC TESTS

Sputum specimens were decontaminated with N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) by adding equal volumes of NALC-NaOH and sputum sample into a 50 ml centrifuge tube (Kent and Kubica, 1985). The samples were vortexed for 30 sec and left to stand at room temperature for 15 min. PBS (pH 6.8) was added and the specimen was inverted several times to mix the contents. This was followed by centrifugation at 3000 rpm for 20 min, and removal of the supernatant. The pellet was suspended in 3 ml PBS and 0.5 ml was inoculated into a mycobacteria growth indicator tube (MGIT) and incubated at 37ºC in the MGIT 960 instrument. Positive cultures were confirmed using the Ziehl-Neelsen staining method (Isenberg, 2007).
M. tuberculosis H37Rv ATCC 25177 was used as a control culture for all assays as it has a drug susceptibility profile fairly representative of most drug susceptible clinical

isolates. *M. tuberculosis* was grown on Lowenstein Jensen slants. Cultures were incubated at 37°C until growth was observed (4 – 6 weeks).

2.4 ANTIBIOTIC SUSCEPTIBILITY TESTS

First line drug susceptibility testing for INH and RMP was originally performed using Middlebrooks 7H11 solid medium, indirect proportion method. However, the HAIN GenoType MTBDR*plus* kit was introduced in 2009 at the Port Elizabeth TB Laboratory and this molecular test was then used to detect mutations conferring drug resistance in the *rpo*B region for rifampicin resistance and *kat*G and *inh*A regions for isoniazid resistance.

Second line drug susceptibility testing was done using Middlebrooks 7H11 solid medium, indirect proportion method, up until July 2012. Hence for Feb – June 2012 period, there are results for 6 drugs (ethambutol, streptomycin, ethionamide, ofloxacin, capreomycin and amikacin) tested. The MGIT 960 DST method was introduced in August 2012, and after negotiations with local health authorities, the panel of drugs for testing was reduced to 3 (viz. ofloxacin, amikacin and capreomycin).

2.4.1 Solid media Drug Sensitivity Test (DST)

Samples collected for Feb-June 2012 [46% (459/1004)] were tested using the phenotypic Middlebrooks 7H11 solid medium method which included a panel of six drugs. DST was performed using the indirect proportion method (Kent and Kubica, 1985; Isenberg, 2007) at the following concentrations: ethambutol (7.5 µg/ml), streptomycin (2 µg/ml), ethionamide (10 µg/ml), ofloxacin (2 µg/ml), amikacin (2 µg/ml) and capreomycin (10 µg/ml). Two standardized inocula representing the entire culture population were prepared from the positive culture at a 1/10 and 1/100 dilution and inoculated onto the Middlebrook 7H11 agar slants. The slants contained the respective drug at the stipulated concentration. Two growth control slants were included (1/10 and 1/100). A 0,1 ml aliquot of the 1/10 dilution was flooded aseptically onto both the 1/10 growth control slant and the antibiotic containing media, and 0.1 ml of a 1/100 dilution was flooded over the second growth control slant. The slants were incubated at 37°C for 3 weeks, after which they were

compared to the growth on the 1/100 growth control slope. A susceptible strain would have no growth or less than 1% of growth compared to the 1/100 growth control slant. A resistant result would have 1% or more growth compared to the 1/100 growth control slant (Isenberg, 2007).

2.4.2 BACTEC MGIT (mycobacteria growth indicator tube) 960

Samples collected from August 2012 – Feb 2013 [54% (544/1004)] were tested using the gold standard, MGIT 960 DST method against three drugs (amikacin, capreomycin and ofloxacin). Testing of the specimen was done within the first 5 days of obtaining a positive culture as described in section 2.3. Samples were vortexed in order to break up clumps and left to stand for 5 – 10 min. A MGIT tube for the growth control (GC), as well as one for each drug, was labelled. OADC (Oleic Albumin Dextrose Catalase) supplement (0.8 ml) was added aseptically to each tube. The reconstituted drug (0.1 ml) was added aseptically at the following concentrations: amikacin (1 µg/ml), capreomycin (2.5 µg/ml) and ofloxacin (2 µg/ml). A 0.5 ml aliquot of the well mixed culture sample was added aseptically to each of the drug containing tubes only while 0.5 ml of a 1:100 dilution (0.1ml of the culture sample to 9.9 ml sterile saline) of the culture was added aseptically to the GC tube. The tubes were capped and loaded into susceptibility “Set carriers”, depending on different drug combinations and incubated in the MGIT 960 instrument at 37°C for 5 – 13 days. The instrument indicated when the drug susceptibility results were completed (Siddiqi *et al.* 2006).

2.5 GENOTYPE MTBDRplus ASSAY

Samples were analysed with the Genotype MTBDR*plus* assay kit (HAIN Lifescience, Version 2 product insert) for detection of resistance to RMP and INH according to the manufacturer’s instructions. The test is based on DNA strip technology and has three steps: DNA extraction, multiplex PCR amplification and hybridisation.

2.5.1 DNA extraction

A volume of 500 µl decontaminated (NALC-NaOH treated) sample, which included respiratory (sputum, bronchial lavage /aspirate & tracheal aspirate) and extra-pulmonary samples (samples collected outside the lungs), was centrifuged at 10 000 x g for 15 min. The supernatant was discarded and the pellet resuspended in 100 µl

of Lysis buffer (A-LYS, HAIN Genotype® MTBDR*plus* kit) by vortexing, incubated at 95˚C for 5 min and centrifuged for 5 min. Neutralization buffer (A-NB) (100 µl) was added to the suspension and vortexed for 5 seconds. The sample was centrifuged at maximum speed for 5 min. The supernatant containing the extracted DNA was used directly or stored separately until further use (GenoLyse® product insert).

2.5.2 Amplification

DNA amplification was performed according to the instructions outlined in the kit manual using a thermal cycler. The amplification mix contained 35 µl primer nucleotide mix (PNM), 5 µl 10x PCR buffer, 2 µl 25 mM $MgCl_2$, 0.2 µl HotStar Taq DNA polymerase (Qiagen), 3 µl of molecular grade water and 5 µl template bacterial DNA (added in a separate room) in a final volume of 50 µl. The amplification protocol for cultivated samples consisted of 15 min of denaturation at 95˚C, followed by 10 cycles comprising 30 sec at 95˚C and 2 min at 65˚C; an additional 20 cycles comprising 25 sec at 95˚C, 40s at 50˚C and 40s at 70˚C; and a final extension at 70˚C for 8 min.

For the sputum specimens, an altered amplification protocol was applied which consisted of 15 min of denaturation at 95˚C, followed by 20 cycles comprising 30 sec at 95˚C and 2 min at 65˚C; an additional 30 cycles comprising 25 sec at 95˚C, 40s at 50˚C and 40s at 70˚C; and a final extension at 70˚C for 8 min (HAIN Lifescience, Version 2 product insert).

2.5.3 Hybridization procedure

Hybridization and detection were performed using the automated hybridization instrument GT-Blot 48 (HAIN Lifescience GmbH). Hybridization involved the addition of 20 µl denaturation (DEN) solution to a strip well followed by the addition of 20 µl amplified DNA. The two solutions were mixed well by repeated pipetting and incubated at room temperature for 5 min. Pre-warmed hybridization solution (HYB) (1 ml) was added to the well and gently mixed. A labelled strip was then added and submerged in the solution and incubated at 45°C for 30 min. After incubation, the wells were aspirated and 1 ml of stringent wash (STR) solution was added and incubated at 45°C for 15 min. The STR solution was removed and 1 ml rinse solution (RIN) added for 1 min at room temperature. This solution was removed and

1 ml of diluted conjugate was added and incubated at room temperature for 30 min. The conjugate solution was discarded, followed by a 3-step wash procedure which included two 1 min washes with RIN solution, and 1 min rinse with distilled water. Diluted substrate (1 ml) was added, incubated under dark conditions at room temperature, without shaking for 3 - 20 min. The reaction was finally stopped by rinsing twice with distilled water. The strips were dried and mounted for evaluation and interpretation (HAIN Genotype® MTBDR*plus* product insert).

2.5.4 Interpretation of results

The MTBDR strip contains 27 reaction zones coated with specific probes (Fig 2.1). Evaluation and interpretation of the MTBDR strips were performed using the automated GenoScan® system.

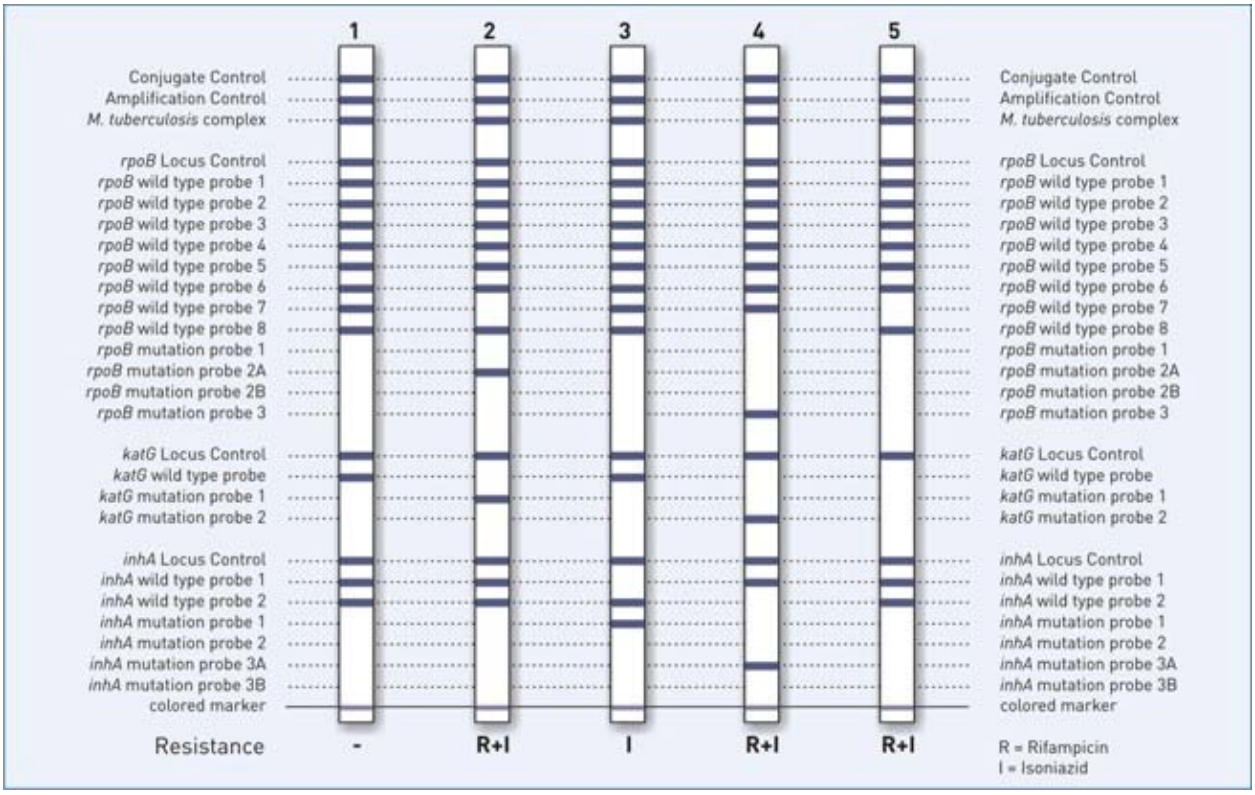

Figure 2.1: Membrane strip coated with specific probes which bind to amplicons. (-): sensitive; (R+I): resistant to rifampicin and isoniazid; (I): resistance to isoniazid (taken from the HAIN Genotype® MTBDR*plus* assay kit manual).

These include six controls: conjugate (CC), amplification (AC), *M. tuberculosis* complex (TUB), *rpo*B, *kat*G and *inh*A (locus controls), eight *rpo*B wild-type (WT0 and four mutant (MUT) probes, one *kat*G wild-type and two mutant probes, and two *inh*A

wild-type and four mutant probes. A band (indicates a positive result) must develop in the amplification control (AC) to rule out errors occurring during the extraction and amplification procedures. *M. tuberculosis* complex (TUB) must be positive to confirm the presence of members of the *M. tuberculosis* complex. If negative, evaluation of the test cannot continue as the bacterium does not belong to the *M. tuberculosis* complex. Locus controls (*rpoB, katG* and *inhA*) must also be positive as they detect gene regions specific for the respective loci for *M. tuberculosis* strains. Wild type probes represent the most important resistance areas for the respective genes. If all the wild type probes are present and the mutation probes absent, the organism is deemed susceptible to that specific antibiotic. If a mutation probe is present, its corresponding wild type probe will be absent due to the amplicon not being able to bind. The organism is therefore resistant to the antibiotic. Bands have to be as strong as or stronger than the AC control to be interpretable. The *rpoB* probes indicate rifampicin resistance, while *katG* and *inhA* probes indicate high and low resistance levels to isoniazid respectively (HAIN Genotype® MTBDR*plus* product insert).

For the detection of RMP resistance, eight *rpoB* wild-type probes (probes WT1 to WT8) encompass the region of the *rpoB* gene encoding amino acids 505 – 533. Four probes (*rpoB* MUT1, *rpoB* MUT2A, *rpoB* MUT2B and *rpoB* MUT3) specifically target the most common mutations indicated in Table 2.1.

Table 2.1: Mutations in the *rpoB* gene and the corresponding wild type and mutation bands (taken from the HAIN GenoType MTBDR*plus* kit manual).

Fading wild type bands	Codons analyzed	Developing mutation band	Mutation
rpoB WT1	505-509		F505L
			T508A
			S509T
*rpoB*WT2	510-513		L511P*
rpoB	510-517		Q513L*
WT2/WT3			Q513P
			Qdel514-516
rpoB	513-519	*rpoB* MUT1	D516V
WT3/WT4			D516Y
			del515
rpoB	516-522		del518*
WT4/WT5			N518I
rpoB	518-525		S522L
WT5/WT6			S522Q
rpoB WT7	526-529	*rpoB* MUT2A	H526Y
		rpoB MUT2B	H526D
			H526R
			H526P*
			H526Q*
			H526N
			H526L
			H526S
			H526C
rpoB WT8	530-533	*rpoB* MUT3	S531L
			S531Q*
			S531W
			L533P

*This rare mutation has only been detected theoretically (*in silico*). It is therefore possible that it cannot be detected *in vitro*.

For the detection of INH resistance, 6 probes (*kat*G MUT1, *kat*G MUT2, *inh*A MUT1, *inh*A MUT2, *inh*A MUT3A, *inh*A MUT3B) specifically target the mutations indicated in Table 2.2.

Table 2.2: Mutations in the *katG* and *inhA genes* and the corresponding wild type and mutation bands (taken from the HAIN GenoType MTBDR*plus* kit manual).

Fading wild type bands	Codons analyzed	Developing mutation band	Mutation
katG WT	315	*katG* MUTI	S315T1
		katG MUT2	S315T2
inhA WT1	-15	*inhA* MUT1	C15T
	-16	*inhA* MUT2	A16G
inhA WT2	-8	*inhA* MUT3A	T8C
		inhA MUT3B	T8A

2.6 ANALYSIS OF TARGET GENES IN XDR-TB

XDR-TB is only identified up to phenotypic level at NHLS, PE as a molecular assay has not yet been validated. The MDR-TB DNA from patients not responding to second line drugs were screened for mutations in *gyrA*, (encode subunit of DNA topoisomerase gyrase), *rrs* (16S rRNA) and *tlyA* (encodes a 2'-*O*-methyltransferase) genes as mutations in these genes could be responsible for resistance to fluoroquinolones and second-line injectable drugs. Amplification conditions and primers are indicated in Table 2.3 below.

Table 2.3: Primers and PCR conditions for detection of mutations in XDR-TB.

Gene	Primer	Sequence	Size (bp)
tlyA (2'-*O*-methyltransferase)	tlyAF3 tlyAR3	AAGGCATCGCACGTCGTCTTTCC TGTCGCCCAATACTTTTTCTACGC	981 bp
rrs gene (16S rRNA)	RRS2-F RRS2-R	TGCCGGGGTCAACTCGGAGG GAACCCCTCACGGCCTACGC	439 bp
gyrA (topoisomerase gyrase)	gyrA-F gyrA-R	CAGCTACATCGACTATGCGA GGGCTTCGGTGTACCTCAT	320 bp
PCR conditions Perdigao *et al.* (2010)		Cycling conditions: denaturation at 94 for 4 min; 35 cycles of denaturation at 94 for 1 min, annealing at 58 (55 for *gyr*A) for 1 min and extension at 72 for 2 min and 30 sec (1 min for *gyr*A) and, final extension step at 72 for 10 min.	

