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‘The evaluation of rapid screening of M/XDR-TB patients within a dedicated M/XDR-TB hospital in Gauteng, South Africa’.

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine in Clinical Microbiology and Infectious Diseases

Johannesburg, 2013

Declaration

I, Lynsey Elizabeth Isherwood, declare that this research report is my own work. It is being submitted for the degree of Master of Science in Medicine in Clinical Microbiology and Infectious Diseases, in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Signature: Lynsey Elizabeth Isherwood

_____ day of _____ 2013.

Dedication

I dedicate this dissertation to:

My three beautiful children

James, Andrew and Emma

To my sister

Mhairi Jerga (neé Stewart)

my forever loving parents

Ron and Margaret Stewart

And my beautiful man:

Dave John Pleasants

You have given me the inspiration to keep going on.

Lastly, to all the patients at Sizwe Tropical Diseases Hospital who fight this dreaded disease and to those who have succumbed to it.

Poster presentations arising from this study

1. **'Evaluation of the GenoType® MTBDR*s*l assay in a cohort of M/XDR-TB patients in South Africa'.**

Lynsey E. Isherwood, Francesca Conradie, Riana Louw, Adriano Duse, Natalie Beylis,Chrisna Veldsman, Pamela Diniso, Amanda Axcell, John Dewar. Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa. Right-to-Care, Johannesburg, South Africa. Sizwe Tropical Diseases Hospital, Gauteng Department of Health, South Africa. Department of Clinical Microbiology and Infectious Diseases, School of Pathology National Health Laboratory Service & University of the Witwatersrand, Johannesburg, South Africa. Hain Lifescience SA (Pty) Ltd, Johannesburg, South Africa. Centre for Tuberculosis, National Institute of Communicable Diseases, a Division of the National Health Laboratory Services, Johannesburg, South Africa. Department of Life and Consumer Sciences, University of South Africa, Florida Campus, Johannesburg, South Africa. **43rd Union World Conference on Lung Health, Kuala Lumpur, Malaysia on 13-17 November 2012. PC-718-17**.

2. **'Sequencing of drug-resistant *Mycobacterium tuberculosis* samples from a cohort of patients in a TB hospital in Gauteng, South Africa'.**

Lynsey E. Isherwood**,** Nicole L. Kushner, Francesca Conradie, Dorothy Fallows, Riana Louw, Chrisna Veldsman, Amanda Axcell, Pamela Diniso, Yvonee Nkosi, Adriano Duse. Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa. Right-to-Care, Johannesburg, South Africa. Sizwe Tropical Diseases Hospital, Gauteng Department of Health, South Africa. Laboratory of Mycobacterial Immunity & Pathogenesis, PHRI – University of Medicine and Dentistry of New Jersey, Newark, USA. Department of Clinical Microbiology and Infectious Diseases, School of Pathology National Health Laboratory Service & University of the Witwatersrand, Johannesburg, South Africa. Hain Lifescience SA (Pty) Ltd, Johannesburg, South Africa. Centre for Tuberculosis, National Institute of Communicable Diseases, a Division of the National Health Laboratory Services, Johannesburg, South Africa. **20th Conference on Retroviruses and Opportunistic Infections, Atlanta, USA. 3-6 March 2013. Abstract # S-158**.

Abstract

Background

In 2006, South Africa documented its first outbreak of extensively drug-resistant (XDR-TB) in Tugela Ferry, Kwa-Zulu Natal. Delayed diagnosis of XDR-TB increases mortality, emphasizing the need or rapid diagnostics. The MTBDR*sl* assay rapidly identifies resistance to fluoroquinolones (FLQ) and aminoglycosides/capreomycin (AG/CP). Hospitalization provides the ideal opportunity for nosocomial infections of TB, including M/XDR-TB, with its associated morbidity and mortality. Occupational exposures amongst healthcare workers are also of concern. Rapid knowledge of second-line resistance patterns of these in-patients would allow for quick stratification and administration of individualized anti-chemotherapy treatment by clinicians, thereby reducing morbidity and mortality within this population.

Methods `

A prospective cohort study was performed from admission sputum specimens collected from patients admitted to Sizwe Tropical Disease Hospital (Sizwe Hospital), the only M/XDR-TB referral hospital in Gauteng, South Africa. MTBDR*plus (version 1)* and MTBDR*sl* line probe assays (LPAs) were performed on all sputa, irrespective of smear positivity and subsequently on Mycobacteria Growth Indicator Tube (MGIT) isolates. The results from the LPAs were compared to the gold standard MGIT 960 culture and direct susceptibility testing (DST) results.

Results

From April 2011 to January 2012, 150 participants were recruited. Seventy-five (50.0%) were female, 91 (60.7%) were HIV-positive and 9 (6.00%) had an unknown HIV status. Of the MGIT cultures performed 71 (47.3%) were positive for *Mycobacterium tuberculosis* (MTB), 31 (20.7%) negative, 40 (26.7%) were contaminated and 8 (5.63%) were inadvertently discarded by the routine

laboratory. The proportion of smear-negative specimens in HIV- positive patients was not statistically significantly different to the smear-negatives in HIV-negative patients (57.1% vs. 46.0%; *p*=0.139). On phenotypic drug susceptibility testing, 47 (66.2%) were resistant to only isoniazid and rifampicin (MDR-TB), 10 (14.0%) were MDR-TB with added resistance to ofloxacin (pre-XDR-TB FLQ), 4 (5.63%) were MDR-TB with added resistance to kanamycin (pre-XDR-TB AG/CP), 4 (5.63%) were MDR-TB with added resistance to both ofloxacin and kanamycin (XDR-TB), 3 (4.23%) were mono-isoniazid resistant, 2 (2.82%) mono-rifampicin resistant, and 1 (1.41%) was TB-drug sensitive.

No TB drug-sensitive sputum specimens were collected and used as a control group for the MTBDR*plus (version 1)* assay, thus only sensitivity indices and positive predictive values (PPVs) could be interpreted.

All specimens collected were used as an inherent control group for the performance of the MTBDR*sl* assay, thus sensitivity and specificity indices together with the PPVs and NPVs, were calculated.

From all sputum samples collected, including both smear-positives and smear-negative specimens, the sensitivity of the MTBDR*plus (version 1)* for RIF and INH was 98.1% for both drugs. Overall sensitivity for the MTBDR*sl* assay to detect ofloxacin (OFX) and kanamycin (KAN) resistance was 90.9% and 42.9%, respectively. The specificity was 100.0% and 95.7%, respectively.

From smear-positive specimens, the sensitivity for OFX and KAN was 100.0% and 50.0%, respectively. The specificity was 100.0% and 96.8%, respectively. From smear-negative specimens the sensitivity was 50.0% and 33.3%, respectively. The specificity was 100.0% and 92.9%, respectively.

From MGIT culture isolates, sensitivity for RIF and INH was 98.2% and 94.5% respectively. The sensitivity for OFX and KAN were 100.0% and 42.9%, respectively, whereas the specificity was 95.6% and 100.0%, respectively.

Conclusion

The performances of both LPAs correlate with other local and international publications. On direct, smear-positive sputa both LPAs perform well. From these, the MTBDR*sl* assay is a good rule-out test for detection of resistance to FLQ and AG/CP. For FLQ, it's a good rule-in test: this indicates that the MTBDR*sl* assay can be used as a rapid screen for the onset of XDR-TB. As predicted, MTBDR*sl* does not perform well on smear-negative specimens, whereas the MTBDR*plus (version 1)* does. On cultures, both LPAs perform well.

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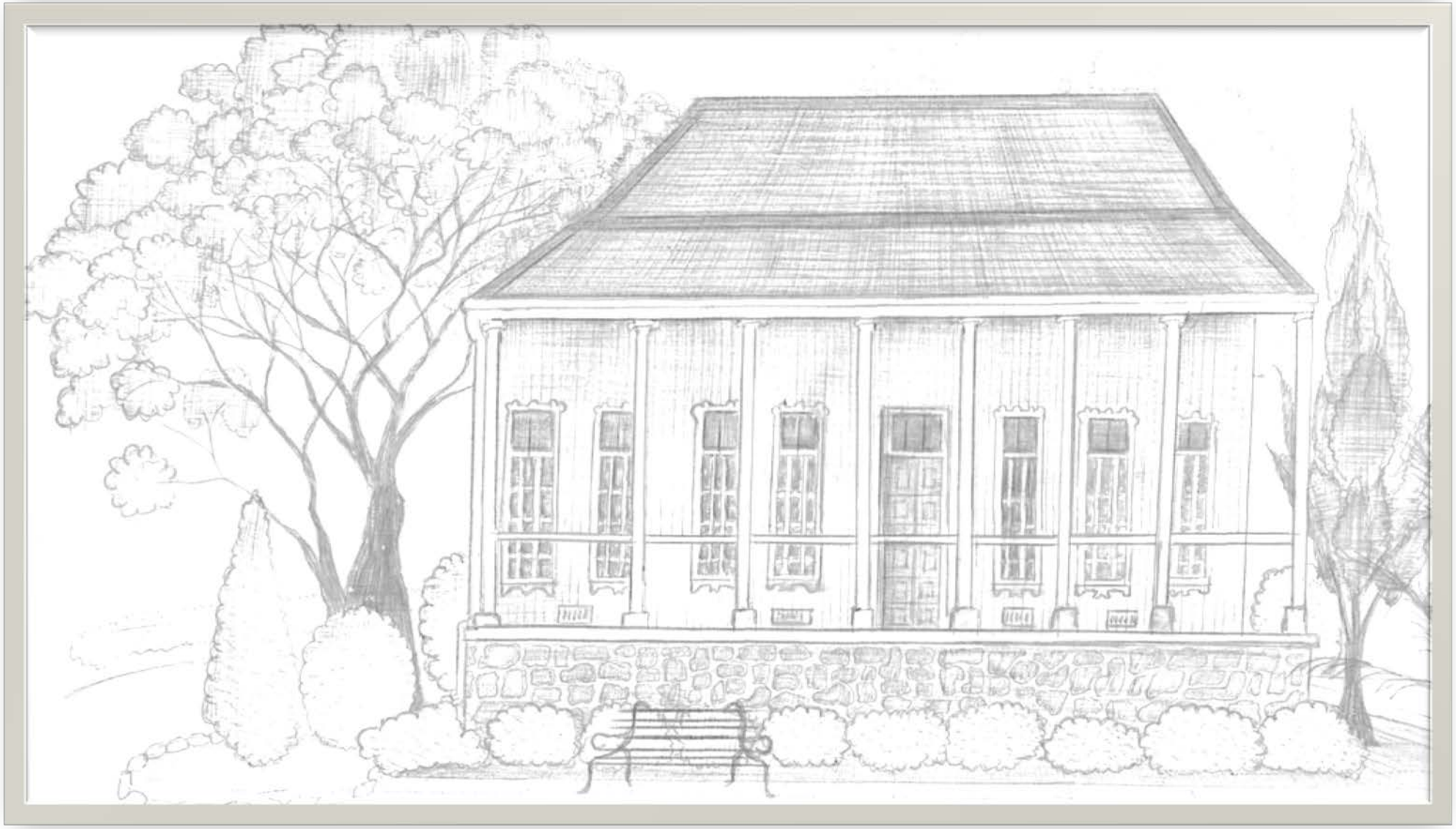

A sketch of a ward at Sizwe Hospital that one of the MDR-TB patients, from this project, stayed in for several months. The sketch was given to Lynsey E. Isherwood and her team, by the patient, on the day of his discharge in 2011.

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List of abbreviations

AC	Amplification Control
AFB	Acid Fast Bacilli
AG/CP	Aminoglycoside/capreomycin
AM	Amikacin
CC	Conjugate Control
CI	Confidence Interval
CM	Capreomycin
CRFs	Clinical Research Forms
dH_20	Distilled water
DR	Drug Resistant
DSTs	Drug susceptibility tests
EMB	Ethambutol
FLQ	Fluoroquinolone
GS	Gold Standard
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
ICF	Informed Consent Form
ID	Infectious Diseases
INH	Isoniazid
IPT	Isoniazid Prevention Therapy
KAN	Kanamycin
LPAs	Line Probe Assays (e.g. GenoType ® MTBDR*plus (version 1)* and MTBDR*sl* assays)

MDR-TB	Multi-drug Resistant Tuberculosis
MGIT	Mycobacterium Growth Indicator Tubes
MUT	Mutation
MTB	*Mycobacterium tuberculosis*
M/XDR-TB	Multi-drug and Extensively Drug-Resistant Tuberculosis
NHLS	National Health Laboratory Service
NPV	Negative Predictive Value
NTBRL	National Tuberculosis Reference Laboratory
OFX	Ofloxacin
RIF	Rifampicin
PCR	Polymerase Chain Reaction
PPV	Positive Predictive Value
TAT	Turn-around-Time
TB	Tuberculosis
TDR-TB	Total Drug-resistant Tuberculosis
TUB	The band that identifies MTB on the LPAs
USA	United States of America
WHO	World Health Organization
WT	Wild Type
XDR-TB	Extensively Drug-resistant Tuberculosis

CHAPTER 1

1.0 Background

It is estimated that mycobacteria have been evolving from environmental bacteria for the last 2-3 million years and have adapted well over time to mammalian niches (1). *Mycobacterium tuberculosis* (MTB) may be the most prolific bacterial killer known to mankind (2). Archeological evidence depicting tuberculosis (TB) dates back to 5,000 B.C. (1).

1.1 History of tuberculosis

TB is an age-old infectious disease that is caused by the pathogen *Mycobacterium tuberculosis.* It is a complex and chronic disease requiring prolonged treatment and because of its large infectious pool, is difficult to control. It is also one of the three major infectious disease killers in our modern world, causing the death of nearly 2 million people each year. Some historic names given to TB include '*The captain of all these men of death*', '*white plague'*, '*consumption*', '*king's evil*', '*lupus vulgaris*' and' *phthisis*' (3) (4).

By the end of the nineteenth century, TB became fully established and had become the principal cause of death, claiming the lives of world-renowned people such as John Keats, Emily Bronte, Robert Louis Stevenson, and D.H. Lawrence, and more recently: Eleanor Roosevelt and among many others, Vivian Leigh, famous for her role in the classic film "*Gone with the Wind*" (5). Also, TB was romanticized when the pale and thin faces of TB sufferers became pictures of beauty depicted through fine pieces of art (6).

TB was convincingly shown to be ‘contagious’ in 1865 by a military surgeon, Jean-Antoine Villemin, who transmitted the disease by injecting TB specimens from humans and cattle into rabbits (7). However, his concept of contagiousness and the consequences thereof, based on solid evidence in animal experiments, was ignored by his contemporaries, until Koch’s discovery of the tubercle bacillus in 1882, described below (8).

1.1.1 Discovery of causative agent

In 1882, Robert Heinrich Hermann Koch isolated and cultured the tubercle bacillus from fresh growing tubercles of animals that died due to TB-infection (9). Now known as *Mycobacterium tuberculosis*, it is, together with the human immunodeficiency virus (HIV), one of the principal etiological agents causing disease and death in humans. Koch determined the causative relationship between the host and microbe, thus defining the so called Koch’s postulates for classifying microbes as a pathogen (8).The principles of Koch’s postulates are: (a) the putative pathogen must be in abundance within the host and cause an infectious condition, (b) there must be a credible correlation between microbiological, pathological and clinical features, (c) the pathogen can be isolated from the diseased host, inoculated onto pure culture and grow, (d) the pathogen should reproduce and cause disease within a new host that has been inoculated with the isolated pathogen (10). Ironically, the requirements for microbiological, pathological and clinical correlations are not met in the case of an asymptomatic host with latent MTB infection (10).



Figure 1.1 Coloured scanning electron microscopy image of *Mycobacterium tuberculosis* bacilli. (Source: Bioquell. Image of M. tuberculosis bacilli [Image on internet]. United Kingdom: Bioquell; 2012 [cited 2012 Oct 16]. Available from: http://www.bioquell.com/technology/microbiology/mycobacterium-tuberculosis).

1.1.2 The history of the treatment of tuberculosis

1.1.2.1 Pre-chemotherapy era: Sanatoria

'*Sanare*' is the Latin word meaning 'to heal' and forms the root word for 'sanatorium' (11). Prior to the introduction of the chemotherapy era in 1944, treatment prescribed for TB included re-location to better climates, horseback riding, walking outdoors or other activities or measures thought to improve the management of TB (12) (13). This lead to Hermann Brehmer's proposal in 1854 to build sanatoria to provide abundance of fresh air, graduated exercise, bed-rest and wholesome nutrition (Figure 1.2). This marked the beginning of the 100.0-year sanatorium era (14).

Brehmer's ideas were rejected by eminent physicians until 1884 when Edward Livingston Trudeau established a sanatorium in the United States at the scenically beautiful Saranac Lake which he earmarked for treatment and research after treating and curing himself with rest, fresh air and good nutrition (13). His

sanatorium was duly converted into the now famous Trudeau Institute devoted to biomedical research including TB and other diseases.

Sanatoria were usually located in mountain resorts where patients received carefully monitored exposure to sunlight, fresh air, bed-rest and exercise. Adherence to diet according to the nurses' cook book which featured prominently on patients' prescription charts was an important factor in the care of TB patients (12). During the sanatorium era, it was believed that sunlight improved patient recovery. Today, it is understood that sunlight improves vitamin D synthesis and absorption, which in turn stimulates macrophage function thereby reducing the risk of TB infection and enhances TB recovery (15). Additionally bed-rest and attenuated exercise both improve lung perfusion in the cavities of the lungs where the bacilli dominate (12).



Figure 1.2 TB-infected patients resting on cane recliners, with moderated sunlight, in a sanatorium (1892). This image represents the BASF sanatorium for consumptives (Dannenfels, German. BASF Aktiengesellschaft, BASF AG).(Source: Campbell M. What tuberculosis did for modernism: The influence of a curative environment on modernist design and architecture. Med His. 2005;49(4):463).



Figure 1.3 Image of a sanatorium still in operation today. Sizwe Tropical Disease Hospital is located in South East of Johannesburg, South Africa. This facility adopts the sanatorium concept of fresh air, sunlight and bed-rest, together with anti-TB chemotherapy. Each ward has its own, separate building. (Source: photograph by Lynsey E. Isherwood).

1.1.2.2 Advent of tuberculosis chemotherapy

Several decades after Koch's discovery, research into therapeutic drugs led to the development of successful antimicrobial agents such as sulphonamides in the 1930's and penicillins a decade later. However, these agents were not effective in combating MTB (16). It was not until the 1940 when Stanley Waksman and colleagues from Rutgers University in New Jersey, U.S.A. discovered an anti-TB drug called actinomycin, produced by actinomycetes organisms (17). This drug however, proved to be too toxic for antimicrobial treatment in humans (16).

1.1.2.2.1 Discovery of streptomycin first mono-therapy for TB

Waksman was inspired by his observations that 'soil microbes' were able to inhibit the growth of bacterial species. Hence, a treatment breakthrough occurred in 1943 when streptomycin, isolated from *Streptomyces griseus*, was identified as an effective antimicrobial drug. Streptomycin was effective in combating TB and was

fairly low in toxicity. However, ototoxicity associated with this drug remains a concern (18).

1.1.2.2.2 Discovery of more anti-tuberculosis drugs

An influx of anti-TB drugs appeared shortly after the introduction of streptomycin. Isoniazid (INH) was introduced for the treatment of TB in 1952, pyrazinamide (PZA) in 1954, cycloserine in1955, ethambutol (EMB) in1962 and Rifampicin (RIF) in1963 (19). In modern times, INH and RIF are still considered to be the most effective drugs for the treatment of TB and the development of resistance to these two drugs has serious implications for therapeutic outcome (20).

Other classes of antibacterial agents, namely aminoglycosides: kanamycin (KAN) and amikacin (AMK) , cyclic peptides (capreomycin-CAP and viomycin), and fluoroquinolones (moxifloxacin- MOX, levofloxin-LEV, ofloxacin-OFX and ciprofloxacin) are also effective against MTB, but are presently only used as second-line drugs (21).

1.1.2.2.3 Early multi-drug therapies for TB

During 1940's, it was discovered that two drugs, streptomycin coupled with para-aminosalicyclic acid (PAS) when administered in conjunction with each other, could delay the emergence of drug resistance in MTB to either of these agents (22).

