RAPID PREDICTION OF MULTI-DRUG RESISTANCE IN CLINICAL SPECIMENS OF *Mycobacterium tuberculosis*

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Submitted in fulfillment of the requirements for the degree of Master of Medical Science, in the Department of Medical Microbiology, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal.

DECLARATION

This study represents original work by the author and has not been submitted in any form to another university. The research described in this manuscript was carried out at the department of Medical Microbiology, Nelson R. Mandela School of Medicine under the supervision of Dr M. Pillay.

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LIST OF CONTENT

PRESENTATIONS EMANATING FROM THIS DISSERTATION

POSTERS

1. Bongiwe Ndimande, Willem Melchers, Willem Sturm, Patrick Sturm, Sheila Bamber, and Manormoney Pillay. Rapid prediction of multi-drug resistance in clinical specimens of *Mycobacterium tuberculosis.* University of KwaZulu-Natal, College of Health Science Research Symposium, 2007.

2. Bongiwe Ndimande, Willem Melchers, Willem Sturm, Patrick Sturm, Sheila Bamber, and Manormoney Pillay. Rapid prediction of multi-drug resistance in clinical specimens of *Mycobacterium tuberculosis.* Federation of Infectious Diseases Societies of Southern Africa (FIDSSA), 2007.

ORAL

3. Bongiwe Ndimande, Willem Melchers, Willem Sturm, Patrick Sturm, Sheila Bamber, and Manormoney Pillay. Rapid prediction of multi-drug resistance in clinical specimens of *Mycobacterium tuberculosis.* University of KwaZulu-Natal, College of Health Science Research Symposium, 2009.

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CONTENTS

ABBREVIATIONS

AFB	Acid-fast bacillus
AK	Amikacin
ARMS	Amplification refractory mutation system
ASP	Aspartic acid
ATP	Adenosine Triphosphatase
BSA	Bovine Serum Albumin
BSC	Biosafety cabinet
BSL2	Biological safety level 2
CAP	Capreomycin
CDC	Center for Disease Control and Prevention
CFU	Colony forming units
CO_2	Carbon dioxide
CTAB	Hexadecyltrimethylammonium bromide
DCS	D-cycloserine
ddF	Dideoxyfingerprinting
DGGE	Denaturing gradient gel electrophoresis
DNA	Dideoxy Nucleic Acid
dNTP's	Deoxynucleoside triphosphates
DST	Drug Susceptibility Testing
ECL	Enhanced Chemiluminescence
EDAC	1-ethyl-3-(-dimethylaminopropyl) carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EMB	Ethambutol
ETH	Ethionamide
FQs	Flouroquinolones
FRET	Fluorescence resonance energy
GITC	Guanidinium thiocynate
GLY	Glycine
HCl	Hydrochloric acid
HIS	Histidine
HIV	Human Immunodeficiency Virus
IALCH	Inkosi Albert Luthuli Central Hospital

INH	Isoniazid
IRS	Inhibitor removal solution
IS*6110*	Insertion sequence 6110
KCl	Potassium chloride
KGV	King George V
KM	Kanamycin
KZN	KwaZulu-Natal
LEU	Leucine
LJ	Lowenstein Jensen
LRPs	Luciferase reporter phages
M. tuberculosis	*Mycobacterium tuberculosis*
MB	Middlebrook
MDR	Multi-drug resistant
MGB	Minor groove binder
$MgCl_2$	Magnessium Chloride
MGIT	Mycobacteria Growth Indicator Tube
MIC	Minimum inhibitory concentration
Min	Minute
ml	Milliliter
MODS	Microscopic observation Drug Susceptibility
MOTT	Mycobacteria other than tuberculosis
MTT	3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NaCl	Sodium Chloride
$NaHCO_3$	Sodium bicarbonate
NALC	N- Acetyl-L- Cysteine
NaOH	Sodium Hydroxide
NRA	Nitrate reductase assay
PAS	*p*-aminosalicylic acid
PCR	Polymerase Chain Reaction
PRO	Proline
PSC	Positive scanty
PZA	Pyrazinamide

PZAse	Pyrazinamidase
RIF	Rifampicin
RIFO	Rifoligotyping rifampicin oligonucleotide typing
RNA	Ribose Nucleic Acid
RNAse	Ribose Nucleic Acidase
RR	Resistance ratio
SDS	Sodium dodecyl sulphate
SER	Serine
SSCP	Single-strand conformation polymorphism
SSPE	Sodium chloride, Sodium phosphate, EDTA
STR	Streptomycin
TB	Tuberculosis
TE	Tris EDTA
TYR	Tyrosine
UCT	University of Cape Town
UV	Ultraviolet
VAL	Valine
VIO	Viomycin
WHO	World Health Organisation
XDR	Extensive drug resistant

LIST OF FIGURES

LIST OF TABLES

ETHICS

This study was approved by the Biomedical Research Ethics Committee of the University of Kwa-Zulu Natal (**Ref.:H245/05**)

ABSTRACT

Conventional drug susceptibility testing techniques, the 'gold standard' for *M. tuberculosis* are slow, requiring about 3-6 weeks from a positive culture. This diagnostic delay, before initiation of appropriate treatment, contributes to increased transmission rates. Molecular techniques provide rapid results and therefore present an alternative to conventional tests. The aim of this project was to develop an in-house reverse line blot hybridization assay (RIFO assay) that could detect mutations associated with Rifampicin resistance directly in clinical specimens of patients in KwaZulu Natal.

A 437 bp region of the *rpo*B gene was sequenced to ascertain the most frequently occurring mutations conferring resistance to rifampicin in isolates in KwaZulu-Natal. Wildtype and mutant probes designed to target these mutations, were immobilized on a Biodyne C membrane. Hybridization conditions were optimized using biotin labeled PCR products from culture. Detection was performed with peroxidase labeled streptavidin using enhanced chemiluminescence. Four DNA extraction methods were evaluated on sputum specimens to determine the one with the least inhibitory effect on amplification.

A total of 11 mutations were found in 236 clinical isolates: 531TTG (109, 58.3%), 516GTC (26, 13%), 533CCG/516GGC (20, 10%), 533CCG (18, 9.6%), other mutations < 5% each. The chelex extraction method was found to be optimal for removing inhibitors in sputum specimens. Sputum specimens of 404 patients hospitalized at King George V Hospital between 2005 and 2006 were rifoligotyped.

The RIFO assay was optimised on clinical isolates and then applied to sputum specimens. The RIFO assay on culture and sputum correlated well with the DST (sensitivity 92% and 94% respectively). However, the specificity was very low in both culture and sputum specimens compared to DST (38% and 35% respectively). This could be attributed to the presence of silent mutations, mixed infections, mixed populations of bacteria or the small number of susceptible strains used in this study.

The in-house RIFO assay can be used directly on sputum specimens to predict Rifampicin resistance and therefore MDR-TB in less than a week compared to the gold standards. A total of 43 samples can be tested simultaneously at low cost and the membrane is reusable compared to commercial kits such as the Hains test that is expensive and strips are not reusable. A similar assay can be designed to target mutations for the detection of XDR-TB. Future studies should be conducted in a clinical setting on patients with sensitive strains to increase the specificity.

Plagiarism

DECLARATION

I, Bongiwe Olga Ndimande, 201508829 declare that:

(i). The research reported in this dissertation, except where otherwise indicated, is my original work.

(ii). This dissertation has not been submitted for any degree or examination at any other university.

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Signed: Bongiwe Olga Ndimande Date: 13 December 2010

CHAPTER ONE

1. INTRODUCTION

Although effective anti-tuberculosis (TB) drugs have been available for decades in South Africa, TB is still a major problem. Drug resistance, an outcome of non-compliance with drug intake, has been known to be the most significant limitation for the success of TB treatment (Mahmoudi and Iseman, 1993). The recent emergence of extensive drug resistance has greatly increased the significance of rapid susceptibility testing of *Mycobacterium tuberculosis* strains (Pillay and Sturm, 2007). Current methods used in susceptibility testing are slow, resulting in delays, which make it difficult to administer appropriate treatment timely. This is one of the most important aspect contributing to the transmission of TB, including multi-drug resistant (MDR) outbreaks and more specifically, extensively drug resistant (XDR) TB.

During the 1990's, MDR-TB defined as resistance to Isoniazid (INH) and Rifampicin (RIF) (WHO, 1997), was considered to be a threat to TB control. However, emergence of the XDR TB cases has aggravated the situation, raising fear of a future epidemic of untreatable TB (CDC, 2006). New anti-TB drug regimens, improved diagnostic tests, and standardised second line drug susceptibility testing, are needed for rapid detection and effective treatment of drug resistant TB (CDC, 2006). However, with the slow growth rate of *M. tuberculosis*, culture requires weeks or even months to obtain results (Kingsbury *et al,* 1985). Patients that harbour infection for weeks or months while they

receive ineffective treatment while awaiting susceptibility test results, transmit TB. Such delays in susceptibility tests increase the risk of transmission both in the community and in hospitals. It is crucial to detect drug resistance timeously in clinical isolates of *M. tuberculosis* for administering of appropriate treatment to avert the development of further resistance and the spread of resistant strains. Identification of these strains is of paramount importance as it can allow initiation of modified treatment regimens. This will impact positively on both public health and patient outcome by reducing the spread of drug resistant strains.

RIF is one of the most potent first line anti-TB drugs (WHO, 1997). Its resistance heralds a more prolonged treatment for the patient and poor results if the isolate is also resistant to INH (Mitchison and Nunn, 1986). The presence of RIF resistance increases the likelihood of MDR-TB because *M. tuberculosis* strains resistant to RIF are more likely to be resistant to several other anti-TB drugs (Telenti *et al,* 1993, Morris *et al,* 1995). The mechanism of RIF resistance is well characterised and has led to the development of molecular assays for rapid detection of RIF resistance (Telenti *et al,* 1993; Kapur *et al,* 1994; Williams *et al,* 1998; Cooksey *et al,* 1997; Nash *et al,* 1997; Piatek *et al,* 1998 and Torres *et al,* 2000).

The occurrence of RIF monoresistance is rare and more than 90% of RIF-resistant isolates are also resistant to INH (Heep *et al,* 2000). Therefore RIF is a good marker of MDR-TB, and thus can be used as a predictor of MDR-TB (Cole and Telenti, 1995; Drobniewski and Wilson, 1998; Ramaswamy and Musser, 1998). Ninety-five percent of

RIF resistant isolates of *M. tuberculosis* harbour specific mutations within the 81-bp region of the RNA polymerase (Yamada *et al,* 1985, Telenti *et al,* 1993). The presence of these mutations can be detected by a genetic assay and could predict clinical drug resistance in the majority of patients.

While RIF is associated mainly with single point mutations in one region of the RNA polymerase gene (*rpo*B) that can easily be amplified by PCR methods, in contrast INH drug resistance is more complex. It involves mutations in at least four gene complexes and not all mutations result in an alteration of the wild type phenotype (Zhang *et al,* 1992; Barnerjee *et al,* 1994; Heym *et al,* 1995; Drobniewski and Wilson, 1998). Therefore, development of an assay for the detection of mutations involved in INH resistance is more complicated and makes detection of RIF resistance more convenient compared to INH resistance.

Detection of mutations is more rapid than conventional RIF susceptibility testing, which depends on culture and therefore, requires an additional 3 to 6 weeks after the primary isolation to obtain the results. Early detection of MDR-TB can improve treatment outcome for the individual patient and reduce the opportunity for spread of the infection. Since RIF resistance is considered the surrogate marker for the identification of MDR-TB (CDC, 1993; Bloch *et al,* 1994), it would be useful for poorly resourced countries to have simple and inexpensive tests that can rapidly detect resistance to RIF.

The objectives of this study were three-fold:

(i) to identify the *rpo*B mutations associated with RIF resistance in a panel of isolates of *M. tuberculosis* isolated in KwaZulu-Natal (KZN), South Africa,

(ii) to determine the optimal method of DNA extraction resulting in the fewest or no PCR inhibitors and highest sensitivity in PCR based techniques to be used.

(iii) to optimize and evaluate the reverse line blot assay, rifoligotyping or RIFO assay for the identification of RIF resistance that is based on PCR amplification of the *rpoB* gene and the detection of the commonest mutations in KZN.

We hypothesize that the optimised rifoligotyping assay will be able to detect mutations in the *rpo*B gene directly in sputum specimens and this will serve as a marker of RIF resistance and thus will be a predictor of MDR TB.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. GENERAL CHARACTERISTICS OF *Mycobacterium tuberculosis*

2.1.1. DESCRIPTION

The tubercle bacillus belongs to the genus *Mycobacterium*, of the family Mycobacteriaceae (Shinnick, 1996). The *M. tuberculosis* complex includes five species of mycobacteria which can cause tuberculosis (TB): *M. africanum*, *M. bovis, M. canetti* and *M. microti and M. tuberculosis.* The first two rarely cause disease in humans; the second two do not cause human disease while the last is the major cause of the disease TB in humans. Although for many years TB complex organisms were known to grow aerobically, (Shinnick, 1996), it has been also shown to persist in conditions of hypoxia (Wayne and Sohasky, 2001). TB is predominantly the disease of the lungs but it can also affect bones and joints, vascular system, central nervous system, genitourinary systems and the lymphatic system (Salerno *et al,* 2006).

2.1.2. GROWTH AND PHYSICAL CHARACTERISTICS

M. tuberculosis is a slow growing microbe with generation times ranging from 12-24 hours (Iseman, 2000). This is extremely slow compared to other bacteria, which tend to

have division times measured in minutes. It is primarily a facultative, intracellular pathogen which resides within the phagolysosomes of alveolar macrophages (Shinnick, 1996). *M tuberculosis* is typically slightly curved or straight rod shaped. Its typical size when cultured *in vitro* is 1 to 4 mm in length and 0.3 to 0.6 mm in diameter, making it smaller than most bacterial pathogens (Iseman, 2000).

2.1.3. MICROSCOPIC CHARACTERISTICS

M. tuberculosis is an acid-fast bacillus (AFB). AFB are gram positive bacilli that stain poorly and are seldom seen in a gram stain. This is due to the lipid content of their cell wall. The primary cell wall structure of *M. tuberculosis* consists of a plasma membrane, which is supported by a peptidoglycan backbone against the osmotic pressure of the interior (Shinnick, 1996). Attached to the peptidoglycan is an arabinogalactan layer and to which are esterified mycolic acids. The high lipid content of *M. tuberculosis* cell wall makes it highly hydrophobic, which results in the clumping of cells, a characteristic that prevents staining with the usual chemicals.

M. tuberculosis can be identified microscopically by its staining characteristics. It resists decolorisation with acid after being stained. In the most common staining technique, the Ziehl-Neelsen stain, AFB are stained a bright red which stands out clearly against a blue background (Figure 2.1). AFB can also be visualized by fluorescent microscopy using an auramine-rhodamine stain. The mycobacterial cell wall is impermeable towards many drugs used in the chemotherapy of bacterial diseases (Nikaido, 1994).

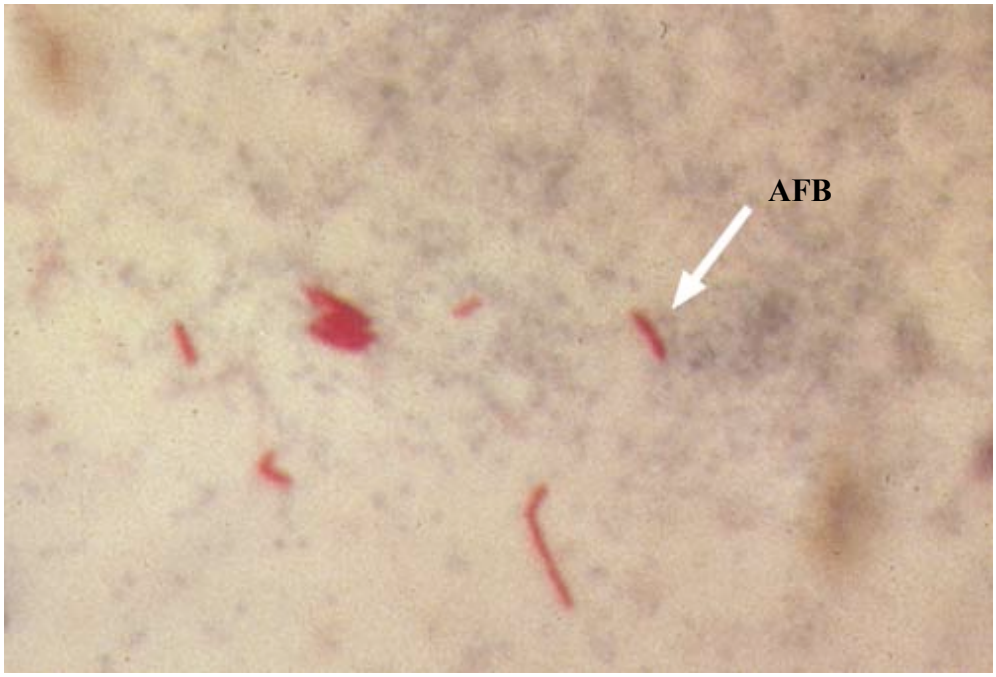

Figure 2.1 shows acid-fast stain of *Mycobacterium tuberculosis* (Textbook of Bacteriology)

2.1.4. TRANSMISSION OF TB

Transmission of TB begins after contact with a human source, almost always an infectious person with cavitary pulmonary TB. The infection is transmitted almost exclusively by the airborne route via a small bacillus-containing particle called a droplet nucleus (Iseman, 2000). When a droplet nucleus containing one or two viable bacilli is inhaled, it is deposited on the alveolar surface where the bacilli begin to multiply. Initially, the infecting organism meets only limited resistance from the host, as phagocytosis by alveolar macrophages has little effect on the bacilli. The earliest evidence of host tissue recognition is dilation of the capillaries, followed by a migration of polymorphonuclear leukocytes and macrophages into the infected area. After several

weeks of infection, the number of leukocytes in the area decreases and the mononuclear cells predominate. These crowd together and contain pale, foamy cytoplasmic material, which is rich in lipids. The resulting unit is called a tubercle, the fundamental lesion of TB (Shinnick, 1996).

When an individual is infected with the tubercle bacilli, the organism is taken up by the alveolar macrophages and carried to the lymph nodes. In the lymph nodes it can spread further to other organs. It takes about 2 to eight weeks after infection for the immune system to elicit a response. During this time, cell mediated immunity and hypersensitivity develops which leads to the characteristic reaction of the tuberculin test (Houben *et al,* 2006). In immunocompetent individuals containment of infection then follows.

About 10% of these infected individuals will develop active disease in their lifetime. The other 90% do not become ill and cannot transmit the organism due to the ability of the normal immune system to contain the infecting organism or even in some instances, to eradicate them. Individuals who are taking immunosuppressive agents or those that are infected with Human Immunodeficiency Virus (HIV) are more likely to develop the disease because of their compromised immunity. Therefore, TB is reported to be rampant in populations that have dual infections with HIV (Barnes *et al,* 1991).

2.2. EPIDEMIOLOGY OF TB

2.2.1. TB INCIDENCE AND PREVALENCE

The World Health Organisation (WHO) estimated that there were 9.4 million incident cases (8.9-9.9 million) of TB globally in 2009 (WHO, 2010). Most of the estimated number of cases occurred in Asia (55%) and Africa (30%), smaller proportions occurred in the Eastern Mediterranean Region (7%), the European region (4%) and the Region of the America's (3%) (Figure 2.2) (WHO, 2010). South Africa is one of the 5 countries with a high burden of incident TB (0.40-0.59 million cases).

Globally, there were an estimated 14 million prevalent cases (12 million- 16 million) of TB in 2009, equivalent to 200 cases per 100 000 population.

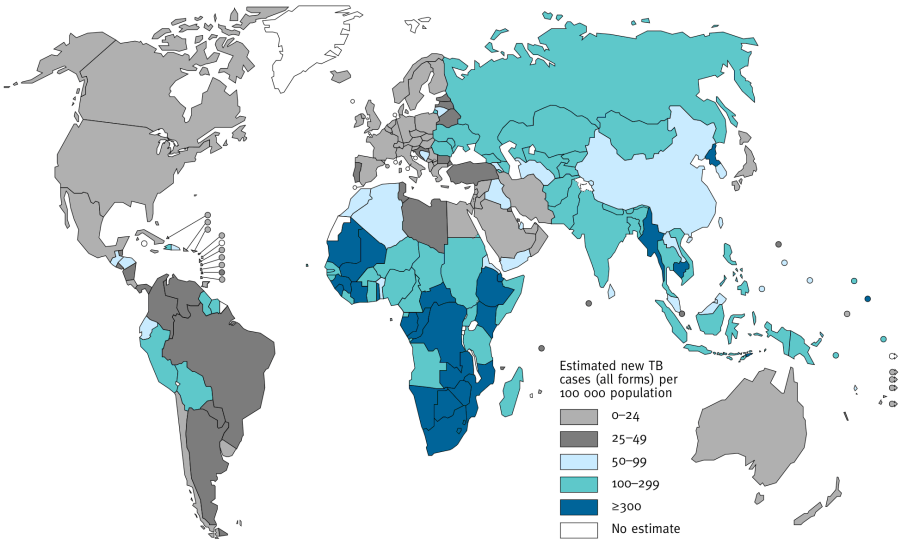

Figure 2.2 Estimated TB incidence rates by country (WHO, 2010)

2.2.2. MDR AND XDR TB

There were an estimated 440 000 cases of MDR TB in 2008 (390 000- 510 000). South Africa is one of the four countries that had the largest number of estimated cases of MDR TB in absolute terms in 2008 (13 000; 10 000-16 000). It is estimated that South Africa had 1.8% MDR among new TB cases and 6.7% amongst previously treated TB cases and 10.5% of XDR cases (WHO, 2010)

2.3. HISTORICAL OVERVIEW OF TB TREATMENT

The treatment of TB goes as far back as the late eighteenth century. However, it was the discovery of streptomycin from *Streptomyces griseus* by Selman Waksman and his colleagues in 1943 that saw the birth of a new era of chemotherapeutic approach to TB therapy or treatment (Keers, 1978). Although streptomycin proved to inhibit the growth of *M. tuberculosis,* it soon became apparent that resistant mutants thrived in the application of streptomycin monotherapy, resulting in treatment failure.

The quest for new drugs to treat TB subsequently lead to the discovery of *p*-aminosalicylic acid (PAS) in 1949, isoniazid (INH) in 1952, pyrazinamide (PZA) in 1954, cycloserine in 1955, ethambutol (EMB) in 1962, and rifampicin (RIF) in 1963 (Rattan *et al,* 1998). In years to follow, more drugs were discovered, such as aminoglycosides which include capreomycin (CAP), viomycin (VIO), kanamycin (KM), amikacin (AK) and quinolones such as ofloxacin (OFX) and ciprofloxacin (CIP) (Rattan *et al,* 1998). Currently, the use of INH, EMB, STR, PZA and RIF constitute the first line drugs for the treatment of TB (Rattan *et al,* 1998). The proper use of these drugs should produce a rapid clinical improvement and a significant decrease in bacterial count (WHO, 1997).

The TB treatment regimen comprises of two phases: the intensive and the continuation phase. The intensive phase takes two months and involves multiple antibiotics such as RIF, INH, PZA and EMB or STR, to ensure that mutants resistant to a single drug do not emerge (WHO, 1997). The following 4 months is the continuation phase and only RIF

and INH are administered to eliminate any uncleared tubercle bacilli. INH and RIF are the two most potent anti-TB drugs and kill more than 99% of tubercle bacilli within 2 months of initiation of therapy (Mitchison, 1985; Iseman, 1989). Therefore, resistance to these two drugs was regarded as MDR in *M. tuberculosis* (WHO, 1997). The use of these drugs in conjunction with each other reduces antitubercular therapy from 18 months to 6 months. When TB results from infection with drug susceptible strains of *M. tuberculosis*, the success rate of this treatment is close to 100%, provided that the patient strictly adheres to the treatment regimen (WHO, 1997)

2.3.1. RIFAMPICIN

RIF, derived from *Streptomyces mediterranei* was first introduced for use as antitubercular therapy in the early 1970's and is still a very important component of current regimens (Musser, 1995). It is the most potent sterilizing antibiotic used for the treatment of TB (Goble *et al,* 1993). The mode of action of RIF is based on the inhibition of the elongation of transcripts by covalently binding to the beta subunit of RNA polymerase in *M. tuberculosis* thus leading to bacterial death (Miller *et al,* 1994). The RNA polymerase beta-subunit is encoded by the *rpo*B gene (Telenti *et al,* 1993).

2.3.2. ISONIAZID

Isoniazid, also called isonicotinic acid hydrazide (INH) was first synthesised in the early part of the 19th century but only introduced as anti-TB drug in the 1950's (Ramaswamy

and Musser, 1998) and its powerful anti-TB activity was discovered in 1951. INH used in treatment of TB is a prodrug and necessitates catalytic activation by KatG to be converted into the active form. KatG is an enzyme with dual activities of catalase and peroxidase (Lei *et al,* 2000). Catalase-peroxidase is a heme containing enzyme encoded by the katG gene of *M. tuberculosis* (Zhang *et al,* 1992), which converts INH to a toxic derivative. INH activation leads to inhibition of mycolic synthesis, a long chain fatty acid-containing component of the mycobacterial cell wall (Winder and Collins, 1970; Takayama *et al,* 1972). Two enzymes involved in the elongation cycle of the fatty acid biosynthesis, namely an enoyl-acyl carrier protein reductase (Banerjee *et al,* 1994) and β-ketoacyl-acyl carrier protein synthase (Mdluli *et al,* 1998) are believed to be the targets of activated inhibitor(s).

2.3.3. ETHAMBUTOL

EMB is a first line *M. tuberculosis* drug with a broad spectrum of activity. Takayama and Kilburn in 1989, demonstrated that the cell wall, and more specifically mycolic acid synthesis process, is the primary target of EMB. They observed effects such as the inhibition of the transfer of precursor molecules in mycolic acid synthesis from the cytoplasm to the cell wall (Takayama *et al*, 1979), the accumulation of the trehalose-, mon- and dimycolates in the cell and the inhibition of the synthesis of arabinogalactan from D-arabinose (Kilburn and Takayama, 1981). Other investigators have suggested that the inhibition of glucose metabolism may be involved (Silve *et al,* 1993). A resistant mutant of *M. tuberculosis* contained less phospholipids and unsaturated fatty acids and

more arabinose, galactose, hexosamine and mycolic acids than the EMB susceptible strain (Sareen and Khuller, 1990). The action and the target of EMB remain less understood.

2.3.4. STREPTOMYCIN

STR is a broad spectrum antibiotic of the aminoglycoside family that is bactericidal in action and was the first drug used in treatment of TB. It is an aminocyclitol glycoside that interferes with prokaryotic protein synthesis. Its main effects are induction of misreading of the genetic code and inhibition of initiation of translation (Moazed and Noller, 1987). The site of action of streptomycin is the small 30s, subunit of the ribosome, especially the ribosomal protein S12 and the 16S rRNA (Garvin *et al,* 1974). STR acts at several stages in protein synthesis and its main effects appear to be the inhibition of initiation of mRNA translation, misreading of genetic code and aberrant proofreading by bacterial ribosome.