2.7 AGAROSE GEL ELECTROPHORESIS

PCR products were separated on 2% (w/v) agarose gels for 45 min at 100 V using Tris-acetate EDTA buffer (40 mM Tris base, 5 mM sodium acetate, 1 mM EDTA, pH 8). Ethidium bromide stained DNA products were visualised by UV transillumination and images captured using an Alpha Imager^{TM}3400 gel system (Alpha Innotech). Control DNA: *M.tuberculosis* H37Rv (ATCC 27294) and Bioline DNA Marker Hyperladder IV (100 bp) (Celtic Diagnostics) was included in each gel to determine approximate sizes of the PCR products.

2.8 SEQUENCING

The Wizard SV gel and PCR clean-up system (Promega) was used to prepare PCR products for sequencing. Purified products were sent for sequencing to the Central Analytical Facility, University of Stellenbosch. Sequence analyses were performed using Chromas 1.45, Bioedit 7.0.5 and Geneious 3.8.5, and sequences compared to mutations included in the TB Drug Resistance Mutation Database (www.tbdreamdb.com). The sequences of these genes were compared to the complete nucleotide sequence of *M. tuberculosis* strain H37Rv. *rpo*B gene (GenBank accession number: L27989), *kat*G (GenBank accession number: X68081) and for the XDR-TB isolates *rrs,* (GenBank accession number: *M. tuberculosis* H37Rv|MTB000019), *tly*A (GenBank accession number: *M. tuberculosis* H37Rv|Rv1694) and *gyrA* (GenBank accession number: JQ699173.1).

CHAPTER THREE

PREVALENCE OF MDR-TB AND XDR-TB IN EASTERN CAPE

3.1 INTRODUCTION

A national and global perspective of the burden of multi and extensively drug resistant tuberculosis and epidemiology of the disease is required, in order to fully understand the challenges and control the drug resistance problem facing the Eastern Cape department of health. In 2011, it was estimated that 3.7% of global new TB cases (Confidence Interval of 2.1–5.2%) and 20% of previously treated TB cases (Confidence Interval 13–26%) had MDR-TB. Approximately 60 000 MDR- TB cases were reported to the WHO in 2011, mostly by European countries and South Africa. This figure, however, represented only 19% of the estimated 310 000 (range 220 000- 400 000) MDR-TB cases among notified TB patients with pulmonary TB. It was also estimated that 9.0% (Confidence Interval 6.7-11.2%) of these MDR-TB cases were extensively drug resistant (WHO, 2012).

According to the SA National Department of Health, the second highest number of MDR-TB cases were diagnosed the Eastern Cape (EC) in 2010, at 1782 cases, with KZN diagnosing the highest number at 2032 cases. The highest number of XDR-TB cases were diagnosed in the EC in the same year at 320 XDR-TB cases as opposed to 201 cases diagnosed in KZN. The Western Cape diagnosed the third highest number of MDR-TB and XDR-TB cases at 1422 and 112 respectively (Directorate Drug- Resistant TB, 2011b).

Differences in treatment outcomes were reported in MDR-TB patients from KwaZulu-Natal (KZN) and the Eastern Cape (EC) provinces between 2005 and 2008 respectively (Odendaal *et al.* 2011). Significantly higher cure rates, lower default rates and lower mortality rates were noted amongst the KZN cohort. Isolates from patients from EC and KZN (64% and 34% of respectively), showed resistance to the 4 first line drugs (isoniazid, rifampicin, pyrazinamide and ethambutol) while 20% and 6% of isolates from EC and KZN were resistant to one flouroquinolone respectively. Fifty one percent of isolates in EC and 11% in KZN were resistant to one injectable,

with 49% and 8% isolates in EC and KZN resistant to all 3 injectable drugs (amikacin, kanamycin and capreomycin). Alarmingly, 22 (17%) patients in EC were found to have XDR-TB with only 6 patients from KZN being infected with XDR-TB (Odendaal *et al*. 2011).

Tuberculosis amongst children is usually an indicator of recent infection and reflects strain diversity and susceptibly patterns circulating in their community (Schaaf *et.al*. 2006). Co-infection with HIV was observed in 48.5% of children with MDR-TB at two Johannesburg hospitals (Fairlie *et al.* 2011). Another interesting finding was the fact that only four of the children with confirmed MDR-TB had been exposed to known household contacts. The remaining children had probably been exposed outside the home, suggesting undiagnosed household or community transmission of MDR-TB (Fairley *et al*. 2011).

The majority of South Africa's TB control budget is spent on treating multi and extreme drug resistant TB, with the estimated cost being in excess of $17 000 (R 170 000) (Schnippel *et al*. 2013). Up until late 2011, all diagnosed MDR and XDR-TB patients were hospitalized in specialized treatment facilities for 6 months or until culture conversion, which was regarded as two consecutive negative TB cultures over two consecutive months (Directorate Tuberculosis control, 2007). Complications arising during this period could result in a delay in discharge of the patient. In an effort to reduce the cost of treating MDR and XDR-TB patients, new TB control guidelines were released in late 2011, which stated that smear negative MDR-TB patients that were clinically not too ill and had access to daily injections, may be treated as out-patients and that those patients that were hospitalized, were eligible for discharge on smear conversion as opposed to the previous criteria requiring culture conversion (Schnippel *et al*. 2013). This implies a reduction in hospital admissions by 30% (Directorate drug resistant TB, 2011a). These new guidelines were estimated by the SA National Department of Health to apply to only 30 – 40% of MDR-TB cases in South Africa. Many challenges face the implementation of these guidelines, such as staff training and patient education, improved infection control education, improved infrastructure and monitoring at clinic level and decentralization of MDR-TB drug supply (Schnippel *et al.* 2013). If not properly

managed, this policy could potentially increase community spread of MDR-TB and ultimately, XDR-TB.

After negotiations with the local MDR/XDR-TB facility, the six drug panel was reduced to a three drug panel. The rationale behind this decision was primarily cost and tailoring the panel to eliminate drugs that were seen to be ineffective in the Eastern Cape. A study conducted in the Western Cape used *emb*B gene analysis to show that 91.4% of ethambutol resistance was missed using routine indirect proportion phenotypic testing in the laboratory and that 87.2% of the EMB resistant isolates were also resistant to isoniazid and rifampicin (Johnson *et al.* 2006a). This evidence raised concern and resulted in the WHO revising the MDR-TB treatment guidelines with the recommendation that at least 4 effective drugs be used to treat MDR-TB and that treatment regimens should not depend on ethambutol DST results (WHO, 2008c). The South African department of Health followed this recommendation by replacing EMB with Terizdone or Cycloserine (Hoek *et al*. 2009). However no data on susceptibility profiles of terizdone or cycloserine were available for this study. These recommendations also led to the decision to remove EMB from the panel of testing in the Port Elizabeth TB Laboratory.

The decision to exclude Ethionamide from the panel of testing was based on the fact that a high incidence of *inh*A mutations, conferring low level resistance to Isoniazid were noticed during routine testing at the Port Elizabeth TB Laboratory. Research has shown *inh*A is targeted by both Ethionamide and INH and that mutations in this region result in cross-resistance to both of these drugs (Banerjee *et al.*1994, Morlock *et al*. 2003; Muller *et al.* 2011). Similarly, Streptomycin was excluded from the panel of testing due to the high frequency of resistance to the drug, using the Middlebrooks solid medium method.

The main challenge facing a province already burdened with HIV is inadequate data on drug resistant TB. Therefore, it was important to assess the prevalence of MDR and XDR-TB in EC tested at the NHLS laboratory.

3.2 RESULTS

3.2.1 Study population

MDR and XDR isolates were classified according to WHO definitions (WHO, 2010c). Pre-XDR-TB isolates were defined as MDR-TB isolates with additional resistance to either fluoroquinolone or a second-line injectable drug (capreomycin, KM or AMI) but not both (Chihota *et al*. 2011). The MDR *sensu stricto* group excluded identified pre-XDR and XDR isolates (Chihota *et al*. 2011).

A total of 1520 MDR isolates that were sequentially tested within the period February 2012 to February 2013 at NHLS were used in this study. A further 89 samples were "repeats" which constituted multiple samples from the same patient who had visited different clinics / hospitals within a week / month. In addition, 140 samples had lost viability and 287 had contamination and were excluded. Samples (n=1004) had interpretable results and were therefore included in the analysis. Resistance to first line anti-TB drugs using the HAIN Genotype MTBDR*plus* kit revealed that 46% (464/1004) of samples were from MDR *sensu stricto* TB patients, with 28% (286/1004) samples from pre-XDR-TB and 26% (258/1004) from XDR-TB patients based on the MGIT 960 liquid culture as well as the Middlebrooks 7H11 solid culture DST methods.

3.2.2 Microscopy and culture

TB microscopy testing is only performed on request at the NHLS TB Laboratory in Port Elizabeth. Of the 1004 samples tested, all were TB Culture positive while 52% (526/1004) had requested smear microscopy with the TB Culture. Of those samples that had smear microscopy requested, 20% (108/526) were scanty (1+) positive, 16% (84/526) were moderate (2+) positive and 22% (115/526) were abundantly (3+) positive. Forty two percent (219/526) were smear negative and 48% (478/1004) did not request smear microscopy. Of the smear positive TB Cultures, 49% (149/307) were female patients and 51% (158/307) were male patients.

3.2.3 Demographic characteristics of patient population

3.2.3.1 Geographical Distribution

The 1004 samples (from MDR-TB and XDR-TB patients) were from 25 hospitals (i.e provincial and TB hospitals, only) and 267 clinics (included small "village hospitals" in rural areas) from the eight health districts in the Eastern Cape (Fig. 3.1). These included, Alfred Nzo, Amathole, Buffalo City Metro, Chris Hani, Nelson Mandela Metro, O.R. Tambo, Cacadu and Joe Gqabi health districts (Appendix: Table A1) with the highest number of cases being recorded from 7 hospitals and 13 clinics (Table 3.1).

Figure 3.1: Geographic distribution of cohort of patients in this study. Map taken from South Africa Yearbook 2012/13, published by the Government Communication and Information System. www.ectourism.co.za; www.ecdc.co.za; www.statssa.gov.za.

Nkqubela Chest hospital had the highest number of MDR and XDR-TB cases. This is a specialized TB treatment facility, situated in the Buffalo City Metropole (BCM) health district in Mdantsane on the outskirts of East London. The majority of specimens received from this facility were MDR 62% (59/96), pre-XDR 25% (24/96) and 14% (13/96) diagnosed with XDR-TB (Table 3.1). The second highest number of MDR and XDR-TB cases came from the Empilweni TB hospital, situated in the Nelson Mandela Bay Metropole (NMM) in Port Elizabeth. Of the specimens received,

35% (10/29) were MDR-TB, 52% (15/29) were pre-XDR-TB with 13% (4/29) being diagnosed with XDR-TB. The Empilweni TB hospital accommodates mostly drug - susceptible TB cases and usually refers MDR-TB and XDR-TB cases to a specialized facility. The Marjory Parrish TB hospital, situated in Port Alfred in the Cacadu district, had the third highest number of cases with the majority being pre-XDR 47% (9/19) and XDR-TB 42% (8/19) (Table 3.1).

Table 3.1: Hospitals and clinics with the highest MDR-TB and XDR-TB cases.

Hospitals/ clinics	**No. of cases within the different groups**			
	MDR-TB	**Pre-XDR-TB**	**XDR-TB**	**Total**
Nkqubela chest hospital (East London)	59	24	13	96
Empilweni TB hospital (Port Elizabeth)	10	15	4	29
Marjorie Parrish TB hospital (Port Alfred)	2	9	8	19
Chatty clinic (Port Elizabeth)	2	6	8	16
Frere Hospital (East London)	10	4	2	16
Gqebega clinic (Port Elizabeth)	3	8	4	15
Laetitia Bam day hosptal (Uitenhage)	8	1	6	15
NU2 clinic (East London)	6	2	7	15
Rosedale clinic (Port Elizabeth)	3	6	6	15
Dora Nginza hospital (Port Elizabeth)	4	5	5	14
GOMPC	7	5	2	14
Booysen Park clinic (Port Elizabeth)	6	6	1	13
Kwazakhele clinic (Port Elizabeth)	7	2	4	13
Cecilia Makiwane hospital (East London)	8	3	1	12
Lunga Kobese clinic (Port Elizabeth)	4	4	3	11
Motherwell NU 2 clinic (Port Elizabeth)	3	4	4	11
Zwide clinic (Port Elizabeth)	2	2	7	11
Motherwell NU11 clinic (Port Elizabeth)	1	4	5	10
NU8 clinic (East London)	4	4	2	10
Tanduxolo clinic (Port Elizabeth)	2	4	4	10

A higher incidence of pre-XDR and XDR-TB was observed among the NMM clinics in Port Elizabeth. Chatty clinic had the highest number at 38% (6/16) pre-XDR and 50% (8/16) XDR-TB cases, followed by Gqebera clinic with 53% (8/15) pre-XDR and 27% XDR-TB cases. Laeticia Bam Day Hospital in Uitenhage, also falling under the NMM had a very high frequency of XDR-TB cases 40% (6/15) as well as Rosedale

clinic in Port Elizabeth, with 40% (6/15) pre-XDR and XDR-TB respectively (Table 3.1).

Three of the seven hospitals recording the highest number of MDR and XDR-TB cases among the 1004 samples tested, were Provincial hospitals. The Frere Hospital in East London (BCM district) had the highest number with 63% (10/16) MDR, 25% (4/16) pre-XDR and 12% (2/16) XDR-TB cases. Dora Nginza Hospital followed with 28% (4/14) MDR and 36 % (5/14) pre-XDR and XDR-TB cases respectively. Cecilia Makiwane Hospital was third highest with 67% (8/12) MDR, 25% (3/12) pre-XDR and 8% (1/12) XDR-TB cases. It must be noted that Dora Nginza Hospital in the Nelson Mandela Bay Metro in Port Elizabeth had the highest frequency of XDR-TB cases.

3.2.3.2 Age and Gender

Of the 1004 samples 28% (148/524) females were pre-XDR and 44% (232/524) MDR only, while 28% (136/480) males were pre-XDR and 48% (232/480) MDR only respectively. For the XDR-TB patients 23% (112/480) were males and 28% (144/524) females. The age of patients, ranged from 5 to 85 years for MDR-TB, from 2 to 71 years for Pre-XDR-TB and from 4 to 74 years for XDR-TB. The majority of patients were in the 26 - 30 and 31 - 35 age groups for MDR-TB, pre-XDR and XDR-TB respectively (Table 3.2).

Table 3.2: Age and gender distribution of MDR, pre-XDR and XDR-TB cases.

	Total				Males				Females			
Age (yrs)	**No.**	**MDR**	**Pre-XDR**	**XDR**	**No.**	**MDR**	**Pre-XDR**	**XDR**	**No.**	**MDR**	**Pre-XDR**	**XDR**
<11 yrs	20	6	5	9	2	0	1	1	18	6	4	8
11 - 15	21	11	8	2	7	2	4	1	14	9	4	1
16 - 20	65	33	17	15	23	12	7	4	42	21	10	11
21 - 25	89	45	18	26	31	17	8	6	58	28	10	20
26 - 30	155	73	33	49	62	31	10	21	93	42	23	28
31 - 35	166	75	54	37	84	39	24	21	82	36	30	16
36 - 40	151	67	44	40	72	30	22	20	79	37	22	20
41 - 45	132	54	50	28	79	34	30	15	53	20	20	13
46 - 50	80	40	19	21	50	27	11	12	30	13	8	9
51 - 55	60	26	16	18	34	15	11	8	26	11	5	10
56 - 60	27	14	11	2	15	9	5	1	12	5	6	1
>60 yrs	38	20	9	9	21	16	3	2	17	4	6	7
Total	**1004**	**464**	**284**	**256**	**480**	**232**	**136**	**112**	**524**	**232**	**148**	**144**

3.2.4 Drug susceptibility profiles

Drug susceptibility testing at the NHLS TB Laboratory in Port Elizabeth was done using Middlebrooks 7H11 solid medium during Feb – June 2012, with results for 6 drugs (ethambutol, streptomycin, ethionamide, ofloxacin, capreomycin and amikacin) tested (Table 3.3). In an effort to align with the WHO gold standard recommendations as well as compelling data from several studies involving TB strains from the Eastern Cape, a decision was made to change methodology. The MGIT 960 DST method was introduced in August 2012 and after negotiations with local health authorities, the panel of drugs for testing was reduced to 3 (viz. ofloxacin, amikacin and capreomycin) (Table 3.4). Of the samples selected for this study, 46% (459/1004) were tested using the Middlebrooks 7H11 solid medium method with the panel of six drugs. Analysis of results revealed a high frequency of resistance to second line drugs streptomycin 70% (235/338), amikacin 54% (131/241), capreomycin and amikacin 48% (31/65), ethambutol 66% (38/58) and ethionamide 63% (42/67) in MDR-TB and an increase in resistance in capreomycin 53% (36/68), streptomycin and ofloxacin 91% (103/113), fluoroquinolones (ofloxacin) and amikacin 100% (110/110), capreomycin and amikacin 52% (34/65) and

ethambutol, streptomycin and amikacin 70% (19/27) in XDR-TB (Tables 3.3 and 3.4).