1.1.3 Emergence of Resistance

1.1.3.1 Development of resistance to Streptomycin

Since the isolation and discovery of MTB, many treatment and diagnostic challenges were posed to biomedical researchers as well as safety concerns

related to its highly contagious nature and small infective dose. Also, the organism's complex metabolism and structure compromised research efforts (23). This is exemplified by MTB's fastidious growth requirements, slow generation time and its thick and complex cell wall with a high lipid content conferring hydrophobic properties which affect the organism's ability to take up stains and allow entry of drugs and disinfectants (24). Also, with regard to treatment of TB with antimicrobial agents, the introduction of these drugs exerted selective pressure leading to the emergence of mutations conferring drug resistance within MTB strains. This is genetically determined and resistance frequency in strains varies between drugs. This was evident when even after the successful administration of streptomycin in 1944, resistance to this drug surfaced within months after its introduction (25).

1.2 Aetiology of tuberculosis

The aetiological agent for TB is the pathogenic species *Mycobacterium tuberculosis* from the genus *Mycobacterium.* MTB belongs to a group of slow-growing *Mycobacterium* called *Mycobacterium tuberculosis complex* (MTBC).This group is responsible TB in humans and animals. Other members of this group are *M. bovis, M. bovis BCG, M. africanum, M. canetti, M. caprae, M. microti, M. pinnipedii* (26).

Mycobacterium tuberculosis is an obligate aerobe. "MTB is found in well aerated areas of the upper lobes of the lungs, as well as in a number of other sites in the body, e.g. the spine. MTB is a facultative intracellular parasite, usually of macrophages (27).

1.2.1 Cell wall

Mycobacteria are protected by a unique thick wall consisting mostly of lipids which possess long-chain fatty acids called mycolic acids (Figure 1.4) (27). The mycolic acids form a permeability barrier that prevents any environmental solutes from entering, thus allowing mycobacteria to easily circumvent antibiotic effectiveness and in turn challenges the therapeutic index and research of current and novel anti-TB drugs (28).

1.2.2 Cord Factor

A unique phenotypic characteristic of MTB is the aggregation of MTB colonies seen on acid-fast smears, giving a serpentine cording appearance caused by ‘cord factor’ (6,6´ trehalose dimycolate). The cord factor is located on the outer layer of the cell wall and was first discovered by Robert Koch who associated this characteristic to the virulence of MTB (27).

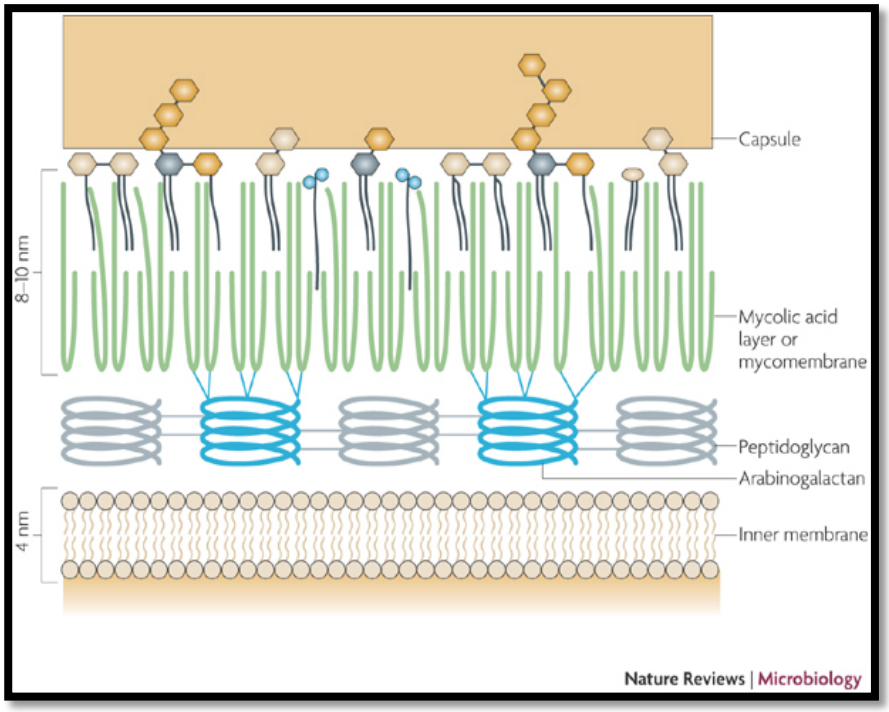


Figure 1.4 Depiction of the cell wall of a *Mycobacterium tuberculosis* bacillus. The cell wall is mainly composed of a complex that contains three different covalently linked structures; peptidoglycan (grey), arabinogalactan (blue) and mycolic acids (green). (Source: Abdallah AM, van Pittius NCG, Champion PADG, Cox J, Luirink J, Vandenbroucke-Grauls CMJE, *et al.*Type VII secretion—mycobacteria show the way. Nat Rev Microbiol. 2007;5(11):883-91).

1.3 Pathogenesis and transmission of TB

Pathogenesis is defined as how an infection or disease develops within the body. Humans serve as the only reservoir for MTB. The degree of pathogenesis in a microorganism pivots on its virulence, durability and persistence within its host. Several characteristics of MTB cause pathogenesis in human. These include a unique cell wall structure and slow cellular metabolic mechanisms (Figure 1.5) (29).

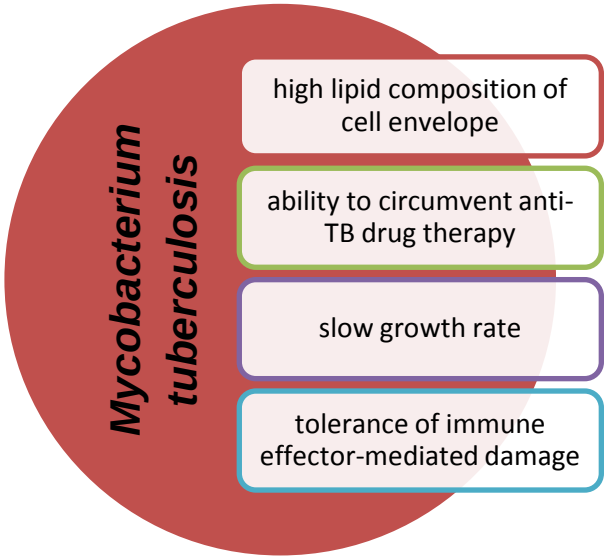


Figure 1.5 Schematic representation of the main causes of pathogenesis of *Mycobacterium tuberculosis*. (Source: Lynsey E. Isherwood).

1.3.1 Growth rate

Mycobacterium tuberculosis has a slow generation time of 15-20 hours (27).

1.3.2 Transmission

Transmission is defined as the spread of disease from one person to another. Droplets from coughing, sneezing, singing or talking of an infected person travel through the air and are inhaled by another person. These droplets contain tubercle bacilli (30). The larger droplets remain lodged in the upper respiratory tract, where infection does not occur. Smaller droplets travel down into the well aerated alveoli (small air sacs) at the ends of the bronchioles of the lungs. These tubercle bacilli then multiply and TB infection begins (Figure 1.6) (31).

Infection occurs as either ‘active disease’ or ‘latent infection’. Active disease occurs when the immune system is unable to contain the tubercle bacilli within granulomas in the alveoli and the disease disseminates throughout the body via

the bloodstream (31). However, not all active TB-disease disseminates throughout the body, but remain confined to the lungs.

Latent infection is inactive TB whereby the immune system is able to contain tubercle bacilli by means of macrophages. These macrophages create granulomas which encapsulate the bacilli and prevent the progression of infection into active disease (31).

The extent to which a person is infected depends on four factors (a) length of exposure, (b) virulence of tubercle bacilli, (c) environment in which infection occurs and (d) infectiousness of the index patient (i.e. the amount of TB bacilli that the person expels in a single cough. The higher the bacillary load in a single cough, the more infectious the index patient is to another person) (29).

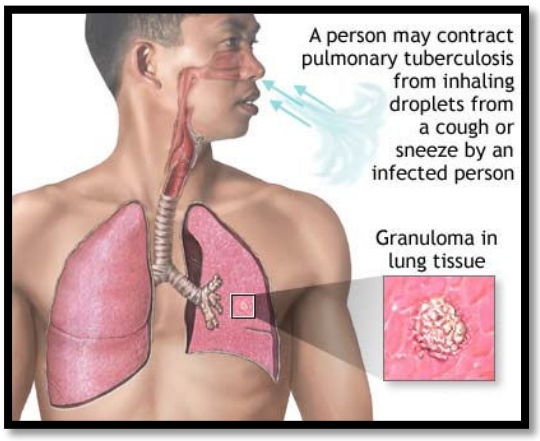


Figure 1.6 Droplet nuclei containing tubercle bacilli from an infectious person travel through the air and are inhaled via the respiratory system of another person. (Source: HIVandHepatitis.com. Inhalation of TB droplet nuclei.[Image on Internet].HIV and Heptatitis.com; 2011 [cited 2013 Mar 6]. Available from: http://www.hivandhepatitis.com/recent/2011/0201_2011_b.html).

1.4 Epidemiology of tuberculosis

1.4.1Global trend of TB cases before the onset of HIV

TB increased dramatically in Europe and America during the seventeenth century with death rates peaking in the 1800s (Figure 1.7). This trend was directly related to poor living conditions, poor nutrition, non-existence of sanitation, sub-standard medical care and lack of hygiene (32). Following this period, and with improved standards of living, death rates dramatically decreased and have been falling for the last 150 years in North America and Europe. Mortality from TB declined even further during the 1940'sand 1950's when effective anti-TB drugs were introduced (13) (32).

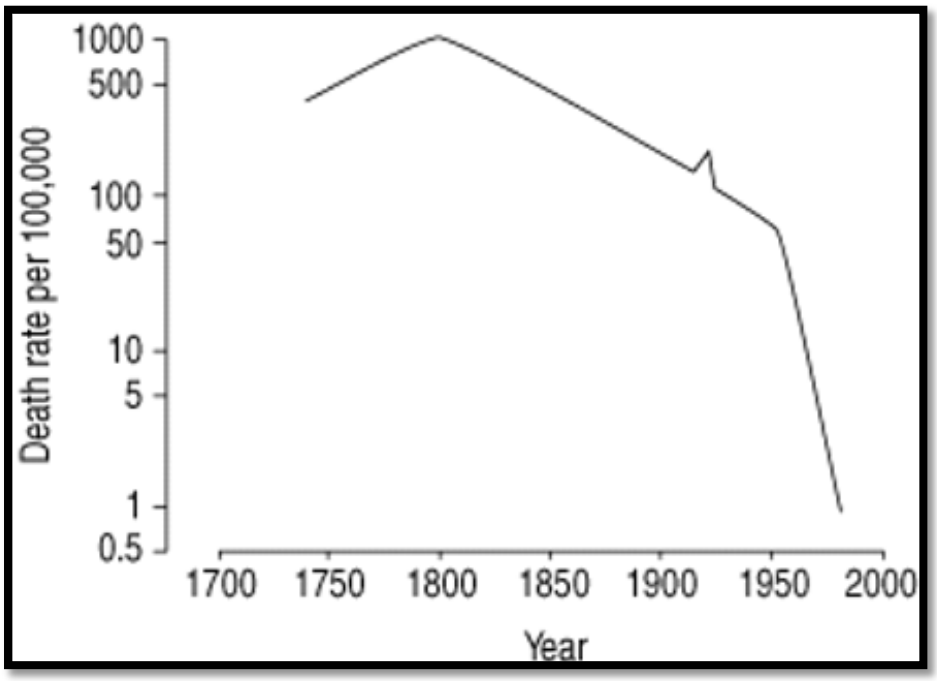


Figure 1.7 Estimated changes in the rates of mortality from tuberculosis in Western Europe. (Source: Murray JF. A century of tuberculosis. Am J Respir Crit Care Med. 2004 Jun 1;169(11):1181-6).

1.4.2 Global trend of TB cases: 1980 onwards

A resurgence of TB cases was noted in the United Sates between 1985 and 1992 (32). This increase was associated with the HIV epidemic, immigration and

outbreaks of TB/HIV co-infection in environments of human accumulation (such as prisons, hospitals and homeless shelters) (32) (13). In 1993, the World Health Organisation (WHO) declared TB as a global public health emergency as there had been no significant progress in the eradication of TB since Koch's era (13).

The first indications of the global emergence of drug-resistant TB, within the HIV-positive population were evident in the early 1990's, when focal outbreaks of multi-drug resistant TB (MDR-TB) were reported in the United States and Europe (33). During the 1990's, global investments were made into the development of directly-observed therapy short-course (DOTS) programmes, this included the global project on anti-TB drug resistance surveillance, initiated in 1994, between the WHO and the International Union against Tuberculosis and Lung Disease (IUATLD) (34) (33). With the widespread use of DOTS, the rate of TB cases slowly declined from about 1993 (34).

The global advent of highly active antiretroviral therapy (HAART) in mid-1990s marked a major milestone in the combat against HIV infection and it fundamentally altered not only the HIV epidemic but also dramatically improved cure rates of TB and reduced the frequency of reactivation and re-infection TB (35). HAART results in an improved immune system in HIV and TB co-infected individuals through partial restoration of CD4 cell numbers and function, and its introduction contributed greatly to the decline in TB in HIV co-infected cases (35). A study conducted in South Africa by Golub, *et al.* demonstrated an effective interaction between HAART and isoniazid preventative therapy (IPT) in HIV- positive patients. This interaction decreased the adjusted risk of TB by 89%, whereas HAART alone decreased the adjusted risk by only 64% (36).

In 1997 a MDR-TB outbreak occurred at Sizwe Hospital for Tropical Diseases, Johannesburg, South Africa involving a strain resistant, not only to INH and RIF also to ethambutol, pyrazinamide, ethionamide, terizidone, thiacetazone and ofloxacin (37).

From January 2004 to January 2007, the National Health Laboratory Service (NHLS) identified 10,000 MDR-TB patients across all provinces, of which 210 patients had XDR-TB. These numbers indicated that XDR-TB was already well-established (38) prior to the 2006 Tugela Ferry outbreak involving a highly resistant XDR-TB strain in Kwa-Zulu Natal, South Africa (39).

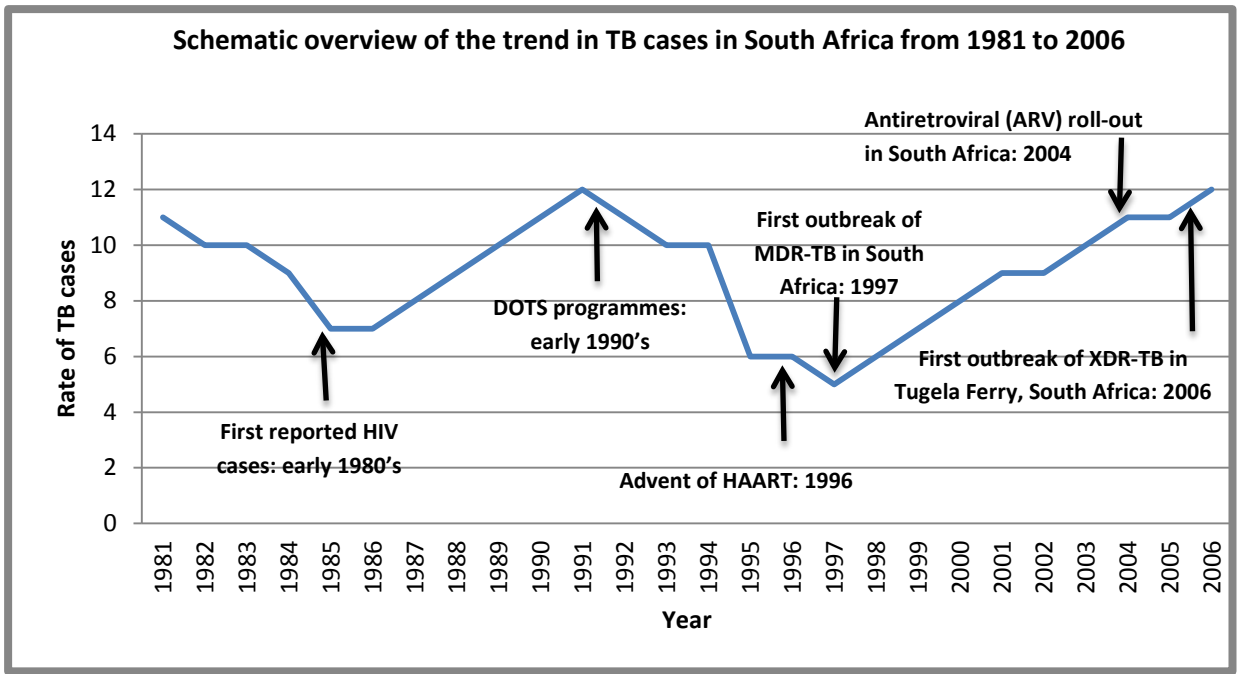


Figure 1.8 Schematic overview of the trend in TB cases in South Africa from 1981 to 2006. (Points on vertical axis are arbitrary and chosen to show trends). Primary causes of TB rate increases and decreases are highlighted with arrows. (Source: Lynsey E. Isherwood).

1.4.3 Epidemiology of drug resistant TB

1.4.3.1Global problem of drug- resistant TB

Drug-resistance is inevitable in MTB as microorganisms including bacteria have evolved mechanisms to resist the impact of chemotherapy on their survival. It has been suggested that mycobacteria have evolved with their hosts over the last 2-3 million years. Hence they have truly learnt to adapt to changing hostile environments including those caused by chemotherapy (40). Drug-resistant TB has become an epidemic that is mostly man-made. It is driven by the mismanagement of patients relating to erratic drug supply, incorrect prescription of TB therapy, inadequate drug adherence, poor infection prevention and control measures (41).

Unfortunately, resistance to anti-TB drugs has evolved into MDR-TB and XDR-TB (collectively known as M/XDR-TB). MDR-TB is defined as disease caused by MTB resistant to at least INH and RIF, both first-line drugs, with or without resistance to other drugs (41). XDR-TB is defined as *in-vitro* resistance to at least INH, RIF, any fluoroquinolone, e.g. OFX and/or MOX and at least one of the aminoglycosides: AM, KAN and/ or CAP (42) (43). Fluoroquinolones and aminoglycosides are second-line drugs used to treat XDR-TB, even though patients are already resistant to these, raising alarm bells that novel drugs are urgently needed to treat this highly resistant strain of TB. The treatment of XDR-TB is more expensive and more toxic, with longer treatment periods, compared to the treatment of MDR-TB (44). Alarmingly, a new pan-resistant TB (TDR-TB) strain which is resistant to all available first- and second-line anti-TB drugs has now emerged (45).

A surveillance project of drug-resistant TB across 35 countries from 1994 to 1997 was conducted by WHO and the IUATLD and published in 1997. The data demonstrated that all 35 nations presented MTB strains resistant to at least one drug, usually INH or streptomycin, concluding that drug-resistant TB is a global problem (33). This survey was followed by two subsequent global surveys one reported in 2001involving 72 countries/settings from 1994-1999 (46). The third global survey report involved 77 geographical areas and sets out in detail the prevalence of resistance patterns globally (47). Eight of the nine South African provinces (no surveillance was done in the Northern Cape) feature in the WHO/IUATLD 2004 global report and the surveillance of each province was conducted over a study period of 12 months during 2001-2002 (47). The mean prevalence rate of primary (new) MDR cases in the eight provinces during this survey was 1.79% (78 out of 4,358 new cases) while 6.70% of retreated patients (101 out of 1,508 cases) and 3.05% (179 out of 5,866) for all cases had MDR-TB (47).

1.4.3.2 Problem of drug-resistant TB in South Africa

The WHO considers TB and HIV co-infection as a catastrophic pandemic (48). When discussing TB in South Africa, one needs to do this in the context of HIV infection. South Africa is ranked the fifth highest DR-TB high-burden country in the world, and M/XDR-TB statistics have increased due to the HIV epidemic and sub-standard management of TB. MDR-TB and XDR-TB have featured in explosive nosocomial outbreaks in HIV-infected populations(44) and M/XDR-TB is the leading cause of death among HIV/TB co-infected patients in South Africa (49).

With the implementation of antiretroviral therapy, the mortality rates in HIV/TB co-infected patients have improved, but drug-resistant TB has emerged as a major cause of death (39).