2.3.5. PYRAZINAMIDE

Currently, PZA is one of the essential drugs in short course chemotherapy of TB. PZA is a synthetic derivative of nicotinamide that has been used in short course anti-TB treatment regimens (Musser, 1995). This drug is the least studied of all the drugs of treatment because its activity depends upon an acid pH environment which leads to discrepancies between the in vivo and in vitro investigations. It acts in the acidic extracelluar micro-environment found during acute inflammation and kills at least 95% of

bacilli during the first two weeks of treatment (Mitchison, 1985). The target of action of PZA in *M. tuberculosis* is thought to be *fasI* (Zimhony *et al,* 2000). It is known that *M. tuberculosis* converts PZA to its active form, pyrazinoic acid, by using enzyme pyrazinamidase (PZAse) (Konno *et al,* 1967).

2.4. GENETIC MECHANISMS OF RESISTANCE

Generally, resistance arises by the process of mutation and adaptation. In *M. tuberculosis* drug resistance occurs spontaneously i.e. sites for resistance are chromosomally located and not linked (Iseman, 2000). It differs from most other bacteria in its cell wall composition, and as a result exchange of genes across this cell wall is difficult. Acquisition of drug resistance in *M. tuberculosis* does not involve plasmids or transposable elements. However, drug resistance is an outcome of changes in protein structure involved in drug uptake or in activation of prodrugs (Middlebrook *et al,* 1954, Telenti *et al,* 1993; Takayama *et al*, 1979, Kilburn and Takayama, 1981). These changes are due to mutations in genes coding for such proteins. The mutations that occur randomly at chromosomal loci include nucleotide changes such as point mutations, small deletions or insertions which confer resistance to single drugs. *M. tuberculosis* accumulates these mutations in a stepwise manner and this leads to drug resistant TB. Drug resistant strains also emerge when treatment is discontinued or otherwise insufficient, emphasizing the importance of early detection of drug resistance.

Resistant organisms (or mutants) evolve in the absence of drug exposure but they are diluted within the vast majority of the drug-susceptible bacilli. During bacterial multiplication, resistance develops through spontaneous mutations at a defined rate e.g. mutations resulting in resistance to RIF occurs at a rate of 10^{-8} in *M. tuberculosis* in vitro and is thought to be a one step mutational event (Tsukamura, 1972, Iseman *et al,* 1989, Musser, 1995). For INH and EMB, resistance rate occurs at a rate 10^{-6} and for STR 10^{-5} (Musser, 1995). During antibiotic treatment, the susceptible sub-population of bacilli are killed and allow the resistant mutants to be selected.

Table. 2.1 Genetic loci conferring drug-resistance in *Mycobacterium tuberculosis*

Drug	Gene	Product	Frequency of mutations in resistant strains (%)
Rifampicin	*rpo*B	B-subunit of RNA polymerase	> 90
Isoniazid	*kat*G	Catalase-peroxidase	60-70
	*inh*A	Enoyl-ACP reductase	< 10
	*ahp*C-*oxy*R	Alky hydro-reductase	~ 20
Streptomycin	*rps*L	Ribosomal protein S12	60
	rrs	16s rRNA	< 10
Pyrazinamide	*pnc*A	Amidase	70-100
Ethambutol	*emb*B	EmbCAB	69

Table from Rattan *et al,* 1998

2.4.1. RESISTANCE TO FIRST LINE DRUGS

2.4.1.1. RIF RESISTANCE

Resistance to RIF has been shown to be due to the alteration of the beta subunit of the RNA polymerase encoded by the *rpo*B gene (Telenti *et al,* 1993). Several authors have reported specific mutations, insertions, and deletions in this gene (Telenti *et al,* 1993; Williams *et al,* 1998; Yuen *et al,* 1999). About 96% of RIF resistant isolates of *M. tuberculosis* have point mutations in an 81-bp region of this gene. These mutations are not present in susceptible isolates and are thus an ideal target for development of genetic susceptibility testing methods (Telenti *et al,* 1993). Mutations in the *rpo*B gene lead to an altered structure of RNA polymerase and hence to a largely impaired affinity to RIF for this enzyme (Musser, 1995).

2.4.1.2. INH RESISTANCE

Complex metabolic changes have been described for INH resistant organisms. Resistance to this drug is associated with a range of mutations affecting one or more genes such as those encoding catalase-peroxidase (*katG*) (Zhang *et al,* 1992; Cockerill *et al,* 1995), the two gene operon (*inhA* locus) encoding the enoyl-acyl carrier protein reductase involved in mycolic biosynthesis (Banerjee *et al,* 1994), the alkyl hydroperoxidase reductase (*ahpC*), which is involved in the cellular response to oxidative stress (Wilson and Collins, 1996; Kelley *et al,* 1997), the β-ketocyl acyl carrier protein synthase (*kasA*) which is important in fatty acid elongation (Mdluli *et al,* 1998) and the *ndh* gene which encodes an

NADH dehydrogenase, causing defects in the enzyme activity that results in an increased $NADH/NAD^+$ ratio and co-resistance to INH and ethionamide (Lee *et al,* 2001). However, most studies have demonstrated that INH is most frequently associated with mutations in *katG* gene.

2.4.1.3. EMB RESISTANCE

Resistance to EMB is a result of mutations in the *embB* gene, coding for arabinosyl transferase, which is involved in mycolic acids metabolism (Takayama *et al,* 1979). The substitutions on codon 306 in the *M. tuberculosis* gene *embB* have been shown to be the most frequent and most predictive mutations for EMB resistance (Sreevatsan *et al,* 1998).

2.4.1.4. STR RESISTANCE

Resistance to STR has been shown to result from three different mechanisms. Firstly, the S12 protein is encoded by the *rpsL* gene and missense mutations in this gene have been shown to confer STR resistance. Presence of mutations results in changes in the conformational structure of the ribosomal subunits. This affects the binding of STR to the ribosome and as a result, the effects of the drug when bound are diminished (Meier *et al,* 1994). Secondly, resistance occurs due to changes in the 16S rRNA (Finken *et al,* 1993). This protein is encoded by the *rrs* gene and mutations in this gene results in changes in the ribosomal structure. Isolates that do not possess mutations in these two genes are

believed to possess an alternate unknown mechanism of resistance (Yamada *et al,* 1985, Nair *et al,* 1993).

2.4.1.5. PZA RESISTANCE

Numerous investigations have reported that PZA-resistant organisms have lost the pyrazinamidase activity possessed by PZA susceptible strains (Scorpio and Zhang, 1996). Resistance to pyrazinamide is usually caused by mutations in the gene *pncA* encoding the enzyme pyrazinamidase (PZAse) (Butler and Kilburn 1983; Ramaswamy and Musser, 1998). This enzyme metabolises PZA to pyrazinoic acid and PZA resistant organisms have lost PZAse activity (McClatchy *et al,* 1981; Butler and Kilburn, 1983). Therefore, it is suggested that PZA resistance may be a result of molecular mechanisms such as loss of PZAse structural gene or missense mutation resulting in an altered allele (Ramaswamy and Musser, 1998). However, some highly PZA resistant strains do not always lack PZA activity and thus an alternate resistance mechanism may exist (Butler and Kilburn, 1983).

2.5. SECOND-LINE DRUGS FOR TREATMENT OF RESISTANT TB

2.5.1. FLUOROQUINOLONES (FQs)

FQ therapy is associated with an improved outcome in MDR-TB. OFX and CIP have been shown to be the most active of the quinolone drugs against *M. tuberculosis*. OFX has been more frequently used compared to CIP because of its increased absorption and half life (Trimble *et al,* 1987). The primary target of FQs in many bacterial species is DNA gyrase which is involved in DNA replication (Hooper and Wolfson, 1993).

2.5.2. CAPREOMYCIN AND VIOMYCIN

CAP and VIO are mostly used together for treatment of drug resistance *M. tuberculosis* strains (Herr *et al,* 1966). They are polypeptide antimicrobial agents and are structurally similar, such that cross-resistance between the two drugs in *M. tuberculosis* has been shown (MacClathy *et al,* 1977). CAP is a macrocyclic peptide antibiotic produced by *Saccharothrix mutabolis* subspecies *capreolus* (Sutton *et al,* 1966; Ziersky, 1969). This appears to hinder the process of translation in mycobacteria. It was shown to inhibit phenylalanine synthesis in an in vitro translation assay using mycobacterial ribosome's (Trnka and Smith, 1970). CAP is very expensive but very useful in cases with tubercle bacilli resistant to STR, KM and AK. VIO affects the dissociation of the 70S ribosome of *M. smegmatis* subunit by binding to both the 30S and 50S ribosome subunits (Yamada *et al,* 1976). Furthermore, it inhibits ribosomal translocation by stopping peptidyl-tRNA in the ribosomal acceptor site (Modolell and Vazquez, 1977).

2.5.3. KANAMYCIN AND AMIKACIN

KM is an aminoglycoside that is used in treatment of organisms that are resistant to first line drugs. KM is the least expensive, but largely used for indications other than TB in some countries. Amikacin (AK) is as active as KM and better tolerated but much more expensive. KM and the closely related AK are commonly used for treatment of MDR-TB (Iseman, 2000). Although strains that acquire resistance to CAP also generally remain susceptible to other anti-TB medication, cross resistance with KM and VIO can occur.

2.5.4. ETHIONAMIDE

ETH, one of the most frequently used second-line drugs in the treatment of MDR TB and is a structural analog of INH (Blanchard, 1996). Both compounds are known to target the enoyl-acyl carrier protein reductase enzyme involved in biosynthesis of mycolic acid and is encoded by the *inh*A gene (Takayama *et al,* 1972). The structural similarity and existence of cross-resistant phenotypes suggests that ETH and INH share a common molecular target (Banerjee *et al,* 1994)

2.5.5. D-CYCLOSERINE

DCS is an effective antimycobacterial agent but is rarely prescribed and is seldomly used due to its adverse effects. Few studies have been conducted on the mode of action and mechanisms of DCS resistance in mycobacteria. It is believed that DCS inhibits the formation of peptidoglycan (David, 2001). It is also an inhibitor of D-alanine: D alanine branch (Lambert and Neuhaus, 1972)

2.6. DRUG SUSCEPTIBILITY TESTING IN *M. tuberculosis*

Diagnostic mycobacteriology plays a significant role in the control of the spread of TB especially MDR-TB. Rapid methods of diagnosis and determination of drug susceptibility are particularly important. Conventional methods using solid media, either agar or egg-based, require long incubation periods before the results are available. Rüsch-Gerdes *et al,* 1999 reported a significant reduction of turnaround times for susceptibility results (from 3-6 weeks to 3-15 days) as a result of introducing manual and automated methods for susceptibility testing in liquid media.

2.6.1. SUSCEPTIBILITY TESTING USING SOLID MEDIA

Three conventional methods that utilise solid media to determine whether an *M. tuberculosis* isolate is susceptible or resistant have been established, namely: the absolute concentration method, the resistant ratio (RR) method and the proportion method (Drobniewski and Balabanova, 2002).

2.6.1.1. ABSOLUTE CONCENTRATION METHOD

Drug free media and media containing graded concentrations of the drug to be tested are inoculated with a standardised inoculum. The drug is included into solid agar or Lowenstein-Jensen medium or in broth as two-fold dilutions. Resistance is defined as the

lowest concentration of the drug that inhibits growth. The major limitation in this method is the variability in inoculum size (Vareldzis *et al,* 1994, Heifets, 1996).

2.6.1.2. RESISTANCE RATIO (RR) METHOD

This method is the refinement of the absolute concentration method that controls for variations in the Minimum Inhibitory Concentration (MIC) of a given isolate when tested on different batches of media containing drugs. It is defined as the MIC of the test isolate divided by the MIC of a standard susceptible strain such as H37Rv. Testing is conducted at three concentrations of the drug and is greatly affected by the inoculum size as well as the viability of the strains. In addition, any variation in the susceptibility of the control strain also affects the RR of the test strain (Vareldzis *et al,* 1994).

2.6.1.3. PROPORTION METHOD

The proportion method based on culture is the 'gold standard' for the detection of resistance in *M. tuberculosis* isolates (Canetti, 1965). In this method, the isolate is classified as susceptible below a critical proportion of resistant bacteria and as resistant above it. The proportion of drug resistant mutants in a population is calculated from a ratio of the number of colonies growing in drug containing medium and on drug free medium (Vareldzis *et al,* 1994). It is an inexpensive and relatively simple technique, which provides results in 3 weeks from a cultured isolate or from AFB smear positive sputum specimen.

2.6.2. SUSCEPTIBILITY TESTING USING LIQIUD MEDIA

It is widely known that drug susceptibility testing of *M. tuberculosis* is more rapid in liquid compared to solid media. Liquid media systems use an increase in biomass as a growth marker of TB and hence predicting resistance to the drug by the test organism. Radiometric methods were first introduced in mycobacteriology by Cummings *et al,* 1975. A major advancement was made in 1977, when Middlebrook introduced a liquid 7H12 medium containing ^{14}C-labelled palmitic acid for radiometric detection of mycobacterial growth (Middlebrook *et al,* 1977).

2.6.2.1. BACTEC 460 TB METHOD

The BACTEC 460 method is a radiometric variant of the proportion method and was first described by Siddiqi *et al,* in 1981. The method is based on detection of growth radiometrically. During growth, bacteria utilize a radiolabelled source of carbon (^{14}C) and produces radiolabeled CO_2. The instrument records the level of radioactivity as growth index. This method has shortened the duration of susceptibility testing to 5 to 7 days. The BACTEC system provides results in only 5 days but it is costly and requires disposal of radioactive material. In 1983, Roberts *et al* reported 98% agreement for BACTEC 460TB in comparison with conventional susceptibility testing of *M. tuberculosis* with a turnaround time of approximately 5 days. These results were concordant with those of Siddiqi *et al* (1981).

2.6.2.2. MYCOBACTERIA GROWTH INDICATOR TUBE (MGIT) METHOD

Palaci *et al,* (1996) reported the Mycobacteria Growth Indicator Tube (MGIT) to be time saving, safe, simple and reliable in the detection of drug resistance. The method uses tubes containing 4.0 ml of Middlebrook 7H9 broth with an oxygen sensitive fluorescence sensor embedded in silicone to indicate microbial growth. The broth is enriched 7H9 (modified Middlebrook) broth base with 0.25% glycerol and 10% CO_2 (Walters and Hanna, 1996).

Actively respiring mycobacteria use the oxygen available within the tube, thereby exciting the fluorescence reaction. In the presence of oxygen the fluorescent indicator is quenched. Fluorescence indicating mycobacterial growth is then detected when the MGIT is viewed with a 365-nm ultraviolet (UV) light from transilluminator. Antimycobacterial susceptibility testing can be performed by comparing the growth in an antibiotic-containing MGIT with that of a growth control MGIT without antibiotics (Walters and Hanna, 1996).

2.6.2.3. MB/BacT SYSTEM

The MB/BacT system has been reported to be a rapid, sensitive method for the growth and detection of mycobacteria from clinical specimens and has shown good performance in comparison with BACTEC 460 TB (Rohner *et al,* 1997; Brunello *et al,* 1999). In this closed system, mycobacterial growth is indicated by a colorimetric CO_2 detection device. Unlike the BACTEC 460 TB, the cumbersome handling of bottles during incubation is avoided. Brunello and Fontana in 2000 concluded that MB/BacT system was suitable to test the antimicrobial susceptibility of *M. tuberculosis* to first line drugs. The advantage of this method is the absence of radioactivity when compared to BACTEC 460 TB method.

2.6.3. MICROSCOPY BASED DRUG SUSCEPTIBILITY TESTING

2.6.3.1. Microscopic observation Drug Susceptibility (MODS) Assay

The simple microscopic observation drug susceptibility assay was developed by Caviedes *et al,* in 2000. This method uses the two properties of *M. tuberculosis*: (i) the growth rate of *M. tuberculosis* is rapid in liquid medium compared to the growth rate in solid medium, and (ii) its morphology in liquid culture is recognizable as consisting of tangles or cords of organisms. Middlebrook 7H9 broth inoculated with decontaminated sputum in 24-well plates is examined under an inverted light microscope. Direct susceptibility testing on clinical specimens is performed by incorporating anti-TB drugs at the onset.

Park *et al,* 2002 and Moore *et al,* 2004 demonstrated similar results when comparing the MODS assay to gold standard proportion method showing a 100% agreement between the two methods.

2.6.4. COLORIMETRIC METHODS

Bacterial species have the ability to reduce an indicator and produce a change of visual colour. This has enabled researchers to exploit this characteristic to develop methodologies for DST such as the Alamar blue (Yajko *et al,* 1995; Franzblau *et al,* 1998), MTT assay (Abate *et al,* 1998; Mshana *et al,* 1998; Foongladda *et al,* 2002), the nitrate reductase assay (Angeby *et al*, 2002), and resazurin (Palomino *et al,* 2002; Banfi and Monti-Bragadin, 2003).

2.6.4.1. ALAMAR BLUE ASSAY

Alamar blue is a dye that indicates cellular growth-viability due to the oxidation-reduction process during metabolism of viable organisms. During metabolic activity the blue oxidised form becomes pink upon reduction. This method was first used by Pfaller *et al* (1994) for drug susceptibility testing of yeast and was first used on mycobacteria by Yajko *et al* (1995). Collins and Franzblau (1997) modified the assay format to create the microplate alamar blue assay or MABA. This assay has been used for DST of *M. tuberculosis* to RIF and INH (Reis *et al,* 2004).

2.6.4.2. 3-(4,5DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYL TETRAZOLIUM BROMIDE (MTT)

MTT is a yellow tetrazolium salt that is converted into blue formazan by dehydrogenases of live cells (Mossman, 1983). The amount of blue or purple formazan formation is proportional to the number of live mycobacteria in a sample (Mshana *et al,* 1998). Abate *et al* (1998) and Moore *et al* (1998) applied this assay to detect RIF resistance directly from sputum specimens. They found it to shorten the turnaround time for detection of RIF resistance.

2.6.4.3. NITRATE REDUCTASE ASSAY

The nitrate reductase assay (NRA) was initially developed at the Central Tuberculosis Research Institute in Moscow Russia (Golyshevskaia *et al,* 1996). It was then called the Griess method, after J. P. Griess, who discovered the chemistry of the detection method (Griess and Bemerkungen, 1879). *M. tuberculosis* has the ability to reduce nitrate to nitrite and this is routinely used for biochemical identification of mycobacterial species. The presence of nitrite can easily be detected with specific reagents, which produce colour change (Kent and Kubica, 1985). Angeby *et al* (2002) evaluated this method for DST of *M. tuberculosis* and found it to be rapid, inexpensive and easy to perform.

2.6.4.4. RESAZURIN ASSAY

The resazurin reduction test was first used to demonstrate bacterial and yeast contamination in milk (Khomenko and Matuzenko, 1976; Guerin *et al,* 2001). Resazurin is a blue dye that does not flouresce but becomes pink and fluoresces as it is reduced to resofurin by oxidoreductases within live cells. This method produced the most reliable and accurate results for DST of INH and RIF (Palomino *et al*, 2002; Banfi and Monti-Bragadin, 2003). It has been used for detection of both first line (Palomino *et al,* 2002) and second line antibiotic resistance of *M. tuberculosis* (Martin *et al,* 2003)

2.7. MOLECULAR TECHNIQUES

Molecular drug assays can be divided into phenotypic and genetic assays. Phenotypic assays are based on measuring an outcome e.g. death of the bacillus and do not require prior knowledge of the primary resistance mechanism. Genotypic assays are based on understanding the drug target and nature of the gene involved in the mechanism of resistance.

2.7.1. PHENOTYPIC MOLECULAR TECHNIQUES

Phenotypic molecular methods use markers of viability instead of an increase in biomass as in the case of liquid media based susceptibility testing. Anti-TB drugs also inhibit bacterial metabolic activities in susceptible isolates but not in resistant isolates.

Strategies for the detection of resistance that have been used include mycobacteriophages such as the phage assay (Albert *et al,* 2002; Galí *et al,* 2003) and the luciferase reporter phages (LRPs) (Jacobs *et al,* 1993). Drug susceptibility is assessed without reference to the genetic basis of resistance.

2.7.1.1. PHAGE ASSAY

The phage assay is fast and easy to perform, yielding results in clinical specimens within 48 hours (Albert *et al,* 2002; Krishnamurthy *et al,* 2002). Bacteriophages can infect and replicate inside mycobacteria. Once mycobacteria are infected, the number of internalised phages determined indicates the original number of *M. tuberculosis*. This is determined after a number of cycles of infection, replication and release in rapidly growing mycobacteria such as *M. smegmatis*.

When drug resistant *M. tuberculosis* is infected with a mycobacteriophage, it remains viable and protected within the bacilli. It then replicates within viable bacilli and eventually lyses its host and lysis is easily seen as clear areas or plaques in a lawn of *M. smegmatis* culture. The number of plaques generated is directly proportional to the number of protected mycobacteriophages, which is dependent on the number of tubercle bacilli that remain viable after drug treatment (Gingeras *et al,* 1998). The sensitivity of this assay as well as the presence of inhibitors in sputum poses major problems.

2.7.1.2. LUCIFERASE REPORTER ASSAY (LRP)

Jacobs *et al* (1993) demonstrated that luciferase reporter mycobacteriophages can be used as a simple tools for rapid determination of drug susceptibility profiles of *M. tuberculosis*. Luciferase activity can be monitored with a luminometer or with a photographic film. The luminometer offers higher sensitivity and quantitative results in 54 hours. It is based on the efficient production of photons by viable mycobacteria infected with specific reporter phages expressing the firefly luciferase gene (*lux*). Some reporter phages possess the firefly gene. Riska *et al* (1999) reported that these phages can inject their *lux* bearing genes in viable mycobacteria and this would result in production of photons. The injected *lux* gene, when transcribed, using the mycobacterial ATP, catalyses the production of light. This activity can be detected by using the luminometric instrument. Mycobacterial cells killed by anti-TB drugs can not be infected and thus do not produce light.

The shortcoming of this assay is that the mycobacteriophage has a broad host array and will infect many non-tuberculous mycobacteria and therefore may lead to false positive reports of drug resistant *M. tuberculosis*. Although there are techniques for increasing species specificity for this assay, this comes at great cost and complexity. This procedure has shown promising results with clinical isolates and sputum samples (Banaiee *et al,* 2001; Bardorov *et al,* 2003). Photographic detection is achieved by using the 'Bronx box', which is an expensive light tight box with a Polaroid cassette. This carries a photographic film which is able to detect light emitted by the infected cells carried out in microtiter plates. Qualitative results can be obtained in 94 hours.

2.7.2. GENETIC MOLECULAR TECHNIQUES

The genes coding for the targets of the first-line anti-tuberculosis drugs as well as the mutations associated with the resistant phenotypes have been identified. The detection of these mutations is facilitated since they are localised in limited regions of the genes encoding the drug targets. After PCR amplification, the mutation is identified by sequencing the PCR products or other mutation detecting methods. Molecular studies have indicated that the genome of *M. tuberculosis*, including mutations in the drug resistant genes, is stable in follow-up samples from MDR-TB patients (Victor *et al,* 1997). This favours the use of molecular techniques to predict drug resistance by mutation analysis. Generally, resistance arises by a process of mutation and adaptation. Detection of genotypic drug resistance in *M. tuberculosis* isolates provides a rapid and easy alternative to conventional phenotypic susceptibility testing.

MDR results from stepwise acquisition of mutations in the genes encoding drug targets or drug converting enzymes (Gillespie, 2002). This knowledge has paved the way for molecular assays that have potential to provide rapid detection of resistance in *M. tuberculosis* isolates. These techniques are highly sensitive, specific and the fact that they do not rely on mycobacterial growth has shortened the time between detection and the commencement of effective treatment. Genetic loci have been established as sources of resistance by targeting and sequencing these regions believed to be sources of resistance

to these drugs. Moreover, the genetic basis of resistance has been exploited to develop rapid tests for drug susceptibility.

Molecular assays that have been used to monitor the *rpo*B gene for RIF resistance mutations consist of: DNA sequencing (Kapur *et al,* 1994), heteroduplex analysis (William *et al,* 1998), polymerase chain reaction-single strand conformation polymorphism (Telenti *et al,* 1993, Heym *et al,* 1994), dideoxy fingerprinting (Femlee *et al,* 1995), line probe assay (Cooksey *et al,* 1997, Goyal *et al,* 1997, Hirano *et al,* 1999, Srivastava *et al,* 2004), molecular beacon (Piatek *et al,* 1998, El-Hajj *et al,* 2001), mismatch analysis (Nash *et al,* 1997), real-time PCR (Garcia de Viedma *et al,* 2002, Torres *et al,* 2003), high density probes assays (Troesch *et al,* 1999, Sougakouff *et al,* 2004, Wada *et al,* 2004) and micro arrays (Yue *et al,* 2004).

2.7.2.1. DNA SEQUENCING

DNA sequencing is the most accurate and reliable method for detection of mutations. It is used as the gold standard for detection of mutations which are believed to be predictive of resistance (Kapur *et al,* 1994). The added advantage of this method is that it has been automated, which has simplified the process. Accurate sequence data can be obtained within 48 hours from automated analyzers that use fluorescent chemistry methods. Except for RIF, DNA sequencing is unlikely to be used in the routine detection of drug resistance mutations because it requires several sequencing reactions per isolate which makes it labour-intensive and expensive. However, this method is impractical for use in

routine detection of drug resistant mutations because it requires specialised equipment and is labour-intensive and expensive.

2.7.2.2. HETERODUPLEX ANALYSIS

Heteroduplex analysis is a method of detecting gene mutations by mixing PCR- amplified mutants followed by denaturation and reannealing (Williams *et al,* 1998; Bahrmand *et al,* 2000). The resultant products are resolved by gel electrophoresis. When two single strand DNA molecules differing in their base matching form a heteroduplex, the resultant conformation is altered, accompanied by a reduced electrophoretic mobility compared to its corresponding homoduplex with no mismatch.

2.7.2.3. DIDEOXYFINGERPRINTING

Dideoxyfingerprinting (ddF) involves dideoxy sequencing followed by non-denaturing electrophoresis and was first described by Sarkar *et al*, in 1992. "Fingerprint" or bandshifts where changes in sequences are present is observed in mutated DNA sequences. The theoretical basis for identification of DNA sequence changes is twofold: (i) secondary structural differences of single-strand DNA (full length and strand termination) and (ii) alterations of the termination site of the primer extension product. The time required for detection of RIF-susceptibility using this method was reduced to 2 days (Femlee *et al*, 1995). They compared this method to SSCP and found that the increased molecular complexity inherent in ddF increases the efficiency of detecting and

discriminating base changes. This same increased complexity contributed to the observation that ddF analysis was less influenced by the location of the mutation, size of the DNA fragment being investigated, temperature and other experimental conditions. These attributes of ddF allowed for greater accuracy in the identification and characterisation of mutations in a reference laboratory setting. This method is not favoured since the use of radioactivity is involved and thus is costly in terms of waste disposal.

2.7.2.4. DENATURING GRADIENT GEL ELECTROPHORESIS

Denaturing gradient gel electrophoresis (DGGE) is a molecular technique that distinguishes mutant amplicons from their wildtype equivalents. This method is more sensitive when compared to DNA sequencing as it can detect point mutations and small insertions and deletions. The principle of this technique is that the melting temperature of DNA changes as its fragments migrate through a gradient of denaturants (McCammon *et al,* 2005). A molecular fingerprint is generated based on the characteristic denaturation pattern of different mutational variants within a DNA fragment. Scarpellini *et al,* (1999) successfully applied this technique in the detection of mutations associated with RIF resistance within the *rpo*B gene.