The MGIT 960 DST method was used to test 54% (544/1004) of samples against amikacin, capreomycin and ofloxacin which were included in the panel of three drugs. These samples were consecutively collected from the time that this method was implemented in the Port Elizabeth TB Laboratory. Analysis of results revealed a higher frequency of resistance to the injectables amikacin and capreomycin at 55% amongst the XDR-TB cohort as well as ofloxacin at 89% (144/162). Interestingly, a higher frequency of capreomycin resistance was observed using the MGIT 960 DST method with 45% (116/260) and 55% (144/260) among MDR and XDR-TB strains, respectively.

Table 3.3: Drug susceptibility tests for six anti-TB drugs.

HAIN Genotype MTBDR*plus* kit Resistance to 1st line drugs (RMP + INH)	**Solid media Middlebrooks 7H11 Drug susceptibility tests Resistance to 2nd line drugs (EMB, STR, ETHIO, OFL, CAP, AMI)**	**MDR-TB**	**PRE-XDR**	**XDR-TB**	**Total no. of strains**
+	STR	94	142	103	339
+	STR+EMB	21	11	19	51
+	STR+ETH+OFL+AMI	0	0	21	21
+	STR+ETH+AMI	0	19	21	40
+	STR+OFL	0	10	103	113
+	EMB+OFL	0	4	20	24
+	STR+AMI	0	131	101	232
+	AMI	0	131	110	241
+	CAP	0	32	36	68
+	STR+CAP+AMI	0	31	34	65
+	ETH	18	24	25	67
+	EMB	26	12	20	58
+	STR+OFL+AMI	0	0	103	103
+	STR+OFL+CAP	0	0	36	36
+	CAP+AMI	0	31	34	65
+	OFL+AMI	0	0	110	110
+	EMB+STR+CAP+AMI	0	6	10	16
+	STR+OFL+CAP+AMI	0	0	34	34
+	EMB+STR+ETH	8	6	8	22
+	EMB+STR+AMI	0	8	19	27
+	EMB+STR+ETH+OFL	0	2	8	10
+	EMB+STR+OFL+CAP+AMI	0	0	10	10
+	STR+ETH	15	23	22	60
+	EMB+STR+ETH+OFL+CAP+AMI	0	0	4	4

RMP- Rifampicin, INH- Isoniazid, EMB- Ethambutol, STR-Streptomycin, ETH- Ethionamide, OFL- Ofloxacin, CAP-Capreomycin, AMI-Amikacin

Table 3.4: Drug susceptibility tests for three anti-TB drugs.

HAIN Genotype MTBDR*plus* kit Resistance to 1st line drugs (RMP + INH)	MGIT 960 Resistance to 2nd line drugs (OFL, CAP, AMI)	PRE-XDR	XDR-TB	Total no. of strains
+	OFL	18	144	162
+	CAP	106	130	236
+	AMI	117	144	261
+	OFL+CAP	0	130	130
+	OFL+AMI	0	144	144
+	OFL+CAP+AMI	0	130	130
+	CAP+AMI	100	130	230

RMP- Rifampicin, INH- Isoniazid, OFL- Ofloxacin, CAP-Capreomycin, AMI-Amikacin

3.3 DISCUSSION

Geographic distribution data suggests that the cohort of this study may be representative of the entire Eastern Cape Province. This assumption is based on the Stats SA report, 2013; and the fact that NHLS, PE is the Reference lab for the province, apart from NHLS, Umtata lab, which tests only the immediate vicinity. The majority of samples were from the Amathole, Nelson Mandela Metropolitan and Cacadu health districts (Fig. 3.1). The majority of patients included in this study, came from TB hospitals in the Eastern Cape (EC), with the Nkqubela Chest TB hospital in Mdantsane township (second largest in South Africa) outside East London providing the majority of specimens followed by Empilweni TB hospital in Port Elizabeth providing the second largest number of specimens. Previous studies conducted in the Eastern Cape included samples supplied by the NHLS TB Laboratory in Port Elizabeth that were routinely received from health care facilities throughout the EC and therefore deemed to be representative of the entire EC province. One of the studies investigated the emergence and spread of XDR-TB in the EC and reported that the atypical Beijing strains of pre-XDR and XDR-TB in the EC contained specific clusters of mutation patterns (Klopper *et al*. 2013). This study also reported that these strains were widely distributed in the EC supporting the claim that the atypical Beijing strain had been circulating over a long period of time and being due to the vast geographical distances between sites in the province was inevitably being spread by transmission within the community (Klopper *et al.* 2013).

A higher percentage of samples were collected from female patients (52%), with 48% being from male patients. Twenty eight percent of both male and female patients had pre-XDR TB, with XDR TB being slightly higher among females at 28% and 23% among the male cohort. Interestingly, the data showed the opposite for the MDR *sensu stricto* group of patients with a higher number of males at 48% and 44% being female. The data also revealed that the disease affected all age groups with the majority of patients falling into the 26 – 35 year old age group. Pregnancy, poverty and HIV are likely to be the reasons why a higher percentage of women (52%) have XDR-TB in the EC (WHO global report, 2011). According to the WHO, TB ranks third as the leading cause of death among women between the ages of 15 – 44. Pregnant women are particularly at risk (WHO global report, 2011). According to the Stats SA mid-year population estimates for 2013, 51% of South Africa's population is female and the overall HIV prevalence rate for South Africa is approximately 10%. It is also estimated that 17% of women in their reproductive years, in South Africa, are HIV positive (Stats SA, 2013). Data from the 2011 annual antenatal survey revealed that the Buffalo City district in the Border region of the Eastern Cape, recorded the highest prevalence of HIV at 34.1%, which coincides with the region recording the highest number of MDR and XDR-TB cases recorded for this study. However, no HIV status for the cohort was available for this study.

Studies have shown that the MDR epidemic in South Africa is being driven primarily by transmission of strains within the community. Strain clusters have been detected by DNA fingerprinting and gene sequencing and data indicates that each province in South Africa has unique strain clusters present (Streicher *et al.* 2011). The Eastern Cape strains are predominantly of the atypical Beijing lineage (cluster 86) (Strauss *et al.* 2008). More than 90% of the XDR-TB cases in the Eastern Cape are represented by this strain (Klopper *et al.* 2013). The Western Cape is predominantly represented by the R220 Beijing cluster (Johnson *et al.* 2010) and Kwa-Zulu Natal is represented by the F15/LAM4/KZN strain (Pillay and Sturm, 2007). Gauteng is represented by a variety of strains suggesting the movement of clusters from other provinces, probably due to human migration (Kok *et al.* 2003).

The South African 2011 national TB guidelines for treating drug resistant TB recommend a standardized approach for treating MDR and XDR-TB. For MDR-TB,

this includes an intensive phase of 6 months of treatment with five drugs. The drugs include kanamycin or amikacin, moxifloxacin, ethionamide, terizidone and pyrazinamide, taken at least six times per week during the injectable phase. This is followed by a continuation phase of treatment which includes four drugs, moxifloxacin, ethionamide, terizidone and pyrazinamide, also taken at least six times per week. Levofloxacin may be used in patients who do not tolerate moxifloxacin (Directorate drug-resistant TB, 2011b).

This study data included 46% (459/1004) of the samples which were tested using the Middlebrooks 7H11 solid medium method and included a panel of six drugs (streptomycin, ethionamide, ethambutol, ofloxacin, amikacin and capreomycin) and 54% (544/1004) of the study samples which were tested using the gold standard, MGIT 960 DST method and was reduced to three drugs (amikacin, capreomycin and ofloxacin). Sensitivity of MGIT 960 and Middlebrooks 7H11 methods could not be determined, as the two methods were applied to different batches of samples. However, sensitivity for MGIT 960 has been reported to be good at 98.9% and 98.2% for INH and RMP, with specificities at 98.2% and 99.6%, respectively (Horne *et al*. 2013). SM, EMB and OFL have reported sensitivities of 99.7%, 83.9% and 99.2% and specificities of 94.3%, 95.8% and 99.9%, respectively (Horne *et al*. 2013). According to results obtained from proficiency testing conducted by the WHO and the IUATLD Supranational Reference Laboratory Network, between 1994 and 2002, cumulative sensitivities for Middlebrooks 7H11 solid media DST for INH and RMP were 99% and 97% respectively, but lower for SM and EMB, at 91% and 89% respectively. Specificities were similar, at 98% and 97% for INH and RMP and 94% for both SM and EMB (Richter *et al.* 2009). Sirgel *et al*, (2011) found poor correlation with capreomycin, between MGIT 960 and Middlebrooks 7H11 solid media; therefore MGIT is considered to be the more reliable method.

The results for patients tested using the 6 drug panel revealed a high frequency of resistance to streptomycin at 70% (235/338) among the MDR *sensu stricto* group and 30% (103/338) among the XDR group. Ethambutol followed with 66% (38/58) and 24% (20/58) resistance among the MDR and XDR-TB groups respectively. Ethionamide showed 63% (42/67) and 37% (25/67) respectively and the injectables

revealed amikacin 54% (131/241) and 46% (110/241) respectively, capreomycin 47% (32/68) and 53% (36/68) respectively.

Ethionamide is a drug used in both the injectable phase as well as the continuation phase of MDR-TB treatment. It is well documented that the Eastern Cape has an over representation of the atypical Beijing strain of TB among pre-XDR and XDR-TB isolates which include *inh*A mutations that confer cross-resistance to INH and ethionamide (Banerjee *et al*. 1994, Morlock *et al*. 2003; Muller *et al.* 2011). This study data shows 63% resistance to ethionamide among the MDR isolates and 37% resistance among the XDR isolates which questions the effectiveness of including this drug in the 5 drug regimen in the Eastern Cape.

Amikacin and kanamycin inclusion in the MDR regimen is also questionable with the data revealing that among the samples tested using 6 drugs, 54% (121/241) of the MDR isolates and 46 %(110/241) of the XDR isolates were resistant to amikacin and among the 3 drug panel using the MGIT 960 DST method, similar levels of resistance were observed at 45% (116/206) and 55%(144/260) among MDR and XDR isolates respectively. It is well documented that high levels of cross-resistance exists between amikacin and kanamycin (Alangadan *et al.* 1998; Jugheli *et al*. 2009; Maus *et al.* 2005).

The high incidence of amikacin/ kanamycin resistance and ineffectiveness of ethionamide along with the known pyrazinamide resistance among MDR-TB in South Africa (Louw *et al.* 2006; Mpahlele *et al*. 2008), effectively limits the recommended standardized regimen for treating MDR TB in the Eastern Cape to two drugs (moxifloxacin and terizadone).

The 2011 TB guidelines for XDR TB treatment suggest that at least 4 known effective drugs or drugs that the patient has not been exposed to before, should be used (Directorate drug-resistant TB, 2011b). They also suggest that at least one injectable with known susceptibility be used. Drugs with known cross-resistance should be avoided as well as drugs known to be harmful to the patient. Other drugs should be included in the regimen based on side effect, drug susceptibility and use of moxifloxacin instead of ofloxacin is recommended due to evidence of improved

outcomes. Group 5 drugs (clofazamine, amoxicillin/clavulanate, clarithromycin, azithromycin, linezolid, thiacetazone, imipenem and high dose INH) may be used when necessary with clofazimine being the drug of choice in this group (Directorate drug-resistant TB, 2011b).

The recommended standard regimen for treating adult XDR TB in South Africa is capreomycin, moxifloxacin, ethionamide, terizidone, pyrazinamide, PAS and clofazimine. Ethionamide and pyrazinamide have already been shown to be ineffective in treating MDR-TB in the Eastern Cape, thus limiting the available drugs to capreomycin, moxifloxacin, terizidone, PAS and clofazimine.

Cross resistance between amikacin, kanamycin and capreomycin is well documented due to the shared A1401G mutation in the *rrs* gene (Maus *et al*. 2005; Jugheli *et al*. 2009, Sirgel *et al*. 2011). Data from this study supports published data from the Eastern Cape (Sirgel *et al*. 2011) showing reduced susceptibility to capreomycin with 53% and 55% among XDR-TB isolates using the solid and liquid media for DST, respectively.

Co-infection with HIV has a devastating impact on TB treatment outcomes (Wells *et al*. 2007; Friedland *et al.* 2007). The prevalence of HIV amongst a cohort of MDR patients from eight South African provinces between 2000 and 2004, reported that patients co-infected with HIV compared to those who were HIV negative, were less likely to have a successful treatment outcome (40.0 versus 49.6; $P<0.05$) and more likely to die (35.2 versus 16.2; $P<0.0001$), particularly those associated with low body weight (Farley *et al*. 2011). Other significant findings from this study were that MDR-TB diagnosis to initiation of treatment was delayed by an average of more than 2 months and that many patients from the cohort were self-administering MDR therapy during the continuation phase, which could also explain the low treatment outcomes (Farley *et al*. 2011). However, a major limitation in this study has been that the clinical information with regard to HIV status of the patients could not be obtained.

The new generation fluoroquinolone, moxifloxacin was incorporated in the standardized XDR-TB treatment regimen as a replacement drug for ofloxacin. Cross-resistance among flouroquinolones due to shared mutations in the *gyrA* region is

also well documented (Devasia *et al.* 2009; von Groll *et al*. 2009). Routine DST testing for moxifloxacin is not currently done in South Africa but published data suggest reduced susceptibility to moxifloxacin (Devasia *et al*. 2009; von Groll *et al*. 2009) thus limiting the drugs available for treatment of XDR-TB to terizidone, PAS and clofazimine.

Routine DST testing for terizidone, PAS and clofazamine is not currently done in South Africa, however, recent studies done on 45 isolates from the Eastern Cape confirmed resistance to PAS at a level of >4.0 µg/ml in 9/45 (20%) of isolates (Klopper *et al*. 2013). The effectiveness of the three remaining drugs, terizidone, a bacteriostatic agent, PAS, known to be poorly tolerated, and clofazimine, documented to be successful in a meta-analysis that showed 65% and 66% success in MDR and XDR-TB cases respectively (Gopal *et al.* 2013), is unknown in South Africa and in particular, the Eastern Cape.

As suggested in a recent study involving isolates from the Eastern Cape (Klopper *et al.* 2013), the continued standardized treatment approach and the lack of individualised treatment regimens for treating MDR and XDR-TB in the Eastern Cape, poor drug tolerance and compliance, poor infection control, delay in initiation of treatment due to limited bed space in MDR and XDR-facilities, the potential for drug resistant TB strains in the Eastern Cape evolving and becoming Totally Drug Resistant (TDR) in the Eastern Cape is inevitable.

Previous studies have already shown a strong association between *inh*A promoter mutations and XDR-TB (Muller *et al.* 2011) and that the spread of XDR-TB due to poor management of these cases is resulting in fewer effective drugs being available to treat the disease (Streicher *et al*. 2011). This study data included a large cohort (n=1004) where >50% of isolates tested were either pre-XDR (28%) or XDR (26%). The major limitation was the absence of HIV status of patients which may exacerbate the TB epidemic in the province. This emphasises the seriousness of the MDR and XDR-TB problem in the EC supporting claims from previous studies that Total Drug Resistance will be becoming a reality in the EC.