The emergence and spread of MDR-TB and XDR-TB in South Africa are hampering efforts for the control and management of TB and are threatening WHO's target of diagnosing and treating 80% of estimated M/XDR-TB by 2015 (50). South Africa reports the highest number of cases of MDR-TB and XDR-TB in the Sub-Saharan region of Africa (49).

The first recorded outbreak of MDR-TB in South Africa occurred in 1997, with a report of 6 patients infected with an MDR-TB strain at Sizwe Tropical Diseases Hospital, Gauteng (37). At that time the problem of MDR-TB in South Africa was well recognised, but had not been effectively managed, also with regard to infection control measures (38).

An XDR-TB outbreak followed in 2006 at the Church of Scotland Hospital in Tugela Ferry, KwaZulu-Natal. The nosocomial significance of the outbreak was emphasised in a presentation of the cases at the International AIDS Conference in Toronto in August 2006 (51) (52). It demonstrated that out of the 536 TB patients admitted to the hospital, 221 were found to have MDR-TB and of these 53 had XDR-TB. Fifty-two of these patients died within 25 days with a median survival time of 16 days. About half of the XDR-TB patients had not received prior TB treatment which suggested that most of these XDR-TB infections were acquired in hospital. Another interesting aspect of the outbreak is that the XDR-TB patients were all on antiretroviral therapy and responding well but despite this, they still succumbed to XDR-TB (53) (54).

In 2010 Ebonwu, *et al.* analysed NHLS laboratory-confirmed MDR-TB and XDR-TB cases across eight of the nine provinces in South Africa, from 1 January 2004 to 6th October 2010 (55). Nationally, 32,038 MDR-TB cases were identified of which 1,378 were XDR-TB cases (56), demonstrating that MDR-TB and XDR-TB is firmly established in South Africa. In 2010 alone, the NHLS of South Africa diagnosed 5,441 MDR-TB and 322 XDR-TB cases (56).

The increase in M/XDR-TB cases between 2006 and 2009 may to a degree be due to increased case detections. Also, the initiation of DR-TB treatment in each province depends on the prevalence of DR-TB as well as the accessibility and efficacy of diagnostic assays and treatment (Tables 1.1 and 1.2) (57).

Table 1.1 Number of M/XDR-TB Patients Diagnosed at the National Health Laboratory Service (NHLS) from 2007 to 2010. (Gauteng Province, in which Sizwe Hospital is located, is highlighted in orange). (Source: Department of Health, Republic of South Africa. Management of drug-resistant tuberculosis. Policy guidelines. South Africa; August 2011. 161p.)

PROVINCE	2007		2008		2009		2010	
	MDR	XDR	MDR	XDR	MDR	XDR	MDR	XDR
Eastern Cape	1092	108	1501	175	1858	123	1782	320
Free State	179	4	381	3	253	3	267	7
Gauteng	986	38	1028	30	1307	65	934	37
Kwazulu-Natal	2208	241	1573	181	1773	254	2032	201
Limpopo	91	2	185	2	204	6	126	6
Mpumalanga	506	12	657	14	446	18	312	5
Northern Cape	199	7	290	19	631	40	353	39
North West	397	4	363	4	520	13	158	14
Western Cape	1771	42	2220	60	2078	72	1422	112
SOUTH AFRICA	7429	458	8198	488	9070	594	7386	741

Gandhi, *et al.* undertook a surveillance of prevalence and consequences of MDR-TB and XDR-TB in rural areas of KwaZulu-Natal covering the period January 2005 to March 2006. From this study, 221 MDR-TB cases were detected, of which 53 had XDR-TB (52 of the 53 died within 16 days). The prevalence was 39% and 6% of MDR-TB and XDR-TB, respectively. All XDR-TB patients tested for HIV were co-infected with HIV. This study further underpinned the notion that XDR-TB transmission in HIV-positive patients was associated with high mortality (39).

Table 1.2 Number of MDR-TB and XDR-TB Patients Started on Treatment, 2007-2010. (Gauteng Province, in which Sizwe Hospital is located, is highlighted in orange).(Source: Department of Health, Republic of South Africa. Management of drug-resistant tuberculosis. Policy guidelines. South Africa; August 2011. 161p.)

PROVINCE	2007		2008		2009		2010	
	MDR	XDR	MDR	XDR	MDR	XDR	MDR	XDR
Eastern Cape	932	171	772	135	847	135	927	224
Free State	158	7	233	7	148	6	167	5
Gauteng	497	45	414	40	512	25	607	30
Kwazulu-Natal	788	170	1039	163	927	177	1788	235
Limpopo	71	2	104	0	88	3	119	3
Mpumalanga	148	0	272	3	198	5	298	6
Northern Cape	145	11	148	8	253	13	230	37
North West	156	4	159	1	175	9	143	14
Western Cape	439	64	890	34	995	58	1034	61
SOUTH AFRICA	**3334**	**474**	**4031**	**391**	**4143**	**431**	**5313**	**615**

Each of the nine provinces in South Africa has one dedicated TB hospital to manage and treat M/XDR-TB. The policy of these hospitals is to admit the patients until they have converted. (Conversion is when their cultures convert from positive

to negative usually 2 consecutive cultures are required before discharge. Over and above this, efforts are being made to trace contacts to enable to early diagnosis and treatment of M/XDR-TB (51).

1.4.3.3 Hospitalisation of drug-resistant TB Gauteng: Sizwe Tropical Diseases Hospital

Sizwe Tropical Diseases Hospital (Sizwe Hospital) in Sandringham, Johannesburg, is the dedicated M/XDR-TB hospital for the Gauteng province of South Africa the same hospital in which the 1997 MDR-TB outbreak occurred (Figure 1.3). Eighty percent of the patients admitted to Sizwe Hospital are co-infected with HIV, and consequently have immune-compromised systems. Patients admitted to Sizwe Hospital have a confirmed multi-drug resistant diagnosis, either by MTBDR*plus (version 1)*, Xpert® MTB/RIF assay (Cepheid, CA, USA), or by culture (58).

In 2011, 572 MDR-TB cases were admitted to Sizwe Hospital: 31 of these being XDR-TB cases. In the same time, 974 cases were diagnosed in the province. Over 100.0 patients died in the interval between specimen collection, result and tracing of the patients. Of concern was that 125 cases were not traced at all. The reasons for these non-traced patients have not yet been investigated. The balance were referred to other provinces for treatment or found on further investigation not to have MDR-TB (58). This data underpins the need for more rapid diagnosis and better tracing systems.

Furthermore, patients at Sizwe Hospital are separated to a ward according to increasing drug- resistance. However, this procedure does not occur until a definitive, updated TB culture and DST result is available from a sputum sample

collected the early morning after admission. This implies that all *de-novo* or re-admitted patients are allocated to a single admissions ward (ward 4) upon admission, until these results are known**.**

Another implication is that patients are treated using the standard MDR-TB anti-TB regimen of kanamycin, ofloxacin, ethionamide, ethambutol and pyrazinamide (and terizidone for ethambutol-resistant patients) (59), even if patients are potentially resistant to one or more of these drugs. Laboratory turn-around-times (TAT) for TB culture and DST are 4 to 6 weeks and thus there is delayed access to appropriate M/XDR-TB treatment and increased risk of nosocomial infections to other patients and health care workers alike.

1.5 Laboratory diagnosis of M/XDR-TB in South Africa

1.5.1. Conventional methods

Historically, routine laboratory approach for the diagnosis of TB, MDR-TB, XDR-TB and treatment monitoring of TB patients is based on sputum smear microscopy, culture of sputum for MTB, and drug susceptibility testing (DST) on culture isolates. Although smear microscopy can still be used as an initial indicator assay for TB, nowadays, the Xpert MTB/RIF is used as a point-of-care assay to rapidly detect TB, with or without resistance to RIF, followed by phenotypic DSTs. DST includes testing specimens against INH, RIF, streptomycin, EMB (first-line drugs) and the second-line drugs ethionamide (ETH), KAN and OFX (41).

Sputum smear microscopy is quick for the bacteriological confirmation of TB (specificity >97%) however, the sensitivity of sputum smear microscopy varies

from 70 to 80%. Sensitivity may decrease in HIV/TB co-infected and paediatric populations (60). TB cultures are the gold standard for TB diagnosis and are highly sensitive but expensive and require advanced laboratory set-ups, together with highly skilled staff. Thus this assay is not accessible to all populations in developing countries (41). Most importantly, when TB culture and DST with their extended laboratory testing times are implemented using sputum samples collected from patients at a high HIV-prevalence, dedicated M/XDR-TB hospital, it results in lengthy exposure of patients whose isolates exhibit various degrees drug-resistance. This problem is aggravated for those patients who are already immune-compromised due to their HIV/TB co-infection. The TAT for TB culture and DST performed on admission sputum specimens are 4 to 6 weeks. Thus, there is delayed access to laboratory-based information for patient-directed M/XDR-TB treatment, as well as an increased risk of nosocomial infections involving other patients and health care workers alike.

Paucibacillary TB (low yield of acid-fast bacilli [AFB]) in HIV/TB co-infected patients) is common. Therefore, sputum smear microscopy often demonstrates false-negative results (61). A possible consequence of false-negative smears is the misidentification of TB including primary M/XDR-TB cases. Thus, patients may not be referred for dedicated TB treatment and in the case of primary X/MDR-TB cases to an M/XDR-TB hospital on time and therefore not receive correct treatment regimens. This may well lead to undetected spread of TB, as well as M/XDR-TB.

1.5.2 Xpert ® MTB/RIF assay

The WHO issued a strong recommendation that the new automated DNA test, Cepheid Xpert® MTB/RIF(GeneXpert-GXP) (Cepheid, Sunnyvale, CA) for TB, should be used as the initial diagnostic test in individuals suspected of having M/XDR-TB or HIV/TB (62). The GXP, not only has a faster TAT than conventional culture which takes up to 6 weeks, but can also detect mutations in the *rpoB* gene, which confers resistance to RIF. The National Department of Health (NDoH) of South Africa moved very rapidly on this recommendation in 2011. According to Prof Wendy Stevens (head of the National Priorities Programs of the National Health Laboratory Service), as at the end of October 2012: 720,713 GXP assays had been performed. Professor Stevens presented data at the 43rd Union World Conference on Lung Health in Malaysia in November 2012. Of the GXP tests performed in TB suspects, 15.01% detected *Mycobacterium tuberculosis*. RIF resistance was detected in 7.04% of these positive tests, diagnosing 7,721 cases of possible MDR-TB.

1.5.2 Line Probe Assays

The bleak situations described above together with the increased number of M/XDR-TB cases in South Africa, highlight the need for alternative and less time consuming assays, such as genotypic LPAs. These assays have become a research priority for the screening of MDR-TB and XDR-TB (63) at both local clinic and hospital levels. The LPAs include the GenoType® MTBDR*plus (version 1)* and MTBDR*sl* (Hain Lifescience GmbH, Nehren, Germany).LPAs utilize the DNA•STRIP® technology that are based on polymerase chain reaction (PCR) with reverse hybridization (64) (65). LPAs can produce a result within 1 to 2 days, as compared to 4 to 6 weeks for gold standard TB culture and DST (65).

1.5.2.1 GenoType® MTBDRplus (version 1)

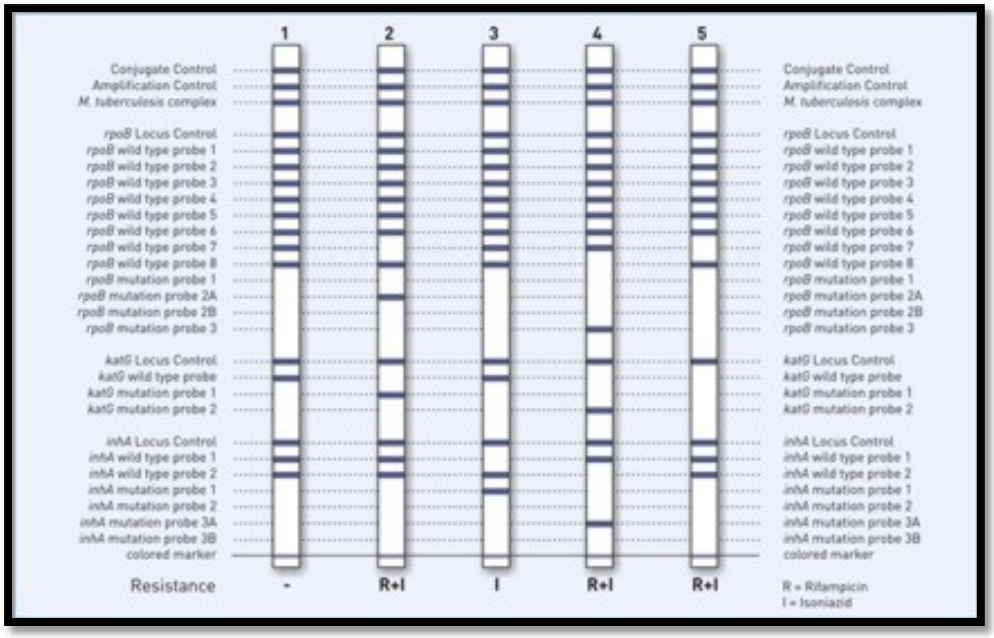
Using PCR and reverse hybridization technology, the GenoType® MTBDR*plus (version 1)* assay rapidly identifies mutations associated with resistance to INH in the *katG* and *inhA* genes and resistance to RIF due to mutations in the “core

region" of the *rpoB* gene. The mutations that cause RIF resistance occur predominantly in an 81 base-pair "core region" of the *rpoB* gene, whereas INH resistance-related mutations occur in several genes and regions. The most frequent mutations (50-95%) occur in codon 315 of the *katG* gene and the *inhA* regulatory region (20-35%), while fewer are located in the *ahpC-oxyR* intergenic region (10-15%) (64).

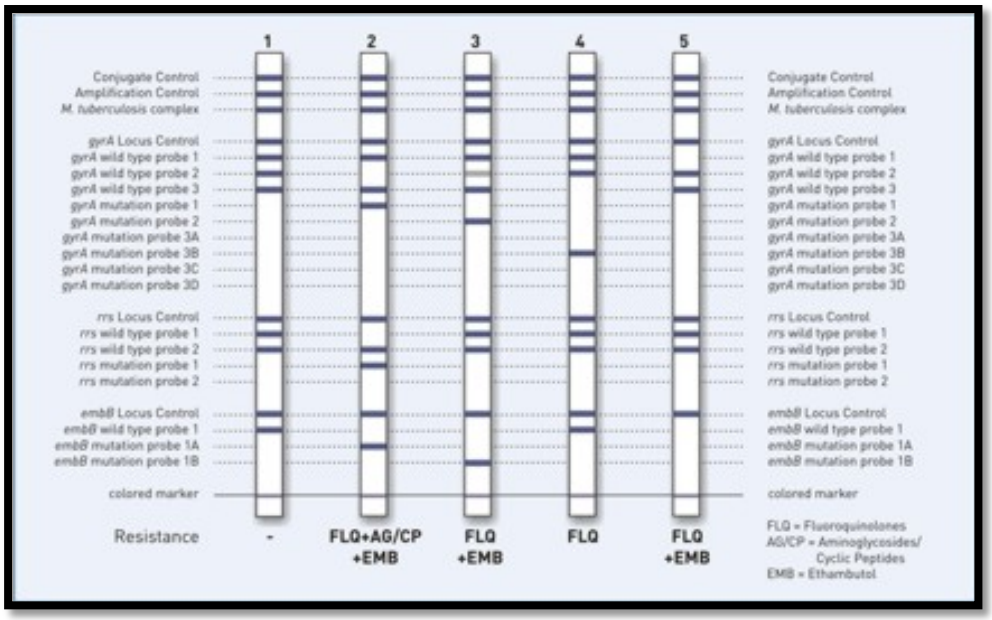
1.5.2.2 GenoType® MTBDRsl

The GenoType® MTBDR*sl* assay rapidly identifies resistance to selected second-line anti-TB drugs, namely fluoroquinolones (FLQs) and the injectable aminoglycosides amikacin (AM), KAN and capreomycin (CM). FLQ resistance is identified by the detection of mutations to the *gyrA* gene, which encodes for DNA gyrase. AM-CM resistance is detected by the identification resistance defining mutations in the *rrs* (16S rRNA) gene conferring resistance to aminoglycoside/cyclic peptide antimicrobial agents. This assay also detects resistance to ethambutol (EMB), through the identification of *embB* gene mutations, but because EMB has a well-documented history of unreliable susceptibility test results, it was not analyzed in this study (65).

Each DNA strip contains 22 probes which includes 16 for mutation detection. The other six are the controls i.e. conjugate (CC), amplification (AC), *Mycobacterium tuberculosis* complex (TUB) and three locus bands (*gyrA*, *rrs* and *embB*) (Figures 1.9 and 1.10) (66).



MTBDR*plus* *(version 1)*



MTBDR*sl*

Figure 1.9 Images of the MTBDR*plus (version 1)* and MTBDR*sl* assay DNA strips with their assigned wild type and mutation probes. Images obtained from Hain Lifescience, GmbH, Nehren, Germany. (Source: Hain Lifescience. MTBDR*plus* and MTBDR*sl* assay strips. [Image on internet]. Hain Lifescience, GmbH, Germany; [cited 2013 Mar 4]. Available from: **http://www.hain-lifescience.co.za/en/products/microbiology/mycobacteria/genotype-MTBdrplus.html** and **http://www.hain-lifescience.co.za/products/microbiology/mycobacteria/genotype-MTBdrsl.html**).

1.5.2.3 Limitations of the LPAs

The LPA assays mentioned above have limitations of their own in that they have only been validated on sputum smear-positive specimens (64) (65). Thus, smear-negatives still require culturing, but LPAs can be performed on the correlating culture-positive isolate, instead of phenotypic DST methods, which then reduces the TAT to MDR-TB diagnosis. Improved DNA extraction methods, especially for paucibacillary tuberculosis, are still being investigated. Although the WHO is recommending further research into LPAs in different epidemiological settings, the use of the MTBDR*plus (version 1)* assay for rapid TAT and cost effectiveness, is recommended by WHO in low to middle income settings (63) (64) (65) (67). However, the MTBDR*sl* assay still needs to be validated in the setting of a dedicated M/XDR-TB hospital in South Africa.

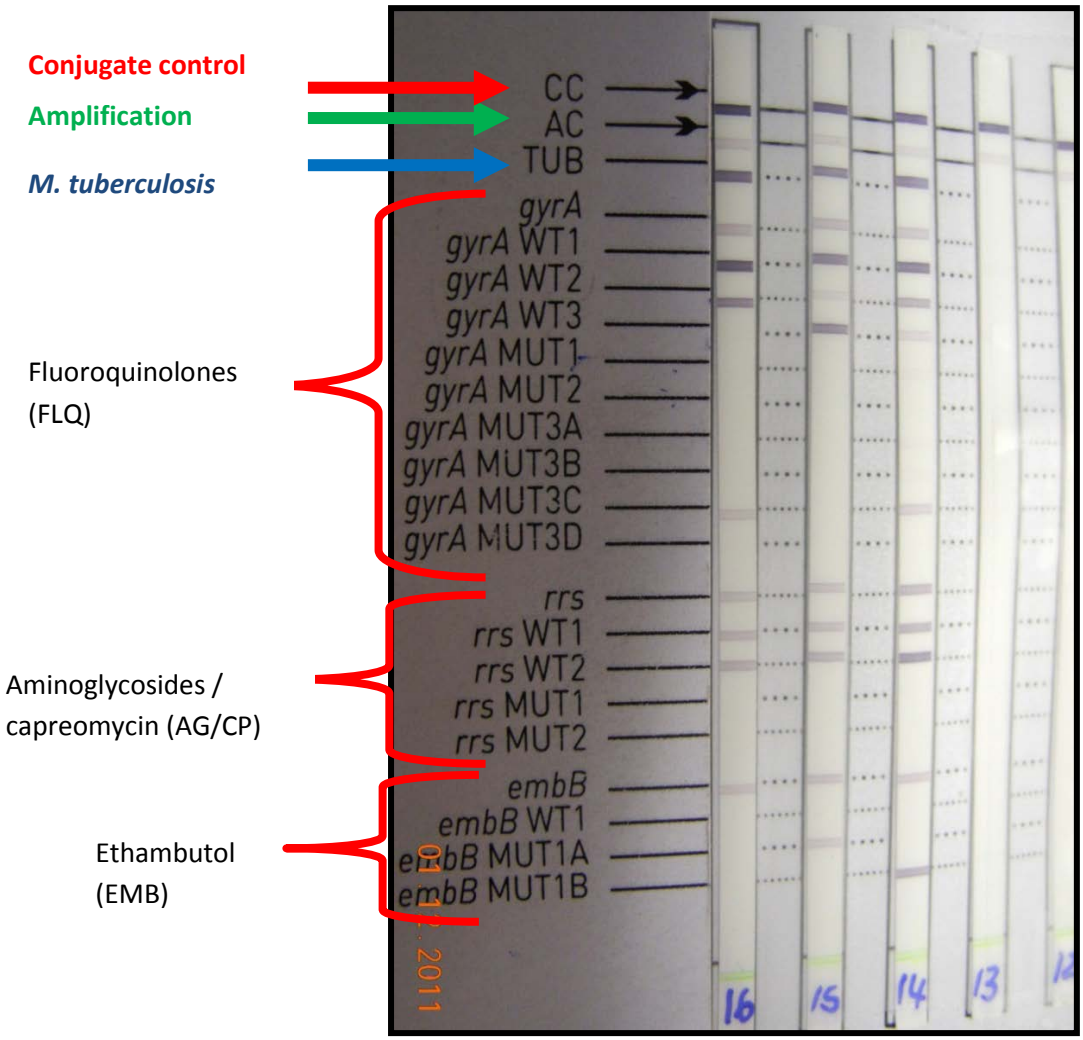


Figure 1.10 Image of actual MTBDR*sl* DNA strips with their assigned wild type and mutation probes. (Source: Photograph of a result sheet from this study: Lynsey. E. Isherwood).