2.7.2.5. LINE PROBE ASSAY

By examining the sequence data in sensitive and resistant strains, probes can be designed and immobilised onto a membrane. Detection of susceptibility or resistance can be done by a reverse hybridisation principle (Saiki *et al,* 1989). PCR is performed on the region of DNA in which mutations are associated with drug resistance. The PCR product is then allowed to hybridize with the probes on a membrane. Failure to bind the wild type probe is due to the presence of a mutation and thus is predictive of drug resistance. The commercially available line probe assay for detection of mutations in the *rpo*B core region (De Beenhouwer *et al,* 1995; Cooksey *et al,* 1997; Bartfai *et al,* 2001) is based on this principle. The region is amplified and biotin labelled by PCR. The DNA is detected after hybridisation with a strip in which 5 probes for wild type *rpo*B sequences, 4 for specific *rpo*B mutations, a conjugate control and *M. tuberculosis* control probes are immobilised. The bound DNA is detected with a colour reaction.

The advantage of this method is that it can detect mutations directly from clinical specimens. However, its limitations are that it is costly and also detects only four mutations. Oliveira *et al* (2002) reported an overall accuracy of 97.6% for detection of susceptibility by this assay when compared to conventional resistance testing. These results were concordant with those of Rossau *et al* (1997). The price of this test limits its use in developing countries where the largest pool of resistant isolates exist (Morcillo *et al,* 2002).

2.7.2.6. RNA/RNA MISMATCH ANALYSIS

RNA/RNA mismatch analysis is based on the ability of double stranded RNA to withstand digestion with RNAse A (Nash *et al,* 1997). Target DNA is amplified by using primers which incorporate T7 RNA polymerase and SP6 RNA polymerase promoters in opposite directions, allowing RNA to be transcribed by using the PCR product as a template. A RIF sensitive strain is also amplified by using the same primers but with the SP 6 and T7 promoters incorporated in the strands complimentary to the test strain. The test PCR product and the reference PCR product are combined in a transcription reaction using either T7 or SP6 RNA polymerase. The complimentary transcripts from the test and PCR products are allowed to hybridise and the resulting hybrids are treated with RNAse. Any mutations in the test transcript will not pair with the reference transcript and so the hybrid will be cleaved at that point. Undigested transcripts and cleavage products can be detected using agarose gel electrophoresis.

2.7.2.7. REAL TIME PCR

Real time PCR combines both rapid-cycle PCR and real time monitoring of the processing and generation of mutation profiles. Mutations can be monitored using fluorescent probe melting profiles. This method is very expensive and also requires sophisticated equipment. However, real-time based PCR has been shown to be highly specific and sensitive in detection of drug resistant *M. tuberculosis*. This has been applied directly on sputum samples from TB patients, giving results within 3 hours from DNA

preparation. In 2000, Torres *et al* described real-time PCR for the detection of resistance associated mutations in *M. tuberculosis* using fluorescence.

Methods based on real-time PCR have utilised fluorescence resonance energy (FRET) probes (Torres *et al,* 2000; Garcia de Viedma *et al,* 2002), molecular beacons (El-Hajj *et al,* 2001; Piatek *et al,* 1998; Piatek *et al,* 2000) and Taqman Minor Groove Binding (MGB) probes (van Doorn *et al,* 2003). Wada *et al* (2004) investigated a real time PCR based system with Taqman MGB probes to detect mutations associated with resistance of *M. tuberculosis* to INH, RIF and EMB. Taqman MGB probes can distinguish single base mismatches. The specificity of MGB probes was shown to be quite high (Kutyavin *et al,* 2000). Drug resistant *M. tuberculosis* can be detected by changes in the cycle threshold values (ΔCt) with Taqman MGB probes in real-time PCR (Kutyavin *et al,* 2000). The shortcoming of this method is its inability to define the exact nucleotide substitution involved in the mutation. However, results can be obtained in 30 min.

2.7.2.8. SINGLE-STRAND CONFORMATION POLYMORPHISM

Single-strand conformation polymorphism (SSCP) analysis is used to identify genetic locations encoding mutations without the need for DNA sequencing of the entire gene. The basis of this technique is that electrophoretic mobility of DNA in a non denaturing gel is sensitive to both size and shape. Based on intra-molecular interactions and base stacking, single stranded DNA can adopt a conformation which is dependent on sequence composition. When even a single base is changed the conformation is affected, thus changes can be detected as alterations in the electrophoretic mobility of the single-stranded DNA in non denaturing polyacrylamide gel (Telenti *et al,* 1993a). Although this technique is cost-effective, simple and sensitive, it has its drawbacks in that it may lead to false positive results due to silent mutations (Kim *et al,* 1997). In addition, it has a high-risk of contamination due to extensive post-PCR manipulation required.

2.7.2.9. MOLECULAR BEACONS

Molecular beacons are hairpin-shaped probes able to detect the presence of specific nucleic acids (Piatek *et al,* 2000). In this assay, the loop portion of the molecule is designed to complement the target nucleic acid molecule. Complementary arm sequences at the ends of probe sequence can anneal to form a stem. The end of one arm of the molecule has a quenching moiety attached to it whilst the other end has a fluorescent moiety attached to it. Fluorescence of the fluorophore is reduced by transfer of energy which results from close proximity of the two moieties to each other. A longer and more

stable hybrid than the stem hybrid is formed when the probe encounters a target molecule.

Fluorescence can be detected when the molecular beacon undergoes a spontaneous conformational reorganisation that forces the stem apart. This causes the fluorophore and the quencher to move away from each other restoring fluorescence. This is monitored in real-time, where the fluorescence increases with every cycle in proportion to the amplification of the hybridising target, which is not detected in cases when the target is not complementary to the beacon (Marras *et al,* 1999). Beacon assays are performed in sealed wells thus preventing contamination. They are easily implemented, automated and can be used in high throughput analyses. In the case of RIF, a set of 5 beacons has been designed to cover the *rpo*B core region in a single reaction with excellent results (El-Hajj *et al,* 2001). The results for RIF resistance were obtained directly from sputum specimens in less than 3 hrs. The assay is sensitive enough to detect 2 bacilli, with results available within 3 hours from sputum collection.

Piatek *et al* (2000) designed a set of beacons to screen for mutations in the regions associated with drug resistance for INH [katG gene (position 315), the promoter region of inhA, the oxyR-ahpC intergenic region and position 66, 269, 312, and 413 of kasA]. The beacons are highly sensitive and specific: a single mismatch in the target sequence diminishes the beacon-target hybrid stability, allowing the detection of point mutations.

2.7.2.10. DNA MICROARRAYS

DNA microarrays are based on the principle of hybridisation (Brown *et al,* 2006). They allow analysis of large numbers of DNA sequences in a single hybridisation step. PCR amplicons labelled with fluorophore moieties are generated from the sample to be hybridised to a large collection of probes bound to a solid surface. The bound amplicons emit fluorescent signals that are scanned with an epifluorescent microscope. Probes are designed to hybridise to fully complementary amplicons. Wildtype and mutant probes are included in the array to determine the presence of specific mutations. Microarrays have been used mainly for species identification and for detection of mutations associated with RIF-resistance, with excellent concordance with sequencing results (Gingeras *et al,* 1998; Troesch *et al,* 1999).

In 2004, Sougakoff and others detected *rpo*B mutations associated with RIF-resistance using a high density probe array. DNA microarray systems are composed of oligonucleotides synthesized onto a silica slide. Thousands of specific DNA or RNA sequences can be detected simultaneously using this system. Yue *et al* (2004) developed an in-house oligonucleotide based microarray for detection of *M. tuberculosis* resistant to RIF in clinical isolates. They reported this assay to be inexpensive, flexible and easy to perform when compared to high density DNA probe. The widespread application of microarrays is limited to the research setting because they require expertise, sophisticated equipment and are costly.

2.7.2.11. AMPLIFICATION REFRACTORY MUTATION SYSTEM

The amplification refractory mutation system (ARMS) was first introduced for the detection of any point mutations by Newton *et al,* in 1989. Fan *et al,* 2004 modified this method for the detection of the *rpoB* gene mutations in *M. tuberculosis*. It is simple, rapid and inexpensive. The principle of ARMS is that oligonucleotides which are complimentary to a given DNA sequence except for a mismatch at their 3'-OH residue will not function as primers in PCR under appropriate conditions. This is due to the absence of the 3'- exonuclease proofreading activity associated with Taq DNA polymerase.

In this method, the 3'-end of a wild type PCR primer is located at the mutation site. In the case of the wild type template, the DNA polymerase will amplify the DNA efficiently, yielding a clear band during gel electrophoresis. In the case of mutant template, the 3'-end of the primer will not hybridise and the DNA polymerase will not amplify resulting in the absence of the band during gel electrophoresis. The major advantages of this method are that it is convenient, relatively inexpensive and easy to perform since it utilises commonly available reagents and equipment. It can be performed in one day as it requires only PCR and electrophoresis of PCR products in an agarose gel. Its sensitivity is comparable to the above mentioned molecular assays.

2.7.2.12. GeneXpert MTB/RIF assay (Cepheid)

The GeneXpert system is fully automated and intergrates all the steps required for PCR-based DNA testing. It is designed to purify, concentrate, detect and identify targeted nucleic acid sequences directly from unprocessed samples. The system consists of an instrument, personal computer, barcode scanner and preloaded software for running tests on collected samples and viewing the results. It requires the use of single disposable GeneXpert cartridges that hold the PCR reagents and host the PCR process.

The Xpert MTB/RIF includes reagents for the detection of TB and RIF resistance as well. A sample processing control ensures adequate processing of the target bacteria and to monitors the presence of inhibitor's in the PCR reaction. The primers in the Xpert MTB/RIF assay amplify a portion of the *rpo*B gene containing the 81 base pair "core" region. The probes are able to differentiate between the conserved wild-type sequence and mutations in the core region that are associated with RIF resistance.

Helb *et al,* 2010 developed and performed the first analysis of the GeneXpert MTB/RIF assay demonstrating a high sensitivity and specificity to both MTB and RIF resistance detection. In this study they reported a detection sensitivity of 71.7% and specificity of 84.6% of smear negative culture positive clinical samples from Vietnam. In a study of re-treatment cases in Uganda, the Xpert MTB/RIF detected 98.4% culture positives and 100% RIF resistance. Boehme *et al,* 2010 reported a sensitivity of 98.2% among smear-positive tuberculosis, 72.5% of smear negative and a specificity of 99.2%. For RIF

resistance detection, a sensitivity of 97.6% was reported and a specificity of 98.1%. In South Africa, the assay was evaluated in a high HIV prevalence region. A sensitivity and specificity of 86% and 95% in HIV negative patients while 85% and 93% for HIV positive patients. Also 100% detection in smear positive culture positive and 65% detection in smear negative culture positive specimens (Scott, 2010).

Although this technique provides quite a number of advantages such as less hands on time on specimen preparation, results are obtained in less than 2 hours and concurrent detection of *M. tuberculosis* and RIF resistance, however it requires sophisticated equipment which is expensive for developing countries. Butkus (2010) in his review estimated the cost of this system to be $30 000 and the cost per test to be about $64.

2.7.2.13. REVERSE LINE BLOT HYBRIDISATION

The reverse line blot hybridisation method is another molecular method that rapidly detects mutations. Morcillo *et al,* 2002 applied this method for detection of *rpoB* mutations hence it was called rifampicin oligonucleotide typing (rifoligotyping in short). A combination of DNA amplification by PCR and reverse line blot hybridisation is involved in this assay. Specific primers are used to amplify the *rpo*B gene of *M. tuberculosis* by PCR. PCR products are then hybridised to oligonucleotide probes (Saiki *et al,* 1989). The membrane has oligonucleotide probes encoding consecutive parts of the *rpo*B gene sequence with the most frequently occurring mutations in RIF resistant strains (Morcillo *et al,* 2002).

The *rpo*B PCR products of the RIF resistant strains will fail to hybridise to one or more of the wild type oligonucleotides and will in most cases show affinity to mutant oligonucleotides (Kremer *et al,* 2002; Morcillo *et al,* 2002). Resistance is detected within a few hours and 43 samples can be tested at once. Application of these methods directly to clinical specimens will greatly decrease the detection time for rifampicin resistance and will help in predicting multi-drug resistance in *M. tuberculosis*.

CHAPTER THREE

3. EVALUATION OF DNA EXTRACTION METHODS

3.1. INTRODUCTION

Timely detection *M. tuberculosis* is important because of the need to make decisions regarding management such as commencement of anti-tuberculosis drug therapy, isolation precautions, and prophylaxis. Delayed diagnosis due to delays in identification of the *M. tuberculosis* complex was one of the problems identified during investigations of outbreaks of MDR TB (CDC, 1993). Although a presumptive diagnosis of pulmonary tuberculosis can be made on the basis of patient history, clinical and radiological findings, the definitive bacteriological diagnosis of TB continues to depend on the microscopic examination of acid fast stained sputum smears followed by culture confirmation (Kocagöz *et al,* 1993). Direct microscopy by Ziehl-Neelsen staining to identify acid-fast bacilli (AFB) is the most rapid method (24hrs) but it lacks sensitivity and specificity.

The Center for Disease Control and Prevention (CDC) recommended the use of both liquid and solid media for mycobacterial culture and a rapid method for identification of *M tuberculosis* complex. These results should be obtained within 21 days of specimen receipt in the laboratory. Although this achievement represented a marked improvement in the laboratory turnaround time, a delay of 21 days for a definitive diagnosis of TB was still not optimal. Thus, the Polymerase Chain Reaction (PCR) assay was developed in

response to the need for a more reliable, rapid diagnostic test targeting unique or specific sequences in *M. tuberculosis* (Brisson-Noël *et al,* 1989; Thierry *et al,* 1990; Eisenach *et al,* 1991; Cousins *et al,* 1992)

The efficiency of the PCR assays in clinical specimens is dependent on both the target sequence selected and on the efficiency of the DNA extraction procedure (Aldous *et al,* 2005)**.** However, the major disadvantage of PCR is the presence of inhibitors in clinical specimens, which interferes with amplification-based techniques (Nolte *et al,* 1993). This may result in up to 20% false negatives (Clarridge *et al,* 1993; Nolte *et al,* 1993). Insertion sequence IS*6110* is the commonest target for PCR based detection of *M. tuberculosis* (Thierry *et al,* 1990). This insertion sequence is reported to be present in multiple copies in *M. tuberculosis* chromosomes and to be specific for *M. tuberculosis* complex. The repetitive nature of this target sequence amplified by PCR contributes to the high sensitivity. Therefore, the test is able to theoretically detect a single *M. tuberculosis* organism and its sensitivity is close to 100% in clinical isolates (Eisenach *et al,* 1990).

The isolation of amplifiable DNA template from clinical material is a crucial step in the direct detection of *M. tuberculosis* by PCR. However, there are several difficulties associated with PCR. The tough and complex cell wall complicates purification of DNA in comparison to that of bacteria with relatively fragile walls (Brennan and Naikado, 1995). The mycobacteria have cell walls with copious amounts of polysaccharides, which can adversely influence subsequent manipulation of DNA. Endogenous inhibitors such as

tissues and cell constituents present in sputum as well as inhibitors introduced by reagents during DNA isolation have been reported to occur in 32-52 % of respiratory samples (Forbes and Hicks, 1996; Kearns *et al,* 1998).

Numerous protocols have been proposed for DNA extraction of mycobacteria for PCR, most of which have included use of detergents, proteolytic enzymes and/or organic solvents (Boom *et al,* 1990; Perera *et al,* 1994; Noordhoek *et al,* 1995; Singh *et al,* 2000). Chakravorty and Tyagi (2001) showed that the introduction of a cleaning step before lysis results in effective removal of biological constituents and potential PCR inhibitors present in the specimen. This creates a better environment for efficient lysis of mycobacteria rendering further purification of DNA unnecessary. In this study, four DNA extraction techniques: the Chelex based extraction method, IDI lysis kit, Qiagen extraction kit and silica based extraction method were evaluated to determine the one that optimally removes PCR inhibitors, thus improving the sensitivity of the subsequent PCR based assays.

3.2. MATERIALS AND METHODS

3.2.1. PATIENT RECRUITMENT AND SPECIMEN COLLECTION

Informed consent was obtained from patients with respiratory disease other than TB, attending the respiratory clinic at Inkosi Albert Luthuli Central Hospital (IALCH). Patients were requested to expectorate large volumes of phlegm in a container over a 1 hour period. Sputa were collected and processed at the department of Medical Microbiology laboratory at the Nelson R Mandela School of Medicine.

3.2.2. PROCESSING OF SPUTUM SPECIMEN

3.2.2.1. LIQUEFACTION OF SPECIMENS

The external surface of the container was wiped with phenol disinfectant. The sputum specimen was transferred into a 50ml tube using a sterile wooden stick. The amount of sputum in the tube was estimated and an equal volume of N- acetyl-L-cysteine (NALC) was added to the sputum. The cap was tightened firmly and tube vortexed until the specimen was liquefied (~15 min). For excessively mucoid sputa, extra NALC was added. Approximately 90% of the liquefied sample was aliquotted into 1ml amounts in 1.5 ml eppendorf tubes. The rest was decontaminated as in 3.2.2.2. The processing of the sputum specimens was carried out in a Biological Safety Cabinet (BSC) in a biosafety

level 2 (BSL2) laboratory and ensuring minimal aerosol generation to prevent cross contamination.

3.2.2.2. DECONTAMINATION OF SPECIMENS

Approximately 1ml of the liquefied sputum was added to 0.5ml of 4% NaOH, the mixture was vortexed and allowed to stand for 15 min (not more than 20 min). Phosphate buffer was added to the 50ml mark of the polypropylene tube. This was followed by centrifugation at 3000g for 20 min after which the supernatant was decanted into concentrated phenol disinfectant carefully to minimize aerosol generation. The pellet was resuspended in 1ml of phosphate buffer and mixed well by vortexing. *E. coli* and decontamination reagents were simulateneously processed to serve as negative controls and to control for contamination respectively. Contamination was also controlled by use of a single Pasteur pipettes per sample and the processing of small batches at a time.

3.2.2.3. SMEAR MICROSCOPY

One drop, equivalent to 100 µl of decontaminated suspension, was dispensed into a slide which was irradiated under UV light on a hot plate for a minimum of 30 minutes. This was followed by Ziehl Nielsen staining of the slides. Briefly; the slides were flooded with 0.3% Carbol Fuchsin. The slides were then flamed using a rod with cotton wool dipped in 70% alcohol for 5 minutes. The process was repeated three times after which the slides were rinsed with water and decolorized with 3% acid alcohol for 2 minutes. This was followed by counter staining with methylene blue for 2 minutes. The slides were rinsed with tap water and allowed to air dry. Examination for acid fast bacilli that appeared red was done under oil immersion at 100 x. Quality control slides were performed using *M.*

tuberculosis H37Rv as a positive control and *E. coli* as a negative control and these controls were processed in the same way as the specimen slides.

3.2.2.4. CULTURE

A sterile Pasteur pipette was used to inoculate 500 µl of decontaminated deposit into MB broth and incubated aerobically at 37ºC for 5 days. This was used to inoculate Lowenstein-Jensen (LJ) slants, which were rotated to ensure even distribution of the inoculum. Slants were incubated at 37ºC aerobically for 3 weeks.

3.2.2.5. IS*6110* PCR

The remaining 400µl suspension was centrifuged at 12 000 g for 15 min. The supernatant was discarded and pellet resuspended in 200µl of 1 x TE buffer. This was stored at -4ºC until required. DNA was extracted by the Qiagen method. A 123 bp sequence within the IS*6110* insertion sequence element was amplified by PCR to determine whether the sputum specimens were negative or positive for TB. Amplification was carried out in a thermal cycler (Gene Amp PCR System 9700) in a total reaction volume of 50 µl.

The PCR reaction mixture contained 10.0 µl of template DNA, 50 mg/ml of T4 primer, 50 mg/ml of T5 primer, 1.0 µl of 25 mM deoxynucleoside triphosphates (dNTP's), 0.5 µl (5 U/µl) of Taq DNA polymerase, 5.5 µl of PE buffer, 1.5 µl of 25 mM $MgCl_2$, 3.73 µl of BSA and 27.6 µl of nuclease free water. The reaction mixture was incubated at 94°C for

2 min to denature the DNA. This was followed by 35 cycles of amplification, each of which consisted of three steps in the following order: denaturation at 94°C for 45 sec, annealing at 68°C for 45 sec and extension of the primers at 72°C for 2 min. Final extension was conducted at 72°C for 5 min. Amplification products were electrophoresed in a 1.5 % Agarose gel containing ethidium bromide, at 100 V for 60 min. They were then visualized using a UV transilluminator. The same PCR was used to identify the DNA extraction method that was optimal for removal of inhibitors in PCR as well as to determine the sensitivity of detection. Different rooms were used for DNA extraction, preparation of reagents for PCR, addition of DNA to the mastermix reagents and PCR amplification; to reduce the risk of cross-contamination of DNA. The risk of contamination was also reduced by the use of plugged pipette tips and liberal use of 5% hypochlorite.

3.2.2.6. STORAGE OF SPUTUM SPECIMENS

Sputum specimens were stored at 4ºC until PCR results were available. These results were then compared to the smear results and if both were negative, the sputa was pooled and used in the spiking experiments.

3.2.3. PREPARATION OF INOCULUM

M. tuberculosis was grown to mid log phase on Lowenstein-Jensen slopes until confluent. A wooden stick was used to transfer a loopful of culture into a tube containing phosphate

buffer, 0.05% Tween 20 and 5-10 glass beads (3 mm). The tube was vortexed for 5 min to break down clumps which were allowed to settle for 45 min. Thereafter, the upper part of the bacterial suspension (3 ml) was transferred to a sterile tube. The bacterial suspension was aspirated ten times with a syringe and a 26 inch gauge needle. The cell suspension was then passed through a 5 µm filter. The turbidity of the bacterial suspension was adjusted to a McFarland standard number 1 (~ 10^7 CFU/ml) with phosphate buffer containing 0.05 % Tween 20. This adjusted bacterial suspension was used to spike TB negative sputum.

3.2.4. SPIKING OF NEGATIVE SPUTUM WITH STANDARDIZED *M. tuberculosis*

A 10-fold serial dilution was prepared by aliquoting 3.6 ml of the pooled, liquefied TB-negative sputum into eight 10 ml tubes. Four hundred µl of the standardised bacterial suspension was added to the first tube. This was thoroughly vortexed, following which 400µl of the spiked sputum was transferred to the next tube. This process was repeated until the last tube. From each dilution, 500µl each was aliquoted into four 2 ml eppendorf tubes, which were stored at 4ºC for evaluation of the four DNA extraction methods. The remaining aliqout in each dilution was decontaminated using 4% NaOH as mentioned in 3.2.2.2 and 20µl were plated out in triplicate on a Middlebrook 7H11 agar plates. These plates were then sealed in CO_2-permeable plastic bags and incubated aerobically for 3 weeks at 37°C. Thereafter, colony counts were performed and the initial bacterial density was calculated using the following formula:

Bacterial density (CFU/ml) = 50 x Colony count x Dilution factor

3.3. DNA EXTRACTION

Four DNA extraction methods, the Chelex based extraction method, the IDI lysis kit, the Qiagen extraction kit and the silica based extraction method, were used to extract DNA from liquefied, spiked sputum specimens to determine which was most effective in removing inhibitors of PCR in the sputum. Water was simultaneously processed with the spiked sputum specimens to serve as a negative control and also to control for contamination. The resultant DNA was amplified by the IS*6110* PCR to confirm the absence of inhibitors and to determine the least number of organisms that can be detected.

3.3.1. CHELEX EXTRACTION METHOD (Chakravorty and Tyagi, 2001)

An aliquot of liquefied, spiked sputum specimen was heat killed for 15 min at 100°C. An equal volume (500 µl) of inhibitor removal solution (IRS) (Appendix I) was added. The mixture was vortexed, incubated for 10 min at 37°C and then centrifuged at 12 000 g for 10 min. The supernatant was discarded and the pellet was washed with 1 ml sterile water. Five volumes of DNA extraction solution (50 µl) (Appendix I) was added, followed by incubation at 95°C for 15 min. The cell debris was pelleted by centrifugation for 10 min at 12 000 g. The supernatant was transferred to a new tube and stored at -20°C for further use.

3.3.2. SILICA BASED METHOD (Boom *et al,* 1989)

An aliquot of 500 µl liquefied, spiked sputum specimen was heat killed for 15 min at 100°C. One milliliter (ml) of lysis buffer (Appendix I) and 40 µl of acid washed silica (Appendix I) respectively, were added to the tubes. These were incubated for 15 min at room temperature with constant mixing. This was followed by centrifugation at 12 000 g for 30 s and the supernatant was discarded. The pellet was washed twice with 1ml wash buffer (Appendix I) at room temperature, centrifuged for 30 s and supernatant discarded. This was followed by two washes with 1ml and 500µl respectively of 70% ethanol. After centrifugation at 12 000 g, the supernatant was discarded. This was followed by one wash with 1ml acetone and centrifugation at 12 000 g for 30 s. After the supernatant was discarded, the pellet was dried in the biosafety cabinet (BSC) with Eppendorf tube caps left open. Thereafter, 75 µl of 10mM Tris-HCl and 1 mM EDTA (1 x TE buffer), ph 8.0 was added. The tubes were vortexed briefly and then incubated at 55ºC for 10 min to elute the DNA. They were then centrifuged at 12 000 g for 2 min. The supernatant was transferred to a new tube and stored at -20°C for further use.

3.3.3. IDI EXTRACTION (Infectio Diagnostic, Inc., Quebec, Canada)

DNA was extracted from an aliquot of liquefied, spiked sputum specimen according to the manufacturer's instructions. Briefly, 500µl of the spiked sputum specimen was transferred to a lysis tube which was vigorously mixed for 5 min in a Vortex Genie 2. The mixture was centrifuged at 1000g for 5 min followed by heating at 95°C for 2 min. The supernatant was discarded using a pipette. The pellet was washed twice in 100µl of sample buffer and centrifuged at 14 000 g for 5 min. It was resuspended in TE buffer. The lysis tube was immediately placed on ice and then stored at -20°C for further use.

3.3.4. QIAGEN QIAamp DNA EXTRACTION (QIAGEN, Valencia, CA)

An aliquot of liquefied, spiked sputum specimen was heat killed for 15 min at 100°C. To this, 180 µl of lysozyme (30 mg/ml) dissolved in tissue lysis buffer was added and incubated for at least 30 min at 37°C. Following this, 20µl Proteinase K (20 mg/ml) and 200µl buffer AL were added. The tubes were mixed by vortexing and then incubated first at 56°C for 30 min, followed by 95°C for 15 min. The microcentrifuge tube was then briefly centrifuged in order to collect drops from the inside of the lid. This was followed by the addition of 400 µl of ethanol (96-100%), and pulse-vortexing for 15 sec. The microcentrifuge tube was briefly centrifuged and the mixture was added to the QIAamp spin column (in a 2 ml collection tube) without wetting the rim. The columns were centrifuged at 6000 x *g* for 1 min in a 2 ml tube to collect the filtrate, which was discarded. The column content was washed twice with buffer AW1 and AW2

respectively. To elute the DNA, the column was incubated at room temperature for 1 min in 100 μl of Buffer AE followed by centrifugation at 8000 x *g* for 1 min. The DNA was stored at -20°C for further use.

3.4. DATA ANALYSIS

Samples were considered positive for *M. tuberculosis* if a single band of 123 bp was present after gel electrophoresis and no similar bands were seen for the negative control. The sensitivity and specificity of the four DNA extraction methods was determined by comparing the PCR with culture results as the "gold standard". The detection limit for visualization was determined using the spiked 10-fold serial dilutions of H37Rv *M. tuberculosis* strains in liquefied sputum specimens. The cost effectiveness of each DNA extraction method was evaluated by calculating the cost per sample, the time it took to perform the extraction and the stability of the solutions used in the extraction method.