CHAPTER FOUR

ANALYSIS OF GENE MUTATION PATTERNS BASED ON HAIN GENOTYPE MTBDRPLUS KIT

4.1 INTRODUCTION

The Genotype MTBDR*plus* version 2.0 assay is a qualitative *in vitro* test used for the identification of *Mycobacterium tuberculosis* complex and detection of resistance to Rifampicin (RMP) and Isoniazid (INH) from both clinical samples and culture isolates (HAIN MTBDR*plus* kit manual). The complex includes species of *M. tuberculosis, M. africanum, M. bovis subsp. bovis, M. bovis subsp. caprae, M. bovis BCG, M. microti, M. canettii,* and *M. pinnipedii.* Mutations in the *rpo*B gene which encodes the β-subunit of RNA polymerase indicates RMP resistance. Mutations in the *kat*G gene, which encodes catalase peroxidase, confer high level resistance to INH and mutations in the *inh*A promoter region which encodes NADH enoyl ACP reductase, confer low level resistance to INH (HAIN Genotype MTBDR*plus* VER 2.0 package insert).

The test is based on DNA•STRIP® technology. This process is carried out in three stages. The first stage involves extraction and isolation of DNA from the sample (clinical sample or culture isolate). The second stage involves replication of selected nucleic acids in the DNA strand, during the amplification procedure. The third stage involves chemical denaturation of the double stranded DNA to single stranded DNA, which is required as the DNA strip contains specific probes which complement the selected amplicons. This is followed by the specific single stranded amplicons binding to the probes during the hybridization process, while the non-specific amplicons are removed during the wash process. A streptavidin-conjugated alkaline phosphatase is then added during the conjugation reaction which binds to the bound amplicon. Finally, a substrate is added, which is transformed into a dye by the enzyme alkaline phosphatase, making it visible in a colorimetric detection reaction. Specific banding patterns can be visualised and read manually, using a specifically designed template or by using the automated GenoScan®, which scans and interprets the DNA strips (HAIN Genotype MTBDR*plus* VER 2.0 package insert).

In 2007, a memorandum of Understanding (MOU) was established between the Foundation for Innovative New Diagnostics (FIND), the National Health Laboratory Service (NHLS) and the South African Medical Research Council (SAMRC) to investigate selected tests for the rapid diagnosis of MDR-TB (SAMRC, 2007). Patients from four South African provinces (Western Cape, Northern Cape, Northwest province and Gauteng) were included in the project, with the aim of testing 20000 sputum samples from high risk MDR patients (WHO, 2008b). The HAIN Genotype MTBDR*plus* VER 1.0 assay was compared with the gold standard MGIT culture in both laboratories but MGIT 960 DST was used in the Johannesburg laboratory and the indirect proportion method on Middlebrook 7H11 solid medium was used in the Cape Town laboratory. The overall sensitivity for resistance to RMP, INH and MDR-TB detected by HAIN Genotype MTBDR*plus* VER 1.0 assay was 98.4%, 91.4% and 96.5% respectively and the specificity was 99.1%, 99.7% and 99.7% respectively (WHO, 2008b).

In addition, a preliminary validation study of the HAIN GenoType MTBDR*plus* VER 1.0 assay on a random selection of 100 smear negative specimens showed a higher proportion (96.8%) of interpretable results using the MTBDR*plus* VER 1.0 assay compared to conventional culture and DST methodology (86.6%). Smear grading of samples did not affect interpretation of results with 1+, 2+ or 3+ smear positive samples having 94.6%, 98.2% and 97.1% interpretable results respectively. Contaminated MGIT cultures (92.7%) gave interpretable results on the MTBDR*plus* VER 1.0 assay. Turnaround time for conventional TB cultured samples ranged from 23 to 99 days as opposed to the 1 to 2 day turnaround time for both smear positive and smear negative samples tested using the MTBDR*plus* VER 1.0 assay. The sensitivity for detecting RMP resistance, INH resistance and MDR-TB was 99%, 94% and 99% respectively and the specificity was 99%, 100% and 100% respectively (Barnard *et al.* 2008).

The sensitivity of smear microscopy is low, particularly in areas with a high HIV prevalence (Getahun *et al.* 2007). Approximately 50 - 60% of pulmonary TB cases can be diagnosed using smear microscopy in well-equipped laboratories. These figures can be even lower in poor income countries and countries with a high prevalence of HIV (Siddiqi *et al*. 2003). The version 1.0 of the HAIN Genotype

MTBDR*plus* assay was only recommended for smear positive sputum samples or culture positive samples (HAIN Genotype MTBDR*plus* VER 1.0 package insert). The HAIN GenoType MTBDR*plus* VER 2.0 assay was developed and introduced as an improvement to the version 1.0 of this assay with notable improvements in the DNA strip technology, allowing testing on smear negative samples, which was previously not recommended on version 1.0 of this assay as well as the inclusion of ready-made master mix solutions for ease of use and improved cost effectiveness (HAIN lifeScience). A recent South African study compared the new version 2.0 of the MTBDR*plus* assay with the Xpert MTB/RIF assay, recently endorsed by the WHO. Consecutive smear positive and smear negative samples were tested using both assays and compared with conventional MGIT 960 culture and the GenoType MTBR*plus* VER 1.0 assay (Barnard *et al*. 2012). The sensitivity and specificity of the MTBDR*plus* VER 2.0 assay was 73.1% and 100% respectively.

Therefore the objective of this chapter was to analyze the pattern of gene mutations in resistant *Mycobacterium tuberculosis* isolates using the Genotype MTBDR*plus* Assay kit (HAIN Lifescience, Version 2.0).

4.2 RESULTS

4.2.1 Genotype membrane strips

Evaluation and interpretation of mutations detected by the assay kit were done as outlined in section 2.5.4 and interpreted according to Fig. 2.1 (Chapter Two). Examples of membrane strips are shown below in Figs. 4.1 – 4.5.

Fig 4.1 shows an example of a positive and a negative control strip. All control bands (conjugate, amplification and M. *tuberculosis*) and all ΔWT bands for *rpo*B, *kat*G and *inh*A are present with no mutation bands present (Fig 4.1A) while the negative control strip only had the conjugate and amplification controls (Fig 4.1B).

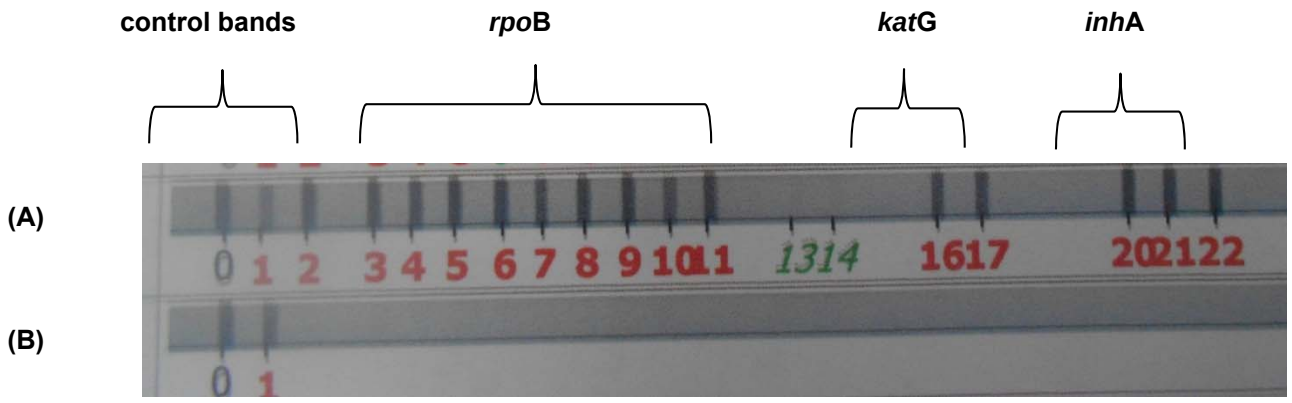

Figure 4.1: Membrane strips coated with specific probes which bind to amplicons. (A): positive control (*M. tuberculosis* ATCC 25177) . (B): negative control (water).

Figure 4.2 shows examples of MDR–TB. Strip (A) shows MUT3 present at band 15, in the *rpo*B region with a weaker ΔWT8 at band 11; absent ΔWT at band 17 and MUT1 alongside in the *kat*G region and ΔWT present, no MUT at bands 21 and 22 in the *inh*A region of the strip. Strip (B) shows missing ΔWT 3 and 4 at bands 6 and 7 and MUT1 present in the *rpo*B region; missing ΔWT and MUT1 at band 18 in the *kat*G region and a missing ΔWT1, no MUT at band 21 in the *inh*A region of the strip.

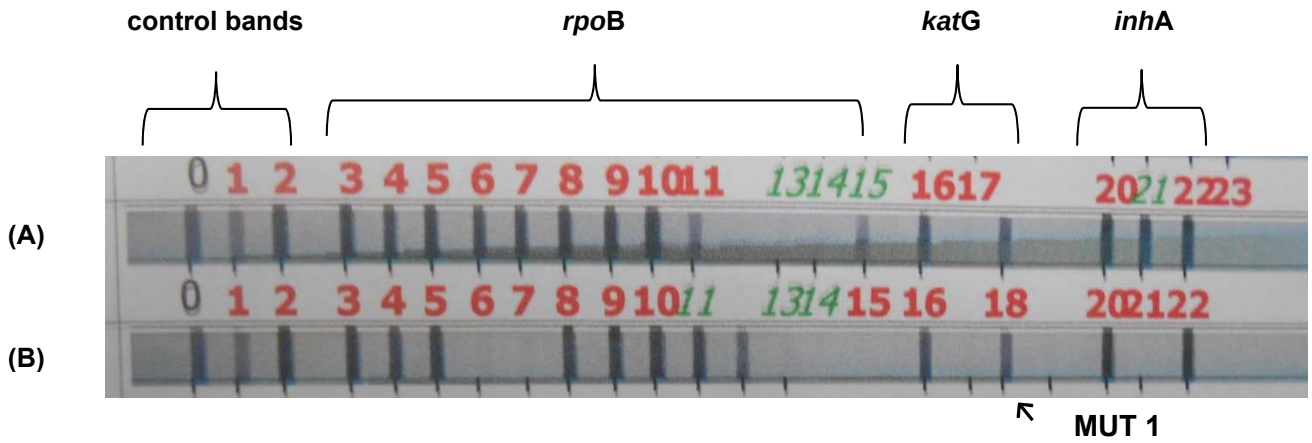

Figure 4.2: MDR-TB Membrane strips. (A): *rpo*B region; MUT3+ΔWT8+ΔWT, *kat*G region; MUT1+ΔWT. (B): *rpo*B region; ΔWT 3+4 + MUT1; *kat*G region ΔWT+MUT1; *inh*A region; ΔWT1+MUT.

Figure 4.3 shows an example of *hetero-resistance* to isoniazid and rifampicin. Here, all ΔWT bands are present in the *rpo*B, *kat*G and *inh*A regions of the strip, however, MUT 3 at band 15 in the *rpo*B region and MUT 1 at band 18 in the *kat*G region are also present, indicating *hetero-resistance* to both INH and RMP.

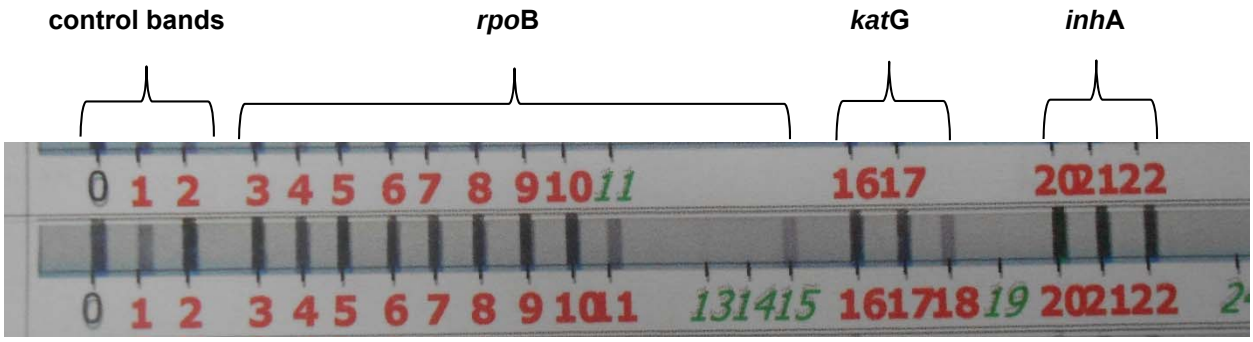

Fig 4.3: Membrane strip with hetero-resistance to INH and RMP.

Figure 4.4 shows an example of two MDR-TB isolates. Strip (A) with ΔWT missing at band 21 and MUT1 present at band 23, seen in the *inh*A region and confers low level resistance to INH and strip (B) with ΔWT missing and MUT1 present at band 18, seen in the *kat*G region and confers high level resistance to INH.

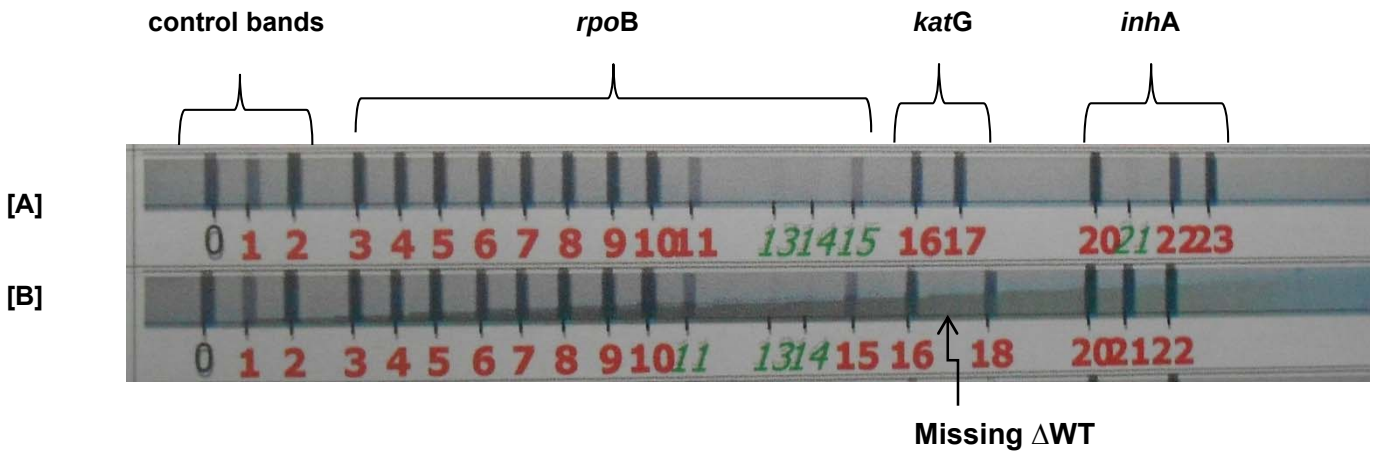

Fig 4.4: Membrane strips indicating high (B) and low (A) level resistance to INH.

Figure 4.5 shows an example of two different strains of MDR-TB. Both strains have a weak ΔWT8 at band 11 as well as a MUT3 at band 15 in the *rpo*B region, conferring resistance to RIF. Strip (A), however, has a missing ΔWT and MUT1 present at band 18 in the *kat*G region as well a missing ΔWT at band 21 and a MUT1 at band 23 in the *inh*A region of the strip. This confers both high and low level resistance to INH. Strip (B) shows missing ΔWT and MUT1 present at band 18 in the *kat*G region only, conferring high level resistance to INH.