1.5.2.4 Interpretation of the LPAs

The interpretation of either the MTBDR*plus (version 1)* or MTBDR*sl*, works on the same principle, except for the labelling of different gene loci to represent susceptibility or resistant to RIF, INH and FLQ, AM-CM and/or EMB, respectively (Figure 1.11). Confirmation of the presence of the *M. tuberculosis* organism is confirmed with the presence of the 'TUB' band on the strips, i.e. A '+' confirms MTB, whereas'-' confirms the absence.

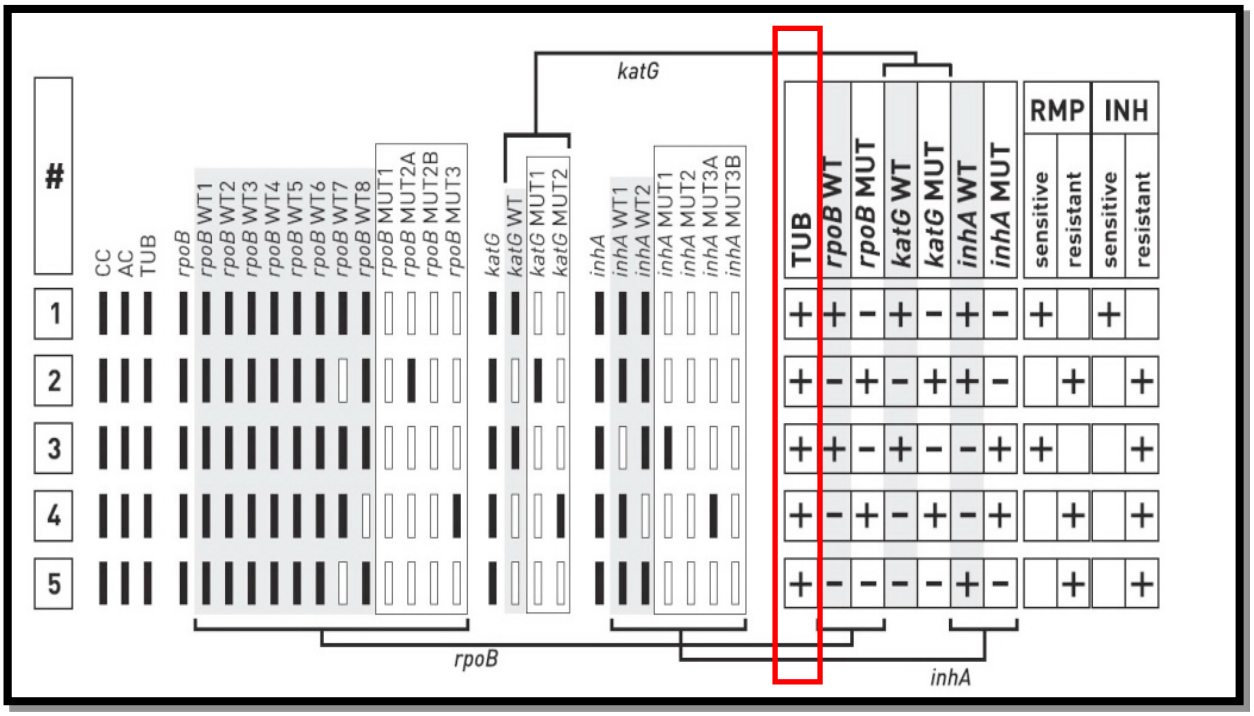


Figure 1.11 Image demonstrating the presence or absence of wild type (WT) and mutation (MUT) bands on the MTBDR*plus(version 1)* DNA strips. A '+' indicates the presence or a WT or MUT band. '-'indicates the absence of a WT or MUT band. (Source:Hain Lifescience. MTBDR*plus (version 1)* and MTBDR*sl* interpretation.[Image on internet]. Hain Lifescience, GmbH, Germany; [cited 2013 Jan 21]. Available from:http://www.moleculartb.org/gb/pdf/protocols/Hain.pdf).

1.5.2.4.1 Isoniazid

INH susceptibility or resistance is indicated by the presence or absence of *katG* and/or *inhA*, respectively. A specimen susceptible to INH will have '+' indicated on the WT bands, with a '-'on the MUT bands (figure 1.11, sample 1).

INH resistance are indicated by the below scenarios

- *katG* MUT band marked '+' with WT band marked '-'. Also, *inhA* MUT band marked '-' with *inhA* WT band marked '+'(figure 1.11, sample 2)
- *inhA* MUT band marked '+' with WT band marked '-'. Also *katG* MUT band marked '-' and *katG* WT band marked '+' (figure 1.11,sample 3)

- *katG* and *inhA* MUT bands are marked with '+' whilst their corresponding WT marked with '-' (figure 1.11, sample 4)
- *katG* MUT band marked '-'with WT band marked '-'. Also, *inhA* MUT band marked '-' and *inhA* WT band marked '+' (figure 1.11, sample 5)

1.5.2.4.2 Rifampicin

In the figure 1.11 above, RIF resistance is indicated by samples 2, 4 and 5 whereas RIF susceptibility is shown in samples 1 and 3. RIF resistance is indicated against the presence or absence of the *rpoB* mutation and wild type bands.

1.5.2.4.3 Fluoroquinolones, aminoglycosides and ethambutol

To identify resistance or sensitivity to aminoglycosides, FLQs and/or EMB, the same principles of '+' and '-' markings as the MTBDR*plus (version 1)* assay, apply to the MTBDRs*l* assays, only difference is that difference loci of the TB genome is interpreted (i.e*. gyrA* for FLQ *rrs* for AG/CP and *embB* for EMB).

1.6 Treatment of TB

1.6.1 Treatment of drug sensitive TB

The algorithm for the treatment of drug sensitive TB includes 2 months of INH, RIF, EMB and PZA, followed by 4 months of INH and RIF (68). Adherence to the therapy usually cures drug-sensitive TB. However, compliance of treatment is usually difficult due to the lengthy period and can thus lead to drug-resistant strains of TB (1).

1.6.2 Treatment of drug-resistant TB

Treatment of M/XDR-TB is more expensive and laborious and is usually for a minimum of 18 to 24 months as compared to the six months for drug-sensitive TB. The toxic side-effects are also more severe (1).

New anti-TB agents are currently being developed, namely Bedaquiline (TMC207) and the nitroimadazoles, PA824 and OPC67683. TMC207 inhibits the ATP synthase complex of the MTB bacilli. OPC-67863 and PA-824 are pro-drugs the ultimate targets of these compounds remain unknown (69) these drugs are currently being tested in new combination regimens for drug-susceptible and M/XDR-TB.

Results from a randomized, pilot study of Bedaquiline, of which Sizwe was the largest recruitment site proved that when Bedaquiline was added to routine MDR-TB regimen for the first eight weeks of treatment, patients converted to culture-negative earlier, compared to those patients who did not receive Bedaquiline (70).

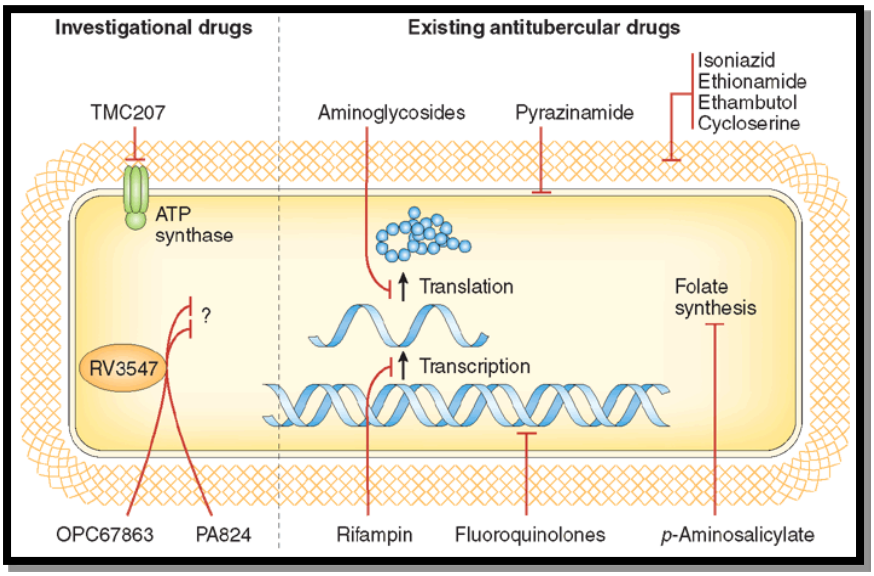


Figure 1.12 Mechanisms of action of investigational drugs, compared to currently available anti-TB drugs. (Source: Sassetti CM, Rubin EJ. The open book of infectious diseases. Nat Med [Internet].2007. [cited 2012 Sep 28]; 13: 279-80. Available from:http://www.nature.com/nm/journal/v13/n3/abs/nm0307-279.htmldoi:10.1038/nm0307-279).

1.7 Whole genome sequencing of H37Rv

The virulent strain of Mycobacterium tuberculosis was first isolated in 1905 namely the H37Rv stain. In 1998 Cole, *et al.* published his work from the sequencing of the complete *Mycobacterium tuberculosis* H37Rv strain (71). Cole's work disclosed that the H37Rv genome is an integrated 4.4 Mb circular chromosome. Today, the sequencing of TB strains is based on the H37Rv integrated genomic map (71) (72) (73).

1.8 Drug resistant tuberculosis

1.8.1 An overview of development of antibiotic drug-resistance

Antibiotic resistance is the ability of sub-populations of bacteria to survive in the presence of one or more antimicrobial drugs (e.g. INH and RIF). TB-infected patients may have a total bacillary population of 10^7 to 10^8 bacteria in a single open cavity (74). Multiple-drug therapy allows for the suppression of drug-specific

resistance. Thus, it is crucial for these patients to be treated with more than one antibiotic at a time to avoid the emergence of specific drug-resistant TB (74).

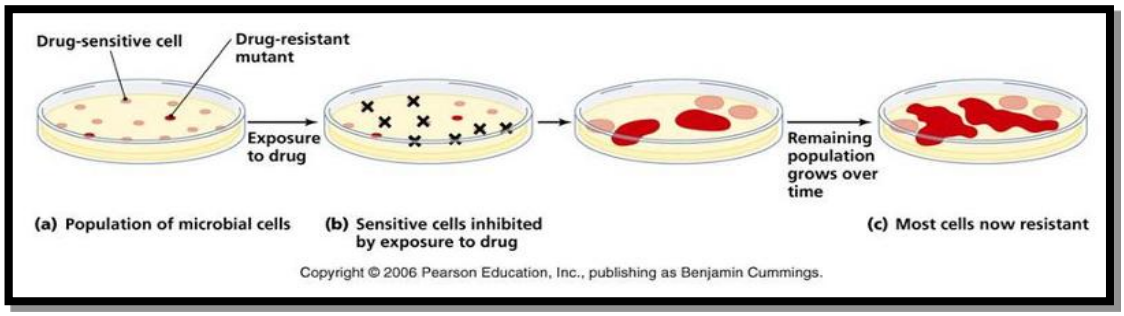


Figure 1.13 Depiction of the general development of drug-resistant bacilli. (Source: Cowan Microbiology Student Study Site. Development of drug-resistant bacilli.[Image on internet]. Pearson Education Inc.; 2006 [cited 2012 Oct 17]. Available from: http://www.pc.maricopa.edu/Biology/rcotter/BIO%20205/LessonBuilders/Chapter%2012%20LB/RevisedCh12LessonBuilder_print.html)

1.8.2 Selection of resistant mutants

A deeper understanding into the interrelationship between phenotypic MTB (virulence, transmissibility and/or clinical manifestations) and genotypic MTB (different strains) characteristics is important for the development of new vaccines, drugs and diagnostic assays. Over the last decade, numerous drug targets and resistance mechanisms have been identified. MTB develops most of its mutations in the form of non-synonymous single nucleotide polymorphisms (nsSNPs), where the encoding amino acid is changed these are usually caused by selective forces, for example, due to the administration of anti-TB drugs (75). Resistance related mutations spontaneously evolve slowly, but constantly. The frequency of drug resistance-related mutations varies between drugs (Table 1.3) (76).

As exposure to an additional drug to which the infecting MTB strain is susceptible will markedly reduce the frequency of selection of an MTB mutant resistant to both drugs, combination therapy has become the cornerstone of the treatment of TB

world-wide. The probability of spontaneous resistance to individual first-line anti-TB drugs is as follows (77):

Isoniazid:	1 in every 10^6 cell divisions (mutation rate: 10^{-6})
Rifampicin:	1 in every 10^9 cell divisions (mutation rate: 10^{-9})
Streptomycin:	1 in every 10^6 cell divisions (mutation rate: 10^{-6})
Ethambutol:	1 in every 10^5 cell divisions (mutation rate: 10^{-5})
Pyrazinamide:	1 in every 10^5 cell divisions (mutation rate: 10^{-5})

The above implies that the probability of selection for resistance in combination therapy should be rare for example, simultaneous resistance to INH and RIF is the sum of both probabilities: (10^{-6}+ 10^{-9}= 10^{-15}) (57).

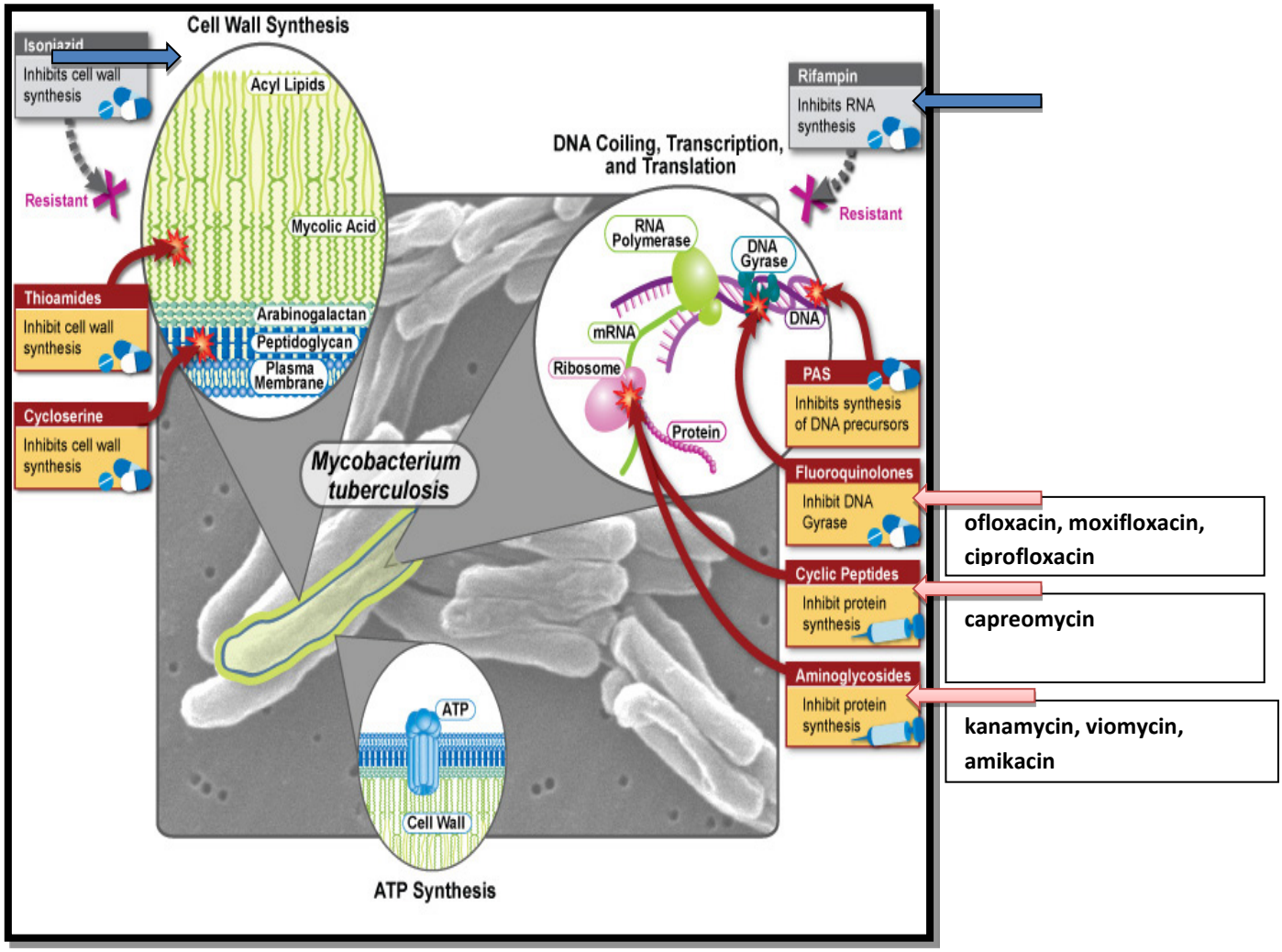


Figure 1.14 An illustration of the mechanisms of action for anti-TB drugs on *M. tuberculosis* bacilli. First-line drugs; isoniazid and rifampicin (blue arrows). Second-line drugs; fluoroquinolones, cyclic peptides, aminoglycosides (red arrows). The drugs of importance in this project are highlighted with arrows. (Adapted from McGraw Hill Companies Inc.). (Source: Cowan Microbiology Student Study Site. [Image on internet]. Pearson Education Inc.; 2006 [cited 2012 Oct 15]. Available from: http://www.pc.maricopa.edu/Biology/rcotter/BIO%20205/LessonBuilders/Chapter%2012%20LB/RevisedCh12LessonBuilder_print.html).

1.8.3 Mechanisms of drug resistance in *M. tuberculosis*

Each drug has its own 'mechanism of action' on the MTB bacilli. i.e. resistance to each drug involves different genetic mutations in the MTB genome (Figure 1.14). These mechanisms of actions are driven by genes that encode for them. As each drug has its own genetic variability, the mechanism of drug-resistance will differ between drugs. Table 1.3 summarisesthe common first and second-line anti-TB

drugs together with their gene(s) involved in resistance, it gene function and mechanism of action (40).Only those genes applicable to this project, is summarised in Table 1.3.

In 2008, Sandgren, *et al.* launched an interactive TB gene mutation database called, TB Drug Resistance Mutation Database (TBDReamMDB). The authors published their work on PLoS in 2009 and were quoted saying "*By providing a comprehensive, single resource of drug resistance mutations in TB, we hope to accelerate and encourage new discoveries that will have applications ranging from diagnostics to drug discovery. We envision that this database will expand as additional mutations are identified in the coming years and will serve as a platform for diverse analyses and projects"* (78).

Table 1.3 Mechanisms of drug action for the common first and second-line anti-TB drugs of *M. tuberculosis*; the genes involved, and their functions. (Genes highlighted in purple are investigated in this study). (Source: Adapted from: Kaufmann HE, Rubbin E. Handbook of Tuberculosis: Molecular Biology and Biochemistry. KGaA, Weinheim: WILEY-VCH Verlag GmbH & Co.; 2008. p. 323-79).