3.5. RESULTS

3.5.1. SMEAR, CULTURE AND PCR FOR SPUTUM SPECIMENS

A total of 43 sputum specimens were collected from IALCH. For all 43 specimens, no AFB was detected under light microscopy. PCR results showed faint bands for 8 specimens which were later determined to be false positives as the culture and smear results were both negative. One specimen was smear negative but positive by PCR and culture. All the sputum specimens that showed faint bands by PCR but were culture negative were not pooled since they were old when the culture results became available. All the sputum specimens that were negative by PCR and smear were pooled and used for spiking experiments.

3.5.2. PCR OF SAMPLES USING THE 4 DNA EXTRACTION METHODS

The experiments were repeated three times. The PCR endpoint was at a dilution of 10^0 when Chelex extracted DNA was used (Figure 3.1), while for the silica based extraction method, it was at 10^{-1} (Figure 3.2). Extractions using the IDI Lysis kit and the Qiagen DNA extraction kit produced higher endpoints of 10^4 and 10^3 respectively (Figure 3.3 and 3.4) suggesting the presence of inhibition. No amplification was observed for the pooled and unspiked specimens for all DNA extraction methods and also the negative control.

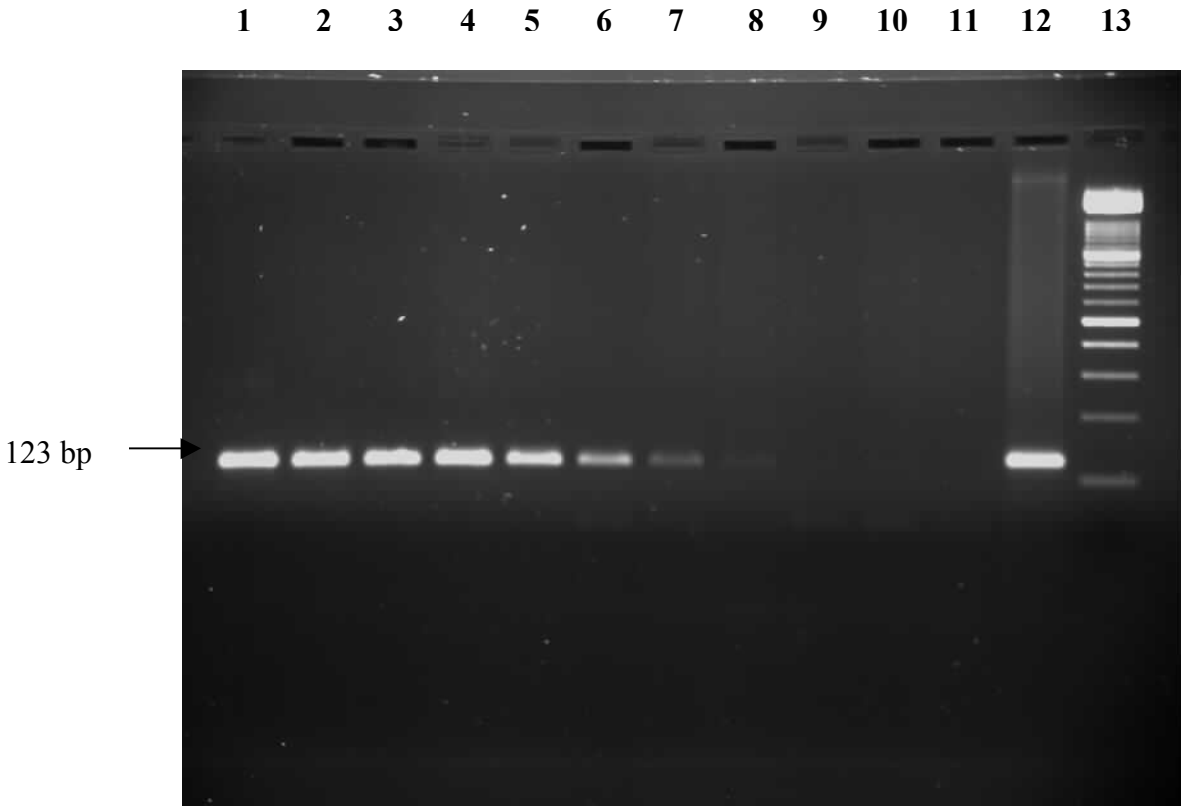

Figure 3.1 IS*6110* amplification of DNA extracted from serial dilutions of *M. tuberculosis* H37Rv using the Chelex extraction method. Lanes 1 to 9 contain 10^{7} ranging down to 10^{-1} organism/ml. Lane 10 contains pooled and unspiked sputum. Lanes 11 and 12 contain the negative and positive controls respectively while molecular weight marker XIV is in lane 13.

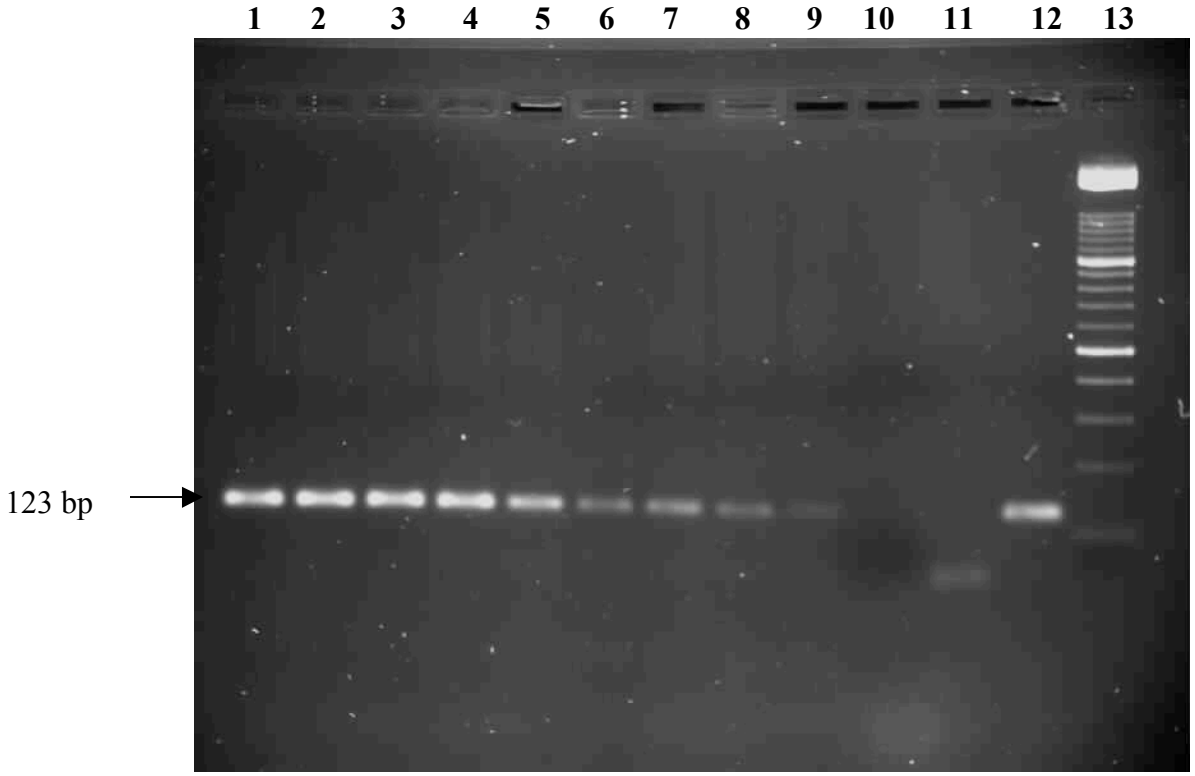

Figure 3.2 IS*6110* amplification of DNA extracted from serial dilutions of *M. tuberculosis* H37Rv using the silica based extraction method. Lanes 1 to 9 contain 10^{7} ranging down to 10^{-1} organism/ml. Lane 10 contains pooled and unspiked sputum. Lanes 11 and 12 contain the negative and positive controls respectively while molecular weight marker XIV is in lane 13.

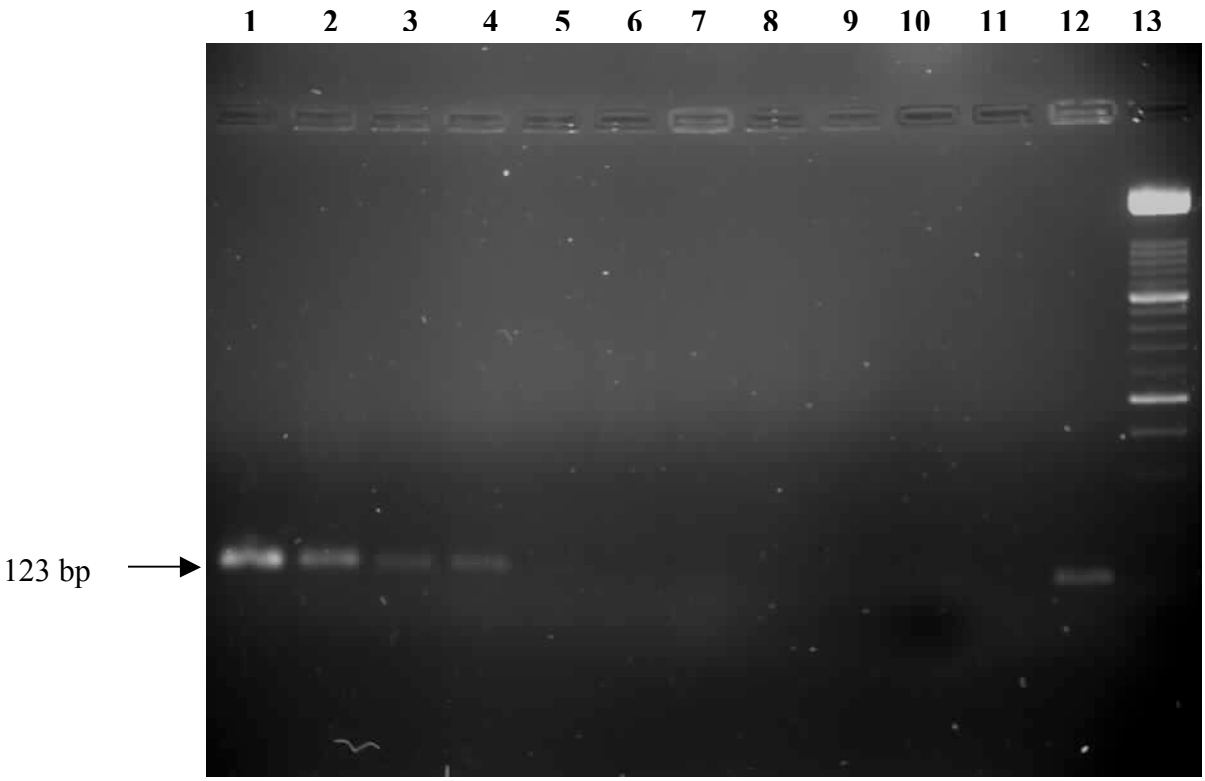

Figure 3.3 IS*6110* amplification of DNA extracted from serial dilutions of *M. tuberculosis* H37Rv using the IDI Lysis Kit extraction method. Lanes 1 to 9 contain 10^{7} ranging down to 10^{-1} organism/ml. Lane 10 contains pooled and unspiked sputum. Lanes 11 and 12 contain the negative and positive controls respectively while molecular weight marker XIV is in lane 13.

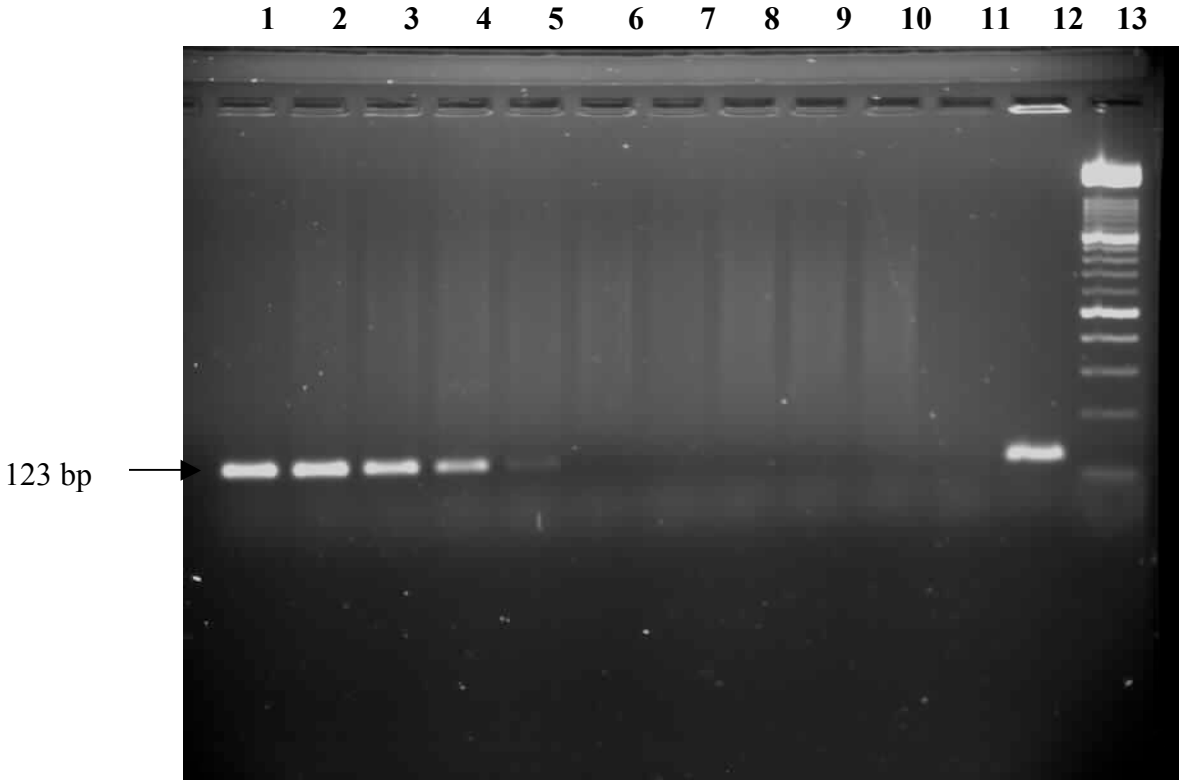

Figure 3.4 IS*6110* amplification of DNA extracted from serial dilutions of *M. tuberculosis* H37Rv using the Qiagen extraction kit. Lanes 1 to 9 contain 10^{7} ranging down to 10^{-1} organism/ml. Lane 10 contains pooled and unspiked sputum. Lanes 11 and 12 contain the negative and positive controls respectively while molecular weight marker XIV is in lane 13.

Table 3.1 Colony counts of spiked sputum dilutions and corresponding estimated bacterial density

		Replicate 1		Replicate 2		Replicate 3	
Bacterial conc[#]	**Dilution factor**	**Colony counts**	**Bacterial density**	**Colony counts**	**Bacterial density**	**Colony counts**	**Bacterial density**
10^7	10^0	ND	-	*	-	*	-
10^6	10^{-1}	*	-	*	-	*	-
10^5	10^{-2}	*	-	*	-	*	-
10^4	10^{-3}	96	0.48×10^4	233	1.17×10^4	238	1.2×10^4
10^3	10^{-4}	16	0.8×10^3	33	1.65×10^3	26	1.3×10^3
10^2	10^{-5}	3	0.15×10^2	3	0.15×10^2	7	0.35×10^2
10^1	10^{-6}	0	0	0	0	2	10×10^1
10^0	10^{-7}	0	0	0	0	0	0
10^{-1}	10^{-8}	0	0	0	0	0	0
-	PU	ND	0	0	0	0	0

ND = Not done, * = the plates were to dense too count, conc[#] = concentration, PU = Pooled and unspiked

Culture is considered the gold standard for the diagnosis of infections with *M. tuberculosis*. Therefore, the PCR results of the serial dilutions were compared with colony counts of the corresponding dilution. For the lower dilutions (10^7, 10^6 and 10^5) the number of colonies could not be counted as plates showed dense growth of *M. tuberculosis* H37Rv strain (Table 3.1). There were no colonies present in the highest dilutions i.e. 10^1, 10^0 and 10^{-1} for replicate 1 and replicate 2 while for replicate 3 colonies were counted at 10^1. Only dilutions 10^4, 10^3 and 10^2 for all 3 replicates and dilution 10^1 in replicate 3 showed colonies that could be counted. Therefore, the PCR was expected to

produce bands until dilution 10^1 for replicate 3 of DNA extraction methods. However both the Chelex and the Silica extraction methods showed bands beyond this dilution.

Table 3.2 Cost-effectiveness of the 4 DNA extraction methods

Extraction method	Time (min)	Stability of solutions	Cost per sample
Chelex	90	2 months in dark	R0.45
IDI	45	1 year	R60.00
Qiagen	180	1 year	R25.36
Silica	60	3 weeks in dark	R2.28

The cost effectiveness of the DNA extraction protocols was evaluated by considering the time and effort required during the procedure, the shelf life of the solutions and the cost of each DNA extraction method (Table 3.2).

3.6. DISCUSSION

DNA amplification by PCR is a rapid and sensitive method for detection of *M. tuberculosis* in sputum specimens. The methods currently used for isolation of mycobacterial DNA from clinical samples suffer from one or more drawbacks that include long processing times, use of organic solvents and enzymes and multiple steps resulting in either DNA loss or in the inefficient removal of PCR inhibitors (Chakravorty and Tyagi, 2001). Multistep methods increase false positive results due to the risk of cross-contamination and also false negative reporting due to losses in DNA recovery.

Hashimoto *et al* (1995) reported a decreased sensitivity in the detection of *M. tuberculosis* by PCR in sputum specimens compared to clinical isolates. They suggested that this was due to loss of the DNA during isolation and contamination with inhibitors such as phenol during the extraction and purification steps. Incompleteness of the bacterial lysis during sample processing has also been reported to significantly lower the sensitivity of PCR methods (Bahador *et al,* 2004). Guanidinium thiocynate (GITC) has been reported to effectively remove inhibiting substances in clinical samples (Boom *et al*, 1990; Chakravorty and Tyagi, 2001). Similarly, in this study good results were obtained with the Chelex and Silica extraction method both of which use GITC.

Chakravorty and Tyagi (2001) suggested that introduction of a "cleaning-before-lysis" step resulted in effective removal of biological constituents and potential PCR inhibitors present in the specimen. The remaining inhibitors, if present at all, are adsorbed by Chelex-100 resin during the DNA isolation step.

In this study, the silica extracted DNA was amplified in samples where culture results were negative. We suggest that this could have been due to cross contamination and also that the PCR is more sensitive than culture. It has been suggested that substances such as alpha casein and bovine serum albumin (BSA), can enhance *Taq* polymerase activity, and thus should be evaluated (Amicosante *et al,* 1995). In future studies, these will improve PCR yield in sputum samples after processing with GITC or other DNA extraction protocols.

The labour required to extract DNA differs from one method to the other. Multistep methods increase false positive results due to the risk of cross-contamination and also augment false negative reporting due to losses in DNA recovery. These drawbacks have been overcome in the IRS method. The Chelex extraction method was found to be the least expensive compared to the other methods evaluated in this study and was the most efficient in performing DNA extraction for *M. tuberculosis* PCR with less hands on time.

The IDI and Qiagen extraction methods showed significant inhibition of the PCR and the cost per sample was also high. Thus, they were excluded as the methods of choice for optimal DNA extraction methods. The Chelex and Silica showed significantly less inhibition of PCR products. Chelex was chosen as the method of choice since it cost 5 times less than the Silica. Also, although the Silica method took less time than the Chelex, the silica was more hands on while the slightly long duration of the Chelex, the former was more labour intensive. The longer duration (30 min more) of the Chelex was

due incubation and centrifugation times and required less hands on time. Based on these reasons, the Chelex extraction method was found to be the most cost effective and therefore, the method of choice for the extraction of DNA directly from sputum specimens. The boiling method, although very rapid, was not considered an option since inhibitors of PCR are not removed.

CHAPTER FOUR

4. DRUG SUSCEPTIBILITY TESTING AND DETERMINATION OF MUTATIONS ASSOCIATED WITH RIF RESISTANCE BY SEQUENCING OF THE *rpo*B GENE

4.1. INTRODUCTION

In most developing countries, drug susceptibility testing (DST) involves culture of isolates on solid media containing the test drug. Although this method provides definitive results, delays of at least 14 days are incurred from the time of primary isolation (Collins and Franzblau, 1997). This leads to prolonged infectivity of the patients including those with drug resistant *M. tuberculosis*, particularly MDR-TB, which compromises clinical success and the effectiveness of TB control programmes (Iseman, 1993). Rapid DST results allow the patient to be treated timeously with appropriate drug regimens. In addition, early detection of drug resistance and the use of the most effective drugs for treatment are therefore very important for proper management of TB patients (Mathew *et al,* 2000).

Molecular techniques have provided an alternative to the conventional susceptibility testing of drug resistance. These techniques exploit the genetic mechanisms involved in the acquisition of drug resistance by *M. tuberculosis*. They also enable detection of drug resistance within days or even a few hours. *M. tuberculosis* becomes drug resistant by the accumulation of mutations at chromosomal loci. Plasmids or transposable elements are not involved in this process. Mutations within an 81-bp region, codon 507-533, (Figure

4.1) of the *rpo*B gene encoding the B chain of the DNA dependent RNA polymerase, confers RIF resistance in approximately 90-95 % of all clinical isolates of *M. tuberculosis* (Telenti *et al,* 1993). These mutations are absent in susceptible isolates, making this region an ideal target for molecular drug susceptibility testing. Since RIF monoresistance is rare, it can be used as a marker for MDR-TB (Telenti *et al,* 1993). Therefore, in this study the *rpoB* gene was sequenced to ascertain the most frequently occurring mutations in this gene that confers resistance to RIF in isolates in KZN. The ultimate goal was to utilize these mutations to design a reverse line blot assay to predict RIF resistance.

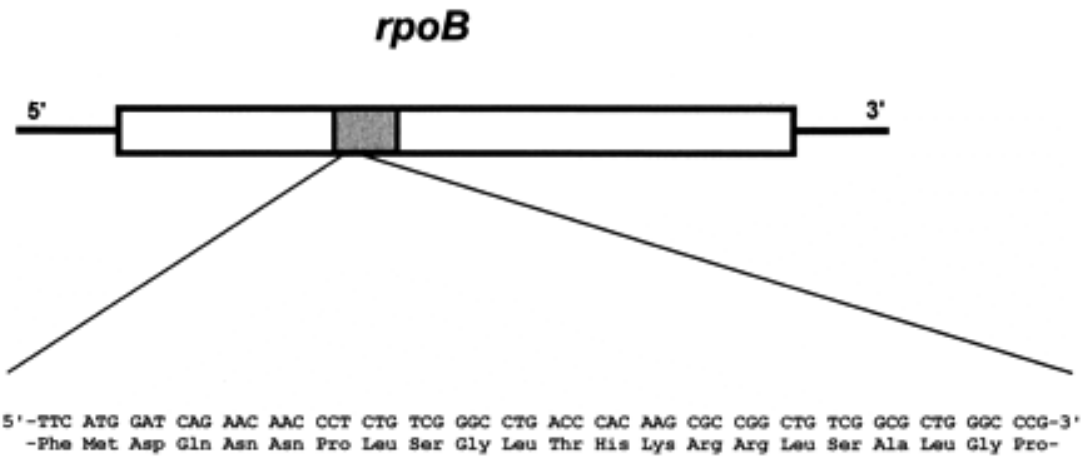

Figure 4.1 The 81-bp region of the *rpo*B gene that harbours mutations conferring resistance to RIF modified from Kremer *et al,* 2002

4.2. MATERIALS AND METHODS

4.2.1. SPECIMEN COLLECTION

Sputum specimens were collected from 425 MDR-TB patients with informed consent at King George V (KGV) hospital. This sputum was used to compare the two molecular methods in predicting drug resistance in *M. tuberculosis*. They were decontaminated by the NALC/NaOH method in a BSC and concentrated by centrifugation. The samples were portioned into two. One half of the deposit was subjected to routine mycobacteriology at the TB laboratory IALCH and the other half was aliquoted in 500 µl ml amounts and stored at -70°C for molecular analysis of drug resistance. Cross contamination was controlled for by the processing of small numbers of specimens at a time

4.2.2. PHENOTYPIC ANALYSIS OF DRUG RESISTANCE

Indirect susceptibility testing of *M. tuberculosis* isolates was performed on all smear positive specimens using the agar proportion method on Middlebrook (MB) 7H10 agar plates containing RIF 1mg/ml, INH 1mg/ml, EMB 5mg/ml, OFX 2mg/ml, STR 2mg/ml and KM 0.5mg/ml**.** The isolates were considered resistant if > 1% of colonies grew on the antibiotic containing medium compared with the drug free medium.

4.2.3. EXTRACTION OF DNA FROM CLINICAL ISOLATES

Confluent cultures of *M. tuberculosis* were harvested for MB 7H11 agar and heat killed at 80ºC for 30 min. DNA was extracted in a BSC according to CTAB method (van Embden

et al, 1993). Briefly, cells were lysed by the addition of lysozyme (10mg/ml) at 37°C for 1 hour. This was followed by deproteinization by adding 75 µl of 10% SDS/proteinase K solution at 65°C for 10 min. The denatured proteins, cell debris and polysaccharides were complexed by 100 µl of 5 M NaCl followed by 100µl CTAB/NaCl solution prewarmed to 65°C for 10 min at 65°C. These complexes were extracted by 750 µl of chloroform/isoamyl alcohol. After centrifugation at room temperature at 12 000 g for 10 min, the DNA present in the aqueous supernatant was precipitated by adding 500 µl of isopropanol at -20 C overnight. DNA was pelleted by centrifugation for 25 min at room temperature. The pellet was washed twice with 1 ml 70 % ethanol, air dried and redissolved in appropriate amount of 1 x TE buffer. It was then stored at -20°C until used.

4.2.4. ANALYSIS OF THE *rpoB* GENE BY SEQUENCING

Sequencing was performed in 2 sites, Netherlands and then in the University of Cape Town (UCT) when the Dutch collaborators experienced technical difficulties. Ninety nine DNA samples were sent to our collaborators in Netherlands after ethanol precipitation to preserve the integrity of the DNA shipping. Briefly, 30 µl of DNA was added to 170 µl of 1 x TE buffer. This was mixed well by tapping and then 20 µl of Na Acetate (3M, pH 5.2) and mixed gently. This was followed by adding 700 µl of 100% ethanol and mixed well. One hundred and thirty eight of the DNA samples were amplified by PCR and sent to the UCT for sequencing.

4.2.5. PCR

A 437 bp fragment of the *rpoB* gene was amplified using the forward primer *rpo*B for1 5'- TGG TCC GCT TGC ACG AGG GTC AGA-3' and the reverse primer *rpo*B-rev1 5'-CTC AGG GGT TTC GAT CGG GCA CAT-3' according to van der Zanden *et al,* (2003). Water was used as negative control and to control for contamination. Different rooms were used for preparation of reagents for PCR, addition of DNA to the mastermix reagents and PCR amplification. Cross contamination was also controlled for by the use of plugged pipette tips. The reaction mixture is shown in Table 4.1. Thermal cycling conditions are shown in Table 4.2. The PCR products were electrophoresed in 1.5% agarose gel for confirmation.