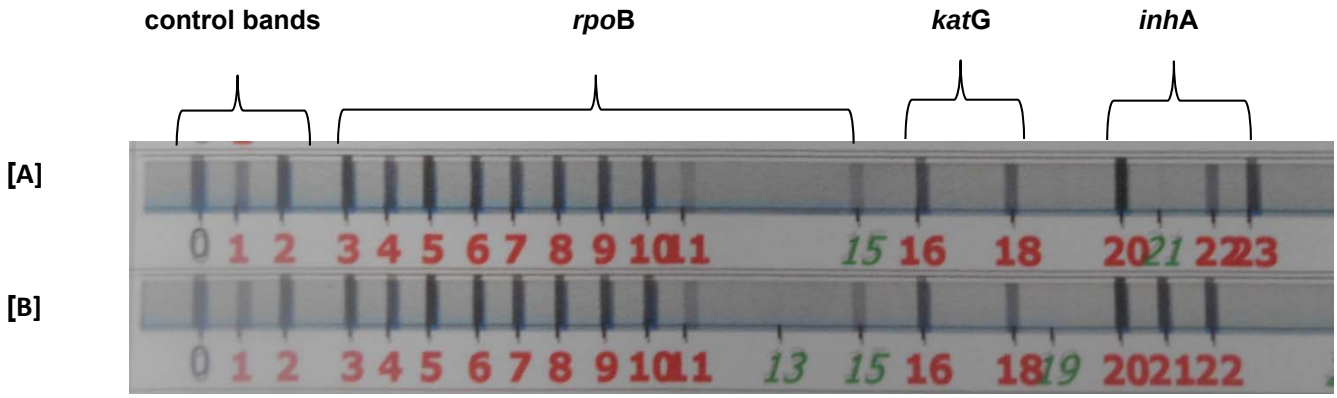

Fig 4.5: Membrane strips with two different strains of MDR-TB.

4.2.2 Gene Mutations

The most common mutation patterns (Table 4.1) observed were: **(i)** 46.0% (457/1004) ΔWT 3, 4, MUT1 [D516V+del515] (*rpo*B); ΔWT, MUT1 [S315T1] (*kat*G); ΔWT1 [C15T] (*inh*A) [39 MDR, 204 XDR-TB and 214 pre XDR-TB isolates]. **(ii)** 6.2% (62/1004) ΔWT8, MUT3 [S531L] (*rpo*B); ΔWT, MUT1 [S315T1] (*kat*G); ΔWT1, MUT1 [C15T] (*inh*A) [19 MDR, 25 pre-XDR and 18 XDR-TB isolates]. **(iii)** 4.6% (46/1004) ΔWT8, MUT3 [S531L] (*rpo*B); ΔWT1, MUT1 [C15T] (*inh*A) [37 MDR, 7 pre-XDR and 2 XDR-TB isolates]. **(iv)** 3.5% (35/1004) ΔWT8, MUT3 [S531L] (*rpo*B); ΔWT, MUT1 [S315T1] (*kat*G); ΔWT2, MUT3B [T8A] (*inh*A) [30 MDR, 3 pre-XDR and 2 XDR-TB isolates]. **(v)** 2.8% (28/1004) ΔWT 3, 4, MUT1 [D516V+del515] (*rpo*B); ΔWT, MUT1 [S315T1] (*kat*G) [21 MDR, 2 pre-XDR and 5 XDR-TB isolates].

Mutations in the *inh*A promoter region were found in 66.6% (669/1004) of the isolates. 25.4% of *inh*A mutations (170/669) were found among the MDR isolates, 39.2% (262/669) among the pre-XDR isolates and 35.4% (237/669) among the XDR-TB isolates. Mutations in the *kat*G region were detected in 33.4% (335/1004) of the isolates. 94.3% of *kat*G mutations (316/335) were found among the MDR isolates with 6.6% (22/335) among the pre-XDR isolates and only 5.7% (19/335) were among the XDR-TB isolates.

Table 4.1: Pattern of gene mutations in resistant *Mycobacterium tuberculosis* isolates using Genotype MTBDR*plus* Assay kit (HAIN Lifescience, Version 2).

RMP pattern (*rpoB*)	INH pattern (*kat*G)	INH pattern (*inh*A)	Result	No. isolates (n=1004)	(%)# isolates	Total no. of isolates
ΔWT 3,4, MUT1	ΔWT, MUT1	ΔWT	MDR	39	8.0	
			XDR	204	45.0	n=457
			PRE-XDR	214	47.0	
ΔWT 3,4, MUT1	ΔWT, MUT1		MDR	21	75.0	
			XDR	5	18.0	n=28
			PRE-XDR	2	7.0	
ΔWT8, MUT3	ΔWT, MUT1	ΔWT, MUT1	MDR	19	31.0	
			XDR	18	29.0	n=62
			PRE-XDR	25	40.0	
ΔWT8, MUT3	ΔWT, MUT2		MDR	12	92.0	
			XDR	1	8.0	n=13
			PRE-XDR	0	-	
ΔWT8, MUT3		ΔWT, MUT1	MDR	37	80.4	
			XDR	2	4.4	n=46
			PRE-XDR	7	15.2	
ΔWT8, MUT3	ΔWT, MUT1	ΔWT2, MUT3B	MDR	30	86.0	
			XDR	2	6.0	n=35
			PRE-XDR	3	8.0	
ΔWT7,MUT2A	ΔWT, MUT1	ΔWT, MUT1	MDR	2	67.0	n=3
			XDR	1	33.0	
ΔWT7,MUT2A	ΔWT, MUT1		MDR	8	89.0	
			XDR	0	-	n=9
			PRE-XDR	1	11.0	
ΔWT7,MUT2A		ΔWT, MUT1	MDR	5	100.0	n=5
ΔWT7,MUT2B	ΔWT, MUT1	ΔWT, MUT1	MDR	0	-	
			XDR	0	-	n=3
			PRE-XDR	3	100.0	
ΔWT7,MUT2B	ΔWT, MUT1	MUT3B	MDR	2	100.0	n=2
ΔWT7,MUT2B	ΔWT, MUT1		MDR	13	1.3%	n=13
ΔWT7,MUT2B		ΔWT, MUT1	MDR	8	89.0	n=9
			XDR	1	11.0	
ΔWT7	ΔWT, MUT1		MDR	7	88.0	
			XDR	0	-	n=8
			PRE-XDR	1	12.0	
ΔWT7		ΔWT, MUT1	MDR	4	100.0	n=4
ΔWT2	ΔWT, MUT1		MDR	1	100.0	n=1
ΔWT2		ΔWT, MUT1	MDR	1	100.0	n=1
ΔWT4	ΔWT, MUT1		MDR	1	100.0	n=1

Δ: deletion; WT: wild type; MUT: mutation; RMP: rifampiin; INH: isoniazid; #: % isolates was calculated using no. of isolates for MDR, XDR and Pre-XDR, out of total no. of isolates (n=1004) for each specific pattern.

4.2.3 Mutation patterns according to geographic locations

The gene mutation patterns from 27 health care facilities in the EC with the highest number of MDR and XDR-TB cases were analysed. The data was also divided into the different health districts in an effort to observe whether the most common mutation patterns were epidemiologically or geographically linked (Table 4.2). The majority of isolates from the Buffalo City Metro (BCM) had the *inh*A promoter mutation present, with Nkqubela Chest Hospital having 60% *inh*A mutations and 40% *kat*G mutations and Frere Hospital having 56% and 44% *inh*A and *kat*G mutations respectively. The exception in this area however, was Pefferville clinic, where the opposite was seen where the majority of isolates (75%) had the *kat*G mutation with only 25% of isolates having an *inh*A promoter mutation.

79% (22/28) of isolates from the two sites with the highest number of MDR-TB and XDR-TB cases in the Cacadu health district had the *inh*A promoter mutation present, with 78% and 79% in isolates from the Addo Fixed clinic and Marjory Parrish TB Hospital respectively. The Nelson Mandela Metropolitan health district also had significantly higher numbers of isolates with *inh*A mutations, with some health care facilities having *inh*A promoter mutations in 100% of isolates (Mabandla clinic, Motherwell Community Health Centre and Veeplaas clinic).

Interestingly, isolates from Zithulele Hospital in the Oliver Tambo health district in the former Transkei area, and geographically far removed from the other health districts, had *kat*G and *inh*A mutations in 70% and 30% of isolates respectively.

Table 4.2: Summary of *kat*G and *inh*A mutations according to geographic locations.

Location	Health district	*kat*G mutations		*inh*A mutations		Total
		% of isolates	No. of isolates	% of isolates	No. of isolates	
Cecilia Makewani Hospital	Buffalo City Metro	25	3	75	9	12
Frere Hospital	Buffalo City Metro	44	7	56	9	16
Gompo C clinic	Buffalo City Metro	36	5	64	9	14
Nkqubela Chest Hospital	Buffalo City Metro	40	38	60	58	96
NU2 clinic	Buffalo City Metro	27	4	73	11	15
NU8 clinic	Buffalo City Metro	40	4	60	6	10
Pefferville clinic	Buffalo City Metro	75	6	25	2	8
Addo fixed clinic	CACADU	22	2	78	7	9
Margery Parrish TB hospital	CACADU	21	4	79	15	19
Booysens Park clinic	Nelson Mandela Bay Metro	31	4	69	9	13
Chatty clinic	Nelson Mandela Bay Metro	12	2	88	14	16
Dora Nginza Hospital	Nelson Mandela Bay Metro	21	3	79	11	14
Empilweni Hospital	Nelson Mandela Bay Metro	21	6	79	23	29
Gqebega clinic	Nelson Mandela Bay Metro	20	3	80	12	15
Kwazakhele clinic	Nelson Mandela Bay Metro	31	4	69	9	13
Laeticia Bam Day Hospital	Nelson Mandela Bay Metro	40	6	60	9	15
Lunga Kobese clinic	Nelson Mandela Bay Metro	27	3	73	8	11
Mabandla clinic	Nelson Mandela Bay Metro	0	0	100	9	9
Missionvale clinic	Nelson Mandela Bay Metro	10	1	90	9	10
Mother NU11 clinic	Nelson Mandela Bay Metro	10	1	90	9	10
Motherwell Community Health Centre	Nelson Mandela Bay Metro	0	0	100	9	9
Motherwell NU2 clinic	Nelson Mandela Bay Metro	18	2	82	9	11
Rosedale clinic	Nelson Mandela Bay Metro	13	2	87	13	15
Tanduxolo clinic	Nelson Mandela Bay Metro	20	2	80	8	10
Veeplaas clinic	Nelson Mandela Bay Metro	0	0	100	8	8
Zwide clinic	Nelson Mandela Bay Metro	27	3	73	8	11
Zithulele Hospital	Oliver Tambo	70	7	30	3	10

Of the 46.0% (457/1004) of isolates that exhibited the mutation pattern ΔWT 3, 4, MUT1 [D516V+del515] (*rpo*B); ΔWT, MUT1 [S315T1] (*kat*G); ΔWT1 [C15T] (*inh*A), the majority (52% (238/457)) were from the Nelson Mandela Metropole (NMM) (Table 4.3). 26% (119/457) were MDR, 26% (119/457) were XDR and 24% (109/457) were pre-XDR. The largest number of isolates from this region came from

Empilweni TB Hospital (8/119) in Port Elizabeth and all were XDR-TB. 4/62 of the 6.2% (62/1004) of isolates that exhibited the mutation pattern ΔWT8, MUT3 [S531L] (*rpo*B); ΔWT, MUT1 [S315T1] (*kat*G); ΔWT1, MUT1 [C15T] (*inh*A), were from the NU2 clinic in the Buffalo City Metropole. Two of the isolates were pre-XDR and two were XDR-TB.

6/46 of the 4.6% (46/1004) isolates that exhibited the mutation pattern ΔWT8, MUT3 [S531L] (*rpo*B); ΔWT1, MUT1 [C15T] (*inh*A) were from the Nkqubela Chest Hospital in the Buffalo City Metropole, East London. All 6 isolates were MDR.

9/35 of the 3.5% (35/1004) of isolates that exhibited the mutation pattern ΔWT8, MUT3 [S531L] (*rpo*B); ΔWT, MUT1 [S315T1] (*kat*G); ΔWT2, MUT3B [T8A] (*inh*A) were from the Nkqubela Chest Hospital in the Buffalo City Metropole, East London. All but one of these isolates had MDR-TB and one isolate had pre-XDR-TB. The majority (4/28) of the 2.8% (28/1004) of isolates that had ΔWT 3, 4, MUT1 [D516V+del515] (*rpo*B); ΔWT, MUT1 [S315T1] (*kat*G) were also from Nkqubela Chest Hospital in the Buffalo City Metropole, East London. One isolate had XDR-TB and the remaining 3 isolates were MDR.

Table 4.3: Pattern of gene mutations in resistant *Mycobacterium tuberculosis* isolates and geographic locations

RMP pattern (*rpoB*)	INH pattern (*kat*G)	INH pattern (*inh*A)	Result	Geographic location with the highest no. of isolates exhibiting mutation pattern
ΔWT 3,4, MUT1	ΔWT, MUT1	ΔWT	MDR	NMM (119)
			XDR	NMM (119)
			PRE-XDR	NMM (109)
ΔWT 3,4, MUT1	ΔWT, MUT1		MDR	BCM (9)
			XDR	AM (3)
			PRE-XDR	NMM (1)
ΔWT8, MUT3	ΔWT, MUT1	ΔWT, MUT1	MDR	BCM (15)
			XDR	BCM (7)
			PRE-XDR	BCM (10)
ΔWT8, MUT3	ΔWT, MUT2		MDR	NMM (6)
			XDR	CH (1)
			PRE-XDR	-
ΔWT8, MUT3		ΔWT, MUT1	MDR	BCM (11)
			XDR	CAC (1); AM (1)
			PRE-XDR	NMM (1);CAC (1); AM (1)
ΔWT8, MUT3	ΔWT, MUT1	ΔWT2, MUT3B	MDR	BCM (17)
			XDR	AM (2)
			PRE-XDR	BCM (2)
ΔWT7,MUT2A	ΔWT, MUT1	ΔWT, MUT1	MDR	BCM (1); AM (1)
			XDR	AM (1)
ΔWT7,MUT2A	ΔWT, MUT1		MDR	BCM (5)
			XDR	-
			PRE-XDR	AM (1)
ΔWT7,MUT2A		ΔWT, MUT1	MDR	NMM (2)
ΔWT7,MUT2B	ΔWT, MUT1	ΔWT, MUT1	MDR	BCM (3)
			XDR	-
			PRE-XDR	BCM (3)
ΔWT7,MUT2B	ΔWT, MUT1	MUT3B	MDR	AM (1); JG (1)
ΔWT7,MUT2B	ΔWT, MUT1		MDR	AM (3)
ΔWT7,MUT2B		ΔWT, MUT1	MDR	NMM (3)
			XDR	AM (1)
ΔWT7	ΔWT, MUT1		MDR	BCM (2); CAC (2)
			XDR	-
			PRE-XDR	BCM (1)
ΔWT7		ΔWT, MUT1	MDR	BCM (2)
ΔWT2	ΔWT, MUT1		MDR	OT (1)
ΔWT2		ΔWT, MUT1	MDR	BCM (1)
ΔWT4	ΔWT, MUT1		MDR	CH (1)

Δ**:** deletion; WT: wild type; MUT: mutation; RMP: rifampiin; INH: isoniazid; NMM: Nelson Mandela Metro; BCM: Buffalo City Metro; AM: Amathole; CH: Chris Hani; JG: Joe Gqabi; CAC: Cacadu

4.3 DISCUSSION

The World Health Organization (WHO) approved the use of the HAIN Genotype MTBDR*plus* test for the rapid diagnosis of INH and RMP resistance, which also allows for the simultaneous identification of *M. tuberculosis* complex (MTBC) strains in clinical isolates (WHO, 2008b; Warren *et al*. 2009). However, there are no reports of the performance of HAIN Genotype MTBDR*plus* assay kit, for the screening of MDR-TB in the Port Elizabeth region.

The Genotype MTBDR*plus* version 1.0 of the assay kit had already been extensively validated and the fact that the version 2.0 of this assay was regarded as an improvement of existing technology, no large scale validation was required. The Port Elizabeth TB Laboratory chose to do an "in-house" validation; comparing 20 smear positive isolates against both versions 1.0 and 2.0 of the assay. This exercise was to confirm that the assay was fit for use under local laboratory conditions. Slight changes in interpretation were noted in the version 2.0 of the assay, notably that if ∆WT8 is weaker than the AC control band and no MUT3 band is present in the *rpo*B region of the strip, the ∆WT8 was still to be considered as present on the VER 2.0, where previously on VER 1.0, it was considered to be missing if weaker than the amplification control band. However, if the MUT3 band was present in the *rpo*B region of the strip and the ∆WT8 was still weakly present, the ∆WT8 was considered as missing in the version 2.0 of this assay (HAIN Genotype MTBDR*plus* VER 2.0 package insert). The results of the "in-house" validation showed 100% concordance and the assay was deemed fit for use. It must be noted that due to the high number of smear negative samples received for TB Culture and first line sensitivity testing, in the Port Elizabeth TB Laboratory, probably due to the high HIV prevalence in the area, a decision was made not to include smear negative samples routinely for testing.