Drug	**MIC (µg/ml)**	**Gene(s) involved in resistance**	**Gene function**	**Role**	**Mechanism of Action**	**Mutation frequency (%)**
INH**(F-L)**[b]	0.02-0.2	*katG*	Catalase-peroxidase	Pro-drug conversion	Inhibition of Mycolic acid	50-80
		inhA	Enoyl-ACP reductase	Drug target	Effects on DNA, lipids, carbohydrates and NAD metabolism	15-43
		ahpC	Alkylhydroperoxide reductase	Marker of resistance	Carbohydrate and NAD metabolism	10-15
RIF**(F-L)**[b]	0.5-2	*rpoB*	RNA polymerase	Drug target	Inhibition of RNA synthesis	96
EMB**(F-L)**[b]	1-5	*embB*	Arabinosyl transferase	Drug target	Inhibition of arabinogalactan synthesis	47-65
SM**(F-L)**[b]	2-8	*rpsL*	S12 ribosomal protein	Drug target	Inhibition of protein synthesis	52-59
		Rrs	16s rRNA	Drug target	Inhibition of protein synthesis	8-21
PZA**(F-L)**[b]	16-50 (pH5.5)	*pncA*	Nicotinamidase / pyrazinamidase	Pro-drug conversion	Depletion of membrane energy. Inhibition of FASI	72-97
Amikacin/kanamycin**(S-L)**[c]	2-4	*Rrs*	16s rRNA	Drug target	Inhibition of protein synthesis	76
Capreomycin **(S-L)**[c]	1-4	*Rrs*	16s rRNA	Drug target	Inhibition of protein synthesis	**NA**[a]
Fluoroquinolones (FQ)**(S-L)**[c]	0.5-2.5	*gyrA*	DNA gyrase subunit A	Drug target	Inhibition of DNA gyrase	75-94
		gyrB	DNA gyrase subunit B	Drug target	Inhibition of DNA gyrase	*In-vitro*

NA[a]: Not available**(F-L)**[b]: First-line anti-TB drugs **(S-L)**[c]: Second-line anti-TB drugs

1.8.4 First-Line Drugs

1.8.4.1 Isoniazid (Discovered: 1952)

Isoniazid (INH) is an important pro-drug that was first chemically synthesized in 1912, but was only registered as an anti-TB drug in 1952. INH, together with RIF, is prescribed globally and is the backbone for the treatment of TB. Although INH

has been extensively prescribed over the last 50 years, neither its bacterial target nor mode of action is well understood (79).

However, early insights into its mode of action were alluded to in 1954 when Middlebrook isolated the first INH drug-resistant microorganism. Through this discovery, Middlebrook, as well as Hedgecock and Faucher demonstrated that INH-resistant organisms had decreased catalase activity, and, that there was an inverse correlation between INH minimum inhibitory concentration (MIC) and catalase-peroxidase activity (80) (81).

1.8.4.1.1 katG encodes for catalase-peroxidase

In 1992 Zhang, *et al.* found that the *katG* gene, which encodes catalase peroxidase, was abnormal in many INH-resistant strains and also reported that the deletion of the *katG* from the MTB chromosome resulted in INH drug resistance in two clinical isolates (82). However, Stoeckle, *et al.* (1993) sequenced 80 randomly selected *M. tuberculosis* isolates from New York City to determine the proportion of *katG* deletions. It was realized that ten multidrug and high-level INH-resistant strains did in fact have an intact *katG* gene, indicating that INH-resistant strains did indeed have grossly intact *katG* genes (83). In 1995, a study by Goto, *et al.* confirmed that the deletion of *katG* was not the major cause of resistance (84). The mutation frequency is about 50 to 80%, with the majority localized to codon 315 (85).

1.8.4.1.2 inhA encodes for enoyl-ACP reductase

INH has inhibitory effects on DNA, mycolic acid biosynthesis, carbohydrates and NAD metabolism by encoding for enoyl-ACP reductase. Banerjee, *et al.*

discovered that the *inhA* locus of an INH resistant strain of *M. smegamatis*, coded for products participating in resistance in INH (86).

Prior to research conducted between 1994 and 1997 by Telenti, *et al.*, Musser, *et al.* and Heym, *et al.*, it was unclear as to whether the *inhA* and *katG* loci interact, and if so, would the interaction result in altered resistance (87) (88) (89). However, the above authors discovered that mutations in the promoter region of *inhA* occur in 20-34% of resistant isolates either alone or in combination with *katG*. It was discovered that *inhA* in the absence of *katG* resulted in low-level resistance to INH, whereas the presence of both *inhA* and *katG* resulted in high-level resistance to INH (85).

1.8.4.2 Rifampicin (Discovered: 1961)

RIF, a rifamycin compound, is the most effective drug in anti-TB therapy. It forms the major component of the short course chemotherapy of 6 months for the treatment of TB. Initial resistance to RIF has proven to have profound clinical relapses in TB using the standard drug-sensitive TB regimen (85).

1.8.4.2.1 rpoB encodes for RNA polymerase ß subunit

In 1978 McClure, *et al.* discovered that RIF binds to the ß subunit of the RNA polymerase to inhibit transcription, thereby inhibiting the synthesis of tubercle bacilli (90). Ninety-six percent of RIF resistance is related to mutations of the *rpoB* locus. In a study by Telenti, *et al.*, 66 RIF-resistant strains and 56 RIF-sensitive strains were sequenced. It was discovered that missense mutations and amino acid substitutions were found at codons 526 and 531 of 80% of the RIF resistant strains (88).

1.8.4.3 Streptomycin (Discovered: 1944)

Streptomycin (SM) belongs to the aminoglycoside group of drugs (Figure 1.14). SM is a bactericidal drug that kills a broad spectrum of bacteria, including MTB.

1.8.4.3.1 rpsL encodes for the ribosomal S12 protein

It mechanism of action involves the inhibition of protein synthesis by binding to the 30S subunit of the bacterial ribosome. The locus of the TB genome (strain: H37Rv) that is mostly responsible for SM resistance is *rpsL*. This gene targets SM at the ribosomal site of action by disrupting mRNA translation and thus damaging the MTB cell wall (i.e. *rpsL* encodes for the ribosomal S12 protein) (40) (91).

1.8.4.4 Ethambutol (Discovered: 1961)

EMB is a bacteriostatic drug that is only active against viable bacteria. This drug is used in combination with other drugs including PZA, RIF and INH, to reduce EMB resistance (40). In South Africa, EMB is an optional drug for the treatment of MDR-TB patients within areas of low prevalence of EMB resistance. However, EMB is no longer part of the South African standardised treatment program for MDR-TB. This is because, phenotypic DSTs of EMB have a history of too many false-sensitive's (57).

This observation was supported by work published from Johnson, *et al.* The study was conducted from two sets of isolates (235 drug-resistant-to any drug and 117 fully susceptible isolates, respectively), collected between November 2000 and June 2003, from 72 rural clinics in the Western Cape, South Africa. Johnson, *et al.* concluded that more effective and reliable phenotypic DSTs need to be established and that genotypic resistance testing of EMB would enhance the management of MDR-TB in South Africa (92).

1.8.4.4.1 embCAB encodes for arabinosyltransferases

Gene mutations responsible for EMB resistance occur in *iniB, iniA, iniC, embR, rmlAZ, hddC, manB, rmlD*as well as the *embA*, *embB* and *embC*loci (i.e. *embCAB* operon) the most common mutations being *embB* found at codon 306 of the MTB genome (78). The *embCAB* operon controls the synthesis of arabinosyltransferases the enzymes that attribute to the building of arabinogalactan in the cell wall (40).

1.8.4.5 Pyrazinamide (Discovered: 1952)

Pyrazinamide (PZA) is an important first line drug and is effective in reducing treatment time from 9-12 months to 6 months, when used in combination with INH and RIF (40). In South Africa, PZA forms part of the second-line drugs, used in the treatment of MDR-TB (57).

1.8.4.5.1 pncA encodes for pyrazinamidase

The *pncA*gene encodes for the enzyme pyrazinamidase, which converts PZA from a pro-drug to an active drug (pyrazinoic acid POA). Thus, the loss of production of POA increases PZA resistance with the MTB cell (40). PZA resistance occurs very slowly it only targets non-growing tubercle bacilli. This in turn creates difficulties in the detection of resistance to PZA, either by phenotypic or genotypic DST. However, it creates a situation whereby resistance is difficult to acquire (57).

The *pncA* gene is diverse (93) and is thus difficult to determine genotypic resistance as, for example, a single DNA strip would not be able to accommodate all the gene loci probes. Hain Lifescience (GmbH, Nehren, Germany) are currently working on a technology to improve genotypic detection of PZA resistance (94).

1.8.5 Second-line Drugs

1.8.5.1 Fluoroquinolones (ofloxacin, moxifloxacin, levofloxacin, ciprofloxacin) (First quinolone discovered: early 1960's)

Mutations of two short gene regions, known as the quinolone resistant-determining region (QRDR), are responsible for most of fluoroquinolone (FLQ)-resistant isolates these are the *gyrA* and *gyrB* regions. In South Africa, the preferred drug of choice is moxifloxacin in the treatment of M/XDR-TB, as opposed to ofloxacin. Ofloxacin is only used in patients under the age of 8 years, or in patients who are intolerant of moxifloxacin (57).

1.8.5.1.1 gyrA encodes for DNA gryase of the two A subunits of the type II topoisomerase enzyme

The genes *gyrA* and *gyrB* encode for DNA gryase which is a topoisomerase II that regulates DNA supercoiling and unlinks tangled nucleic acid strands. Thus mutations in the gryA and gyrB regions will result in either microbial death or confer to FLQ resistance (93).

In previous studies, it has been shown that most of the mutations occur in the *gyrA* gene, in codons A90, 91 and D94. For example, Mokrousov, *et al.* demonstrated that 83% FLQ resistance occurred from the *gyrA* of the QRDR, in codons 88, 90 94, as compared to 8.3 % in the *gyrB* region (95). In South Africa Said, *et al.* demonstrated this when 18 ofloxacin-resistant isolates were sequenced and showed that 15/18 (83%) isolates had *gyrA* mutations, whereas the rest had *gyrB* mutations. The most common mutation occurred in the D94 codon, followed by A90 (96).

1.8.5.1.2 gyrB encodes for DNA gryase of the two B subunits of the type II topoisomerase enzyme

Previous research indicates that mutations of the *gyrB* region occur less frequently, however, there have been cases whereby heteroresistant isolates have demonstrated both mutant and wild type alleles of both *gyrA* and *gyrB* (42) (65) (97).

1.8.5.2 Aminoglycosides (kanamycin, amikacin, capreomycin, viomycin)

Strains that are resistant to SM are usually susceptible to KAN and AMK. However, there is high cross-resistance between KAN and AMK and it must be assumed that resistance of one of these drugs, infers resistance to the other (57).

1.8.5.2.1 rrs encodes for 16S rRNA

Like SM, CAP, AMK, KAN and viomycin are protein synthesis inhibitors. Resistance to capreomycin, AMK and KAN is caused by mutations occurring in codons, 1400, 1401, 1402 and 1484, respectively of the *rrs* gene. Mutation in codons 1400, 1401 and 1484 demonstrates high-level resistance to all aminoglycosides; a mutation codon 1401 displays low-level resistance to CAP whereas in codon 1402 only causes high-level resistance to CAP and low-level resistance to KAN (65) (96) (98). Refer to figure 1.15 for a schematic representation of the primary site of action of aminoglycosides.

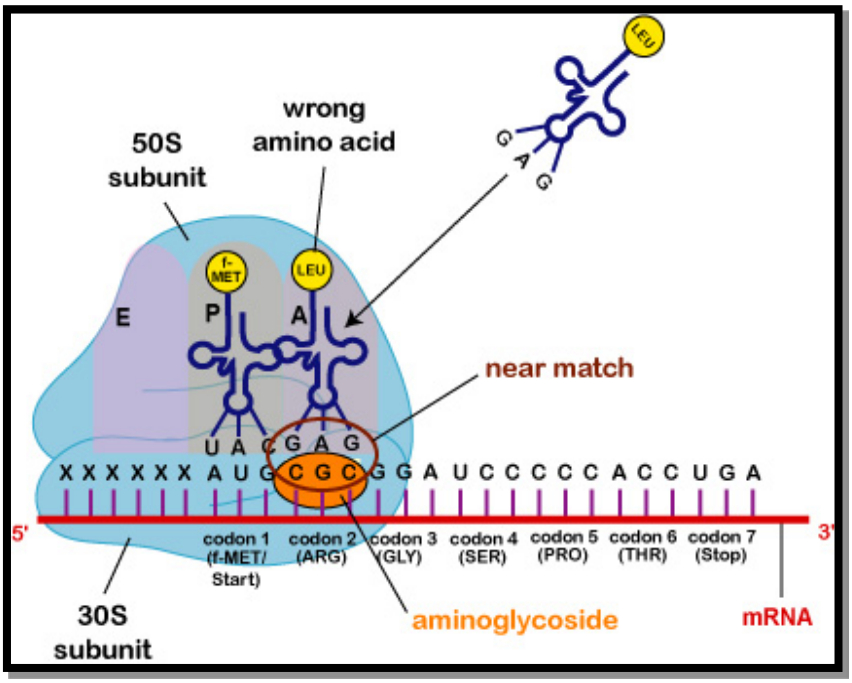


Figure 1.15 Primary site of action of the aminoglycosides is the 30S ribosomal subunit; some aminoglycosides also bind to several sites on the 50S ribosomal subunit. (Source: Tenson T, Mankin A. Antibiotics and the ribosome. Mol Microbiol. 2006 Mar; 59(6): 1664-77).

CHAPTER 2

2.0 Materials and Methods

2.1 Ethics approval

The protocol, patient information leaflet and informed consent form (ICF), together with a demographics-related questionnaire were submitted to the Human Research Ethics Committee (HREC) of the University of the Witwatersrand, Johannesburg, South Africa. The protocol was approved unconditionally on 1 October 2010 (**clearance certificate number: M10931**).

2.2 Study population

The research was conducted on sputum specimens from multidrug-resistant TB (MDR-TB) and extensively drug- resistant TB (XDR-TB) patients, collectively abbreviated to M/XDR-TB patients, who were admitted to the dedicated TB hospital Sizwe Tropical Disease Hospital (Sizwe Hospital), Johannesburg, South Africa. One hundred and fifty patients were recruited upon admission. Each patient entered into the study was admitted with at least one laboratory-confirmed MDR-TB diagnosis from a community clinic or doctor. The laboratory-confirmed diagnosis included either or both the MTBDR*plus (version 1)* and/or GXP, together with a microscopy smear result. These were then follow-on with phenotypic DST results.

As the laboratory diagnosis of M/XDR-TB is central to the present study and as paucibacillary tuberculosis is common in HIV/TB co-infected patients, their HIV serological status was documented upon admission and entered as HIV-positive, HIV-negative, or as unknown.The following recruitment criteria were followed:

2.2.1 Inclusion criteria

- Adults ≥ 18 years of age admitted to Sizwe Hospital
- Patients able and willing to sign informed consent
- Patients able to produce an early morning sputum sample, the day after admission

2.2.2 Exclusion Criteria

- Patients under the age of 18 years
- Patients unwilling or unable to sign informed consent
- Patients unable to produce an early morning sputum sample, the day after admission

2.3 Study sites

2.3.1 Site of recruitment

Sizwe Tropical Disease Hospital, 2 Modderfontein Road, Edenvale, Johannesburg, Gauteng, South Africa was the recruitment site where patients were entered into the study after informed consent had been obtained.

2.3.2 Site of sputum microscopy, culture and drug susceptibility testing

Sputum specimens from tuberculosis (TB) patients entered into the study were processed for routine smear microscopy, culture and drug susceptibility testing (DST) at the Mycobacteriology Referral Laboratory of the National Health Laboratory Service (NHLS),corner de Korte and Hospital Streets, Braamfontein, Johannesburg, Gauteng, South Africa.

2.3.3 Site of performance of line probe assays

The GenoType® MTBDR*plus (version 1)* and MTBDR*sl* line probe assays (LPAs; Hain Lifescience GmbH, Nehren, Germany) for the study were performed at the National Tuberculosis Reference Laboratory (NTBRL), National Institute of Communicable Diseases (NICD), 1 Modderfontein Road, Sandringham, Johannesburg, Gauteng, South Africa.

2.4 Patient confidentiality

Patient data included the results of sputum smear microscopy, TB culture and DST, as well as data on the GenoType® MTBDR*plus (version 1)* and MTBDR*sl* LPA findings. Patients were each allocated a unique study-specific number (participant identification number: PID) and specimens were labelled accordingly. The PID included 3 numbers and up to 3 INITIALS (e.g. 073-LES), which is taken from either their identity documents or medical records. Other data such as demographics and data related to patient management history were also collected. All patient data was recorded on clinical research forms (CRFs) which were kept in research files. These files were locked away at the research unit at Sizwe Hospital. Only personnel involved in the study had access to the files.

2.5 Data collection and storage

A study-specific database was designed by Lynsey E. Isherwood, using Microsoft Office Access 2007. This database was located and backed-up on-site. All data collected CRFs and routine results were transferred into this anonymous database. Data captured included (Addendum A):

- Demographics
- Work environment description

- Home environment description
- History of culture and DST results prior to entry into study
- HIV status and management history
- DNA strip of each LPA performed
- Line probe assay performed and their results
- Records of results from previous PCR assays
- Smear microscopy tests performed with dates and results
- Routine culture and DST results on first-line drugs
- Routine culture and DST results on second-line drugs

Source documentation included the baseline demographic questionnaires, LPA interpretation sheets, routine smear microscopy findings and TB culture and DST result sheets.

2.6 Recruitment and informed consent

Once admitted to ward 4 (admissions ward), patients were approached and invited to participate in the study. Willing patients briefly had the study background, objectives, benefits and risks explained to them. Included in the background of information presented, the disease itself (i.e. M/XDR-TB) was described and was compared to drug-sensitive TB.

Once an overview of the study was presented, an English patient information leaflet and informed consent form (ICF) was given to the patient (Addendum B). The informed consent process was performed in English. If the patient did not understand or speak English an independent person who understood and spoke the patient's native language assisted in the communication between the patient

and study coordinator. The ICFs were then given to the patient overnight to give him/her time to consider his/her participation/non-participation in the study.

The next morning, the patient's questions or queries were answered, as far as possible. If the patient was still willing to participate in the study, they then signed the ICF together with an independent witness, if applicable. A copy of the ICF was given to the patient and the original was kept in a file at the research unit, located at Sizwe Hospital.

The consent procedure took approximately 60 minutes (excluding the time that the patient was left with the ICF to read overnight). Only once the patient had signed the ICF, were sputum samples assigned to the study and residues of their sediments collected at the NHLS laboratory following the decontamination/concentration and routine processing of the sputum specimens, and finally transported to the NTBRL for the performance of project related procedures.

2.7 Algorithm routine versus study-related assays

The genotypic LPAs were performed on the residual sediments of processed sputum specimens scheduled for routine sputum microscopy, MGIT culture and DST (BACTEC™ MGIT™ 960 System. Becton Dickinson Microbiology Systems, Cockeysville, MD, USA). The performance of the LPAs for this study did not interfere in any way with the routine investigations conducted on the specimens. The flow of the sputum processing and the points when the LPAs were "tagged" on are illustrated in the algorithm below (Figure 2.1). For the tests performed directly on sputum, as indicated on the LPA algorithm, DNA was extracted from the portion of the pellet that remained after the routine specimen decontamination

had been performed. Similarly, for the cultures, the LPA algorithm indicates that DNA was extracted from the same culture isolate that was used for routine culture and DST. Thus for each decontaminated pellet and each isolate collected, both the MTBDR*plus (version 1)* and MTBDR*sl* assays were run in tandem to each other

LPAs were performed on all smear-positive and smear-negative specimens, as well as smear-positive and smear-negative, culture-positive isolates. As the LPAs were analyzed on both smear-positive and smear-negative specimens, the candidate (Lynsey E. Isherwood) performing the LPAs was not aware of the sputum smear microscopy status obtained by the NHLS on routine testing of the specimens. Also, Lynsey Isherwood was blinded to the DST results of the patients prior to their admission to Sizwe Hospital.