Table 4.1 Reaction mixture for amplifying the *rpo*B gene from DNA

PCR reagent	Volume (μL)
50 pmol *rpo*B-for1 primer	6.9
50 pmol *rpo*B-rev1primer 5' biotinylated	6.9
14.5 mM Tris (pH 9.0)	7.9
500 mM KCl	5.5
25mM $MgCl_2$	5.0
0.2 mM dNTP's	1.5
Taq DNA polymerase	0.2
Water	19.1
DNA (1:10 dilution)	2
Total reaction	55

Table 4.2 Cycling conditions for amplification of the *rpo*B region for sequencing

Step	Time (min)	Temperature (°C)	No. of cycles
Initial denaturation	3	96	1
Denaturation	1	96	
Annealing	1	95	25
Extension	1	72	
Extension	5	72	1

The PCR products and primers were then sent to UCT for sequencing, which was done using the Big Dye terminator v3.1 cycle kit (Applied Biosytem) and a 3130 Genetic Analyser (Applied Biosystem)

4.2.6. ANALYSIS OF SEQUENCE DATA

The sequences were analysed using the basic alignment search tool BLAST against the GenBank nucleic acid sequence database. The ChromasPro and BioEdit software were used to analyse the sequences by aligning them against that of *M. tuberculosis* H37RV.

4.3. RESULTS

4.3.1. DRUG SUSCEPTIBILITY TESTING

Table 4.3 shows the indirect phenotypic susceptibility results of clinical isolates of *M. tuberculosis* n = 271

No. of isolates	Sensitive to:	Resistant to:
110	EOK	IRS
70	ESOK	IR
16	ES	IROK
14	IRESOK	-
10	RESOK	I
9	OK	IRES
8	E	IRSOK
6	REOK	IS
5	IESOK	R
4	ESO	IRK
3	SOK	IRE
2	EO	IRSK
2	O	IRESK
2	K	IRESO
2	ESK	IRO
2	IREOK	S
2	IEOK	RS
1	-	IRESOK
1	S	IREOK
1	RES	IOK
1	SK	IREO

E-Ethambutol; I-Isoniazid; K-Kanamycin; O- Ofloxacin; R- Rifampicin; S-Streptomycin

Of the 425 sputum specimens that were collected at KGV hospital only 271 had susceptibility results available from conventional testing using the proportion method (Table 4.3). Of the 271, 231 (85.2 %) were MDR, i.e. resistant to RIF and INH alone, or in addition to any of the other drugs. Of these, 26 (9.6%) were XDR (i.e. resistant to OFX and KM in addition). Fourteen (5.1%) were sensitive to all the drugs while one (0.4%) was resistant to all the drugs. Only five (1.8 %) were resistant to RIF alone and ten (3.7%) were resistant to INH alone.

4.3.2. MUTATIONS IN THE *rpo*B GENE

DNA sequencing analysis of 236 isolates showed that 187 (79.2%) isolates harboured mutations in the 81-bp hot spot region of the *rpo*B gene (Table 4.2). Two of these 187 isolates were phenotypically susceptible to RIF. Of the 49 isolates with no mutations, 25 were resistant to RIF and the rest were susceptible on conventional drug susceptibility testing. However, there was a significant association between the presence of the mutations and resistance to RIF ($p = 0.02$).

Mutations were most frequently observed in codons 516, 531 and 533 (Appendix II). The nucleotide substitution and the subsequent amino changes together with the frequency of the different mutations are shown in Table 4.4. Six single mutations and 5 double mutations were observed. The most frequent mutation was a TCG 531 TTG (58%), followed by 516 GAC to GTC (13.8%), then the double mutation 516 GAC to GGC/533 CAG to CCG (10.6%) and codon 533 CAG to CCG (9.5%) alone.

Table 4.4 Frequency of the mutations in the *rpo*B gene of 236 clinical isolates

Codon no. affected	Nucleotide substitution	Amino acid change	No of isolates
516	GAC→TAC	ASP→TYR	1 (0.5%)
516	GAC→GTC	ASP→VAL	26 (13.9%)
526	CAC→GAC	HIS→ASP	6 (3.2%)
526	CAC→TAC	HIS→TYR	3 (1.6%)
531	TCG→TTG	SER→LEU	109 (58%)
533	CTG→CCG	LEU→PRO	18 (9.6%)
510 516	CAG→CAT GAC→TAC	LEU→PRO ASP→TYR	1 (0.5%)
511 516	CTG→CCG GAC→TCC	LEU→PRO ASP→SER	1 (0.5%)
511 516	CTG→CCG GAC→TAC	LEU→PRO ASP→TYR	1 (0.5%)
516 533	GAC→GGC CTG→CCG	ASP→GLY LEU→PRO	21 (11.2%)
531 537	TCG→TTG GGT→GAT	SER→LEU GLY→VAL	1 (0.5%)
-	No mutation	-	49* (20.7%)

*Of these 49, 25 were RIF susceptible and 24 were RIF resistant

4.4. DISCUSSION

More than 96% of RIF resistant isolates of *M. tuberculosis* isolated in different countries present with mutations in the 81-bp region of the *rpoB* gene (Telenti *et al,* 1993; Williams *et al,* 1998; Hirano *et al,* 1999; Scarpellini *et al,* 1999). Different studies have reported specific mutation frequencies and also novel mutations (Bartfai *et al,* 2001; Mani *et al,* 2001). Telenti *et al* (1997), reported mutations in the *rpo*B gene in all RIF resistant isolates and in none of the susceptible isolates tested. Similarly, Garcia *et al* (2001), reported mutations in the *rpo*B region in all clinical isolates that were resistant to RIF. In the present study, 10% of the RIF resistant isolates did not possess any mutations in the *rpo*B region, which suggests the presence of mutations outside the 437 bp region or another resistance mechanism such as efflux pump (Jiang *et al,* 2008). The mutations found in 2 drug susceptible strains may be silent mutations.

Point mutations in the *rpo*B gene are associated with RIF resistance and codons 516, 526 and 531 together are known to be the most mutated codons worldwide (Telenti *et al,* 1993; Williams *et al,* 1998; Hirano *et al,* 1999; Scarpellini *et al,* 1999). We found a total of 11 different genetic alterations with mutations in codons 516, 531 and 533 occurring in 80% of RIF resistant isolates. Schilke *et al* (1999) reported that the most frequently mutated codons among South African isolates were 531 (55%), 526 (20%) and 516 (13%). They also observed a high number of substitutions at codon 526 in contrast to our findings. The frequency of point mutations found in isolates originating from different geographic regions of South Africa were found to be comparable to those from other

countries, mainly the USA and Northern Europe (Schilke *et al,* 1999). This was similar to our observations.

Kiepiela e*t al* (1998) evaluated 113 RIF resistant isolates of *M. tuberculosis* in KZN using sequencing, PCR-HDR analysis and line probe assay. They reported 24 mutations affecting 13 codons of the *rpo*B region. In this study, eleven different types of mutations were identified in 188 RIF resistant *M. tuberculosis* clinical isolates. Contrary to our findings, Kiepiela *et al,* (1998) observed numerous different mutations in the 526 codon while only 2 different mutations for this codon were seen in this study. Similarly to our findings, codon 516, 531 and 533 were the most frequently mutated. Most of the mutations reported by Kiepiela *et al* (1998) were not observed in the isolates of this study. This may be due to the inability of these strains to survive as a result of fitness costs incurred by the mutations (Billington *et al,* 1999).

The frequency of mutations at codons 516, 526 and 531 within the *rpo*B gene vary significantly depending on the geographic location of RIF resistant *M. tuberculosis* isolates. Kapur *et al,* 1994, reported the occurrence of 5%, 43% and 31% of RIF resistant *M. tuberculosis* isolates from New York and Texas carrying mutations at codon positions 516, 526 and 531, respectively. Cooksey *et al,* 1997 have also reported similar frequencies (6%, 43% and 33%) among their isolates. Among the different mutations seen at codon 531, in our study, the change of TCG (Ser) to TTG (Leu) occurred at a frequency of 58%. Matsiota-Bernard *et al* (1998), Pozzi *et al* (1999) and Mani *et al* (2001) also reported similar high frequencies of this mutation, 59, 56 and 49%

respectively. The low frequency (3%) of the CAC to GAC in codon 526 was similar to that reported by Mani *et al* (2001) but significantly lower than the 30% found in Italian isolates (Pozzi *et al*, 1999) and 19% in Greek isolates (Matsiota-Bernard *et al,* 1998). Considering the variety of missense mutations occurring at variable frequency in the core region of the *rpoB* gene, the predominance of the 531(Ser-Leu) mutation suggest a selective advantage possibly as a result of compensatory mutations. The nucleotide substitution at this position may lead to an optimal inhibitory effect on RIF action associated at the same time with minimal loss of function of the RNA polymerase (Billington *et al,* 1999)

Mutations 510CAT, 511CCG, 516 TCC and 516GGC were found in combination with other mutations i.e. as double mutation within the *rpo*B gene and thus the extent to which they contributed to the resistance of the isolates could not be determined. Although, mutations 516TAC and 533CCG were found in combination with some of the above mentioned mutations, they were also detected as single mutations and thus it may be assumed that they contributed to the phenotypic RIF resistance of these isolates. Studies need to be undertaken in future to determine the MICs of these isolates with double and single mutations in order to determine the effect of the added mutation on RIF resistance.

The detection of RIF resistance by molecular techniques fails to correlate with the resistant phenotype in about 5-10% of all cases (Telenti *et al,* 1993). Twenty (9.7%) of our strains had no mutation detected in the 81-bp core region of the *rpoB* gene. In these strains, RIF resistance could have resulted from the presence of mutations outside the

locus sequenced, novel mechanisms, or heteroresistance. Heep *et al,* (2000) reported a mutation in the beginning of the *rpoB* gene conferring resistance to RIF in *Helicobacter pylori* (V149F) and subsequently in *M. tuberculosis* (Heep *et al* 2001). They showed that RIF resistance was associated with the V176F mutation of the *rpo*B gene when cluster I to III mutations were excluded. In their study, it was shown that the mutation V176F conferred high-level resistance in clinical *M. tuberculosis* isolates which could account for more than 1% of all RIF resistant strains.

Alternative mechanisms such as cell wall permeability, plasmids or transposons (which could carry resistant determinants), and mutations elsewhere in the genome are all unlikely, although mutations in other RNA polymerase subunits is a possibility (Heep *et al,* 2000). The inactivation of RIF by ribosylation has been reported in several fast growing mycobacterial species, all of which are naturally resistant to RIF but it is not yet known whether this activity is also present in *M. tuberculosis*. Approximately 10% of our RIF resistant *M. tuberculosis* isolates did not show any mutations in the *rpo*B region. This was similar to findings of Yue *et al* (2003) but much higher than other studies (Hirano *et al,* 1999; Telenti *et al,* 1993), 4, 5 % respectively. In this study other possible mechanisms of drug resistance were not investigated and future studies should be undertaken to explore other reported mechanisms of resistance such as the efflux pump system, inactivation of ribosylation and mutations in the V176F region in order to identify new targets of resistance.

Kapur *et al* (1994) reported rare mutant *rpoB* alleles in isolates from Victoria, Texas. These were also observed in one of our strains that had combination of point mutations in two non- contiguous codons. Novel mutations not previously published, were demonstrated in this study. However, no direct link could be made to RIF resistance as they were always associated with other known mutations. Since the frequency of mutations in the *rpo*B of *M. tuberculosis* can vary according to geographic area, it is important to sequence isolates of that region in order to determine the prevalent mutations. This will allow utilization of molecular techniques that are better suited for detection of these mutations and thus rapid detection of drug resistance.

CHAPTER FIVE

5. RIFOLIGOTYPING OF CLINICAL AND SPUTUM SPECIMENS

5.1. INTRODUCTION

The reverse dot blot assay was first described in 1989 by Saiki *et al* for the detection of the *HLA-DQA* genotype and the Mediterranean β-thalassemia mutations in human DNA. This was later modified to develop the reverse line blot assay by Kaufold *et al,* 1994 for the M protein gene of the group A streptococci. In this assay, the membrane bound probe was hybridised to the PCR product which was free in solution (Zhang *et al,* 1991). Prior to use, the membrane was prepared by activation with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) which in turn activated carboxyl groups on the membrane to form O-acylurea. These intermediates could be linked by amines to form amide bonds (Figure 5.1).

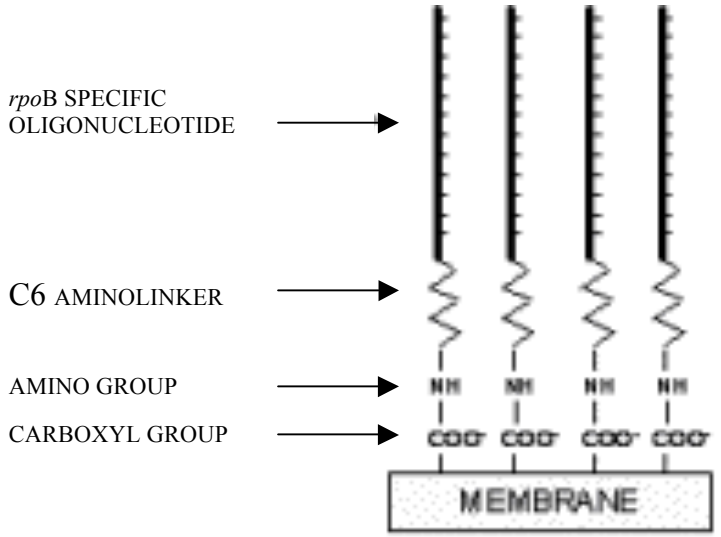

Figure 5.1 Diagrammatic representation of the immobilised probes (google images)

The covalent binding of an amino linked oligonucleotide to a negatively charged membrane pushed the probe outwards, making it accessible to react with the target DNA during hybridisation (Kaufold *et al,* 1994). After immobilisation of the probes, the remaining active carboxyl groups that were reactive to any nucleophile present in solution were quenched with NaOH. This prevents non-specific binding through electrostatic, hydrophobic or chemical interactions which can reduce the sensitivity of the assay without affecting the covalent bonds between oligonucleotide probes and the membrane. Since the bound probes could withstand multiple, stringent hybridisation and washing conditions, the membrane could be reused many times after being stripped (Kamerbreek *et al,* 1997).

The reverse line blot assay has been used to predict RIF resistance by detecting mutations in the *rpo*B region of the RNA polymerase in *M. tuberculosis* (Morcillo *et al,* 2002, van der Zanden *et al,* 2003, Mokrousov *et al,* 2004, Figure 5.2). Since these mutations are associated with resistance to RIF, the assay was termed the Rifampicin oligonucleotide typing assay (Rifoligotyping assay) and in turn was abbreviated: RIFO assay (Kremer *et al,* 2002). Multiple genetic loci, as well as a large number of samples could be analysed in parallel, with considerable savings of cost, time and effort. This assay would be useful as a rapid diagnostic tool for large scale detection of drug resistance in settings with high prevalence of MDR/XDR-TB.

In this study, we aimed to optimise this technique for direct detection of RIF mutations in clinical specimens in KZN, a region with unacceptably high rates of MDR and XDR-TB.

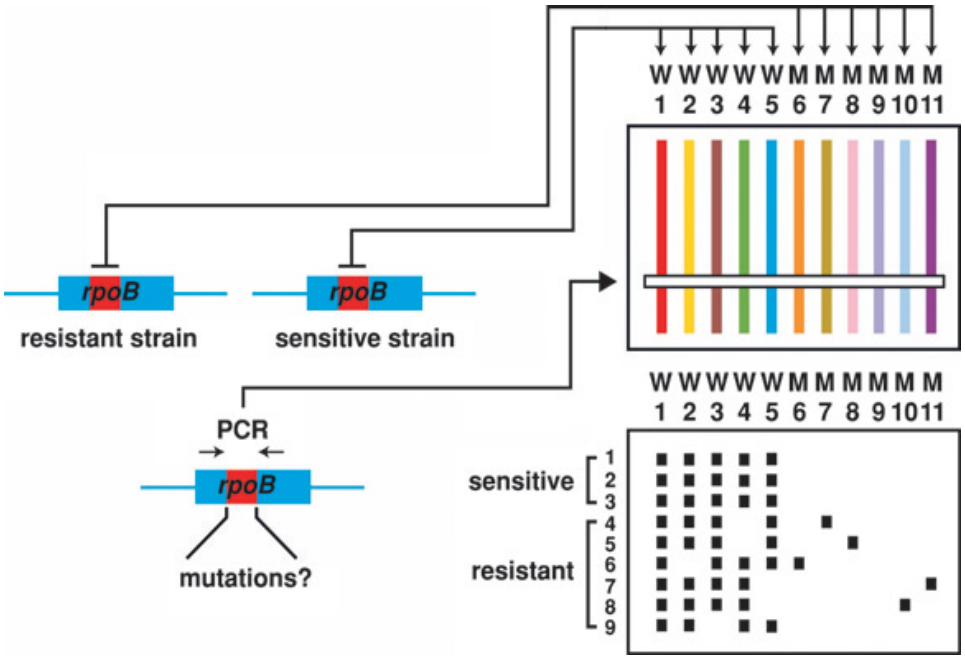

Figure 5.2 Diagrammatic representation of RIFO assay. W= wild type; M = mutant. The wild type oligonucleotides were derived from the sequence of the 'hot spot' region of the *rpoB* gene of a drug susceptible *M. tuberculosis* strain. The mutant oligonucleotides were derived from the sequences of the 'hot spot' region of the *rpoB* gene of various drug resistant *M. tuberculosis* strains (Morcillo *et al,* 2002).

5.2. MATERIALS AND METHOD

5.2.1. PATIENT RECRUITMENT

Patients diagnosed with MDR TB were recruited at KGV Hospital during the period of May 2005 to June 2006. Sputum specimens and demographic data were collected with informed consent.

5.2.2. LABORATORY PROCESSING OF SPUTUM SPECIMENS

Specimens were decontaminated using the NALC-NaOH method. The deposit was aliquoted into 4 portions, 3 of which were stored at -70°C. Auramine smear microscopy, culture on MB 7H11, MGIT and the proportion method of drug susceptibility testing were performed on the fourth aliquot.

5.3. RIFOLIGOTYPING ASSAY/RIFO ASSAY

5.3.1. SEQUENCE OF OLIGONUCLEOTIDE PROBES

The oligonucleotide probe sequences were designed using the OligoAnalyser 3.0 (www.basic.northwestern.edu/biotools/oligocalc.html) from the wild type sequence of H37Rv of *M. tuberculosis* obtained at GenBank while the mutant probes were designed using the sequences obtained by sequencing. Probe sequences were designed such that

they possessed similar theoretical melting temperatures (52.4< Tm < 62.8 °C) to facilitate their use in a single hybridisation assay. Fifteen oligonucleotide probes with a C6 aminolink at the 5" end (Whitehead Scientific Inc) were used to produce a macroarray (Table 5.1). The first five probes represented a scanning array to detect the wildtype genotype and the remaining 10 analysed loci associated with RIF resistance.

Table 5.1 Sequences of the oligonucleotide probes immobilised on membrane

Name	Probe	Sequence	Tm (°C)	Concen-tration*
WT1	*rpoB* 509-514 wt	AGC CAG TCG AGC CAA TTC AT	57.3	0.8
WT2	*rpoB* 514-520 wt	TTC ATG GAC CAG AAC AAC CCG	57.5	1
WT3	*rpoB* 521-525 wt	CTG TCG GGG TTG ACC CG	58.6	25
WT4	*rpoB* 524-529 wt	TTG ACC CAC AAG CGC CGA	60.3	800
WT5	*rpoB* 530-534 wt	CTG TCG GCG CTG GGG	60	12.5
MT1	*rpoB* 510CAT	AGC CA**T** CTG AGC CAA TTC AT	54.9	800
MT2	*rpoB* 511CCG	AGC CAG C**C**G AGC CAA TTC AT	59.9	50
MT3	*rpoB* 516TCC	TTC ATG **TC**C CAG AAC	55.0	1600
MT4	*rpoB* 516TAC	TTC ATG **T**AC CAG AAC	52.4	50
MT5	*rpoB* 516GTC	TTC ATG G**T**C CAG AAC	55.0	12.5
MT6	*rpoB* 516GGC	TTC ATG G**G**C CAG AAC	58.0	25
MT7	*rpoB* 526GAC	TTG ACC **G**AC AAG CGC CGA	60.1	150
MT8	*rpoB* 526TAC	TTG ACC **T**AC AAG CGC CG	55.4	1
MT9	*rpoB* 531TTG	CTG T**T**G GCG CTG GGG	57.2	6
MT10	*rpoB* 533CCG	GCG C**C**G GGG CCC	62.8	6

WT/wt = wildtype, MT= mutant, * = concentration in pmoles. Nucleotides bolded and underlined indicate mutation point on the codon

5.3.2. PREPARATION OF MEMBRANE

A Biodyne C membrane (Pall Biosupport) was activated by incubating with 16% (wt/vol) EDAC (Sigma) at room temperature for 15 min. The membrane was rinsed with tap water and placed together with a PC200 support cushion (Immunetics, Cambridge, Mass) and cling wrap in a Miniblotter 45 manifold (Immunetics). The oligonucleotide probes with a C6 aminolink at the 5′ end (Whitehead Scientific), were diluted in 0.5 M $NaHCO_3$ (pH 8.4) at a concentration ranging from 0.8 to 1600 pmol/150μL. These were applied on the activated membrane in a macroarray format by filling the slots of the miniblotter MN45 (Figure 5.3).

The probes were allowed to bind for 1 min at room temperature, after which the probe solution was aspirated from the slots. The membrane was removed from the manifold using forceps and was then inactivated by incubating with 100 mM NaOH at room temperature for 10 min. This was followed by washing in 2 x SSPE containing 0.1% SDS for 10 min at 50°C with shaking. The membrane was stored in 20mM EDTA in a sealed plastic bag at 4°C to avoid dehydration until required. Optimal probe concentrations were determined by binding varying concentrations of the probe such that all the probes resulted in equally intense signals.

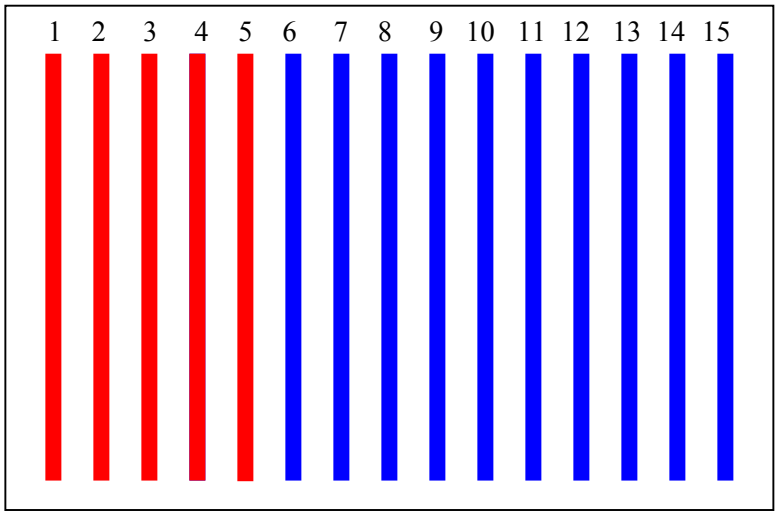

Figure 5.3 Membrane with immobilised oligonucleotide probes. Red lines represent wildtype probes and blue lines represent mutant probes.

5.4. DNA EXTRACTION

Extraction of DNA from clinical specimens and isolates was performed as described in chapter 3 section 3.5.1 and chapter 4 section 4.2.3 respectively.

5.5. AMPLIFICATION OF *rpo*B GENE

5.5.1. CLINICAL ISOLATES

For the detection of the *rpoB* genotype, PCR was performed as described by van der Zanden *et al,* 2003 using the primers described in section 4.2.5 to amplify a 437 bp fragment of the *rpoB* gene using the reaction mixture shown in Table 5.2. The reverse primer was biotinylated at the 5' end.

Table 5.2 Reaction mixture for amplifying the *rpo*B gene from DNA of clinical isolates

PCR reagent	Volume (µL)
50 pmol *rpoB*-for1 primer	6.9
50 pmol *rpoB*-rev1primer 5' biotinylated	6.9
14.5 mM Tris (pH 9.0)	9
500 mM KCl	5.5
25mM $MgCl_2$	5.0
0.2 mM dNTP's	1.3
Taq DNA polymerase	0.2
Water	19.2
DNA	1
Total reaction	55

Cycling was performed by incubation for 3 min at 96°C followed by a touch down PCR (Table 5.3).

Table 5.3 Cycling conditions for amplification of the *rpo*B region

Step	Time (min)	Temperature (°C)	No. of cycles
Initial denaturation	3	96	
Denaturation	1	96	
Annealing	1	72	2
Extension	1	72	
Denaturation	1	96	
Annealing	1	71	2
Extension	1	72	
Denaturation	1	96	
Annealing	1	70	2
Extension	1	72	
Denaturation	1	96	
Annealing	1	69	2
Extension	1	72	
Denaturation	1	96	
Annealing	1	69	45
Extension	1	72	

5.5.2. CLINICAL SPECIMENS

Amplification of a 437 bp fragment of the *rpoB* gene from DNA extracted directly from sputum specimens (van der Zanden *et al,* 2003) was performed with modifications shown in Table 5.4 and thermal cycling was performed as described in Table 5.3.

Table 5.4 Modified PCR reaction mixture for amplifying the *rpo*B gene in DNA from sputum specimens

PCR reagent	Volume (µL)
50 pmol *rpoB*-for1 primer	6.9
50 pmol *rpoB*-rev1 primer 5' biotinylated	6.9
14.5 mM Tris (pH 9.0),	7.9
500 mM KCl	5.5
25mM $MgCl_2$,	7.7
0.2 mM dNTP's	1.3
GoTaq polymerase	0.2
Water	8.6
DNA	10
Total	55

5.6. OPTIMISATION OF HYBRIDISATION AND WASHING CONDITIONS FOR CLINICAL ISOLATES AND SPECIMENS OF *M tuberculosis*

Ten and thirty microliters of PCR products respectively of clinical isolates and specimens were diluted in 150µL and 130 µL of 2 x SSPE/0.1% SDS (Appendix I). After denaturation at 100°C for 10 min, the DNA was chilled on ice. It was then applied on to the membrane in the miniblotter, in a direction perpendicular to the immobilised oligonucleotides. The assay was optimised by testing different hybridisation temperatures (55°C, 58°C, 60°C and 65°C) followed by aspiration of excess PCR product and washing at temperatures of 2°C higher to increase stringency. This stringency made it unlikely that sequences with two or more mismatches or sequences with insertion/deletions will hybridise to the probes and produce non-specific signals.

After washing twice in 2 x SSPE/0.5% SDS at temperatures ranging from 57 to 67°C, the membrane was incubated in 10 ml of 2 x SSPE /0.5% SDS (Appendix I) containing 1.25 U streptavidin-peroxidase conjugate at 42°C for 45 min. This was followed by washing twice in 2 x SSPE/0.5% SDS at 42°C for 10 min and twice in 2 x SSPE (Appendix I) at room temperature for 5 min. The optimal hybridization temperature was found to be 58°C and washing was done at 60°C (increased by 2°C to increase stringency). The membrane was used up to 9 times before the signals became faint and then considered unusable.