Molecular line probe assays for first line DST are WHO endorsed and considered the gold standard for first line DST thus implying that phenotypic testing is not required as confirmation of first line DST (WHO 2010b). Most NHLS TB Culture laboratories are currently using the Genotype MTBDR*plus* VER 2.0 assay for first line DST.

Validation studies of the Genotype MTBDR*plus* VER 1.0 assay was conducted in Gauteng directly on the sputum samples and cultured isolates. Sensitivity of the kit for detection of resistance to RMP, INH and MDR-TB was 95%, 93.4% and 100% respectively, while the specificity was 99.7%, 100% and 100% respectively (Matsoso *et al.* 2010). However, there was a recommendation that the assay not be used for smear negative samples or paucibacillary sputum samples as it did not produce interpretable results (Dorman *et al*. 2012). The Genotype MTBDR*plus* VER 2.0 assay was compared to conventional smear microscopy, MGIT 960 culture and DST and showed a combined sensitivity and specificity of smear negative samples to be 79.8% and 99.2% respectively (Crudu *et al.* 2012).

Previous studies indicate that atypical Beijing strains of pre-XDR and XDR-TB originated in 5 of the 8 district municipalities (Amathole, Cacadu, Chris Hani, Nelson Mandela Bay and O R Tambo) in the Eastern Cape with the largest atypical pre-XDR Beijing cluster (MP5) was also found to be present in 4 adjacent districts (Amathole, Cacadu, Nelson Mandela Bay and Oliver Tambo) and the largest XDR-TB cluster (MP6) in 3 of these districts as well as an extra district (Amathole, Cacadu, Nelson Mandela Bay and Chris Hani) suggesting that these strains have been circulating over an extended period of time similar to findings by Klopper *et al*, (2013).

Two thirds [66.6% (669/1004)] of the isolates in this current study had *inh*A mutations present with 25.4% (170/669) found among the MDR isolates, 39.2% (262/669) among the pre-XDR isolates and 35.4% (237/669) among the XDR-TB isolates. This is of concern as research has shown that these strains are being spread by transmission within the community and circulating in the province (Strauss *et al.* 2008; Müller *et al*. 2011; Klopper *et al.* 2013). The remaining one third [33.4% (335/1004)] of isolates had mutations in the *kat*G region with 94% (316/335) among the MDR isolates with only 7% and 6% among the pre-XDR and XDR-TB isolates respectively.

The mutation distribution data collected from this study highlights differences between the health districts as well as differences between the facilities within the health districts (Table 4.2). 60% of isolates from the Nkqubela chest Hospital situated in the Buffalo City Metropole (BCM), where the majority of study samples

were received from, had *inh*A promoter mutations and 40% had *kat*G mutations. Similarly, other sites in the same area such as Frere Hospital had 56% and 44% *inh*A and *kat*G mutations respectively. In contrast, however, 70% of isolates from Pefferville clinic in the BCM had *kat*G mutations with only 30% of isolates from this clinic having *inh*A mutations. This could be explained by the transmission of a unique/ different strain in the immediate area surrounding the Pefferville clinic. Patients in the immediate area would seek health care from the nearest facility, which could explain why samples sent from Pefferville clinic have predominantly *kat*G mutations.

Samples received from the Margery Parrish TB Hospital in the Cacadu health district showed that a larger percentage of isolates (79%) had the *inh*A promoter mutations present with only 21% of isolates from the region having *kat*G mutations. In contrast, isolates received from the Oliver Tambo health district in the former Transkei area, which is geographically far removed from all other health districts had a very different mutation distribution with 70% of the isolates having *kat*G mutations present and 30% with *inh*A mutations, thus showing that different strains of MDR, pre-XDR and XDR-TB are circulating in these areas.

Samples received from the Nelson Mandela Metropole (NMM); show that the vast majority of isolates in this health district have *inh*A mutations. 79% of isolates received from the Empilweni TB Hospital were found to have *inh*A promoter mutations with 21% having *kat*G mutations. Chatty clinic and Gqebega clinic in the NMM showed similar strain distributions at 88% and 80% *inh*A mutations and with 12% and 20% *ka*tG mutations respectively. Some health care facilities within the NMM such as Mabandla clinic, Missionvale clinic, Motherwell NU11 clinic and Motherwell Health Centre had 90 - 100% of isolates with *inh*A mutations. This data clearly shows that a larger proportion of isolates collected from the Nelson Mandela Metropole have *inh*A mutations if compared to the Buffalo City Metropole.

Mutations in *inh*A promoter regions were strongly associated with XDR-TB isolates. This supports findings by Muller *et al.* (2011) and Chihota *et al.* (2011) where *inh*A promoter mutations were strongly associated with XDR-TB in South Africa. This data is of extreme concern as it indicates that the majority of MDR isolates in the

immediate Port Elizabeth area (NMM) as well as in the Buffalo City Metropole and Cacadu health districts have the *inh*A promoter mutation present and are according to Klopper *et al.* (2013), likely to be of the atypical Beijing genotype.

This potentially suggests a role of these mutations in XDR-TB development in South Africa. It has been proposed by Klopper *et al.* (2013) that these *inh*A mutations detected by the Genotype MTBDR*plus* test could be used as a rapid screening tool to identify patients harbouring XDR atypical Beijing strains. This means that as a result of poor TB control in these areas, these strains continue to spread and most likely to evolve into XDR-TB and as indicated in the previous chapter, resulting in very few drugs being available to treat these patients.

CHAPTER FIVE

DETECTION OF MUTATIONS IN XDR-TB ISOLATES

5.1 INTRODUCTION

Extensively Drug - Resistant TB (XDR-TB) is defined as a form of TB that is resistant to isoniazid and rifampicin (MDR-TB) as well as any fluoroquinolone (ofloxacin and moxifloxacin) and any one of the injectable anti-TB drugs (amikacin, kanamycin and capreomycin) (WHO, 2013a). The World Health Organization estimates that at least 9% of MDR-TB cases globally, are extensively drug - resistant.

Laboratory diagnosis of XDR-TB at the Port Elizabeth TB Laboratory currently involves the use of conventional second-line DST (MGIT 960). This phenotypic test is the gold standard for 2nd line drug susceptibility testing. Fluoroquinolone (ofloxacin and moxifloxacin), polypeptide (capreomycin) and aminoglycoside (amikacin and kanamycin) testing is recommended by the WHO in routine laboratories, as reliability and reproducibility of results allow for quality assured diagnosis of XDR-TB. This is not the case for other second line drugs and they are therefore not recommended for routine testing laboratories (WHO, 2010b).

The urgent need for rapid laboratory diagnostics for XDR-TB has led to the development of a number of phenotypic and molecular assays. These include non-commercial assays such as MODS (Microscopic Observation Drug Susceptibility assay). This is a phenotypic micro-colony assay that operates on the principle of inoculating the patient's sample into drug –free or drug containing liquid media. Early growth of mycobacteria is observed microscopically using an inverted microscope (WHO, 2010b).

Molecular testing for XDR-TB involves detection of mutations in the resistance-determining regions of M. *tuberculosis*. Fluoroquinolones target the DNA gyrase region (encoded by *gyr*A and *gyr*B). Mutations in these Quinolone Resistance – Determining Regions (QRDR) are associated with resistance (von Groll *et al.* 2009, Lau *et al*. 2011). Aminoglycosides, amikacin and kanamycin inhibit protein synthesis

by binding to the16S rRNA (encoded by the *rrs* gene) in the 30S ribosomal subunit (Magnet *et al.* 2005). Capreomycin is thought to interfere with translation and inhibit phenylalanine synthesis in mycobacterial ribosomes (Trnka and Smith, 1970) Mutations conferring resistance to capreomycin lie in the *tly*A gene which encodes a 29-O-methyltransferase that modifies nucleotide C1409 in helix 44 of 16S rRNA and nucleotide C1920 in helix 69 of 23S rRNA (Johansen *et al.* 2006).

Rapid genotypic methods include the HAIN GenoType MTBDR*sl* assay, which is currently the only molecular test that can detect XDR-TB. This test operates on the same principle as the MTBDR*plus* assay (HAIN GenoType MTBDR*sl* package insert). This assay detects resistance to fluoroquinolones (ofloxacin and moxifloxacin), aminoglycocides / cyclic peptides (amikacin, kanamycin and capreomycin) and ethambutol from smear positive clinical samples and/ or culture isolates. Fluoroquinolone resistance is determined by detecting the most significant mutations (C88S, A88T, A90V, S91P, D94A, D94N, D94Y, D94G and the rare mutation D94H which has only been detected *in silico*) in the *gyr*A region (DNA gyrase). Aminoglycocide/ cyclic peptide resistance is determined by detecting mutations (A1401G, C1402T and G1484T) in the 16S rRNA gene (*rrs*) and resistance to ethambutol is determined by detecting mutations (M306I (ATG→ATA, ATG→ATC, ATG→ATT) and M306V) in the *emb*B gene (*emb*B, *embA and embC* genes code for the enzyme arabinosyl transferase which is targeted by ethambutol) (HAIN MTBDR*sl* package insert).

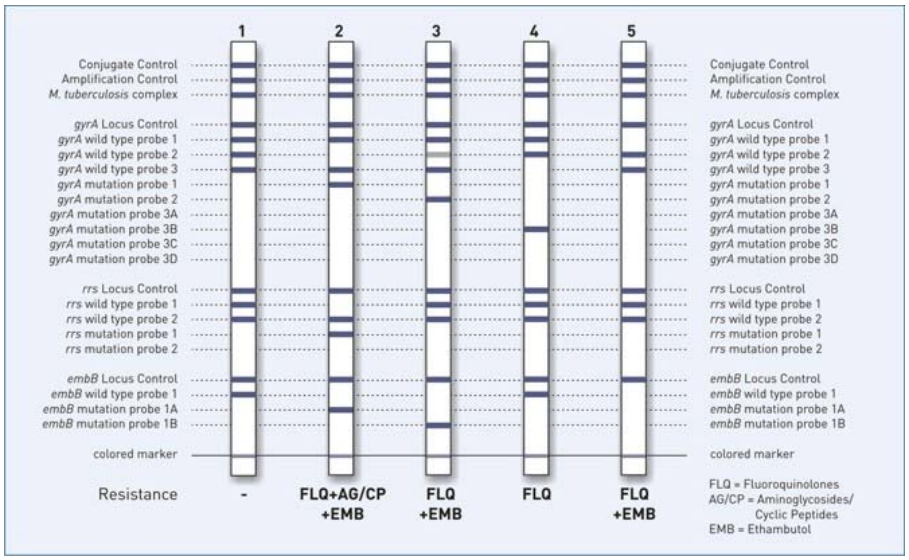

Figure: 5.1: Mutations detected by Genotype kit for identification of XDR-TB. (http://www.hain-lifescience.de/en/products/microbiology/mycobacteria/genotype-mtbdrsl.html)

In March 2012, the WHO expert group convened a meeting to decide whether the GenoType MTBDR*sl* kit could be used to replace the gold standard conventional DST method for second line DST (WHO, 2013b). A review of numerous studies revealed that the assay offered moderate test sensitivity for the detection of resistance to fluoroquinolones and the injectables but high test specificity. There was, however, poor correlation between kanamycin cross-studies which resulted in poor sensitivity to kanamycin, which led to the assay being considered inadequate.

The GenoType MTBDR*sl* assay was considered a good rule-in test but due to the lower sensitivity, negative results for resistance could not reliably rule out resistance. Further issues were the fact that the assay could not differentiate between the injectable drugs as well as the issue of incomplete cross-resistance, which meant that the assay results could not be used to manage XDR treatment. It was finally decided that the GenoType MTBDR*sl* assay may not be used as a replacement test for conventional DST but may be used as a rule in test for XDR-TB to be confirmed by conventional DST (WHO, 2013b).

Currently research is focusing on the development of rapid, affordable, easy to use, molecular tests that can diagnose XDR-TB within a week, in high MDR / XDR-TB burden countries, as mandated by the WHO. The Global Consortium for Drug Resistant TB Diagnostics (GCDD) is an international collaboration of researchers and TB specialists with the aim of reducing the time for detection of XDR-TB. Strains from Chisinau in Moldova, Mumbai, India and Port Elizabeth, South Africa were examined using an array of rapid tests (MODS, line probe assay and pyrosequencing) and compared them to the gold standard MGIT culture and DST. Preliminary results indicate that the line probe assay and MODS are able to produce results within a week (Medpage Today, 2013).

Until there is sufficient understanding of the mutations responsible for resistance to amikacin, kanamycin and capreomycin, rapid diagnosis of XDR-TB will remain a challenge. Therefore this chapter investigated the detection of mutations in 20 selected XDR-TB isolates.

5.2 RESULTS

5.2.1 XDR-TB drug susceptibility test results and sequence analyses

There was good correlation between the twenty samples tested for phenotypic XDR-TB drug susceptibility and sequence analyses of the *gyr*A and *rrs* genes (Table 5.1). Predictably, phenotypic drug resistance to STR, OFL and AMI was observed in 90% (18/20) of the XDR-TB isolates that had mutations in the *rrs* and *gyr*A regions. Thirty percent (6/20) of these isolates were found to have additional resistance to ethionamide. Notably, all 6 of these isolates had corresponding *inh*A promoter mutations, detected using the GenoType MTBDR*plus* VER 2.0 assay, suggesting cross-resistance. Ten percent (2/20) of the isolates had phenotypic resistance to OFL as well as all three aminoglycosides (STR, CAP and AMI) and one isolate was found to be resistant to INH, RMP, STR, ETH, OFL, CAP and AMI.

The majority of the XDR-TB isolates [40% (8/20)] were received from the Buffalo City Metropole (BCM). Fifty percent had additional phenotypic resistance to ethionamide with corresponding *inh*A promoter mutations as indicated earlier in this chapter. The Nelson Mandela Metropole (NMM) had the second highest number of XDR-TB isolates at 35% (7/20). Only one of these isolates had additional resistance to ethionamide, which is contradictory to overall findings in NMM in this study, where the majority of strains were found to have *inh*A promoter mutations. This discrepancy can be explained by the random selection of the small number of isolates for the purposes of gene sequencing. Cacadu health district had the third highest number of isolates at 20% (4/20), with very little variation seen in phenotypic drug resistance patterns.

Table 5.1: Comparison of XDR- TB Drug susceptibility test results and sequence analyses of isolates.

Isolate	Drug susceptibility result	District	Mutation	
			rrs	***gyrA***
1	STR, ETH, OFL, AMI	CAC	+	+
2	STR, ETH, OFL, AMI	NMM	+	+
3	STR, OFL, AMI	CH	+	+
4	STR, OFL, CAP	CAC	-	+
5	STR, OFL, AMI	NMM	+	+
6	EMB, STR, ETH, OFL, AMI	BCM	+	+
7	STR, OFL, AMI	NMM	+	+
8	STR, OFL, AMI	NMM	+	+
9	STR, OFL, AMI	NMM	+	+
10	STR, ETH, OFL, AMI	BCM	+	+
11	STR, OFL, CAP, AMI	BCM	+	+
12	STR, OFL	NMM	-	+
13	STR, OFL, AMI	CAC	+	+
14	STR, OFL, AMI	NMM	+	+
15	STR, OFL, AMI	BCM	+	+
16	STR, OFL, CAP, AMI	BCM	+	+
17	STR, OFL, AMI	BCM	+	+
18	STR, OFL, AMI	CAC	+	+
19	STR, ETH,OFL, CAP, AMI	BCM	+	+
20	STR, ETH, OFL, AMI	BCM	+	+

STR: streptomycin, ETH: ethionamide, OFL: ofloxacin, AMI: amikacin, CAP: capeomycin, EMB: ethambutol, CAC: Cacadu, NMM: Nelson Mandela Metropole, BCM: Buffalo City Metropole, CH: Chris Hani

5.2.2 Amikacin, kanamycin, streptomycin (*rrs* gene); ofloxacin, moxifloxacin (*gyr*A gene) and capreomycin (*tly*A gene) resistance in XDR-TB

PCR amplification of the *rrs, gyr*A and *tly*A genes produced the expected 439 bp, 320 bp and 981 bp size products respectively (Figs. 5.2 to 5.4).