All post-DNA extraction residues were stored overnight at 2^0C-8^0C in case of interpretable LPA results. Also, all remaining DNA was stored at -20^0C, until the end of the study. Thus, if any discordant results occurred between gold standard assays and the genotypic LPAs, then the relevant DNA samples could be re-called for sequencing to ascertain which genes presented with drug-resistant mutations. However, sequencing these genes falls outside the scope of the present study and will be performed in a subsequent study.

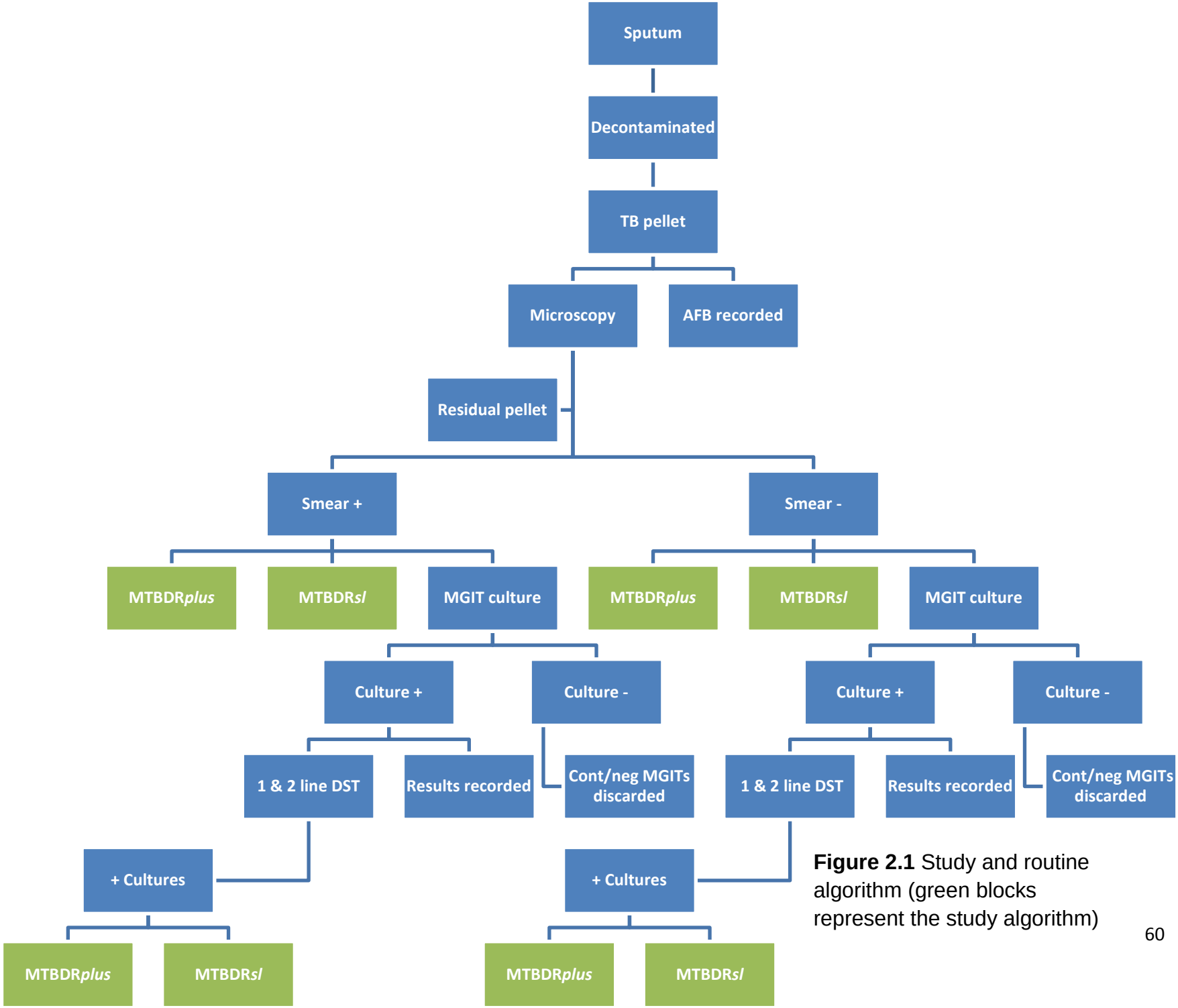


Figure 2.1 Study and routine algorithm (green blocks represent the study algorithm)

2.8 Sputum: collection, transportation and storage

2.8.1 Collection

A good quality, early morning sputum sample was collected from the study participant, in a fresh sample canister, before breakfast and before brushing teeth. All sputum sample canisters and requisition forms were clearly labeled, linking the sputum sample to this study. This included the protocol number and participant PID number.

2.8.2 Transportation

The sputum samples were immediately couriered from Sizwe Hospital to the NHLS, Braamfontein for routine smear microscopy, TB culture and DST. The specimen canisters were transported within a cooler bag that maintained the specimens at a constant low temperature.

2.8.3 Storage

Upon arriving at the NHLS, the specimen canisters were immediately placed into a 4^0C fridge.

2.9 Decontaminated sputum: transportation and storage

2.9.1 Transportation

Residual decontaminated specimens (TB pellets) were transported in Falcon conical tubes according to ‘Category A Infectious substances affecting humans’ (UN 2814) specifications.

2.9.2 Storage

After routine decontamination, each residual TB pellet was storedat 4^0C in its own Falcon tube (BD Falcon* 50mL Conical Centrifuge Tubes) which was used during the decontamination process. The period of storage was no more than a week.

The pellets were collected weekly and transported, by car, to the NTBRL. These pellets were packed within primary, secondary and tertiary containers to ensure optimized refrigeration and minimized contamination.

2.10 MGIT culture isolates: transportation and storage

2.10.1 Transportation

The MGIT culture isolates were transported from the NHLS to the NTBRL in primary, secondary and tertiary containers to ensure safety, minimizing chances of breakage, contamination and optimized refrigeration.

2.10.2 Storage

Upon arriving at the NTBRL, the MGIT culture isolates were stored in a dedicated 4^0C refrigerator.

2.11 Routine algorithm

Routine microscopy, culture and drug susceptibility testing on specimens submitted from patients at the MDR-TB Hospital were performed at the National Health Laboratory Service (NHLS) Mycobacteriology Referral Laboratory in Johannesburg, according to the laboratory's standard operating procedures.

Sputum specimens were processed using N-acetyl-cysteine-sodium hydroxide (NALC-NaOH) decontamination (NaOH final concentration 1%) (99). Following

centrifugation, the pellet in each tube was suspended in 0.5ml phosphate buffer pH6.8 and 0.5ml of the pellet was inoculated into prepared 7ml MGIT culture tubes (also known as culture isolates) for routine culture. The concentrated pellet was also used to prepare the sputum smear for auramine staining which was examined under 400 times magnification using light-emitting diode (LED) fitted fluorescent microscopes. Smear microscopy findings were graded, in terms of the presence of acid-fast bacilli, according to WHO/IUATLD guidelines (100.0).

The inoculated MGIT tubes were incubated for culture for a total of 6 weeks in the automated BACTEC MGIT 960 System (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) instruments. Positive tubes were removed from the incubator rack upon fluorescence for further processing.

Microscopy of a Ziehl Neelsen (ZN) stained smear of the culture was performed to confirm the presence of acid-fast bacilli (AFB) in the culture and the presence of AFB arranged in cords was noted. AFB-positive cultures underwent identification using the MPT64 antigen detection assay (SD Bioline TB Ag MPT64 Rapid Kit (Kat Medical, Roodepoort, Johannesburg, South Africa). Those that were positive for *Mycobacterium tuberculosis* underwent DST by BACTEC MGIT 960 proportion method.

If no fluorescence was detected in the MGIT tube at 6 weeks of incubation, a negative report was issued. Contaminated tubes (the presence of non-acid-fast bacteria or fungi) were identified using the ZN smear on the culture.

The BACTEC MGIT 960 DST for streptomycin (S), isoniazid (I), Rifampicin (R), and ethambutol (E) was performed using the BACTEC MGIT 960 SIRE kit,

according to manufacturer's recommendations and routine laboratory standard operating procedures. The critical drug concentrations used were 1.0µg/ml, 0.1µg/ml, 1.0µg/ml and 5.0µg/ml for Rifampicin, isoniazid, streptomycin and ethambutol respectively.

Drug susceptibility testing to ethionamide, kanamycin and ofloxacin was performed using the BD BACTEC MGIT 960 instrument and drugs prepared by the media department of the NHLS. Critical concentrations used were 2.5µg/ml, 2.0µg/ml and 5.0µg/ml for kanamycin, ofloxacin and ethionamide respectively.

2.12 Line probe assays (GenoType® MTBDRplus (version 1) and MTBDRsl)

Both the MTBDR*plus (version 1)* and MTBDR*sl* genotypic LPAs were conducted according to the manufacturer's guidelines. The DNA extraction, multiplex PCR and reverse hybridization methodology is similar in both LPAs. Only the colorimetric probes on the DNA strips that indicate either wild type (WT) sequences or specific mutations (MUT) to *rpoB*, *katG and inhA* genes (MTBDR*plus* [*version 1]*) or *gyrA, rrs* and *embB* genes (MTBDR*sl*) differ between the two LPAs.

Genotypic LPAs were batched and processed on a weekly basis. Laboratory grade distilled water (dH_20) was used as MTB negative controls for each batch. No MTB positive controls were used. Residual TB pellets and/or MGIT isolates were collected from the NHLS and batched to either a 'direct LPA' run or a 'culture LPA' run of either or both the MTBDR*plus (version 1)* and MTBDR*sl* assays. Each of the two assays could process up to 10 specimens at a single time.

2.12.1 Laboratory set-up

All genotypic LPAs were performed at the NTBRL. The master mix preparation, DNA extraction, amplification and hybridization were performed in separate, sterile, unidirectional rooms, as per recommendation from the World Health Organization (WHO) (63).

2.12.2 Master-mix preparation

A PCR master mix was prepared including primers, nucleotides, $MgCl_2$ and HotStart *Taq* polymerase (QIAGEN, Hilden, Germany). The PCR is a multiplex PCR designed to amplify portions of the relevant genes for either the MTBDR*plus (version 1)* or MTBDR*sl* assays. A separate master mix was prepared for each of the MTBDR*plus (version 1)* and MTBDR*sl* assays, as each assay require their own specific primers and nucleotides. These primers and nucleotides are included in the LPA kits (Figure 2.2).

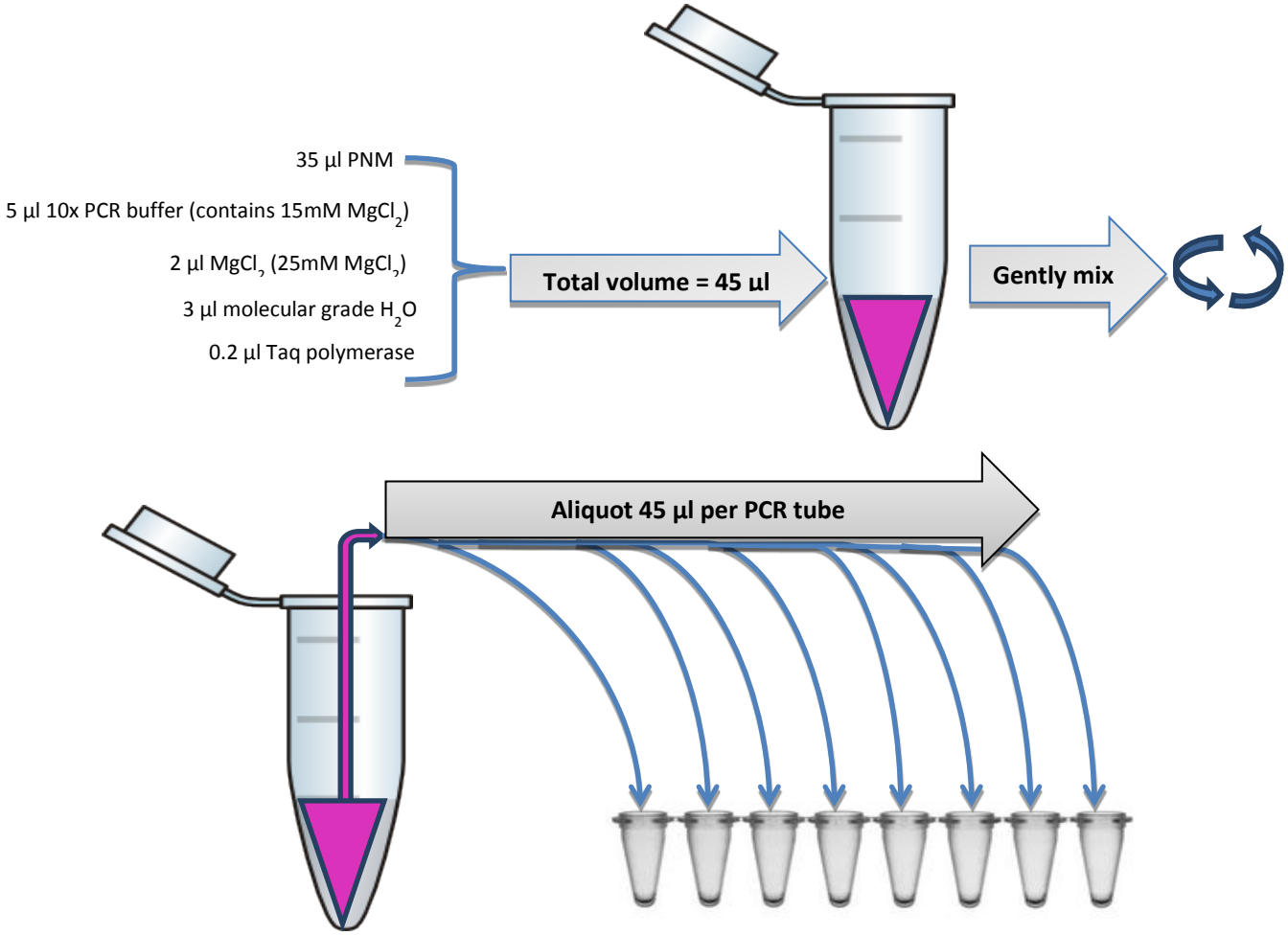


Figure 2.2 Schematic diagram of the preparation of the master mix. The same preparation method was used for both the GenoType® MTBDR*plus (version 1)* and MTBDR*sl* assays. PNM = primer-nucleotide mix (Source: Adapted from Hain Lifescience. Master mix preparation. Hain Lifescience GmbH, Germany; [cited 2012 Sep 26]. Available from: www.hain-lifescience.com).

2.12.3 DNA extraction

DNA extraction includes heating, sonification and centrifugation. A single DNA extraction was performed from either the direct specimen (decontaminated pellet known as direct DNA) or from the MGIT culture isolates (known as culture DNA). Direct and culture DNA extracts were used for both the MTBDR*plus (version 1)* and MTBDR*sl* assays (Figure 2.3).

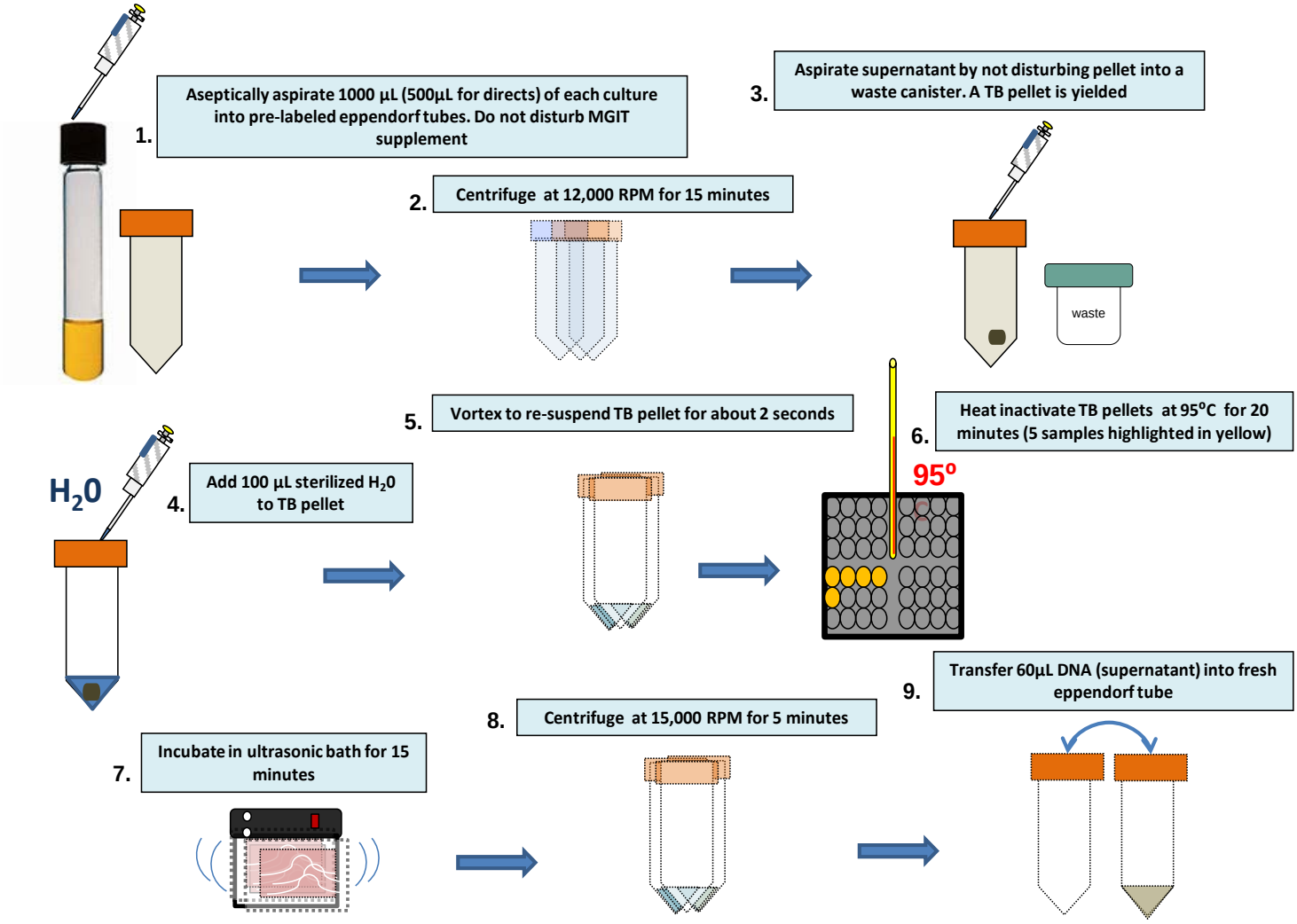


Figure 2.3 Schematic representation of the DNA extraction method used in this study. Extraction of either MGIT cultures or direct specimens is the same except that 100.00µL or 500µL of either culture or direct specimen respectively, is required. (Source: Lynsey E. Isherwood).

2.12.3.1 Direct specimens

500 µl (500 µl per genotypic LPA is required) of the decontaminated pellet, collected from the NHLS, was used for the DNA extraction in preparation for the genotypic LPAs.

2.12.3.2 Cultures

100.00 µl (100.00 µl per genotypic LPA is required) of the culture, collected from the NHLS, was used for the DNA extraction in preparation for the genotypic LPAs.

2.12.4 Multiplex PCR amplification

5µl of each DNA supernatant (extracted from either a direct specimen or a culture isolate) was added into a corresponding, labeled PCR tube containing the 45 ul master mix. Residual DNA was stored at -20^{0}C.

Amplification of the TB DNA was performed within a thermal cycler (GTQ-Cycler 96, Hain Lifescience) involving various cycles of denaturing, annealing and elongation of the TB DNA. Amplification of the MTBDR*plus (version 1)* and MTBDR*sl* samples were processed separately.