5.7. DETECTION OF HYBRIDISED DNA

The hybridised DNA was detected using enhanced chemiluminescence (ECL) (Amersham) under red safety light. After exposure to detection reagents one and two, the membrane was initially exposed to a light sensitive ECL Hyperfilm (Amersham) for 20 min. Subsequent exposures were increased to 60 min to increase the intensity of the signals. Susceptible strains were expected to hybridise with wild type probes but with none of the resistant probes, while the resistant strains would hybridise with the mutant probe but not its corresponding wild type probe. Strains would also be characterised as resistant, if they did not hybridise with any one of the wild type probes and with none of the mutant probes, assuming that the probe with that particular mutation has not been immobilised.

5.8. INTERPRETATION OF RESULTS

(i) Presence of a mutation defined an isolate or specimen as resistant to RIF. This was characterized by absence of a hybridization signal for one or more of the wildtype probes and presence of a signal for one or more of the mutant probes. However, absence of a hybridization signal for one of the wildtype probes and no hybridization with any of the mutant probes was also defined as resistance as this could mean that a different mutation to that of the immobilised probe was present.

(ii) Absence of a mutation defined an isolate or specimen as susceptible to Rifampicin. This was characterized by presence of a hybridization signal for all

the wildtype probes and absence of a hybridization signal for all the mutant probes.

(iii) An isolate or specimen that gave a hybridization signal for all the wildtype probes and a hybridization signal for one or more mutant probe was defined as discrepant.

5.9. STATISTICAL ANALYSIS OF RESULTS

The RIFO assay results were compared with those of the 1% proportion method of drug susceptibility testing as the gold standard. Comparison was also done between RIFO assay results of clinical isolates and that of clinical specimens. SPSS version 15.0 (SPSS Inc. Chicago Illinois) was used to generate frequency tables and cross tabulations. Sensitivity, specificity, Predictive Positive Values (PPV), Negative Predictive Values (NPV) and their 95% confidence intervals were calculated using EpiCalc 2001 version 1.02 (Gillman and Myatt, 1998).

5.10. RESULTS

5.10.1. SPUTUM SMEAR AND CULTURE RESULTS

Of the 425 patients enrolled at KGV hospital between 2005 and 2006, 195 (46%) were smear and culture positive while 84 (20%) were smear negative and culture positive (Fig 5.4).

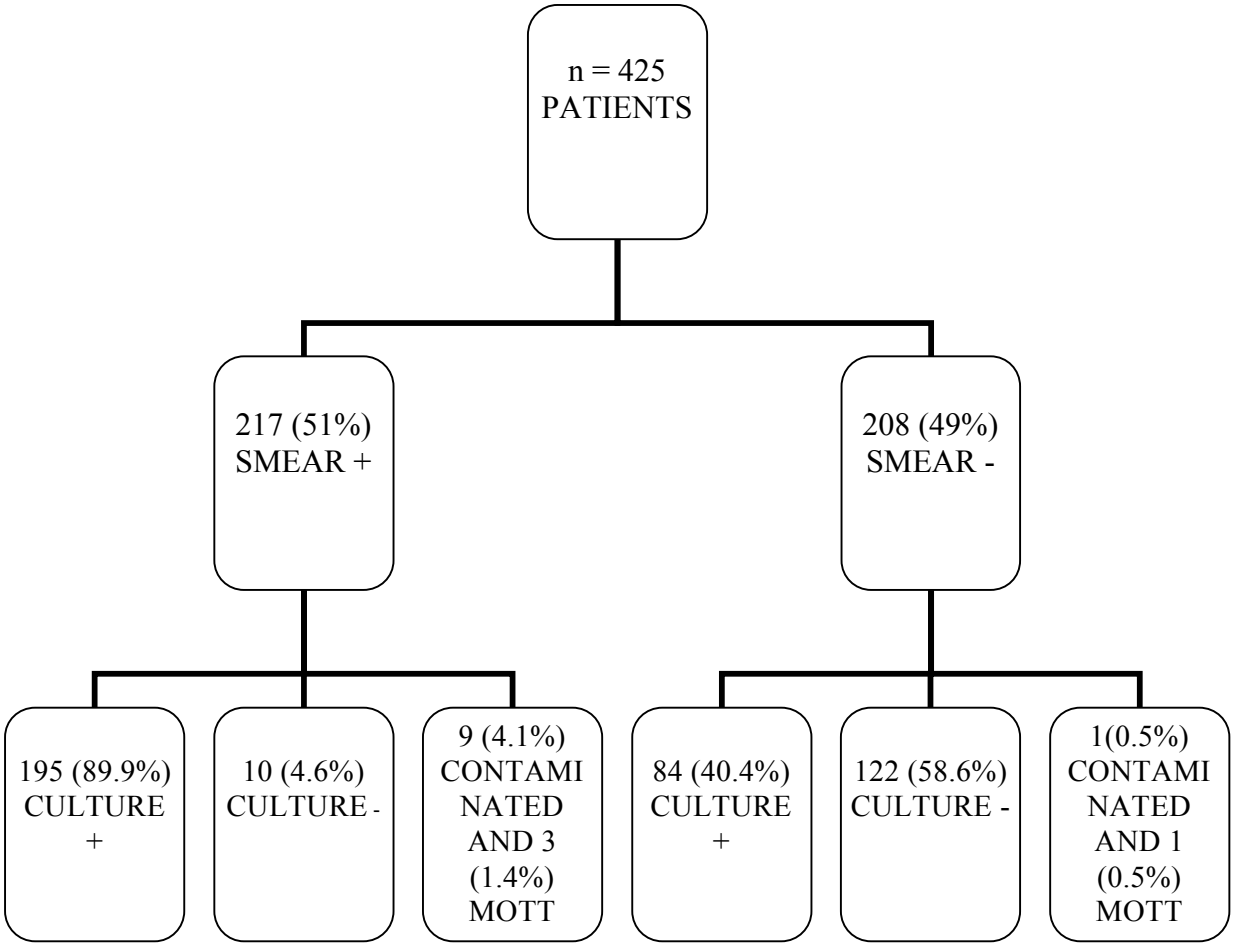

Figure 5.4 Smear and culture results of 425 patients hospitalised at KGV hospital between 2005 and 2006

5.10.2. RIFOLIGOTYPING OF CLINICAL ISOLATES

5.10.2.1. RIFO PATTERNS

Of the 425 clinical specimens, 279 (65.6%) were culture positive. RIFO patterns (Figure 5.5) were obtained in 258 (92%) of the isolates of *M. tuberculosis* while 19 (7%) RIFO was not done as DNA was unavailable and 2 (1%) gave no patterns. Ten mutations were detected in 219 isolates and 37 isolates did not have any mutations. Two isolates showed no hybridization with one wildtype probe and no hybridization with the corresponding mutant probes suggesting that a mutation different to the immobilised mutant probe was present. The mutations detected in the RIFO assay are listed in Table 5.5. The most common site of point mutation leading to an amino acid substitution was Ser531Leu (117/219, 53.4%).

Five double mutations were found in 25 isolates involving codons Glu510Hist, Leu511Pro, Asp516Gly, Asp516Tyr, Asp516Ser and 533, while 1 isolate showed a triple mutation in codon Asp516Val, Ser531Leu and Leu533Pro (Appendix III). Thirteen isolates showed discrepant results i.e. hybridization signal for all the wild type probes and in addition hybridization signal for one or more mutant probes. When culture RIFO assay results were compared to sequencing results, 197 of the 279 were comparable as 82 isolates were not sequenced. Of the 197 comparable to sequencing results, 180 (91.4%) were concordant, 8 (4%) were discordant and 9 (4.6%) were discrepant (hybridization signal for all wildtype and one or more mutant probes).

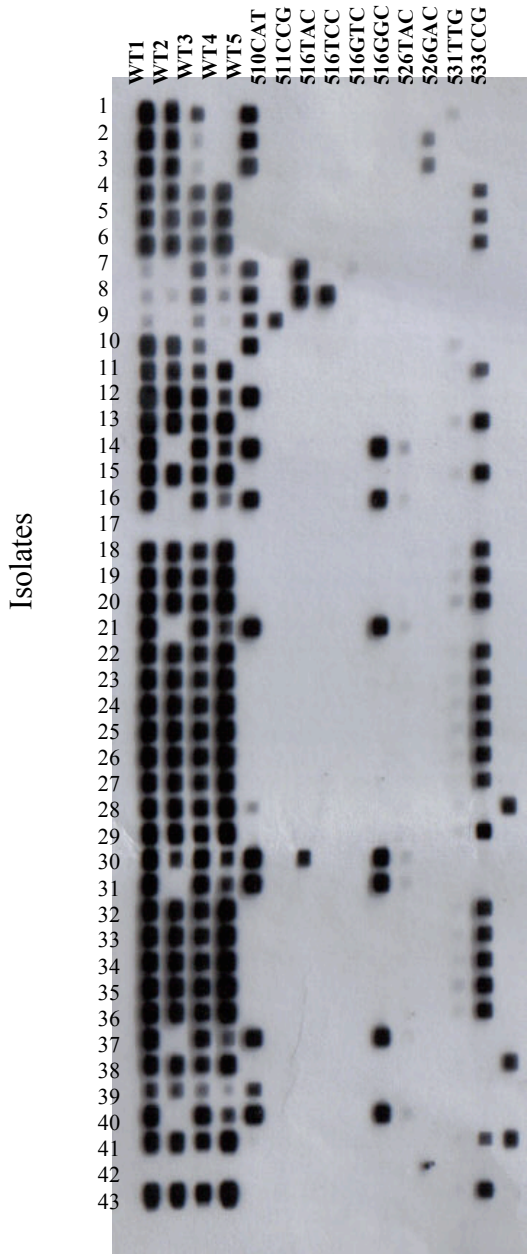

Figure 5.5 RIFO assay results for detection of mutations in clinical isolates. Lanes 1 to 5 across contain oligonucleotide probes corresponding to the wildtype sequence of the *rpo*B gene and lanes 6 to 11 contain the mutant oligonucleotide probes. Lanes 1 and 10 vertically: mutations in codon 526 from CAC to GAC. Lanes 2 and 3: mutation in codon 526 from CAC to TAC. Lanes 7, 8 and 9: double mutations 511CCG/516TAC, 511CCG/516TCC and 510CAT/516TAC respectively. Lanes 14, 16, 21, 30, 31, 37 and 40: mutation 516GTC. Lanes 28 and 38: mutation 533CCG. Lanes 30 and 41: discrepant results. Lane 30: signals for all the wildtype probes as well as signal for 511CCG and 516GTC. Lane 41: no signal for WT5 and signals for 531TTG and 533CCG. Lane 17: no pattern. Lane 42 no pattern but this was a negative control. Lane 39: signals for all of the wildtype probes and no signals for all the mutant probes and this was a positive control. The rest of the lanes showed mutation 531TTG which is the most frequently found mutation.

Table 5.5 Frequency of mutations detected by the RIFO assay in isolates of *M. tuberculosis*

MUTATION TYPES	NO. OF ISOLATES
511CCG	**2**
516GTC	**28**
516TAC	**1**
526GAC	**8**
526TAC	**5**
531TTG	**117**
533CCG	**19**
510CAT/516TAC	**1**
511CCG/516GGC	**1**
511CCG/516TAC	**1**
511CCG/516TCC	**1**
516GGC/533CCG	**21**
No mutation*/516TAC	**1**
No mutation*/516GGC	**1**
No mutation*/516GTC	**2**
No mutation*/531TTG	**3**
531TTG/533CCG/516GTC	**1**
No mutation*/516GTC/531TTG	**3**
No mutation*/526GAC/531TTG	**3**

*** hybridization signal for all wild type probes**

5.10.2.2. COMPARISON OF RIFO PATTERNS TO DST

Of the 279 clinical isolates, 241 (88.6%) had RIFO assay results that could be compared to RIF drug sensitivity results performed by the proportion method (Table 5.6). Of these, 217 were resistant to RIF by the RIFO assay. RIF resistance by both DST and RIFO assay was present in 206 of 224 RIF resistant, demonstrating a sensitivity of 92% [CI: 95%, 0.87, and 0.95]. The specificity (35%) [CI: 95%, 0.15 and 0.61] of the assay was low, with 6 of 17 drug susceptible strains correctly identified as sensitive. The positive predictive value was 95% [CI: 95%, 0.91 and 0.97] and negative predictive value was 25% [CI: 95%, 0.11, 0.47].

Table 5.6 RIFO culture results compared to DST

RIFO ASSAY	DST		TOTAL
	RESISTANT	SUSCEPTIBLE	
RIF^R	206	11	217
RIF^S	18	6	24
TOTAL	224	17	241

RIF^R = Rifampicin resistant, RIF^S = Rifampicin sensitive

5.10.2.3. ANALYSIS OF DISCREPANT RESULTS BETWEEN PROPORTION METHOD OF DST AND RIFO ASSAY

Sequencing was performed for 25 isolates that were either previously sequenced and produced discrepant results from the RIFO assay or not. Thirteen of these were sensitive by the proportion method and resistant by RIFO assay. Eleven of the 13 were not sequenced initially. Subsequent sequencing showed concordance of mutations with RIFO assay in 10 isolates. Additionally, 2 new mutations were identified (526CTC and 505 CTC) in 2 previously unsequenced isolates. The sequencing chromatogram was suggestive of the presence of a mixed infection/ population of bacilli in the eleventh isolate. The remaining 2 were resistant by both sequencing and RIFO assay; however subsequent sequencing gave a mutation different to that of initial sequencing and RIFO assay. No mutation was observed for the last isolate thus suggesting RIF sensitivity.

Twelve isolates were RIF resistant by DST, and the initial sequencing was either not performed or discordant with the RIFO assay. Of these, 11 that were previously sequenced were concordant by the RIFO assay and the second sequencing. This suggests that the results of the first sequencing were incorrect. The second sequencing confirmed that the twelfth isolate that had not been previously sequenced had a mutation that was concordant with the RIFO assay. No hybridization signal was obtained for the wild type probe for codons 509-514 for this isolate and sequencing revealed a mutation in codon 513.

5.10.3. RIFOLIGOTYPING OF SPUTUM SPECIMENS

5.10.3.1. RIFO PATTERNS

Of the 425 sputum specimens that were rifoligotyped, 21 were excluded from the analysis as 10 were contaminated, 4 were MOTT and 7 although culture was positive, no DST results were available. RIFO patterns were produced for 3 of the contaminated, and 4 without DST. RIFO patterns were not obtained for all 4 MOTT.

RIFO patterns were obtained for 236 (58.4%) sputum specimens and no patterns were obtained for 168 (41.6%). Figure 5.6 represents an example of the RIFO assay results obtained from sputum specimens. Of the 236, 32 specimens could not be included in the analysis as 15 were smear and culture negative, 7 were smear positive but culture was negative thus no DST results were available, 10 produced discrepant patterns which included signals for all wildtype probes and in addition one or more mutant probe either with culture RIFO or with sputum RIFO results. The remaining 204 (50.5%) produced RIFO patterns that were analyzable. Of the 168 specimens that did not produce RIFO patterns, 142 were smear negative while 26 were smear positive. Of the 142 that were smear negative, 108 were culture negative and 34 were culture positive. Of the 26 that were smear positive, 3 were culture negative as well while 23 were culture positive.

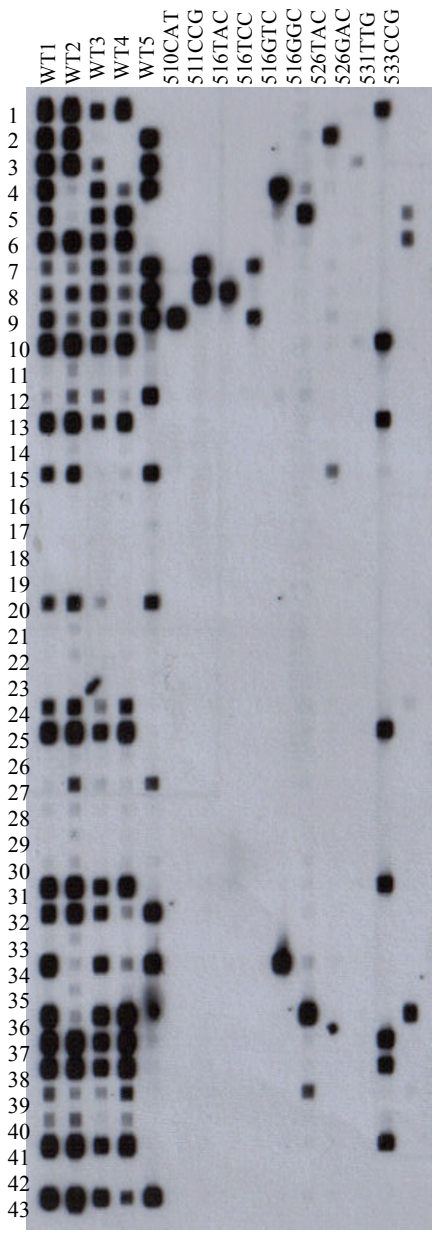

Figure 5.6 RIFO assay results for detection of mutations in sputum specimens with Chelex extracted DNA. Lanes 1 to 10 and lane 43: clinical isolates with all the mutations obtained by sequencing and H37Rv respectively and served as positive controls for the mutations. Lane 32: no mutation pattern. Lanes 13, 25, 31, 37, 38 and 41: mutation 531TTG. Lane 20: no hybridization for WT4 probe but no signal for mutant probe indicating that another mutation not targeted could be present. Lanes 24 and 34: mutations 533CCG and 516GTC respectively. Lane 36: double mutation 516GGC/533CCG. The rest of the lanes showed either incomplete patterns (these were repeated for better results) or no patterns at all.

Table 5.7 Frequency of mutations detected by the RIFO assay in sputum specimens

MUTATION TYPES	NO. OF ISOLATES
511CCG	**1**
516GTC	**21**
516TAC	**4**
526GAC	**7**
526TAC	**4**
531TTG	**127**
533CCG	**16**
511CCG/516GGC	**1**
511CCG/516TAC	**1**
511CCG/516TCC	**1**
516GGC/533CCG	**17**
No mutation*/531TTG	**3**
531TTG/533CCG/516GTC	**2**
No mutation*/511CCG/516TCC	**1**
No mutation*/526GAC/531TTG	**1**
No mutation*/526TAC/531TTG	**1**

*** hybridization signal for all wild type probes**

5.10.3.2. COMPARISON OF SPUTUM RIFO ASSAY RESULTS TO DST

DST of clinical isolates was compared with RIFO assay results of the 204 clinical specimens (Table 5.8). The RIFO assay was able to correctly identify mutations in 176 of the 188 RIF resistant samples, thus demonstrating a sensitivity of 94 % [95% CI: 0.89, 0.97]. The specificity of the RIFO assay on clinical specimens was low with only 6 of the 16 being correctly identified as RIF susceptible (specificity 38% [95% CI: 0.16, 0.64]). The positive predictive value was 95% [CI: 95%, 0.91 and 0.97] and negative predictive value was 33% [CI: 95%, 0.14, 0.59].

Table 5.8 Sputum RIFO assay results compared to DST

SPUTUM RIFO ASSAY	**CULTURE RIFO**		**TOTAL**
	RESISTANT	**SUSCEPTIBLE**	
RIF^R	176	10	**186**
RIF^S	12	6	**18**
TOTAL	**188**	**16**	**204**

5.10.3.3. PERFORMANCE OF THE RIFO ASSAY ON SPUTUM SPECIMENS

The performance of RIFO assay on sputum specimens was evaluated using the smear grading results (Table 5.9). RIFO patterns were obtained in a range of 84 - 95% of all smear positive specimens that produced RIFO patterns (56 of 65, 86% of PSC; 29 of 34, 85.3% of P1-10; 43 of 51, 84% of P11-20 and 55 of 58, 95% of P>20) including those with scanty and one plus AFBs. Of the 84 smear negative and culture positive 45 (53.6%) gave RIFO patterns. Of the 122 smear negative and culture negative 15 (12.3%) gave RIFO patterns.

Table 5.9 Performance of the RIFO assay compared to smear and culture results N= 198

SMEAR GRADING	RIFO PATTERNS OBTAINED FOR:	
	CULTURE POSITIVE	CULTURE NEGATIVE
PSC	51	5
P1-10	28	1
P11-20	42	1
>20	54	1

PSC positive scanty = 2-10 AFB per smear; P1-10 = 10-100 per 100 fields; P11-20 10-100 AFB per fields; P>20 = >100 AFB per field.

5.10.4. COMPARISON OF RIFO ASSAY BETWEEN SPUTUM SPECIMENS AND CLINICAL ISOLATES

The RIFO assay results of 202 (50%) of the 404 specimens were comparable between sputum specimens and clinical isolates (Table 5.10). Of these, 174 clinical isolates were resistant while 28 were susceptible to RIF by RIFO assay. The RIFO assay was able to detect 167 of the 174 resistant and 16 of the 28 susceptible samples, thus giving a sensitivity and specificity of 96% (CI: 95%, 0.92, 0.98] and 57% [CI: 95%, 0.37, 0.75] respectively. The positive predictive value was 93% [CI: 95%, 0.88 and 0.96] and the negative predictive value was 70% [CI: 95%, 0.47, 0.86]

Table 5.10 Sputum RIFO results compared to Culture RIFO results

SPUTUM RIFO ASSAY	**CULTURE RIFO ASSAY**		**TOTAL**
	RIF^R	**RIF^S**	
RIF^R	167	12	**179**
RIF^S	7	16	**23**
TOTAL	**174**	**28**	**202**

5.11. DISCUSSION

In this study, an in-house RIFO assay was optimized for the detection of the 10 commonest mutations in the *rpo*B gene that are associated with RIF resistance in *M. tuberculosis* specifically in KZN. These mutations were first determined by sequencing the hot spot region, codons 507 to 533 since they vary widely according to geographic location (Cavusoglu *et al,* 2002, Mokrousov *et al,* 2004, Jou *et al,* 2005).

The significance of prior sequencing for modification of the RIFO assay for application in different regions is evident from studies undertaken by Senna *et al,* 2006 and Mokrousov *et al,* 2006. In an unmodified assay by the former, 55 (61%) of 90 isolates hybridized with mutant probes and 29 (32%) did not hybridize with the wildtype probes. In contrast, a high sensitivity (92.7%) was reported by Mokrousov *et al*, 2006 for the detection of mutations in Chinese isolates using a modified RIFO assay. This highlights the need to include mutant probes that are able to detect mutations prevalent in a specific area.

In our study, mutations in the *rpo*B gene were correctly detected in 206 of 224 clinical isolates (92% sensitivity) that were confirmed as RIF resistant by the proportion method of DST. This was similar to that reported by Mokrousov *et al,* 2006. High sensitivies were also seen in studies done by Suresh *et al,* in 2006 and Jiao *et al,* in 2007 reporting sensitivities of 95.5 % and 91.5% respectively. High sensitivities were also reported for the detection of RIF resistance using other similar genotypic assays. The GenoType MTBDR*plus* showed sensitivities of 98.7% (Hillemann *et al*, 2007) and 91.7% (Lacoma

et al, 2008), whilst sensitivities of greater than 95% were achieved in several studies conducted in different countries using the INNO LiPA Rif.TB test (Cooksey *et al,* 1997, Martilla *et al,* 1998, Hirano *et al,* 1999, Traore *et al,* 2000, Bartfai *et al,* 2001).

Alternative mechanisms of resistance such as mutations outside the 81-bp segment of the *rpo*B gene (Heep *et al,* 2001), bacterial efflux systems (Choudhuri *et al,* 1999, Banerjee *et al,* 2000; Putman *et al,* 2000) may be responsible for resistance in the remaining 8% of the resistant isolates with no mutations detected by the RIFO assay. Although some of these resistant isolates were sequenced again, their genotypic profile still showed a mutation, suggesting resistance. However, the DST was not repeated, and thus we could not rule out the possibility of false sensitive results by DST. These results concur with those of Mokrousov *et al,* 2006; Senna *et al*, 2006, Suresh *et al,* 2006 and Jiao *et al,* 2007 who reported 4 (7.3 %) of the 55, 6 (6.7%) of 90 and 6 (8.5 %) of 71 respectively of the resistant isolates that do not possess mutations in the *rpo*B region.

In sputum specimens, we observed a similar sensitivity (94%) to that of clinical isolates, demonstrating the ability of this technique to predict MDR directly in sputum specimens. Zhang *et al* (2003) performed RIFO assay on isolates recovered on ZN-stained slides and was able to obtain RIFO patterns in 57% of their slides. They did not calculate the sensitivity and the specificity of the RIFO assay for their study. However, sensitivities of 96.2% (Nikolayevskyy *et al,* 2009), 98.6% (Hillemann *et al,* 2007) and 100% (Lacoma *et al,* 2008 and Miotto et al, 2006) were obtained for sputum smear positive specimens using the GenoType MTBDR*plus*. The sensitivity was reduced to 46.1% and 70.9%

respectively for smear negative sputum specimens (Lacoma *et al,* 2008, Miotto *et al,* 2006).

Mutations could not be detected directly in 12 (6.3%) of the 188 clinical specimens with RIF resistant isolates, possibly due to other resistant mechanisms discussed above. As with culture, the RIFO assay displayed low specificity when performed on sputum specimens, as only 6 of the 16 specimens susceptible by DST were correctly identified as being RIF susceptible. This gave the assay a reduced specificity of 38%. This was low compared to the specificity of the RIFO assay reported by Mokrousov *et al,* 2006 (100%); Senna *et al*, 2006 (100%), Suresh *et al,* 2006 (99.1%) and Jiao *et al,* 2007 (100%) for clinical isolates. Nikolayevskyy *et al,* in 2009 investigated the performance of the GenoType MTBDR*plu*s in sputum specimens and reported a specificity of 90.7%.

The low specificity on both culture and sputum specimens in our study may be attributable to several reasons. The presence of silent mutations in the *rpo*B gene is interpreted as resistant by the RIFO assay. Analysis of discrepant results by sequencing confirmed the presence of the identical mutations identified by the RIFO assay in 10 of 11 isolates classified as susceptible by the proportion method. These strains may be one step from conversion to phenotypic resistance and therefore, may be classified as pre-MDR, as most were INH monoresistant. Thus, the RIFO assay may be useful in the detection of pre-MDR-TB cases. However, the disadvantage would be an over diagnosis of MDR-TB cases if it is genotypically resistant and phenotypically susceptible. The low specificity may also have resulted from an under representation of patients with drug

susceptible TB, since they were recruited from KGV hospital which is a referral hospital for MDR- and XDR-TB patients in Durban.This may be an important limitation which may have introduced bias in our study results. Therefore, this assay should be evaluated in an appropriate clinical setting to ascertain its' true specificity.

Heteroresistance may provide an alternative explanation for the low specificity observed in our study. Rinder *et al*, 2001, reported that the accuracy and reliability of drug susceptibility testing on clinical specimens is adversely affected by heteroresistance. They found that phenotypic results after primary isolation of pure cultures may not be representative of sputum specimens with mixed strains. In TB endemic regions such as in South Africa, concurrent mixed infections or mixed populations of drug resistant and drug susceptible bacilli are commonly encountered (Victor *et al,* 1999).

We observed heteroresistance in 14 of the clinical isolates and 8 sputum specimens that showed a mixed profile by RIFO assay i.e. a wild-type and a resistant genotype. The isolates grown from these specimens were resistant with the proportion method of drug susceptibility testing. Similar observations were reported by de Oliviera *et al,* 2002 using the GenoType MDRTB*plus* assay. Thus, these assays demonstrate their usefulness in the detection of mutations in mixed populations. The sensitivity of detection of resistant subpopulations varied in different studies. Telenti *et al,* 1997 reported a mixed population of susceptible and resistant strains in 1 out of 42 RIF resistant isolates using the SSCP method. The INNO-LiPA test detected resistant subpopulations of strains at a level of 50-75% of the total population (Cooksey *et al,* 1998). In this study, the RIFO assay could

detect 6.4 % and 2.9% from clinical isolates and sputum specimens respectively of mixed populations.