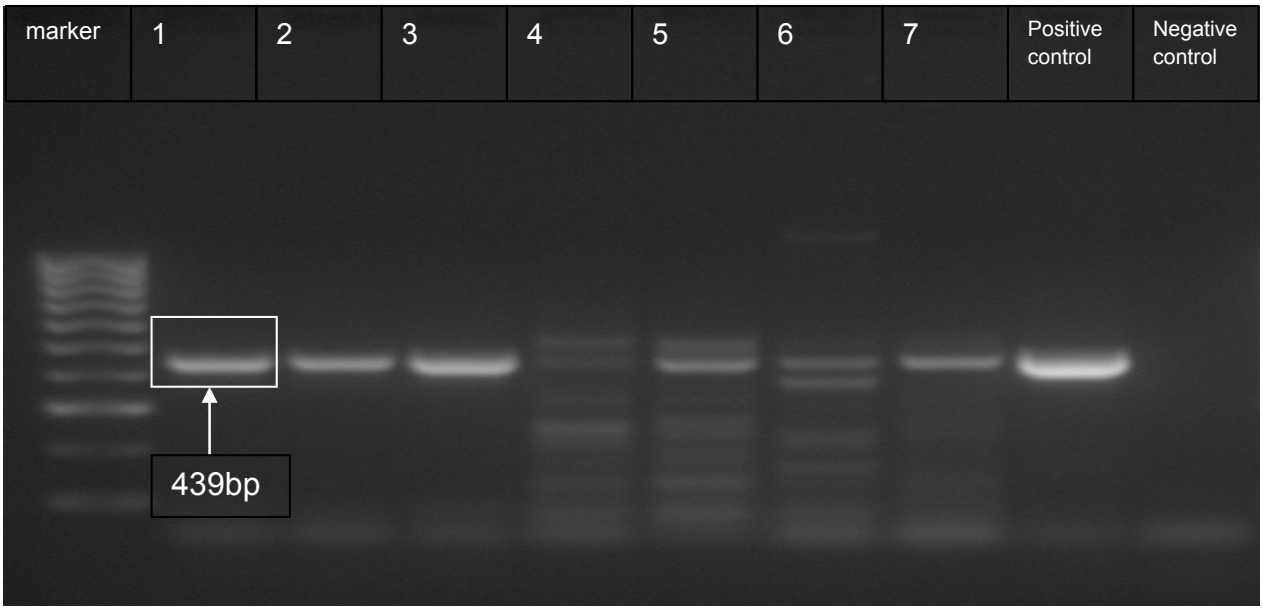

Figure 5.2: PCR amplification of *rrs* gene. Marker: HyperLadder IV (Bioline); Lanes 1-7: *M. tuberculosis* XDR isolates from different patients.

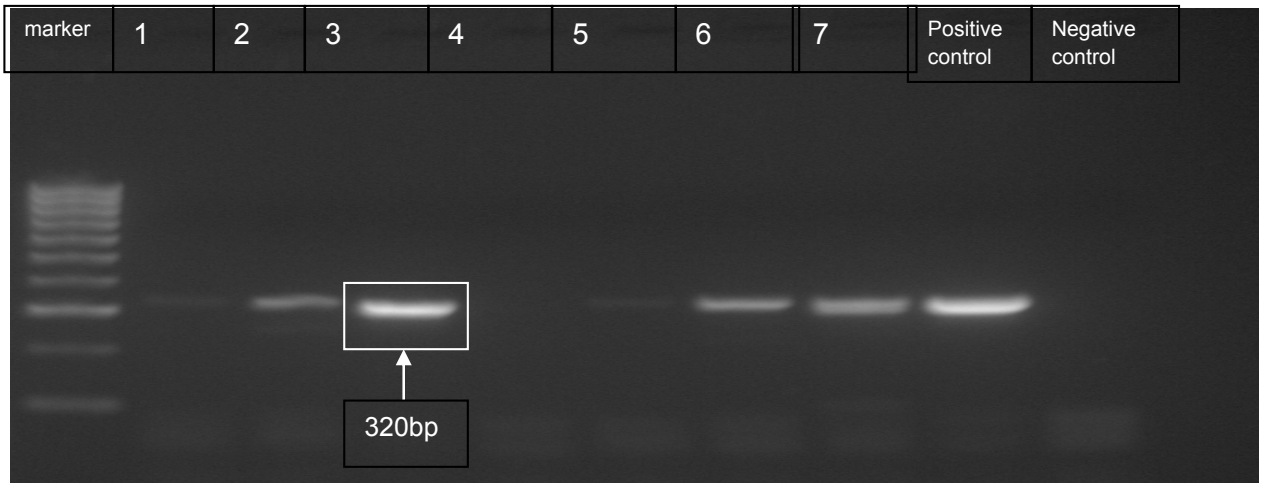

Figure 5.3: PCR amplification of *gyr*A gene. Marker: HyperLadder IV (Bioline); Lanes 1-7: *M. tuberculosis* XDR isolates from different patients.

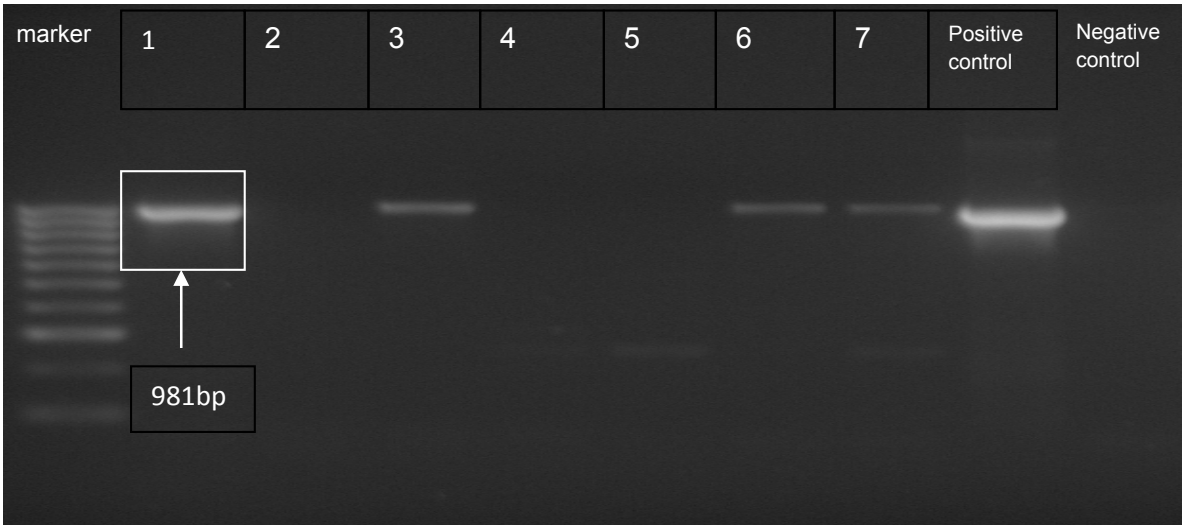

Figure 5.4: PCR amplification of *tlyA* gene. Marker: HyperLadder IV (Bioline). Lanes 1-7: *M. tuberculosis* XDR isolates from different patients.

Common mutations occurring in the *gyr*A gene were: A90V, D94H and del129. Mutation A1401G was found in the *rrs* gene in 18/20 (90%) strains and the C1199-deletion was observed in a few strains (Table 5.2). However, there were no mutations in the short region of the *tly*A gene which was sequenced. The majority of XDR-TB isolates contained mutations at positions C269T [A90V] (6/20) and A1401G (18/20) in *gyr*A and *rrs* genes respectively.

Table 5.2: Mutations detected in *gyrA, rrs and tlyA* genes of XDR-TB strains.

Gene	Mutation (nt)	Amino acids changes	Total No. of strains
*gyr*A	C269T	A90V	6
	G280C	D94H	4
	C387-	Del 129	5
	C387A	Y129 stop	4
	A281G	D94G	2
	G280T	D94Y	1
	G383A	R128K	1
	G262T	G88C	1
	C124-	Del 42	1
	A281C	D94A	1
rrs	A1401G		18
	C1199-		3
	T1197C		1
	T1386A, A1387T, T1399C, A1406C, A1421G, A1427G, C1474T, T1525A, A1524C		1
	T1264G, A1266G, T1276-, A1278G, A1280G, A1281G, C1283G.		1
	-1201A, C1202T, C1210-, C1211G, A1232G		1

5.3 DISCUSSION

Analysis of gene sequencing of the 20 XDR-TB strains selected from this study revealed that the most common mutations in the *gyr*A gene were: A90V, D94H and del129. Mutation A1401G was found in the *rrs* gene in 18/20 (90%) strains and the C1199- deletion was observed in a few strains. Interestingly, no mutations were detected in the short region of the *tly*A gene. This is in agreement with a previous study involving 310 isolates collected in the Eastern Cape, between June 2008 and November 2009 which found that 58% (181/310) of the isolates had the A1401G mutation and that no mutations were found in the *tly*A region in a subset of 50 of these isolates (Sirgel *et al*. 2011).

Another study on the common mutation patterns of drug resistant strains from 4667 drug susceptible, MDR and XDR-TB cases in three neighbouring provinces in South Africa (Western Cape, Eastern Cape (EC) and Kwa-Zulu Natal (KZN) (Muller *et al*. 2013). Mutations in drug target sites in the atypical Beijing R86 sub-group (n=193) of the EC were selected and examined. Mutations in the *inh*A promoter, *kat*G, *rpo*B, *pnc*A, *emb*B, *rrs* and *gyr*A genes were analyzed by gene sequencing. Two main clusters from the R86 sub-group emerged. 69% (133/193) MDR isolates showing identical resistance mutations in *inh*A promoter, *kat*G, *rpo*B, *pnc*A, *emb*B and *rrs* genes, suggesting transmission of a particular genotype within the community (Muller *et al*. 2013).

A variety of *gyr*A mutations were observed among the XDR-TB isolates (D94G, D94H and D94N), suggesting subsequent development of fluoroquinolone resistance (Muller *et al.* 2013). Interestingly, mutations D94G and D94H were also detected among the 20 isolates sequenced in this study. 17% (32/193) MDR isolates in the Müller *et al.* (2013) study, showed identical resistance mutations in the *kat*G, *rpo*B, *pnc*A, *emb*B and *rrs regions.* These two predominant strain clusters were found in 4 and 3 of the health districts respectively (Muller *et al*. 2013). These study results showed *kat*G S315T / *rrs* 513 A→C / *pnc*A 172 G insertion / *inh*A promoter -17 G→T / *emb*B M306I / *rpo*B D516V / rrs 1401 A→G mutations were found in the larger of the two main R86 clusters. With 67.7% (90/133) isolates from the Nelson Mandela Metropole (NMM) health district, 22.6% (30/133) from the Amathole health district (AM), 9% (12//133) from the Cacadu district and 0.8% (1/133) from the Chris Hani

district. *kat*G S315T / *rrs* 513 A→C / *pnc*A C14R / *rpo*B S531L / *emb*B M306I were found in the second of the two main clusters, with 59.4% (19/32) and 37.5% (12/32) from the AM and NMM districts respectively. The remaining R86 clusters showed that *kat*G S315T / *rrs* 513 A→C / *pnc*A 172 G insertion / *inh*A promoter -17 G→T / *emb*B M306I / *rpo*B D516V / *rrs* 1401 A→G / *gyr*A D94G were found among 70.5% (31/44) of isolates from NMB and 20.5% (9/44) of isolates from AM district. *kat*G S315T / *rrs* 513 A→C / *pnc*A C14R / *rpo*B S531L / *emb*B M306I / *inh*A promoter -15 C→T / *rrs* 1401 A→G / *gyr*A A90V were found among 70% (7/10) isolates from AM district, 20% (2/10) NMB and 10% (1/10) from the OR Tambo health district (Muller *et al.* 2013).

A study in KZN found A90V mutation in *gyr*A in 24% of fluoroquinolone resistant isolates and 1400 *rrs* mutations in 60% of kanamycin resistant isolates. This study also suggested that the epidemic in KZN is being driven by clonal expansion of the same strain (Ioeger *et al,* 2009). However, a comparison of resistance mediating mutations among the isolates sequenced in this study and the two studies above was not possible due to the low numbers that were sequenced, however, further studies on XDR-TB isolates from the overall sample population (n=1004) should be done.

Another interesting observation was the fact that there were many common mutations observed between the 20 XDR-TB isolates sequenced for the purposes of this study and those detectable on the HAIN GenoType MTBDR*sl* line probe assay, viz. A90V, D94A, D94Y, D94G, D94H in the *gyr*A gene (fluoroquinolones) and A1401G in the *rrs* gene (aminoglycocides). This perhaps suggests that this assay may be worth using in conjunction with conventional DST as a rule-in test for the rapid diagnosis of XDR-TB in the Eastern Cape.

CHAPTER SIX

CONCLUSIONS

In an effort to gain perspective of the MDR and XDR-TB problem in the Eastern Cape (EC), there are two crucial questions which require answers: (i) Is there rapid laboratory diagnosis of MDR and XDR-TB in the province and (ii) What is the status of the programmatic management of these patients infected with MDR and XDR-TB?

Approximately 13% of South Africa's population live in the EC of which 88% of households are black, due to the incorporation of the former Ciskei and Transkei homelands into the province in post-apartheid South Africa. About 57% of the EC population live in poverty and unemployment in the provinces remains extensive with an estimated 27% of the province's population being economically active and many of the poor relying on social grants for survival. It is also estimated that 20% of the EC province is functionally illiterate (Eastern Cape Socio Economic Consultative Council**,** 2012). This information paints a bleak picture of the socio-economic status of the province and is one of the main driving forces behind the MDR and XDR-TB epidemic in the province.

Specimens included in this study were sequentially received between February 2012 and February 2013. A total of 1 520 specimens were collected in this investigation of which 1004 had interpretable results and were therefore included in the analysis. All the samples were decontaminated and processed for TB Culture. Smear microscopy on TB Culture samples was only done on request, due to local cost constraints, hence not all TB culture samples that could have potentially been smear positive, benefitted immediately from direct testing for first line DST using the Genotype MTBDR*plus* assay. This is usually done within 24 to 72 hours from the time that the smear result is available. This implies that when smear microscopy is not requested, the lab cannot test directly on sputum specimen but will have to wait for a Mtb positive culture result and then be tested using the Genotype MTBDR*plus* assay. These samples would be delayed until they were culture positive for TB,

which could have been any time between a few days to up to 6 weeks, resulting in delays in initiation of treatment and increasing the risk of community transmission.

Fifty two percent of samples collected for the purposes of this study were from female patients. There were also more females with XDR-TB (28%), however, for the MDR *sensu stricto* group; the majority were males at 48%. According to the 2011 WHO global report, pregnancy, HIV and poverty are the main reasons why women are more at risk of becoming infected with XDR-TB. The 2011 antenatal survey revealed that the highest prevalence of HIV amongst pregnant women in South Africa was recorded in the Buffalo City Metropole in the Eastern Cape at 34.1%, which also coincides with the area with the highest number of MDR and XDR-TB cases recorded in this study. With an estimated 17 % of women in their reproductive years being HIV positive (Stats SA, 2013), one can assume that the HIV/TB co-infection rates in this area may be high, even though the HIV status among this study cohort was not available.

The 1004 samples included in this study were considered representative of the entire province. Samples were received from 25 hospitals and 267 clinics in all eight health districts in the EC. These included Alfred Nzo, Amatole, Buffalo City Metro, Chris Hani, Nelson Mandela Metro, O.R. Tambo, Cacadu and Joe Gqabi health districts. Three TB hospitals, Ngqubela Chest, Empilweni and Marjorie Parrish TB Hospitals, from three neighboring health districts, Buffalo City, Nelson Mandela Bay and Cacadu, were the sources of the majority of MDR and XDR-TB samples included in this study. The majority came from Nqqubela Chest TB Hospital which services the second largest township (Mdantsane) in South Africa.

Although the Buffalo City Metropole had the highest number of MDR and XDR-TB cases reported in this study. The majority of isolates collected from the immediate NMM were pre-XDR or XDR-TB. Reports by Muller *et al*. (2011) and Chihota *et al.* (2011) in which *inh*A promoter mutations were strongly associated with XDR-TB in South Africa as well as Klopper *et al.* (2013) where 86.7% pre-XDR and 95.4% XDR-TB isolates were found to belong to the atypical Beijing genotype, are similar to findings in this study where *inh*A promoter mutations were strongly associated with XDR-TB isolates, suggesting that these isolates may also belong to the atypical

Beijing genotype. However, spoligotyping of these isolates is required to confirm this.