Different amplification conditions were applied depending whether the DNA was extracted from a direct specimen (40 cycles), or a culture isolate (30 cycles). The conditions are described below:

Direct specimens

15 minutes at 95˚C	1 cycle
30 seconds at 95˚C 2 minutes at 58˚C	10 cycles
25 seconds at 95˚C 40 seconds at 53˚C 40 seconds at 70˚C	30 cycles
8 minutes at 70˚C	1 cycle

MGIT cultures

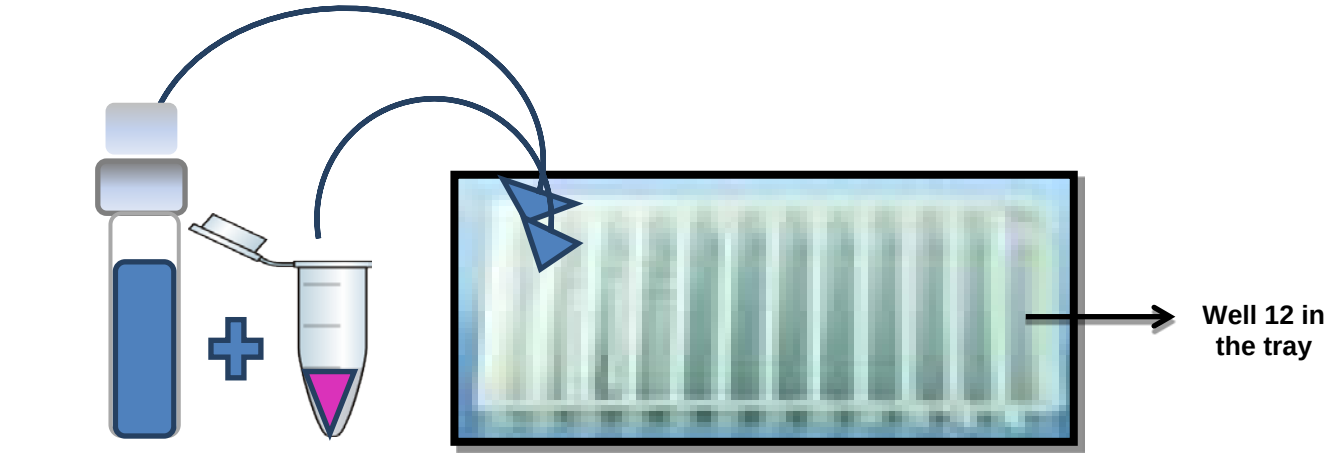
15 minutes at 95˚C		1 cycle
30 seconds at 95˚C 2 minutes at 58˚C	}	10 cycles
25 seconds at 95˚C 40 seconds at 53˚C 40 seconds at 70˚C	}	20 cycles
8 minutes at 70˚C		1 cycle

2.12.5 Reverse Hybridization

Reverse hybridization was performed using two TwinCubator® (Hain Lifescience) a manual hybridization machine. Hybridization of the MTBDR*plus (version 1)* and MTBDR*sl* batches ran in tandem to each other.

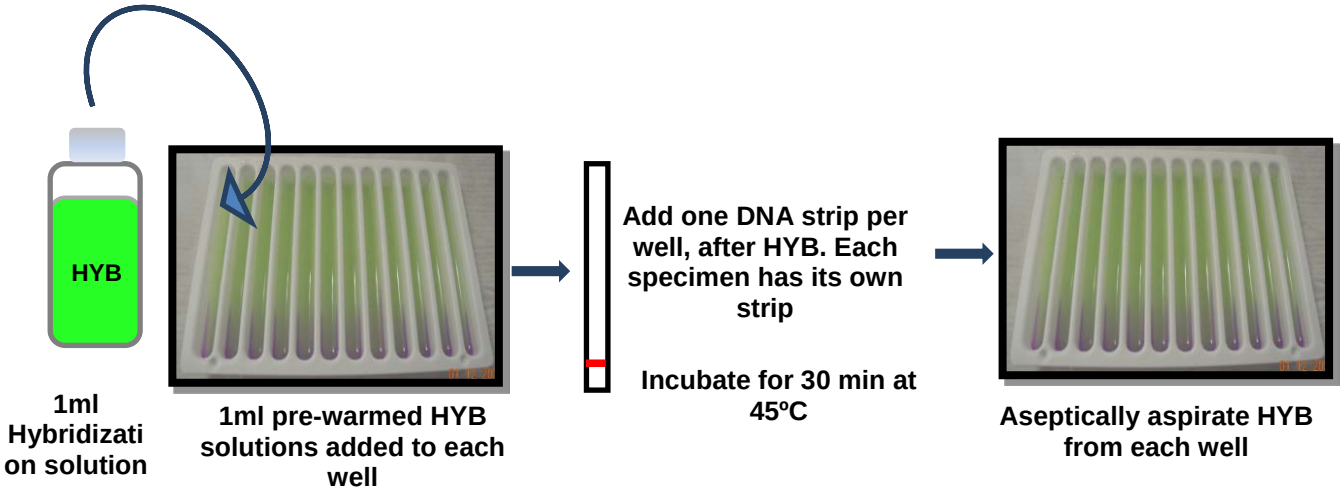
After hybridization, the strips were blotted dry and fixed onto a result sheet with strips of transparent sellotape (Figure 2.4).

Step One: denaturing solution + DNA amplicon

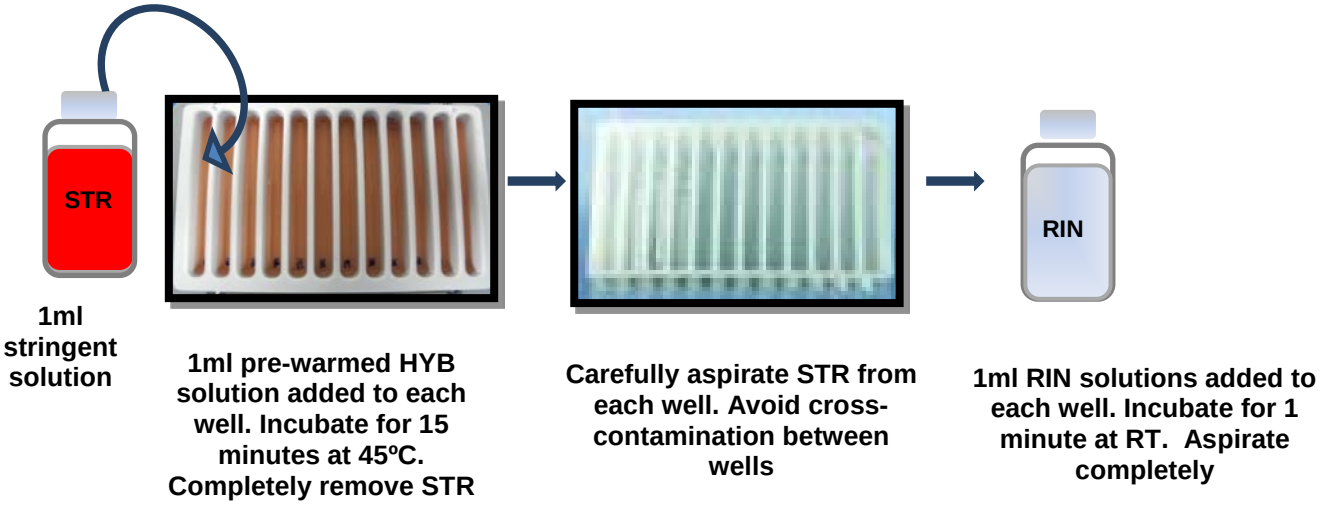


Twelve wells within a tray. Tilt the tray backwards and forwards gently to mix each well. Incubate for 5 minutes at room temperature (RT)

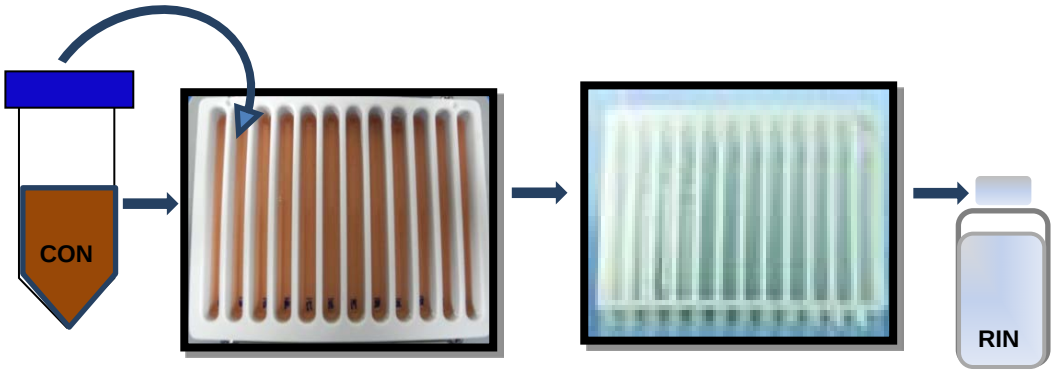
Step Two: hybridization solution + DNA strip



Step Three: stringent solution + rinse



Step Four: conjugate solution + rinse



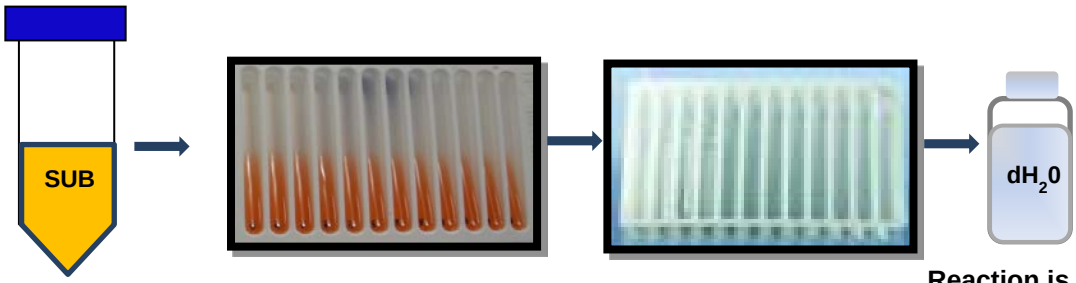
Conjugate (CON) solution prepared in a 50mL Falcon tube:
10µL CON-C + 990µL CON-D per DNA strip

1mL of CON solution added to each well. Incubated for 30 minutes at RT

Carefully pour CON out

1ml RIN solutions added to each well. Incubate for 1 minute at RT. Aspirate completely. Repeat. Then rinse with dH_20

Step Five: substrate solution + rinse + evaluation of results



Substrate (SUB) solution prepared in a 50mL Falcon tube:
10µL SUB-C + 990µL SUB-D per DNA strip

1mL of SUB solution added to each well. Incubated for 2-10 minutes at RT; keep strips in the dark

Carefully pour out SUB

Reaction is stopped by adding 1mL dH20 to each well. Incubate for 1 minute. Repeat

Remove DNA strips from tray and place on absorbent paper

Figure 2.4 Schematic representation of hybridization. (Source: Lynsey E. Isherwood).

2.12.6 Interpretation of DNA strips

The MTBDR*plus (version 1)* and MTBDR*sl* assay results were interpreted according to the manufacturer's guidelines (Figure 2.5). Also refer to section 1.5.7.4 on page 36.

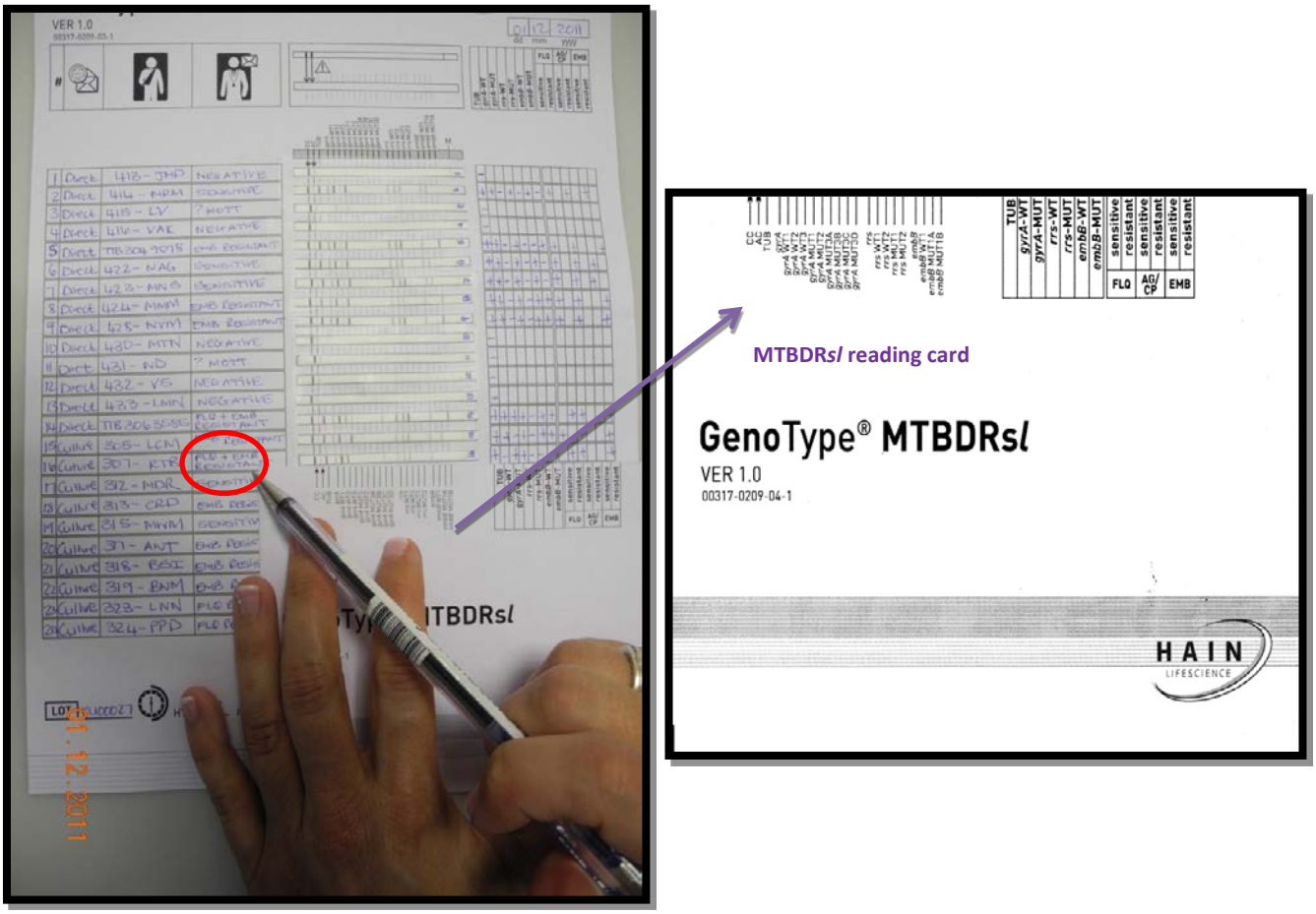


Figure 2.5 Actual result sheet of one of the MTBDR*sl* assay batches comprising both direct and culture specimens. A pre-XDR-TB (FLQ) result is circled in red. This specimen is also resistant to ethambutol. The presence of a wild type (WT) or mutation (MUT) is indicated with a '+'. The absence is indicated with a '-'. (Source: Lynsey E. Isherwood).

2.13 Statistics

2.13.1 Age and gender

The calculations and histograms of the age and gender in chapter 3 were done with XLSTAT 2013.1.01 (descriptive statistics). The p-value of the age difference between the males and females were done with the t-test.

2.13.2 Sensitivity, specificity, PPV and NPV

The sensitivity, specificity, PPV and NPV's were calculated using STATA v12. Ninety-five percent confidence intervals were applied.

Chapter 3

3.0 Results

3.1 Description of study population

One hundred and fifty study participants were new admissions to Sizwe Hospital sequentially recruited onto the study according to inclusion and exclusion criteria (refer to Chapter 2).One sputum sample was received from each participant either on the day of admission, or the day after admission, before anti-TB drugs were administered.

3.2 Demographics of study participants

3.2.1 Age and gender

Of the 150 participants, 75 (50.0%) were female and 75 (50.0%) were male. The males were significantly older than the females (p=0.001).The median ages of the males and females were 41.0 years (IQR: 34.5-49.0) and 35.0 (IQR: 26.0-41.5), respectively. The below scattergrams (figures 3.1 and 3.2) demonstrate the distribution of age between the males and females.

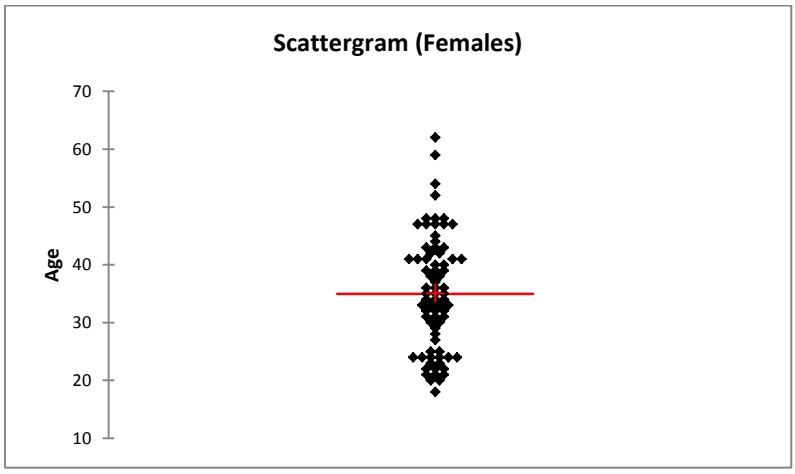


Figure 3.1 Scattergram of the age of the female study participants

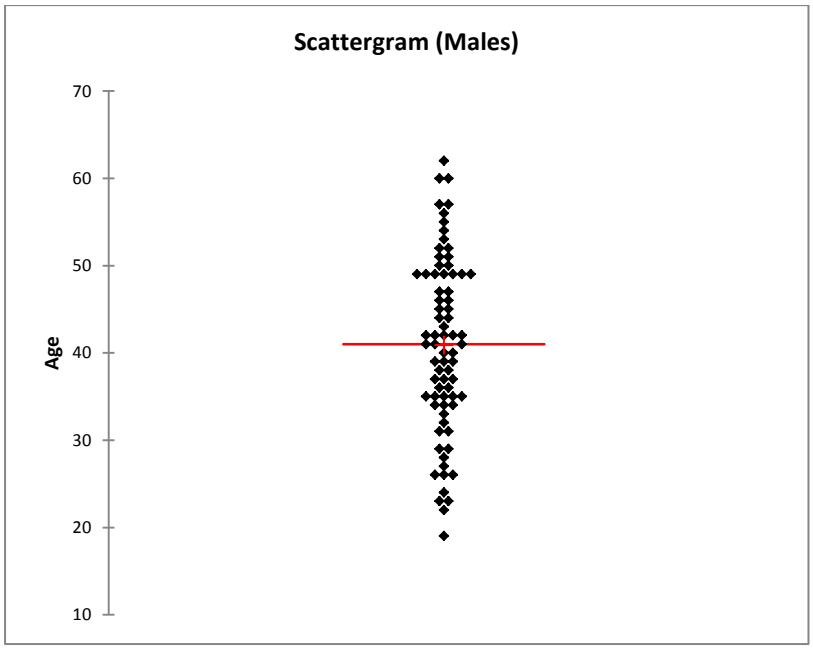


Figure 3.2 Scattergram of the age of the male study participants

3.2.2 HIV status

At the time of admission, 60.7% (n=91) were HIV-positive, 33.3% (n=50) HIV-negative and 6.00% (n=9) had an unknown status.

3.2.3 Ethnicity

Self-identified ethnicity of the study population was recorded: 92.7% (n=139) were black, 4.67% (n=7) coloured, 1.33% (n=2) white, 0.67% (n=1) Asian and 0.67% (n=1) other. 89.3% (n=134) participants were born in South Africa with the majority, 78.4% (n=105), originating from Gauteng.

3.2.4 TB episode and treatment history

Fifty-six percent (n=85) disclosed that they had at least one previous episode of TB of which 35.3% (n=30) had received anti-TB injections as part of their treatment regimen.

3.3 Assay summary

3.3.1 MGITs

Evaluable MGIT culture isolates were defined as those that have been confirm as MTB by the NHLS laboratory together with a complete first and/or second line DST results.

A total of 67/150 MGIT cultures were evaluable and compared to either or both 'direct' and/or 'culture' LPA results.