In agreement with most other studies, we found the most common mutations present at codons 516, 531 and 533. The most frequently occurring mutation in our study was the Ser-531-Leu, and this is in concordance with findings reported in other geographic areas (Telenti *et al,* 1993, Williams *et al,* 1998, Hirano *et al,* 1999, Scarpellini *et al,* 1999; and Cooksey *et al,* 2007). Mutations at codon 526 were less common, which was similar to the findings by Barnard *et al,* 2008, but in contrast to that reported in many other countries (Telenti *et al,* 1993, Williams *et al,* 1998, Hirano *et al,* 1999; Scarpellini *et al,* 1999, Samper *et al,* 2005, and Cooksey *et al,* 2007).

The majority of specimens that produced no RIFO patterns in this study were AFB smear negative. Van der Zanden *et al,* 2003 reported rifoligotyping patterns from positive ZN stained slides that contained 2+ or more AFB. However, in our study, between 85 - 95% of all positive smear specimens produced RIFO patterns, including those with scanty and one plus AFBs. We also observed some smear positive specimens that produced no RIFO patterns. This could have been due to insufficient numbers of bacilli present in the sputum specimens during aliquoting. We also found that some smear and culture negative sputum specimens produced RIFO patterns that may have resulted from the presence of uncleared dead bacilli as most of the specimens were collected from patients who were already on TB treatment.

Rapid, cost effective drug susceptibility testing is crucial for reducing nosocomial and community transmission of TB, especially MDR and XDR-TB. Developing countries, including those in Sub-Saharan Africa, Asia and Eastern Europe experience the highest burden of TB, including MDR and XDR-TB (WHO, 2009). The incidence of TB in South Africa is currently the fifth highest in the world, with a high number of XDR-TB cases reported in the province of KZN (Gandhi *et al*, 2006). Such rapid results would be highly beneficial in regions with high-HIV prevalence, such as in South Africa, where a large proportion of patients with HIV-TB co-infections are smear negative.

The use of this macro-array format on clinical specimens or isolates as a first line adjunct to phenotypic DST is a great advantage, particularly in laboratories that already perform spoligotyping. Both assays utilize the same equipment, technology and chemistry, and are easy to conduct. In addition, results are rapidly generated, within 48 hours after DNA extraction, and large numbers of samples, up to 43, can be processed at a time.

A further attractive feature is that the membrane can be chemically treated for multiple usage. In this study, it was re-used 9 times. These features significantly reduce the cost per sample, which was calculated to be R6, 81 thus rendering it more cost effective than the commercial macroarray methods such as the INNO LiPA Rif.TB (US$45 per sample, Morgan *et al,* 2005) and /or the GenoType MTBDR*plus* assays (R116 per sample) currently used in reference laboratories in Southern Africa (Bernard *et al,* 2008).

Although commercial assays such as the GenoType MTBDR*plus* and the GeneXpert MTB/RIF have been endorsed by the WHO and are currently being used in reference laboratories in South Africa, they are expensive and only a limited number of samples can be tested at a time. Also, only a limited number of selected mutations can be detected by these assays for prediction of RIF resistance. In contrast, the in house RIFO assay detects mutations specific for a geographic region and also allows for modification of the assay as new mutations are discovered. One technician can be employed and be dedicated to this assay and can process at least 43 specimens at once. The thousands of rands that can be saved from using this assay as an alternative to the expensive commercially available assays can be used in patient care and management of the TB disease. Development of this assay for direct detection of RIF resistance in sputum specimens is an initial step towards incorporating other mutations which are markers of resistance for first line as well as second line drugs. This will be a milestone in directly detecting XDR-TB.

CHAPTER SIX

6. DISCUSSION AND CONCLUSION

KZN province has one of the highest TB burdens in the world, including MDR and XDR-TB as was highlighted in Tugela Ferry in 2005 (Ghandi *et al,* 2006). There is an urgent need for diagnostic tests that can rapidly identify TB and detect drug resistance in *M. tuberculosis* directly from clinical specimens in order to reduce transmission rates and reverse these trends. Molecular assays have shown tremendous potential to fulfill the requirements of rapid diagnostics.

Molecular tests based on DNA extracted from cultured isolates require primary isolation from clinical specimens which adds on to diagnostic delays by at least 3 or more weeks. This may be overcome by performing direct detection of *M. tuberculosis* from clinical samples. However, the presence of inhibitors in clinical specimens such as sputum reduces the sensitivity of molecular assays. In this study, we investigated four DNA extraction methods and found the Chelex extraction method to be the most optimal in removing PCR inhibitors. It was also cost effective with respect to price, ease of performance and shelf life of the reagents. The Chelex method was then used to extract DNA directly from sputum samples to optimize an in-house reverse line hybridization assay for detecting mutations in the *rpoB* region of *M. tuberculosis*.

Sequencing of *rpo*B gene of the clinical isolates in our study revealed the presence of 11 mutations, with the highest frequency of mutations occurring in the 531 codon

(TCG→TTG resulting in Ser→Leu) as was reported in numerous studies. This was followed by 516(GAC→GTC causing a Asp→Val) and 533(CTG→CCG; Leu→Pro). We compared the mutations obtained in our study isolates to those of Kiepiela *et al*, in KZN strains in 1998, and did not find 50% of the mutations that were present in their study. It is possible that these mutations were associated with a fitness cost that reduced the survival of strains harbouring such mutations approximately a decade later.

Literature has consistently demonstrated that mutations in the *rpoB* region are not detected in all resistant strains of *M. tuberculosis*. Similarly, in our study, mutations in this locus were not detected in some isolates by sequencing of the *rpoB* region, even though they were resistant by the proportion method of DST. This can be attributed to the presence of mutations outside the resistant locus targeted or other mechanisms of resistance such as efflux pumps that have been demonstrated in other bacterial species and are yet to be elucidated in *M. tuberculosis*. The high percentage (9.7%) of isolates lacking mutations represents an impediment to molecular drug resistance testing. Therefore, although molecular methods may significantly enhance the rapid detection of mutations associated with drug resistance, they should be used as an adjunct to, but not replace phenotypic methods.

The reverse line assay (RIFO assay) was modified to detect 10 specific mutations identified among the KZN isolates, using Chelex extracted DNA directly from clinical specimens. The accuracy (91%) of mutation detection from clinical samples was determined by comparison with mutations detected in isolates cultured from the same

specimens. The sensitivity of the RIFO assay in both clinical isolates and sputum specimens was high (92 and 94% respectively) compared to the phenotypic proportion method as the gold standard. However, the low specificity (35 and 38% respectively) for both isolates and sputum specimens is attributable to the presence of silent mutations in 77% of samples as confirmed by sequencing of the *rpoB* gene of the susceptible strains whose RIFO assay results were discrepant with DST. These findings highlight the significance of silent mutations in genotypic assays in over estimation of MDR cases. However, they may signify the prelude to phenotypic RIF resistance and thus true MDR, which may be confirmed by testing serial isolates of patients in a longitudinal study over time.

The limitation of this study is that the in-house RIFO assay was not evaluated in a clinical setting with a large number of patients with drug susceptible TB and this may also have contributed to the lower specificity. Although relatively easy to perform, the assay is time consuming and can only be performed within a laboratory setup, requiring a certain level of molecular biology expertise.

Despite the low specificity due to silent mutations and the limitations mentioned above, the in-house RIFO assay has numerous advantages. It is capable of detecting the commonest mutations specific to KZN and is cheaper than commercially available assays (R 6.81 compared to R116 for the Haines test. Multiple samples (43) can be tested simultaneously and the membrane is robust so that it is re-usable up to 9 times, thus significantly reducing the costs. The modest equipment requirements such as a thermal

cycler, hybridization oven, waterbath, microcentrifuge and darkroom facility are generally available in a standard laboratory. The RIFO assay is rapid, and can be performed directly on sputum specimens to generate results within 3 days of specimen receipt. The high sensitivity enables prediction of RIF resistance and therefore MDR-TB in 3 days compared to the phenotypic gold standard. These attributes highlight the potential of this assay as a suitable, competitive alternative to expensive commercially available genetic based tests in poorly resourced developing countries with high TB burdens such as in Southern Africa. The Hains GenoType MTBDR*plus* would have missed the double mutations 516GGC/533CCG double and single mutation 533CCG which constituted almost 20% of our isolates.

The WHO endorsed GenoType MTBDR*plus* and the GeneXpert MTB/RIF currently in use in reference laboratories in South Africa are expensive and can detect only a limited number of selected mutations for prediction of RIF resistance. The in house RIFO assay detects mutations specific for a geographic region and also allows for modification of the assay as new mutations are discovered. This assay can be further developed for the rapid detection of mutations associated with resistance to first and second line drugs. This will be a milestone in directly detecting XDR-TB.

Future studies should be conducted in clinical settings to enroll more patients infected with drug susceptible TB to increase the specificity of the assay and to determine its cost effectiveness. Similar assays should be designed for the rapid detection of resistance to drugs defining XDR-TB and to explore the possibility of automation of such assays.

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APPENDICES

APPENDIX I

Preparation of reagents

10% SDS

SDS	10 g
Water	100 ml

The SDS was dissolved in deionised water and stored at room temperature for up to 6 months.

Tris-Cl/Borate/EDTA (TBE Buffer 5X)

Tris base (Sigma)	54 g
Boric acid (H_3BO_3) (BDH)	27.5 g
0.5 M EDTA (pH 8.0)	20 ml

Tris base and boric acid was dissolved in 800 ml distilled water. EDTA was then added and the pH was adjusted to 8.0 with NaOH. The buffer was autoclaved at 121°C for 15 min. A working concentration of 0.5 X was used; i.e. 1:10 dilution of 5X stock.

10 X TE Buffer (pH 8.0)

100 mM Tris/HCl

10 mM EDTA

Tris	1.211 g
EDTA	0.3722 g

Dissolve 1.211 g Tris in 80 ml of distilled water. Add 0.3722 g EDTA. Mix until it dissolves. PH to 8.0 with concentrated HCl. Make up to 100 ml in measuring cylinder. Autoclave at 121°C for 15 min.

5 M NaCl

NaCl	14.6 g
Water	50 ml

Dissolve NaCl in 45 ml distilled water with heating. Bring up to 50 ml. Autoclave at 121°C for 15 min.

CTAB/NaCl

NaCl	4.1 g
CTAB	10 g
Water	100ml

Dissolve 4.1 g of NaCl in 80 ml distilled water. Add 10 g of CTAB and heat solution at 65°C until dissolved. Make up to 100 ml with distilled water.

Lysozyme

10 mg/ml

Dissolve 10 mg (0.01 g) of lysozyme in 1 ml distilled water. Store at -20C. Prepare just before use.

Proteinase K

10 mg/ml

Dissolve 10 mg (0.01 g) of Proteinase K in 1 ml distilled water. Store at -20C. Prepare just before use.

SDS/Proteinase K mix (per sample)

5µl Proteinase K (10mg/ml)

70µl 10% SDS

Prepare just before use

Chloroform/isoamyl alcohol

C: I

24: 1

2 ml isoamyl alcohol brought up to 50 ml chloroform in measuring cylinder

70% Ethanol

70 ml absolute ethanol

30 ml sterile distilled water

0.5 M EDTA

EDTA	186.1 g
Water	800 ml

Add 186.1 g of EDTA to 800 ml of water. Stir vigorously with a magnetic stirrer; adjust pH to 8.0 with NaOH pellets (~20g). Bring volume up to 1000 mls.

Inhibitor Removal Solution

Guanidium thiocynate(GITC)	30 g
EDTA	0.47 g
Sarcosyl	0.25 g
L- mercaptoethanol	0.78 ml
Tris/HCl (pH 7.5)	5 ml

Dissolve GITC, EDTA, sarcosyl and L-mercaptoethanol in 45 ml water. Add to 5 ml of Tris/HCl pH 7.5. (NB: Stable for 2 months at room temperature if stored in dark and airtight container. This solution is prepared in the fume hood and GITC- containing waste was disposed in 10 M NaOH).

Extraction solution

Chelex	1.2 g
Tween 20	36µl
Triton X 100	3.6µl

Add distilled water to make a final volume of 10 ml.

Acid washed silica

Silica (SiO_2)	6 g
Water	50 ml

Add 6 g of SiO_2 in 50 ml distilled water and vortex thoroughly. Allow to settle 24 hrs at room temperature. Remove 43 ml of upper liquid by aspiration and fill up to 50 ml. Allow to settle for 5 hrs at room temperature. Remove the upper liquid by aspiration. Add 60 l of concentrated HCl to adjust the pH to pH 2. Aliquot 4 mls in small bottles and autoclave. Store at room temperature in the dark.

Lysis Buffer (L6)

Guanidium thiocynate	24 g
0.1 M Tris/HCl pH 6.4	20 ml
0.2 M EDTA pH 8.0	4.4 ml
Triton X 100	500 µl

Dissolve 24 g in of GITC in 20 ml of 0.1 M Tris/HCl, pH 6.4 (Heat at 60C to dissolve). Add 4.4 ml of 0.2 M EDTA (pH 8.0) and 500 µl of Triton X 100 (mix by inverting). Store at room temperature, in the dark (Stable for 3 weeks).

Wash Buffer (L2)

Guanidium thiocynate	24 g
0.1 M Tris/HCl pH 6.4	20 ml

Dissolve 24 g GITC in 20 ml of 0.1 M Tris/HCl pH 6.4 at 60 C. Store at room temperature, in the dark (Stable for 3 weeks).

M Tris/HCl pH 6.4

Tris	1.211 g
Water	100 ml

Dissolve 1.211 g of Tris in 80 ml of distilled water. Adjust pH to 6.4 with concentrated HCl.

0.1 M EDTA pH 8.0

EDTA	37.77 g
Water	500 ml

Dissolve 37.22 g of EDTA in 400 ml of distilled water. Adjust pH with NaOH to 8.0.

3M Sodium Acetate

Sodium Hydroxide	40.82 g
Water	100 ml

Dissolve 40.82 g of NaOH pellets in 80 ml of distilled water. Adjust pH to 5.2 with glacial acetic acid. Adjust volume to 100 ml and sterilize by autoclaving at 121 C for 15 min.

500 mM $NaHCO_3$

$NaHCO_3$	4.2 g
Water	80 ml

Dissolve 4.2 g of NaHCO3 in 80 ml of distilled water. Adjust pH to 8.4 and make up to 100 ml by adding water. Store at room temperature for no longer than one year

16% (w/v) 1- ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC)

EDAC	1.6 g
Water	8 ml

Dissolve 1.6 g of EDAC in 8 ml of distilled water. Once dissolved adjust volume to 10 ml with distilled water. Prepare fresh before use.

20 x SSPE

$Na_2HPO_4*2H_2O$	17.8 g
NaCl	105.12 g
EDTA	3.7 g

Place in magnetic stirrer; adjust pH to 7.4 using NaOH and autoclave. Store at room temperature for no longer than 1 year.

APPENDIX II

SEQUENCING RESULTS

No.	Strain no.	Sensitive	Resistant	Sequencing results
1	R01	O K	I R E S	531TTG
2	R02	E O K	I R S	531TTG
3	R05	E O K	I R S	526GAC
4	R09	E O K	I R S	531TTG
5	R10	E O K	I R S	531TTG
6	R11	E O K	I R S	531TTG
7	R12	E O K	I R S	531TTG
8	R15	E O K	I R S	531TTG
9	R17	E O K	I R S	531TTG
10	R20	E S O K	I R	531TTG
11	R21	E O K	I R S	531TTG
12	R22	E O K	I R S	516GTC
13	R23	E S O K	I R	516GTC
14	R25	E O K	I R S	516GTC
15	R26	E S	I R O K	516GGC/533CCG
16	R29	E S O K	I R	531TTG
17	R30	E	I R S O K	531TTG
18	R32	E O K	I R S	531TTG
19	R34	E O K	I R S	516GTC
20	R35	E O K	I R S	533CCG
21	R38	E O K	I R S	531TTG
22	R39	E O K	I R S	526GAC
23	R40	S O K	I R E	533CCG
24	R42	E S O K	I R	516GTC
25	R43	S O K	I R E	533CCG
26	R44	E O K	I R S	531TTG
27	R46	E S O K	I R	531TTG
28	R47	E O	I R S K	533CCG
29	R48	R E S O K	I	531TTG
30	R50	E O K	I R S	531TTG
31	R53	E O K	I R S	516GTC
32	R55	E S O K	I R	516GTC
33	R56	E S O K	I R	531TTG

34	R57	E S O K	I R	531TTG
35	R58	E O K	I R S	531TTG
36	R59	E O K	I R S	516GTC
37	R60	E O K	I R S	516GTC
38	R64	E O K	I R S	531TTG
39	R65	E O K	I R S	516GTC
40	R66	E S O K	I R	531TTG
41	R69	E O K	I R S	531TTG
42	R70	E O K	I R S	531TTG
43	R83	E O K	I R S	531TTG
44	R86	E O K	I R S	531TTG
45	R91	E O K	I R S	531TTG
46	R94	E O K	I R S	516GTC
47	R102	I E S O K	R	533CCG
48	R108	E S O K	I R	533CCG
49	R109	E O K	I R S	531TTG
50	R113	E O K	I R S	531TTG
51	R114	O K	I R S E	533CCG
52	R116	E S O K	I R	526TAC
53	R123	E S O K	I R	533CCG
54	R134	E O K	I R S	526GAC
55	R137	E S O K	I R	516GTC
56	R138	E S O K	I R	531TTG
57	R139	E S O K	I R	516GTC
58	R140	E S O K	I R	531TTG
59	R141	E O K	I R S	516GTC
60	R143	E S O K	I R	531TTG
61	R145	E S O K	I R	531TTG
62	R146	E S O K	I R	516GTC
63	R154	E O K	I R S	531TTG
64	R157	E S O K	I R	533CCG
65	R160	O K	I R S E	531TTG
66	R164	O K	I R E S	531TTG
67	R171	I R E S O K		531TTG
68	R207	E S O K	I R	531TTG
69	R208	E S O	I R O	526TAC
70	R209	E O K	I R S	516GTC
71	R211	E O K	I R S	531TTG

72	R212	E S	I R O K	516GTC
73	R213	E O K	I R S	531TTG
74	R214	E O K	I R S	531TTG
75	R216	E O K	I R S	531TTG
76	R223	E S O K	I R	531TTG
77	R225	E O K	I R S	516GTC
78	R226	E O	I R S K	531TTG
79	R230	E S O K	I R	531TTG
80	R231	E O K	I R S	531TTG
81	R232	E O K	I R S	531TTG
82	R233	O K	I R E S	526GAC
83	R234	I R E S O K		531TTG
84	R242	E O K	I R S	531TTG
85	R243	E S O K	I R	533CCG
86	R248	E O K	I R S	No mutation (s)
87	R251	S	I R E O K	516GGC/533CCG
88	R253	E S	I R O K	516GGC/533CCG
89	R254	E O K	I R S	516GTC
90	R256	E O K	I R S	516GTC
91	R257	E S	I R O K	516GGC/533CCG
92	R268	O K	I R E S	531TTG
93	R269	E S O K	I R	531TTG
94	R271	E S O K	I R	531TTG
95	R273	E O K	I R S	531TTG
96	R276	E S O K	I R	531TTG
97	R277	E O K	I R S	516GTC
98	R281	E S O K	I R	533CCG
99	R283	E S O K	I R	516GTC
100	R284	E S O K	I R	533CCG
101	R285	E O K	I R S	531TTG
102	R287	E S O K	I R	533CCG
103	R293	O K	I R E S	531TTG
104	R298	E S O K	I R	531TTG/537GAT
105	R299	E	I R S O K	533CCG
106	R300		I R E S O K	531TTG
107	R301	E O K	I R S	531TTG
108	R303	E O K	I R S	531TTG
109	R309	E S O K	I R	531TTG

110	R314	E S	I R O K	516GGC/533CCG
111	R325	E O K	I R S	531TTG
112	R327	E O K	I R S	516GTC
113	R328	E O K	I R S	531TTG
114	R332	E O K	I R S	531TTG
115	R334	E S O K	I R	531TTG
116	R336	E O K	I R S	531TTG
117	R339	E S	I R O K	516GGC/533CCG
118	R348	E O K	I R S	531TTG
119	R350	E O K	I R S	516GGC/533CCG
120	R352	E O K	I R S	531TTG
121	R364	E O K	I R S	531TTG
122	R373	E O K	I R S	531TTG
123	R375	E	I R S O K	516GGC/533CCG
124	R376	E S	I R O K	516GGC/533CCG
125	R377	E O K	I R S	516GGC/533CCG
126	R378	E O K	I R S	531TTG
127	R379	E S O K	I R	516GTC
128	R382	E O K	I R S	531TTG
129	R384	E S O K	I R	531TTG
130	R385	E O K	I R S	531TTG
131	R386	O K	I R E S	531TTG
132	R387	E S O K	I R	531TTG
133	R388	E O K	I R S	531TTG
134	R389	E S	I R O K	516GGC/533CCG
135	R391	E S O	I R O	531TTG
136	R392	E S O K	I R	511CCG/516TAC
137	R395	E S O K	I R	531TTG
138	R396	E S O K	I R	531TTG
139	R404	E O K	I R S	531TTG
140	R413	E S O	I R K	531TTG
141	R415	E S O K	I R	511CCG/516TCC
142	R417	E O K	I R S	510CAT/516TAC
143	R421	E O K	I R S	531TTG
144	R425	E S O K	I R	526GAC
145	R426	E S O	I R K	533CCG
146	R427	E S O K	I R	531TTG
147	R428	E S	I R O K	516GGC/533CCG

148	R430	E O K	I R S	531TTG
149	R431	E O K	I R S	531TTG
150	R34	E S	I R O K	516GGC/533CCG
151	R437	E S O K	I R	526TAC
152	R443	O	I R E S K	531TTG
153	R459	E S O K	I R	531TTG
154	R471	E O K	I R S	531TTG
155	R479	E O K	I R S	531TTG
156	R492	E S	I R O K	531TTG
157	R496	E S O K	I R	531TTG
158	R498	E S O K	I R	531TTG
159	R500	E S O K	I R	531TTG
160	R501	E O K	I R S	516GGC/533CCG
161	R502	E S	I R O K	516GGC/533CCG
162	R503	E	I R S O K	516GGC/533CCG
163	R504	E S	I R O K	516GGC/533CCG
164	R506	E	I R S O K	516GGC/533CCG
165	R507	S K	I R E O	531TTG
166	R510	E S O K	I R	531TTG
167	R514	K	I R E S O	531TTG
168	R524	E O K	I R S	531TTG
169	R525	E	I R S O K	516GGC/533CCG
170	R527	I E O K	R S	531TTG
171	R539	O K	I R E S	531TTG
172	R561	E O K	I R S	531TTG
173	R574	E O K	I R S	531TTG
174	R575	E O K	I R S	516GTC
175	R576	O	I R E S K	531TTG
176	R577	E O K	I R S	531TTG
177	R589	E S O K	I R	531TTG
178	R613	E O K	I R S	526GAC
179	R617	E O K	I R S	516GTC
180	R619	O K	I R E S	531TTG
181	R621	E O K	I R S	533CCG
182	R622	E O K	I R S	516GGC/533CCG
183	R626	E O K	I R S	533CCG
184	R627	E O K	I R S	516GTC
185	R629	E O K	I R S	531TTG

186	R670	E O K	I R S	531TTG
187	R693	E O K	I R S	531TTG
188	R62	E S O	I R K	No mutation(s)
189	R87	E S O K	I R	No mutation(s)
190	R88	E O K	I R S	No mutation(s)
191	R210	E S O K	I R	No mutation(s)
192	R258	E S O K	I R	No mutation(s)
193	R274	E S O K	I R	No mutation(s)
194	R282	E S O K	I R	No mutation (s)
195	R330	E S O K	I R	No mutation(s)
196	R349	E S O K	I R	No mutation(s)
197	R380	E O K	I R S	No mutation(s)
198	R383	I R E S O K		No mutation(s)
199	R400	I R E S O K		No mutation(s)
200	R402	E S	I R O K	No mutation(s)
201	R429	E S O K	I R	No mutation(s)
202	R436	I E S O K	R	No mutation(s)
203	R623	E S	I R O K	No mutation(s)
204	R24	E S O K	I R	No mutation(s)
205	R 49	E S O K	I R	No mutation(s)
206	R52	R E S O K	I	No mutation(s)
207	R61	E S O K	I R	No mutation(s)
208	R67	E O K	I R S	No mutation(s)
209	R79	I R E S O K		No mutation(s)
210	R95	O K	I R S E	No mutation(s)
211	R103	I R E S O K		No mutation(s)
212	R104	I R E S O K		No mutation(s)
213	R107	I R E S O K		No mutation(s)
214	R117	E O K	I R S	No mutation(s)
215	R125	I R E S O K		No mutation(s)
216	R126	I R E S O K		No mutation(s)
217	R127	I R E S O K		No mutation(s)
218	R132	I R E S O K		No mutation(s)
219	R149	E O K	I R S	No mutation(s)
220	R152	I R E S O K		No mutation(s)
221	R166	I R E S O K		No mutation(s)
222	R167	I R E S O K		No mutation(s)
223	R172	I R E S O K		No mutation(s)

224	R181	I R E S O K		No mutation(s)
225	R186	I R E S O K		No mutation(s)
226	R187	I R E S O K		No mutation(s)
227	R196	I R E S O K		No mutation(s)
228	R237	E S O K	I R	No mutation(s)
229	R252	E	I R S O K	No mutation(s)
230	R261	I R E S O K		No mutation(s)
231	R262		I R E S O K	No mutation(s)
232	R267	I R E S O K		No mutation(s)
233	R286	I R E S O K		No mutation(s)
234	R288	I R E S O K		No mutation(s)
235	R291	I R E S O K		No mutation(s)
236	R292	I R E S O K		No mutation(s)

Appendix III

RIFO RESULTS

Strain no.	Smear Results	Culture Results	Sensitive	Resistant	Sequencing	Culture RIFO results	Sputum RIFO results
R12	PSC	2wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R20	P11-20	2 wk +	E S O K	I R	531TTG	531TTG	531TTG
R25	P1-10	3wk Sc	E O K	I R S	516GTC	516GTC	531TTG
R9	P1-10	2 wk +	E O K	I R S	531TTG	531TTG	No pattern
R17	P11-20	2wk ++	E O K	I R S	531TTG	531TTG	531TTG
R28	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R7	P1-10	6wk +	failed to grow	failed to grow	**ND**	**ND**	531TTG
R1	P11-20	2wk Sc	O K	I R E S	531TTG	531TTG	531TTG
R4	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R14	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R22	PSC	2 wk +	E O K	I R S	516GTC	516GTC	516GTC
R27	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R24	PSC	1wk Sc	E S O K	I R	No mutation	No mutation	533CCG
R16	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R11	P>20	1wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R6	P11-20	1wk Sc	failed to grow	failed to grow	**ND**	**ND**	No pattern
R2	NEG	2wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R13	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R18	NEG	3wk Sc	R E S O K	I	**ND**	516GTC	No mutation