Klopper *et al.* (2013) was also able to show through DNA sequencing, that the atypical Beijing strains were closely related and found in 5 of the 8 health districts (AM, NMM, CAC, OT and CH), suggesting that these strains were being spread by ongoing transmission within these health districts (Klopper *et al.* 2013). Similar findings are seen in this cohort, where pre-XDR and XDR-TB isolates were found in AM, BCM, NMM, CAC and OT, with the exception of CH. It must be noted that the Buffalo City Metropole is a new health district and was formerly part of the Amathole district.

When considering these findings, one cannot but begin to question the effectiveness of programmatic management of TB in the Eastern Cape. No changes in findings have been observed between research conducted using samples collected between 2008 and 2009, to this study cohort collected between 2012 and 2013. The ongoing, one size fits all policy of using a standardized treatment regimen for MDR and XDR-TB in the Eastern Cape, despite recommendations for an individualized treatment approach (Klopper *et al*. 2013; Müller *et al*. 2013), suggests either a lack of understanding of the severity of the MDR /XDR-TB situation in the province or apathy on behalf of the health authorities in the province. It is the author's experience and opinion that the disease is being driven primarily by the fact that the burden of initial patient management has become the responsibility of nursing staff and is rarely managed by clinicians. This is exacerbated in some instances, due to a poor understanding of and interpretation of laboratory results at initiation of primary health care, which may be responsible for delays or mismanagement of patients.

The WHO recommends that laboratory testing techniques be suitable for the level of service able to be offered by the particular laboratory. It recommends the use of light-emitting diode fluorescent microscopy (LED), the use of liquid culture (BACTEC MGIT 960) medium for conventional TB Culture processing, the use of the Genotype MTBDR*plus* assay for rapid molecular testing for MDR-TB in order to improve programmatic management and the use of phenotypic liquid culture methods, such as the BACTEC MGIT 960 method for second line DST. It also recommends that

only fluoroquinolones (ofloxacin, moxifloxacin), polypeptides (capreomycin) and aminoglycocides (amikacin, kanamycin) be routinely tested for 2nd line DST (WHO 2010). At this point, the only rapid molecular method for the detection of XDR-TB, the Genotype MTBDR*sl* assay, is only recommended as a "rule-in" test by the WHO and needs to be confirmed by phenotypic DST (WHO, 2013b). All these recommendations are currently being followed by the NHLS, Port Elizabeth TB laboratory and are considered "best practice". Until such time as a reliable, affordable, rapid molecular diagnostic test is available for the detection of XDR-TB, current practice for TB diagnostics falls short in the efforts to improve management of the disease.

Two major limitations of this study were the fact that the HIV prevalence amongst this cohort was unknown, which would have provided valuable insights into the current HIV / TB co-infection rates and the fact that only 20 samples were able to be sequenced for the purposes of this study, thus allowing only assumptions to be made and preventing any accurate comparison with previous research done in this province. Further studies would need to focus on HIV/TB co-infection rates and treatment outcomes in light of 2010 treatment policy changes that allowed patients starting on XDR-TB treatment to receive simultaneous ARV treatment regardless of the CD4 count (Kvasnovsky *et al.* 2011), particularly in light of the fact that the highest prevalence (34.1%) of HIV amongst pregnant women in South Africa was recorded in the Buffalo City Metropole in the Eastern Cape in the 2011 National Antenatal Sentinel HIV and Syphilis Prevalence Survey. A thorough analysis through gene sequencing and spoligotyping would also need to be done on a cohort of this size in order to provide an accurate comparison of current isolates with those documented in previous research.

In conclusion, when considering the two questions posed at the beginning of this chapter, it becomes clear that the urgent need for a reliable, affordable, rapid molecular XDR-TB diagnostic test is paramount. Patient management is also of grave concern as current practice is clearly exacerbating transmission of extremely resistant strains of XDR-TB in the Eastern Cape. One wonders, as eluded in Klopper *et al.* (2013), whether the Eastern Cape is becoming the epicentre for the development of Totally Drug-resistant Tuberculosis (TDR-TB) in South Africa.

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LIST OF CONFERENCE PRESENTATION

Poster presentation

Hayes, C. and S. Govender. 2013. Genetic analysis of multi- and extensively-drug resistant *Mycobacterium tuberculosis* isolates from Eastern Cape. 5th FIDSSA (Federation of Infectious Diseases Societies of Southern Africa) Conference, Champagne Sports Resort, Drakensberg. 10 - 12 October.

APPENDIX

Table A1: Hospitals (25) and clinics (267) with MDR-TB and XDR-TB cases in this study.

	No. of cases within the different groups			
Hospitals / clinics	**MDR**	**PRE-XDR**	**XDR**	**TOTAL**
Aberdeen mobile clinic	1			1
Addo fixed clinic	3	4	2	9
Aeroville clinic	3			3
Alexandia clinic	1			1
Algoa park clinic	1	2	1	4
Alice clinic			1	1
Alicedale hospital	1			1
Aliwal North hospital	2			2
All Saints Gateway clinic	1			1
Alphendale clinic		1	1	2
Amahle clinic	1	2		3
Asperanza clinic	1	2		3
Assinamandla clinic		1		1
Bala clinic	1			1
Balfour clinic	2			2
Banzi PHC clinic	1			1
Barkly East hospital	1			1
Bathurst clinic			1	1
Baviaans clinic	1			1
Beatrice Ngwentle clinic			1	1
Bedford clinic	1			1
Bergsig clinic	1			1
Bhele clinic		1		1
Bisho hospital	2	2		4
Booysens Park clinic	5	7	1	13
Braelyn clinic			1	1
Butterworth hospital	3	1	1	5
Canzibe hospital		1		1
Canzibe hospital	1	2		3
Cecelia Makiwane hospital	8	3	1	12
Central clinic		2		2
Chatty clinic	2	6	8	16
Chris Hani clinic	3			3
Cimezile clinc	2			2
Civic center clinic	1			1
CL Bikitsha PHC clinic	1			1

	No. of cases within the different groups			
Hospitals / clinics	**MDR**	**PRE-XDR**	**XDR**	**TOTAL**
Cofinvaba hospital			1	1
Cradock hospital			1	1
Cumakala mobile 2 clinic	1			1
Dimbaza clinic	5	1		6
Dora Nginza hospital	4	5	5	14
Drake road clinic	1		1	2
Du Preez street clinic	1			1
Duncan Village Day Hospital	3	1	1	5
East London central clinic		1		1
Edamini clinic	1			1
Ekiphumleni clinic	1			1
Empilisweni Gateway clinic	1		1	2
Empilisweni hospital		2	1	3
Empilweni clinic	3	2		5
Empilweni Gompo health centre	5			5
Empilweni TB hospital	9	12	8	29
Ethembeni clinic	1		1	2
Extension 7 clinic	2			2
Ezibeleni clinic		1		1
Flagstaff clinic			1	1
Fort Beaufort TB hospital	2	1	1	4
Fort Grey clinic		1	1	2
Fort Grey TB hospital	1	1	1	3
Fransbury satellite clinic	1			1
Frere hospital	10	4	2	16
Frontier hospital	2			2
Gateway clinic	1			1
Gateway clinic, Bisho	1		1	2
Gateway clinic, East London		1		1
Gateway clinic, Holycross	2	1		3
Gateway clinic, SS Gida		1		1
Gateway clinic, Umtata	1			1
Gelvandale clinic	3		2	5
Gilton clinic		1		1
Ginsburg clinic	3			3
Gompo B clinic	1		2	3
Gompo C clinic	7	5	2	14
Govan Mbeki clinic		2	4	6

	No. of cases within the different groups			
Hospitals / clinics	**MDR**	**PRE-XDR**	**XDR**	**TOTAL**
Gqebera clinic	3	8	4	15
Graaf Reinet day hospital	2			2
Grace Hospital Gateway clinic	1			1
Grahamstown prison	1	1		2
Greenfields clinic	4	2		6
Grey hospital		1		1
Gxwederha clinic, Alice		1		1
Helenvale clinic		1		1
Helpmekaar hospital, Griquastad	1			1
Hewu clinic	1			1
Humansdorp hospital		2		2
Ibika clinic. Butterworth	1			1
Ikamvelihle clinic	2	2	1	5
Ilitha clinic	2			2
Imidange clinic	1			1
Imizamo Yethu clinic			1	1
Isolomzi clinic	1		1	2
Jaji clinic, Peddie	1			1
Jansenville clinic	2			2
Joe Slovo clinic		3		3
John Dock clinic	3		1	4
Jose Pearson TB hospital		1	3	4
Joubertina day hospital	1			1
Joza clinic		2	1	3
Kareedouw hospital	2			2
Kenton clinic		1		1
Khotana clinic	3		1	4
Khotsong clinic	1			1
Khuze clinic	1			1
King Williams Town hospital			1	1
Kirkwood clinic	1			1
Kirwood municiple clinic		2	1	3
Korsten clinic	1	1		2
Kruisfontein clinic	2			2
Kubusi clinic		1		1
Kwadwezi clinic	2	1	1	4
Kwanonqubela clinic	1	1	1	3
Kwazakhele clinic	7	2	4	13

	No. of cases within the different groups			
Hospitals / clinics	**MDR**	**PRE-XDR**	**XDR**	**TOTAL**
Kwazakhele day hospital	4	4	1	9
Kwazinzele clinic			1	1
Kwelera clinic	1		1	2
Lady Frere	2			2
Ladismith hosptal	2			2
Laeticia Bam day hospital	4	1	10	15
Langeni clinic, Qumbu	1			1
Lenye clinic	1			1
Life Care Algoa clinic		1		1
Linton Grange clinic			2	2
Livingstone hospital	3	2	2	7
Lizo Ngcana clinic	6			6
Louterwater clinic	1			1
Lukhanyiso clinic	3			3
Lukhanyo clinic			1	1
Lunga Kobese clinic	4	4	3	11
Lusikisiki clinic	2			2
Mabandla clinic	1	3	5	9
Machibini clinic			1	1
Macibe clinic, Centane	1			1
Madwaleni hospital	3		1	4
Marcelle clinic		1	2	3
Margery Parkes TB Hospital	1	2	2	5
Margery Parrish TB hospital	4	7	8	19
Mary Theresa Gateway clinic, Kokstad	1			1
Mary Theresa hospital			1	1
Masakhane clinic Aberdeen	2		1	3
Masakhane clinic Bisho	1			1
Masakhane clinic Greenbushes	4	2		6
Masakhane clinic Hankey			1	1
Max Madlingozi clinic	1	3	1	5
Mbombu clinic		1		1
Mdingi clinic	1			1
Melitafa clinic			1	1
Mercy clinic	1			1
Mevana clinic, Libode	1			1
Mgwali clinic	1			1
Mhlanga clinic	1			1

	No. of cases within the different groups			
Hospitals / clinics	**MDR**	**PRE-XDR**	**XDR**	**TOTAL**
Middelburg clinic	1			1
Middelburg prison	4			4
Middle Street clinic	2	1		3
Middle Terrace clinic		1	1	2
Misgund clinic			2	2
Missionvale clinic	1	2	7	10
Mjanya clinic		1		1
Molteno clinic	1			1
Molteno hospital	1			1
Mooiplass clinic	1		1	2
Moore street clinic	1	1		2
Moses Mabida clinic		1		1
Motherwell community health centre	1	3	6	10
Motherwell NU 11 clinic	1	4	5	10
Motherwell NU2	3	4	4	11
Motherwell NU8	2	1	1	4
Mount Frere clinic	1		1	2
Mpongo clinic	1	1		2
Mqambeli clinic			1	1
Mtambalala clinic	1			1
Mtyolweni clinic		1		1
Mzamomhle clinic	1			1
Mzamomhle clinic, Burgersdorp			1	1
Ncerha clinic		1		1
Ncora clinic	1			1
Ndanya clinic		1		1
Ndevana clinic	5			5
Ndlovini clinic		1		1
New Brighton clinic	2	1	3	6
New Rest clinic	1			1
Newlands clinic	1		1	2
Newton clinic	2			2
Ngangelizwe health centre, Umtata	2			2
Ngqubela Chest hospital	63	20	13	96
Nkqubela PHC clinic		1		1
Nkwenkwezi clinic	1	2	2	5
Nomangezi Jayiya	1	2	2	5

	No. of cases within the different groups			
Hospitals / clinics	**MDR**	**PRE-XDR**	**XDR**	**TOTAL**
Nompumelelo clinic	2	2		4
Nompumelelo PHC clinic, Indwe		1		1
Nomzamo PHC clinic, Izebeleni	1	1		2
Noncampa clinic, Bisho			1	1
Nontsikelelobiko clinic,		1		1
Nora clinic, Peddie		1		1
Nozuko clinic	2	2	1	5
Nqamaqwe health centre		2		2
Nqancula clinic	1			1
Ntafufu clinic, Libode	1			1
NU1 clinic	4	1	3	8
NU12 cinic			1	1
NU13 clinic	2	1		3
NU16 clinc	1	2		3
NU17 clinic			1	1
NU2 clinic	6	2	7	15
NU3 clinic	4	1	2	7
NU5 clinic	1	2	3	6
NU7 clinic	2	1	1	4
NU8 clinic	4	4	2	10
NU9 clinic	2		1	3
Orsmond TB hospital		4		4
Park Centre clinic	1		1	2
Parkvale clinic	2			2
Paterson clinic	1			1
PE occupational health clinic		1		1
Peddie clinic		1		1
Peddie extension clinic	1		3	4
Pefferville clinic	6	1	1	8
Pellsrus clinic		2	1	3
Petros Jobane clinic	3	1		4
Philani clinic Cradock	1			1
Philani clinic, Lady Frere			1	1
Philanic clinic Queenstown	1			1
Pikholi clinic		1		1
Port Alfred clinic		1	1	2
Port Alfred hospital		2		2
Potsdam clinic		1	1	2

	No. of cases within the different groups			
Hospitals / clinics	**MDR**	**PRE-XDR**	**XDR**	**TOTAL**
Povincial hospital PE	4		3	7
PZ Meyer TB hospital	2	2		4
Qibira clinic		1		1
Qobo clinic, Lusikisiki	1			1
Qumanco clinic, Cofimvaba	1			1
Raglan road clinic		2	1	3
Rosedale clinic	3	6	6	15
Sanddrift clinic			1	1
Settlers hospital		3	1	4
Seymore clinic	1			1
Shepherds Hope clinic	2			2
Sheshegu clinic	1			1
Sigidi clinic	1			1
Silvertown clinic	1	2		3
Sinebongo clinic	3	1		4
Soto clinic		1		1
Soweto clinic	4	2	2	8
St Albans prison	1			1
St Barnabas hospital	1			1
St Francis Bay clinic	1			1
St Michaels clinic	2	2		4
Tabankulu clinic		1		1
Tafalofefe clinic	3	2		5
Tanduxolo clinic	2	4	4	10
Taylor Bequest clinic	1			1
Thembalethu clinic, East London	2	2		4
Thembisa satellite clinic	1			1
Thornhill clinic		1		1
Thozamile Madikane clinic	1			1
Tombo clinic	1			1
Trafalga clinic	1	1		2
Tshangana clinic		2	4	6
Tshatshu clinic	1			1
Tshezi clinic	1			1
Tsitsikamma mobile clinic		1		1
Tstsikama clinic	1			1
Twee Rivier clinic			1	1
Tyutyu clinic	2			2

	No. of cases within the different groups			
Hospitals / clinics	**MDR**	**PRE-XDR**	**XDR**	**TOTAL**
Tyutyu village clinic, Bisho	2		1	3
Uitenhage hospital	1		3	4
Umlamli clinic	1	1		2
Umtata hospital	2			2
V Shumane clinic		1	1	2
Veeplaas clinic		2	6	8
Walmer 14th avenue clinic		1	2	3
War memorial clinic	2		1	3
Wells estate clinic	2	3	4	9
Wesley clinic	1	1		2
West end clinic	3	2	2	7
Weston clinic	1			1
Willowmore clinic			1	1
Winterberg TB hospital		1		1
Wongalethu clinic	1			1
Yonda clinic			1	1
Zanempilo clinic	1		1	2
Zazulwana clinic	1			1
Zigodlo clinic	1			1
Zihlaleni clinic	1			1
Zithulele clinic	8	1	1	10
Zola clinic	2			2
Zwelakhe clinic, Engcobo	1			1
Zwelitsha 5 clinic, Bisho			1	1
Zwide clinic	4	2	5	11