R23	P>20	2wk ++	E S O K	I R	516GTC	531TTG	531TTG
R19	P11-20	2wk ++	I R E S O K		**ND**	No mutation	No mutation
R15	PSC	2wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R3	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R8	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R5	P1-10	2wk Sc	E O K	I R S	526GAC	526GAC	526GAC
R21	P>20	2wk +++	E O K	I R S	531TTG	531TTG	531TTG
R10	NEG	3wk +	E O K	I R S	531TTG	531TTG	531TTG
R26	PSC	2wk Sc	E S	I R O K	516GGC/533CCG	516GGC/533CCG	516GGC/533CCG
R30	NEG	3wk Sc	E	I R S O K	531TTG	531TTG	No pattern
R29	NEG	2wk Sc	E O S K	I R	531TTG	531TTG	531TTG
R39	PSC	2wk Sc	E O K	I R S	526GAC	526GAC	No pattern
R44	NEG	3wk Sc	E O K	I R S	531TTG	531TTG	No pattern
R47	NEG	4wk Sc	E O	I R S K	533CCG	533CCG	No pattern
R34	NEG	2wk Sc	E O K	I R S	516GTC	516GTC	516GTC
R52	PSC	1wk Sc	R E S O K	I	**No mutation**	**No pattern**	526?
R57	NEG	2wk Sc	E O S K	I R	531TTG	531TTG	No pattern
R31	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R59	PSC	2wk Sc	E O K	I R S	516GTC	516GTC	516GTC
R36	NEG	6wk +	R E O K	I S	**ND**	531TTG	531TTG
R42	PSC	3wk Sc	E S O K	I R	516GTC	516GTC	531TTG
R50	P11-20	1wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R33	PSC	NEG	NEG	NEG	**ND**	**ND**	533CCG
R46	P1-10	2wk Sc	E S O K	I R	531TTG	531TTG	531TTG
R55	NEG	2wk Sc	E O S K	I R	516GTC	516GTC	No pattern

R49	P>20	1wk Sc	E S O K	I R	No mutation	No mutation	No mutation/526TAC/531TTG
R32	NEG	1 wk Sc	E O K	I R S	531TTG	531TTG	526GAC
R41	NEG	MOTT	NEG	NEG	**ND**	**ND**	No pattern
R54	NEG	6wk Sc	I R E S O K		**ND**	No mutation	No pattern
R38	NEG	2wk +	E O K	I R S	531TTG	531TTG	531TTG
R51	PSC	2wk Sc	R E S O K	I	**ND**	No mutation	No mutation
R37	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R43	PSC	2wk Sc	S O K	I R E	533CCG	533CCG	No pattern
R35	NEG	1 wk Sc	E O K	I R S	533CCG	533CCG	531TTG
R58	P11-20	2wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R53	P11-20	NEG	E O K	I R S	516GTC	516GTC	516GTC
R40	NEG	2wk Sc	E O S K	I R	533CCG	533CCG	533CCG
R45	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R56	PSC	2wk Sc	E S O K	I R	531TTG	531TTG	531TTG
R60	PSC	2wk Sc	E O K	I R S	516GTC	516GTC	516GTC
R48	P11-20	2wk Sc	R E S O K	I	531TTG	531TTG	No mutation/531TTG
R72	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R84	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R66	PSC	1wk Sc	E S O K	I R	531TTG	531TTG	531TTG
R90	NEG	3wk Sc	R E O K	I S	**ND**	No mutation	No mutation
R70	PSC	2wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R61	PSC	1wk Sc	E S O K	I R	No mutation	No mutation	No mutation
R87	P>20	3wk Sc	E S O K	I R	No mutation	No mutation/531TTG	531TTG
R111	NEG	NEG	NEG	NEG	**ND**	**ND**	No mutation

R118	NEG	NEG	NEG	NEG	**ND**	**ND**	516GTC
R85	NEG	4wk Sc	I R E S O K		**ND**	No mutation	No mutation
R71	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R124	PSC	NEG	NEG	NEG	**ND**	**ND**	No pattern
R64	PSC	4wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R136	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R94	NEG	4wk Sc	E O K	I R S	516GTC	516GTC	516GTC
R116	PSC	6wk +	E S O K	I R	526TAC	526TAC	526TAC
R141	P1-10	1wk Sc	E O K	I R S	516TAC	516TAC	516TAC
R62	P1-10	1wk Sc	E S O	I R K	No mutation	No mutation	533CCG
R138	P11-20	2wk ++	E S O K	I R	531TTG	531TTG	No mutation/526GAC/531TTG
R67	PSC	1wk Sc	E O K	I R S	No mutation	No mutation	531TTG
R148?	NEG	NEG	NEG	NEG	**ND**	No mutation	No mutation
R143	P1-10	2wk Sc	E S O K	I R	531TTG	531TTG	531TTG
R120	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R82	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R92	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R68	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R137	P1-10	2wk Sc	E S O K	I R	516GTC	516GTC	516GTC
R65	P>20	1wk Sc	E O K	I R S	516GTC	516GTC	516GTC
R144	P>20	1wk Sc	S O K	I R E	**ND**	511CCG/516GGC	511CCG/516GGC
R109	PSC	2wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R117	PSC	1wk Sc	E O K	I R S	No mutation	No mutation/526GAC/531TTG	531TTG
R147	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern

R132	NEG	2wk Sc	I R E S O K		No mutation	No mutation	No mutation
R69	PSC	1wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R122	PSC	Contaminated	Contaminated		**ND**	**ND**	531TTG
R140	NEG	3wk Sc	E O S K	I R	531TTG	531TTG	No pattern
R89	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R145	P>20	1wk Sc	E S O K	I R	531TTG	531TTG	531TTG
R135	P11-20	NEG	NEG	NEG	**ND**	**ND**	531TTG
R123	NEG	2wk Sc	E O S K	I R	533CCG	533CCG	533CCG
R133	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R81	NEG	NEG	NEG	NEG	**ND**	**ND**	No mutation
R139	NEG	2wk Sc	E O K	I R S	516GTC	516GTC	516GTC
R80	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R108	P>20	1wk +	E S O K	I R	533CCG	533CCG	533CCG
R142	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R121	PSC	1wk Sc	R E S	I O K	**ND**	533CCG	533CCG
R83	P11-20	6wk +	E O K	I R S	531TTG	531TTG	531TTG
R113	NEG	2wk Sc	E O K	I R S	531TTG	531TTG	No mutation
R119	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R112	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R110	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R93	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R115	NEG	NEG	NEG	NEG	**ND**	**ND**	516GTC
R159	NEG	4wk Sc	MOTT	MOTT	**ND**	**ND**	No pattern
R205	NEG	NEG	NEG	NEG	**ND**	**ND**	526TAC
R214	P11-20	2wk Sc	E O K	I R S	531TTG	531TTG	No pattern

R154	P>20	1wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R161	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R213	P>20	2wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R216	P11-20	2wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R223	P>20	2wk Sc	E S O K	I R	531TTG	531TTG	531TTG
R232	P>20	1wk +++	E O K	I R S	531TTG	531TTG	531TTG
R149	PSC	1wk Sc	E O K	I R S	No mutation	531TTG	531TTG
R237	P>20	2wk Sc	E S O K	I R	No mutation	No mutation	No mutation
R155?	PSC	NEG	NEG	NEG	**ND**	**No mutation**	511CCG
R229	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R242	P11-20	4wk +	E O K	I R S	531TTG	531TTG	531TTG
R156	P>20	NEG	NEG	NEG	**ND**	**ND**	531TTG
R219	PSC	3wk Sc	I R E O K	S	**ND**	526GAC	No pattern
R211	P11-20	2wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R208	P>20	1wk Sc	E S K	I R O	526TAC	526TAC	531TTG
R225	P11-20	2wk Sc	E O K	I R S	516GTC	516GTC	No pattern
R157	P11-20	1wk Sc	E S O K	I R	533CCG	533CCG	533CCG
R240	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R163	PSC	1wk Sc	R E S O K	I	**ND**	516GTC	516GTC
R212	NEG	2wk Sc	E S	I R O K	516GTC	516GTC	533CCG
R158	NEG	4wk Sc	I E S O K	R	**ND**	No mutation	No mutation
R234	P11-20	2wk Sc	I R E S O K		531TTG	531TTG	531TTG
R164	P1-10	4wk Sc	O K	I R E S	531TTG	531TTG	531TTG
R215	P1-10	4wk +	R E S O K	I	**ND**	533CCG	No pattern
R227	PSC	NEG	NEG	NEG	**ND**	**ND**	516TAC

R160	P1-10	4wk Sc	O K	I R E S	531TTG	531TTG	531TTG
R235	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R243	P>20	2wk Sc	E S O K	I R	533CCG	533CCG	533CCG
R162	NEG	1wk Sc	I E S O K	R	**ND**	No mutation/516TAC	516GTC
R257	PSC	1wk Sc	E S	I R O K	516GGC/533CCG	516GGC/533CCG	516GGC/533CCG
R248	P1-10	1wk Sc	E O K	I R S	No mutation	No mutation	531TTG
R204	NEG	NEG	NEG	NEG	**ND**	**ND**	531TTG
R228	P>20	2wk Sc	I R E O K	S	**ND**	531TTG	531TTG
R210	P>20	2wk Sc	E S O K	I R	No mutation	No mutation	531TTG
R206	NEG	NEG	NEG	NEG	**ND**	**ND**	516GTC
R221	NEG	NEG	NEG	NEG	**ND**	**ND**	531TTG
R217	NEG	NEG	NEG	NEG	**ND**	**ND**	531TTG
R207	NEG	2wk Sc	E O S K	I R	531TTG	531TTG	531TTG
R220	NEG	NEG	NEG	NEG	**ND**	**ND**	531TTG
R254	P1-10	1wk Sc	E O K	I R S	516GTC	516GTC	No mutation
R209	P>20	2wk Sc	E O K	I R S	516GTC	516GTC	No mutation
R218	NEG	NEG	NEG	NEG	**ND**	**ND**	531TTG
R238	P>20	2wk Sc	R E S O K	I	**ND**	533CCG	533CCG
R222	NEG	NEG	NEG	NEG	**ND**	**ND**	516GTC
R231	PSC	3wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R247	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R251	P>20	3wk++	S	I R E O K	516GGC/533CCG	516GGC/533CCG	516GGC/533CCG
R255	NEG	NEG	NEG	NEG	**ND**	**ND**	526GAC
R249	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R224	P>20	2wk Sc	E S O	I R K	**ND**	531TTG	No mutation

R239	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R226	P11-20	4wk Sc	E O	I R S K	531TTG	531TTG	No pattern
R230	NEG	3wk Sc	E O S K	I R	531TTG	531TTG	531TTG
R241	PSC	Contaminated	NEG	NEG	**ND**	**ND**	533CCG
R233	P11-20	2wk Sc	O K	I R E S	526GAC	526GAC	526GAC
R253	NEG	3wkSc	E S	I R O K	516GGC/533CCG	516GGC/533CCG	531TTG
R256	P1-10	1wk Sc	E O K	I R S	516GTC	516GTC	516GTC
R250	P1-10	1wk Sc	R E O K	I S	**ND**	No mutation	No mutation
R252	PSC	1wk Sc	E	I R S O K	No mutation	No mutation/516GGC	516TAC
R273	P>20	1wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R258	P11-20	1wk Sc	E S O K	I R	No mutation	No mutation/516GTC	516TAC
R283	PSC	1wk Sc	E S O K	I R	516GTC	516GTC	516GTC
R259	NEG	NEG	NEG	NEG	**ND**	**ND**	516GGC/533CCG
R295	P11-20	1wk Sc	E S	I R O K	**ND**	516GGC/533CCG	516GGC/533CCG
R309	PSC	2wk Sc	E S O K	I R	531TTG	531TTG	531TTG
R268	P11-20	1wk Sc	O K	I R E S	531TTG	531TTG	531TTG
R271	NEG	2wk Sc	E O S K	I R	531TTG	531TTG	531TTG
R300	P>20	1wk Sc		I R E S O K	531TTG	531TTG	516GGC/531TTG/533CCG
R354	NEG	2wk Sc	R E O K	I S	**ND**	No mutation	No mutation
R349	P>20	2wk Sc	E S O K	I R	No mutation	No mutation	No mutation
R269	NEG	3wk Sc	E O S K	I R	531TTG	531TTG	No pattern
R274	P1-10	1wk Sc	E S O K	I R	No mutation	No mutation/531TTG	531TTG
R280	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R351	NEG	2wk Sc	E S	I R O K	516GGC/533CCG	516GGC/533CCG	No pattern
R285	PSC	1wk Sc	E O K	I R S	531TTG	531TTG	531TTG

R270	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R297	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R310	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R330	NEG	3wk Sc	E O S K	I R	No mutation	No mutation	No mutation/531TTG
R272	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R276	P>20	1wk Sc	E S O K	I R	531TTG	531TTG	531TTG
R296	P11-20	1wk Sc	E O K	I R S	**ND**	531TG	531TTG
R308	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R326	PSC	NEG	NEG	NEG	**ND**	**ND**	No mutation
R334	P>20	1wk Sc	E S O K	I R	531TTG	531TTG	531TTG
R347	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R277	P11-20	1wk Sc	E O K	I R S	516GTC	516GTC	516GTC
R350	PSC	1wk Sc	E O K	I R S	516GGC/533CCG	516GGC/533CCG	516GGC/533CCG
R353	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R302	P>20	1wk Sc	E S O K	I R	**ND**	531TTG	531TTG
R316	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R312	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R332	P>20	1wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R338	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R278	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R284	NEG	2wk Sc	E O S K	I R	533CCG	533CCG	No pattern
R328	PSC	1wk Sc	E O K	I R S	531TTG	531TTG	No mutation
R337	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R298	P>20	1wk Sc	E S O K	I R	531TTG	531TTG	531TTG
R279	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern

R352	PSC	2wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R281	NEG	2wk Sc	E O S K	I R	533CCG	533CCG	533CCG
R314	NEG	2wk Sc	E S	I R O K	516GGC/533CCG	516GGC/533CCG	No pattern
R335	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R282	PSC	2wk Sc	E S O K	I R	No mutation	No mutation/531TTG	531TTG
R311	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R299	P>20	1wk Sc	E	I R S O K	533CCG	531TTG/533CCG/516GGC	531TTG/533CCG/516GGC
R329	P1-10	1wk sc	R E S O K	I	**ND**	No mutation	533CCG
R317	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R303	P11-20	1wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R294	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R301	P>20	1wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R304	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R331	P1-10	2wk Sc	R E O K	I S	**ND**	No mutation	No mutation
R339	PSC	2wk Sc	E S	I R O K	516GGC/533CCG	516GGC/533CCG	516GGC/533CCG
R333	P1-10	1wk Sc	E O K	I R S	**ND**	No mutation	531TTG
R318	NEG	2wk Sc	I R E S O K		**ND**	No mutation	No mutation
R313	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R336	P>20	1wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R348	PSC	2wk Sc	E O K	I R S	531TTG	531TTG	No pattern
R315	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R327	P>20	1wk Sc	E O K	I R S	516GTC	516GTC	516GTC
R275	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R381	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R362	NEG	4wk Sc	I R E S O K		**ND**	No mutation	No pattern

R389	PSC	1wk Sc	E S	I R O K	516GGC/533CCG	516GGC/533CCG	516GGC/533CCG
R413	NEG	2wk Sc	E O S	I R K	531TTG	531TTG	531TTG
R443	P>20	1wk Sc	O	I R E S K	531TTG	531TTG	531TTG
R432	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R424	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R355	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R435	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R374	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R427	P>20	1wk Sc	E S O K	I R	531TTG	531TTG	516GGC/533CCG
R394	P>20	1wk Sc	E O K	I R S	**ND**	531TTG	531TTG
R418	P1-10	1wk Sc	Contaminated	Contaminated	**ND**	**ND**	No pattern
R436	P>20	4wk Sc	I E S O K	R	No mutation	No mutation	No mutation
R364	P>20	1wk Sc	E O K	I R S	531TTG	531TTG	516GTC
R421	NEG	2wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R441	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R356	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R385	NEG	1wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R430	P11-20	2wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R378	P11-20	1wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R357	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R437	NEG	1wk Sc	E O S K	I R	526TAC	526TAC	526TAC
R392	NEG	1wk Sc	E O S K	I R	511CCG/516TAC	511CCG/516TAC	511CCG/516TAC
R376	NEG	1wk Sc	E S	I R O K	516GGC/533CCG	516GGC/533CCG	516GGC/533CCG
R434	P11-20	1wk Sc	E S	I R O K	516GGC/533CCG	516GGC/533CCG	No mutation
R372	NEG	3wk Sc	I R E S O K		**ND**	No mutation	No pattern

R425	P>20	1wk Sc	E S O K	I R	526GAC	526GAC	526GAC
R358	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R387	P11-20	1wk Sc	E S O K	I R	531TTG	531TTG	531TTG
R411	PSC	3wk	I E S O K	R	**ND**	**ND**	531TTG
R429	NEG	1wk Sc	E O S K	I R	No mutation	No mutation/516GTC/531TTG	531TTG
R373	P>20	1wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R433	NEG	6wk Sc	R E O K	I S	**ND**	**ND**	516GGC/533CCG
R423	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R363	P>20	1wk Sc	E O K	I R S	**ND**	**No mutation**	531TTG
R442	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R383	P>20	1wk Sc	I R E S O K		No mutation	No mutation	526?
R365?	NEG	NEG	NEG	NEG	**ND**	516GTC	No pattern
R375	P>20	1wk Sc	E	I R S O K	516GGC/533CCG	516GGC/533CCG	516GGC/533CCG
R396	NEG	1wk Sc	E O S K	I R	531TTG	531TTG	531TTG
R431	NEG	2wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R377	PSC	1wk Sc	E O K	I R S	516GGC/533CCG	516GGC/533CCG	516GGC/533CCG
R415	P11-20	1wk Sc	E S O K	I R	511CCG/516TCC	511CCG/516TCC	511CCG/516TCC
R420	P>20	1wk Sc	I R E S O K		**ND**	No mutation	No mutation
R412	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R391	NEG	2wk Sc	E S K	I R O	531TTG	531TTG	531TTG
R380	P11-20	1wk Sc	E O K	I R S	No mutation	No mutation	No mutation/511CCG/516TCC
R422	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R386	P>20	1wk Sc	O K	I R E S	531TTG	531TTG	531TTG
R414	PSC	1wk Sc	E O K	I R S	**ND**	**ND**	531TTG

R419	NEG	3wk +	E O K	I R S	**ND**	**ND**	531TTG
R428	P11-20	1wk Sc	E S	I R O K	516GGC/533CCG	516GGC/533CCG	531TTG
R382	P1-10	2wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R395	P11-20	1wk Sc	E S O K	I R	531TTG	531TTG	531TTG
R417	NEG	1wk Sc	E O K	I R S	510CAT/516TAC	510CAT/516TAC	No pattern
R426	P11-20	1wk Sc	E S O	I R K	533CCG	533CCG	533CCG
R379	NEG	3wk Sc	E O S K	I R	516GTC	516GTC	No pattern
R416	P1-10	2wk Sc	E O K	I R S	**ND**	516GTC	531TTG
R384	PSC	1wk Sc	E S O K	I R	531TTG	531TTG	531TTG
R388	P11-20	1wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R410	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R393	NEG	3wk Sc	R E S O K	I	**ND**	533CCG	533CCG
R390	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R505	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R450	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R516	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R456	NEG	6wk Sc	I E S O K	R	**ND**	526GAC	No pattern
R444	PSC	3wk Sc	E S O K	I R	**ND**	No mutation	No pattern
R524	NEG	2wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R502	P11-20	1wk Sc	E S	I R O K	516GGC/533CCG	516GGC/533CCG	516GGC/533CCG
R483	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R495	NEG	1wk Sc	I R E S O K		**ND**	No mutation	516GGC/533CCG
R446	P1-10	NEG	NEG	NEG	**ND**	**ND**	No pattern
R477	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R520	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern

R504	NEG	3wk Sc	E S	I R O K	516GGC/533CCG	516GGC/533CCG	No pattern
R445	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R466	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R499	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R518	NEG	3wk Sc	K	I R E S O	**ND**	No pattern	No pattern
R447	NEG	3wk Sc	Awaiting res	Awaiting res	**ND**	516GTC	531TTG
R491	NEG	3wk Sc	E O S K	I R	**ND**	531TTG	No pattern
R522	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R448	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R458	P11-20	1wk Sc	EOK	I R S	**ND**	533CCG	533CCG
R507	NEG	2wk Sc	S K	I R E O	516GGC/533CCG	516GGC/533CCG	No pattern
R500	P1-10	2wk Sc	E S O K	I R	531TTG	531TTG	531TTG
R523	NEG	NEG	NEG	NEG	**ND**	**ND**	516GGC/533CCG
R449	P1-10	3wk Sc	SOK	I R E	**ND**	531TTG	531TTG
R453	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R473	PSC	2wk Sc	E S O K	I R	**ND**	**531TTG**	No pattern
R514	PSC	2wk Sc	K	I R E S O	531TTG	531TTG	531TTG
R451	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R471	P11-20	1wk Sc	E O K	I R S	531TTG	531TTG	No pattern
R479	P11-20	1wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R493	NEG	1wk Sc	Contaminated	Contaminated	**ND**	**ND**	No pattern
R452	P>20	1wk Sc	contaminated	contaminated	**ND**	**ND**	531TTG
R515	NEG	2wk Sc	E O K	I R S	**ND**	531TTG	No pattern
R454	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R475	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern

R467	NEG	6wk Sc	E	I R S O K	**ND**	**ND**	No pattern
R497	P>20	1wk Sc	E O K	I R S	**ND**	**ND**	531TTG
R481	P11-20	1wk Sc	E O K	R S	**ND**	**ND**	No pattern
R455	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R517	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R459	NEG	3wk Sc	E O S K	I R	531TTG	531TTG	No pattern
R457	P>20	1wk Sc	I R E S O K		**ND**	No mutation	No pattern
R476	PSC	NEG	NEG	NEG	**ND**	**ND**	531TTG
R521	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R501	P11-20	1wk Sc	E O K	I R S	531TTG	531TTG	531TTG
R519	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R478	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R506	NEG	2wk Sc	E	I R S O K	516GGC/533CCG	516GGC/533CCG	No pattern
R470	P11-20	Contaminated	contaminated	Contaminated	**ND**	**ND**	No pattern
R494	PSC	1wk Sc	Contaminated	Contaminated	**ND**	**ND**	No pattern
R498	PSC	1wk Sc	E S O K	I R	531TTG	526GAC	No pattern
R508	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R472	P11-20	NEG	NEG	NEG	**ND**	**ND**	No pattern
R492	P>20	1wk Sc	E S	I R O K	531TTG	531TTG	No pattern
R503	P>20	1wk Sc	E	I R S O K	516GGC/533CCG	516GGC/533CCG	No pattern
R496	P>20	1wk Sc	E S O K	I R	531TTG	531TTG	531TTG
R560	PSC	1wk Sc	MOTT	MOTT	**ND**	**ND**	No pattern
R568	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R629	P1-10	1wk +	E O K	I R S	**531TTG**	531TTG	No pattern
R589	P11-20	1wk Sc	E S O K	I R	531TTG	531TTG	531TTG

R525	NEG	2wk Sc	E	I R S O K	516GGC/533CCG	516GGC/533CCG	No pattern
R620	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R574	PSC	1wk Sc	E O K	I R S	531TTG	531TTG	No pattern
R635	P1-10	2wk Sc	E O K	I R S	**ND**	No mutation/516GTC/531TTG	531TTG
R614	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R526	NEG	2wk Sc	I R E S O K		**ND**	**526?**	No pattern
R627	NEG	2wk Sc	E O K	I R S	**516GTC**	516GTC	No pattern
R693	P>20	1wk +	E O K	I R S	531TTG	531TTG	531TTG
R564	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R633	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R580	NEG	2wk Sc	E O S K	I R	**ND**	**ND**	531TTG
R527	NEG	2wk Sc	I E O K	R S	531TTG	531TTG	531TTG
R689	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R617	P1-10	1wk Sc	E O K	I R S	**516GTC**	**516GTC**	531TTG
R584	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R577	P11-20	1wk Sc	E O K	I R S	531TTG	531TTG	516GTC
R571	P>20	1wk Sc	O K	I R E S	**ND**	**ND**	531TTG
R530	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R624	NEG	4wk +	failed to grow	failed to grow	**ND**	**ND**	No pattern
R586	P1-10	1wk Sc	Contaminated	Contaminated	**ND**	**ND**	No pattern
R691	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R562	P>20	2wk Sc	E O K	I R S	**ND**	526TAC	526GAC
R622	NEG	1wk Sc	E O K	I R S	**533CCG/516GGC**	533CCG	No pattern
R582	NEG	2wk Sc	E O S K	I R	**ND**	531TTG	531TTG

R572	P11-20	2wk ++	E O K	I R S	**ND**	531TTG	531TTG
R529	P11-20	1wk Sc	E O K	I R S	**ND**	**ND**	516GGC/533CCG
R566	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R626	P11-20	1wk +	E O K	I R S	**533CCG**	No mutation	No mutation
R616	P11-20	1wk +	E O K	I R S	**ND**	**ND**	No mut/531TTG
R585	P1-10	1wk Sc	E O K	I R S	**ND**	533CCG	531TTG
R699	NEG	2wk +	I R E O K	S	**ND**	**ND**	No pattern
R565	P1-10	2wk +++	E S O K	I R	**ND**	No mutation/526GAC/531TTG	531TTG
R631	P11-20	3wk Sc	E O K	I R S	**ND**	**ND**	No pattern
R618	P1-10	1wk Sc	E S O K	I R	**ND**	526GAC	526GAC
R528	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R615	P1-10	1wk Sc	R E S O K	I	**ND**	511CCG	511CCG
R588	P1-10	1wk Sc	E S O K	I R	**ND**	531TTG	No pattern
R579	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern
R573	NEG	1wk Sc	MOTT	MOTT	**ND**	**ND**	No pattern
R613	NEG	2wk Sc	E O K	I R S	**526GAC**	531TTG	No pattern
R531	PSC	1wk Sc	E O K	I R S	**ND**	531TTG	531TTG
R567	P>20	1wk Sc	E O K	I R S	**ND**	**ND**	531TTG
R630	P1-10	1wk Sc	failed to grow	failed to grow	**ND**	**ND**	531TTG
R619	NEG	6wk Sc	O K	I R E S	**531TTG**	531TTG	No pattern
R561	P>20	1wk Sc	E O K	I R S	**531TTG**	526TAC	526TAC
R591	NEG	3wk Sc	E O K	I R S	**ND**	511CCG	No pattern
R575	PSC	1wk +	E O K	I R S	516GTC	531TTG	531TTG
R563	PSC	3wk Sc	I R E S O K		**ND**	No mutation/526GAC/531TTG	531TTG

R632	P11-20	3wk Sc	E S O K	I R	**ND**	Mut 509-514	No pattern
R578	NEG	1wk Sc	E O S K	I R	**ND**	**ND**	No pattern
R587	P>20	1wk Sc	Contaminated	Contaminated	**ND**	**ND**	No pattern
R623	NEG	3wk Sc	E S	I R O K	**No mutation**	No mutation	No pattern
R576	P1-10	1wk Sc	O	I R E S K	531TTG	531TTG	No pattern
R581	PSC	NEG	NEG	NEG	**ND**	**ND**	531TTG
R621	P1-10	1wk Sc	E O K	I R S	**533CCG**	533CCG	531TTG
R628	NEG	6wk +	E O K	I R S	**ND**	No mutation/516GTC	No pattern
R634	P>20	1wk Sc	E O K	I R S	**ND**	No mutation/516GTC/531TTG	531TTG
R583	P1-10	Contaminated	Contaminated	Contaminated	**ND**	**ND**	No pattern
R625	NEG	NEG	NEG	NEG	**ND**	**ND**	No pattern