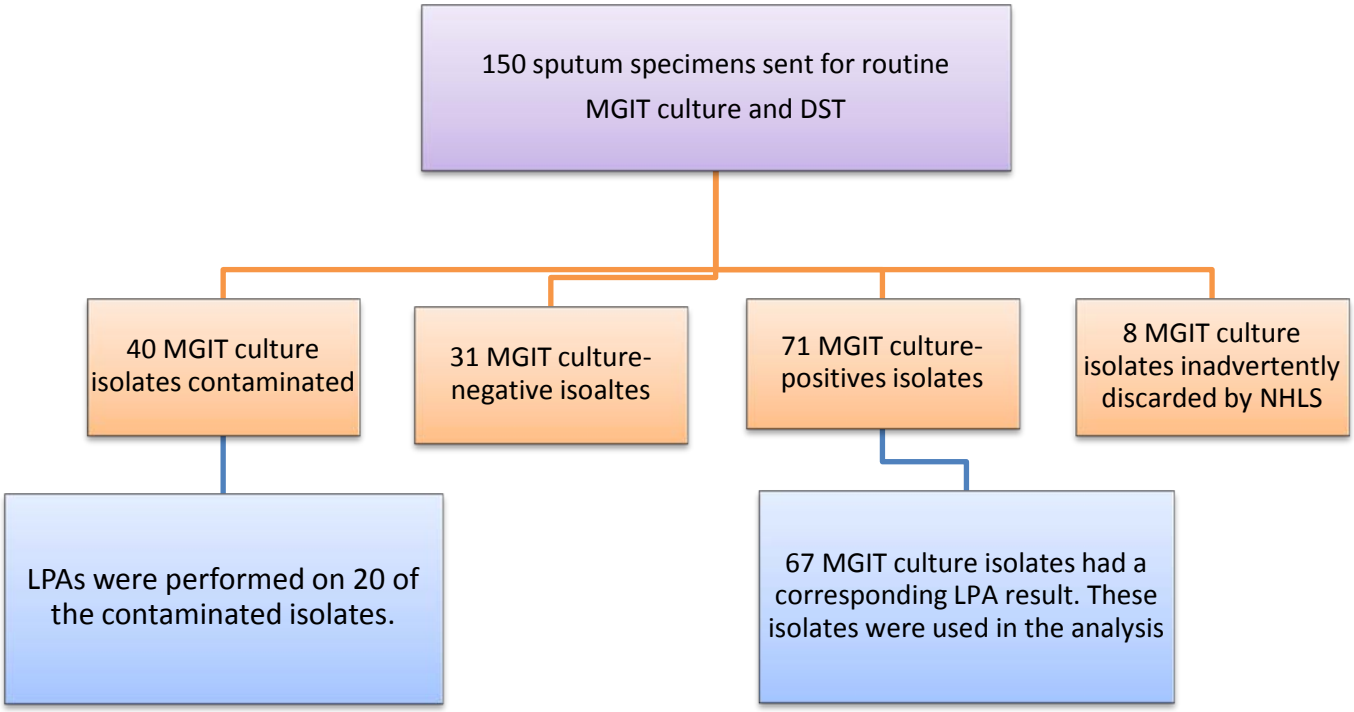


Figure 3.3 Organogram of MGIT culture isolates yielded in the study

3.3.2 LPAs

For ease of reference the 'culture LPA' results were categorized as follows:

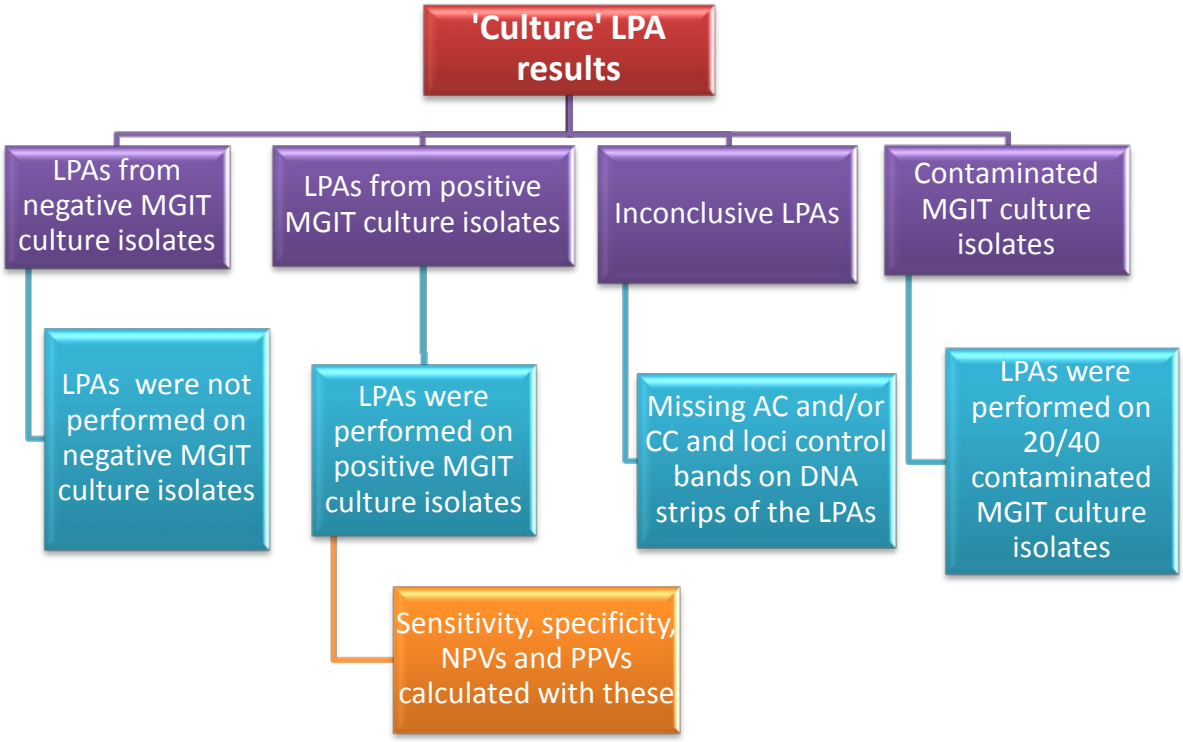


Figure 3.4 Classification of 'culture' LPA results in this study. (Source: Lynsey E. Isherwood).

Both the GenoType® MTBDR*plus* (*version 1*) and MTBDR*sl* assays were performed on 71/150 MGIT positive cultures. 'Culture' LPAs is those LPA strips that were performed from TB DNA extracted from positive culture isolates. However, only 67/71 culture positive isolates had a correlating LPA result.

TB DNA was not extracted from MGIT negative culture isolates, as these isolates would contain a small quantity of the original specimen. MGIT culture-negative isolates are routinely discarded by the NHLS. These were thus excluded from the calculations.

'Direct' LPAs were defined as those LPA strips that were performed on DNA extracted from direct decontaminated sputum specimens (pellets). Both the GenoType® MTBDR*plus (version 1*) and MTBDR*sl* assays were performed on 147/150 sputa specimens. Three pellets were erroneously discarded by the NHLS

laboratory before an LPA could be performed. Also, 10/64 ‘direct’ LPAs were inconclusive and were thus excluded from the calculations.

Table 3.1 Number of of MGIT cultures, ‘culture’ LPAs and ‘direct’ LPAs performed

Event	% (n)
Direct LPAs	98.0 (n=147)
Culture LPAs (from MGIT positive culture isolates)	100.0 (n=71)
Contaminated MGIT culture isolates	26.7 (n=40)
MGIT negative culture isolates	20.7 (n=31)
MGIT culture isolates inadvertently discarded by NHLS	5.3 (n=8)

3.3.3 Microscopy smears

Of the 150 sputum specimens sent for smear, culture and drug susceptibility testing (DST), 145/150 had their respective smear microscopy results recorded on the DISA system at NHLS.

On comparison to the 67evaluable MGIT positive cultures, 97.0% (n=65) had a recorded smear result. Of these, 43.1% (n=28) were smear-negative and 57.0% (n=37) were smear-positive.

3.3.4 Contaminated culture isolates versus LPAs

Twenty of the 40 contaminated culture isolates were tested with on ‘culture’ LPAs to determine whether the results would be interpretable. The other 20 contaminated cultures were inadvertently discarded by the NHLS laboratory. All 100.0% of the contaminated culture isolates demonstrated an interpretable LPA

result. Of the interpretable results, 70.0% (n=14) were MDR-TB 15.0% (n=3) were pre-XDR (FLQ), 10% (n=2) were XDR-TB and 1 was positive for MTB, but fully susceptible to both first and second line drugs. These results were used to inform patient management. However, as these culture isolates were contaminated, no gold standard phenotypic results could be used for comparison for the calculations of the sensitivity, specificity, PPV and NPV of the LPAs.

3.3.5 Smear microscopy, culture and LPAs

3.3.5.1 Smear microscopy

A total of 142/150 microscopy smears were resulted by the NHLS. Of the 91 HIV-positive population, 57.1% (n=52) were smear-negative, 37.0% (n=33) were smear-positive and the remainder 6.59% (n=6) smears were not done. Of the 50 HIV-negative population, 46.0% (n=23) were smear-negative, 52.0% (n=26) were smear-positive and one not done. Of the 9 unknown HIV statuses, 4 each were smear-positive and smear-negative, respectively and one microscopy smear was not performed by NHLS for a reason unknown.

The proportion of smear-negatives in HIV-positive patients was not statistically significantly different to the smear-negatives in HIV-negative patients (57.1% vs. 46.0%; p=0.139).

Table 3.2 Summary of microscopy smears in the HIV-positive and HIV-negative study participants

HIV-positive participants (n=91)	% (n=142)
Smear-negative	36.6 (n=52)
Smear-positive	23.2 (n=33)
Not done	4.23 (n=6)
HIV-negative participants (n=50)	
Smear-negative	16.2 (n=23)
Smear-positive	18.3 (n=26)
Not done	0.70 (n=1)
Unknown HIV status (n=9)	
Smear-negative	2.82 (n=4)
Smear-positive	2.82 (n=4)
Not done	0.70 (n=1)
TOTAL microscopy smears resulted by NHLS	100.0 (n=142)

3.3.5.2 MGIT culture

Of the 150 specimens submitted for MGIT culture, 47.3% (n=71) were culture-positive, 20.6% (n=31) culture-negative, 26.6% (n=40) were contaminated and 5.33% (n=8) cultures were inadvertently discarded by the NHLS. However, 67/71 of the culture-positives were considered as evaluable and these had a corresponding ‘culture’ LPA result.

3.3.5.3 Phenotypic DST

Table 3.3 Summary of the diagnosis of either MDR-TB, pre-XDR-TB or XDR-TB from the 67/71 MGIT culture positive isolates analysed.

MGIT culture result	(n=67)
MDR-TB	67.2% (n=45)
***Pre-XDR-TB (FLQ)**	13.4% (n=9)
****Pre-XDR-TB (AG/CP)**	5.97% (n=4)
XDR-TB	5.97% (n=4)
#Mono-INH	2.99% (n=2)
##Mono-RIF	2.99% (n=2)
Drug Sensitive	1.49% (n=1)

***pre-XDR-TB (FLQ)**: resistant to fluoroquinolones, INH and RIF without resistance to aminoglycosides and capreomycin); ****pre-XDR-TB (AG/CP)**: resistant to aminoglycosides /capreomycin, INH and RIF without resistance to fluoroquinolones; **#Mono-INH:**resistant to isoniazid only (sensitive to rifampicin); **##Mono-RIF:**resistant to rifampicin only (sensitive to isoniazid).

3.3.5.4 LPA DST

Table 3.4 Molecular diagnosis of either MDR-TB, pre-XDR-TB or XDR-TB from both the MTBDR*plus* (*version 1*) and MTBDR*sl* 'direct' and 'culture' LPAs.

LPA Result	% Direct (n=54)	% Culture (n=58)
MDR-TB	74.1 (n=40)	69.0 (n=40)
Pre-XDR (ofloxacin resistant)	14.8 (n=8)	19.0 (n=11)
Pre-XDR (kanamycin resistant)	7.41 (n=4)	1.72 (n=1)
XDR-TB	1.85 (n=1)	1.72 (n=1)
Mono-RIF	1.85 (n=1)	5.17 (n=3)
Mono-INH	0.00 (n=0)	1.72 (n=1)
Drug sensitive	0.00 (n=0)	1.72 (n=1)

The number of LPA results analysed were, 54/147 from direct sputa and from 58/71 MGIT culture positive isolates. The reason being is that these were the only LPAs that had both a direct smear and MGIT positive culture result corresponding to its LPA result.

3.4 Sensitivity, Specificity, PPV and NPV

Four performance indices (sensitivity, specificity, positive predictive values [PPV] and negative predictive values [NPV]), for both the MTBDR*plus* (*version 1*) and MTBDR*sl* assays, were calculated by using 2x2 tables to compare the LPA results to the gold standard phenotypic culture and DST findings. Ninety-five percent confidence intervals were calculated using STATA v12.

3.4.1 Parameters of calculations

- Analysis was only performed on MGIT positive cultures irrespective of microscopy smear result, that had a corresponding 'direct' and/or 'culture' LPA. This totaled to 67 comparable MGIT culture DST results
- LPA results from 54 corresponding direct smears (LPAs performed directly from sputum) and 58 'culture' LPAs (LPAs performed from MGIT positive cultures), respectively, were compared to the 67 MGIT culture DST results
- During the study, Lynsey Isherwood was blinded to the microscopy smear result of each specimen as well as the phenotypic DST of the patient, prior to their admission to Sizwe Hospital.
- As no drug-sensitive specimens were collected as a control group the specificities and negative predictive values (NPVs), respectively for the GenoType® MTBDR*plus* (*version 1*) assay were not determined

- The specificities and NPVs were included in the analysis of the GenoType® MTBDR*sl* assay, as the MDR-TB specimens were used as the control group
- Positive Predictive Value (PPV): Frequency of the detection of drug resistance by the LPAs
- Negative Predictive Value (NPV): Frequency of the detection of drug susceptibility by the LPAs

3.4.2 GenoType® MTBDR*plus* (*version 1*)

3.4.2.1 Rifampicin (RIF)

3.4.2.1.1 Rifampicin: direct LPAs

Table 3.5 (a) The 2x2 tables used for the calculations for RIF resistance/susceptibility

	MGIT (resistant)	**MGIT (sensitive)**	
LPA (resistant)	52	3	55
LPA (sensitive)	1	0	1
	53	3	56

Prevalence (%) **95% CI**	**Sensitivity (%)** **95% CI**	***PPV (%)*** ***95% CI***
94.6 (85.1-98.9)	98.1 (89.9-100.0.0)	94.5 (84.9-98.9)

Sixty-four of the 67 MGIT cultures were resistant to RIF. Of these, 52 were resistant to RIF on 'direct' LPA with 11 not done and one LPA recorded as a sensitive result to RIF.

Thus, for RIF, the sensitivity of all the direct sputum specimens is 98.1%. This makes sense as the study population had an already confirmed MDR-TB diagnosis, from a peripheral clinic, prior to admission to Sizwe Hospital. However,

there were 3 RIF sensitive specimens as per MGIT culture and DST, which is unexpected for a MDR-TB hospital environment. Another factor to consider is that this may be due to phenotypic heterogeneity of the specimens.

This is reaffirmed with the positive predictive value (PPV) of 94.5%, and the 94.6% prevalence of RIF resistance in this population.

3.4.2.1.2 Rifampicin: microscopy smear-negative specimens

Table 3.5 (b) The 2x2 tables used for the calculations for RIF resistance/susceptibility

	MGIT (resistant)	**MGIT (sensitive)**	
LPA (resistant)	16	1	17
LPA (sensitive)	1	0	1
	17	1	18

Prevalence (%) 95% CI	**Sensitivity (%) 95% CI**	**PPV (%) 95% CI**
94.4 (72.7-99.9)	94.1 (71.3-99.9)	94.1 (71.3-99.9)

Of the 56 overall RIF LPA results, a total of 27 were smear-negative. However, 9/27 smear-negative microscopies were inconclusive, leaving 18 evaluable smear-negative specimens. Again, the study population was an already confirmed MDR-TB diagnosed group of participants hence they would be resistant to both RIF and INH, as per the WHO definition of MDR-TB.

The PPV of RIF resistance detection by LPA from direct smear-negative specimens is 94.1% and the prevalence of RIF resistance is 94.4%. This indicates that RIF resistance is indeed prevalent and that the MTBDR*plus* (*version 1*) assay is able to identify RIF resistance from direct, smear-negative sputum specimens.

The clinicians can diagnose, with confidence, that 94.1% (PPV) of the study population diagnosed with RIF resistance, is indeed resistant to RIF.

3.4.2.1.3 Rifampicin: microscopy smear-positive specimens

Table 3.5 (c) The 2x2 tables used for the calculations for RIF resistance/susceptibility

	MGIT (resistant)	**MGIT (sensitive)**	
LPA (resistant)	33	2	35
LPA (sensitive)	0	0	0
	33	2	35

Prevalence (%) **95% CI**	**Sensitivity (%)** **95% CI**	**PPV (%)** **95% CI**
94.3 (80.8-99.3)	100.0.0 (89.4-100.0.0)	94.3% (80.8-99.3)

Of the 56 overall RIF direct LPA results, a total of 35 were smear-positive.
The sensitivity of the MTBDR*plus* (*version 1*) to detect RIF resistance from direct smear-positive specimens is 100.0%. From this, clinicians could be 94.3% sure that those with a positive RIF resistance result on LPA testing, actually do have RIF resistance (PPV = 94.3%).

The PPV of the smear-negative specimens was 94.1% versus 94.3%for smear-positive specimens, indicating that the probability of detecting RIF resistance on the LPA was very high and virtually the same on smear-positive and smear-negative specimens.

The prevalence of MDR-TB disease, based on MGIT DST and LPA, in this study population was high and is in concordance with a population of pre-diagnosed MDR-TB patients.

3.4.2.1.4 Rifampicin: culture isolates

Table 3.5 (d) The 2x2 tables used for the calculations for RIF resistance/susceptibility

	MGIT (resistant)	MGIT (sensitive)	
LPA (resistant)	56	1	57
LPA (sensitive)	1	0	1
	57	1	58

Prevalence (%) 95% CI	Sensitivity (%) 95% CI	PPV (%) 95% CI
98.3 (90.8-100.0.0)	98.2 (90.6-100.0.0)	98.2 (90.6-100.0.0)

There were a total of 67 evaluable MGIT culture results, however, 9/67 isolates were inadvertently discarded by NHLS before a 'culture' LPA could be performed. Thus a total 58 RIF culture LPAs was evaluable. The sensitivity and PPV for the detection of RIF resistance on LPA from culture-positive isolates were both 98.2%, respectively.

3.4.2.2 Isoniazid (INH)

3.4.2.2.1 Isoniazid: direct LPAs

Table 3.6 (a) The 2x2 tables used for the calculations for INH resistance/susceptibility

	MGIT (resistant)	MGIT (sensitive)	
LPA (resistant)	52	2	54
LPA (sensitive)	1	0	1
	53	2	55

Prevalence (%) 95% CI	Sensitivity (%) 95% CI	PPV (%) 95% CI
96.4 (87.5-99.6)	98.1 (89.9-100.0.0)	96.3 (87.3-99.5)

Sixty-four cultures were resistant to INH and only 55/64 had a corresponding direct INH LPA result. As MDR-TB is defined as *Mycobacterium tuberculosis* resistant to at least RIF and INH, it is to be expected that the prevalence of INH resistance

within this particular study population would be very high (96.4%). The sensitivity of the MTBDR*plus* (*version 1*) assay to detect INH resistance is 98.1% whereas the LPA's positive predictive value is 96.3%, inferring that the LPA is a reliable means of diagnosis from direct sputum specimens. However, there are slightly different PPVs between the smear-negative and smear-positive specimens, as indicated below, but the numbers of the tests performed are small and the difference is not significant.

3.4.2.2.2 Isoniazid microscopy smear-negative specimens

Table 3.6 (b) The 2x2 tables used for the calculations for INH resistance/susceptibility

	MGIT (resistant)	**MGIT (sensitive)**	
LPA (resistant)	17	1	18
LPA (sensitive)	0	0	0
	17	1	18

Prevalence (%) 95% CI	**Sensitivity (%) 95% CI**	**PPV (%) 95% CI**
94.4 (72.7-99.9)	100.0.0 (80.5-100.0.0)	94.4 (72.7-99.9)

Twenty-eight out of 67 MGIT cultures had an INH DST with smear-negative result, however, 10/28 did not have a corresponding direct INH LPA result equating to 18 evaluable INH direct, smear-negative LPAs.

The 94.4% prevalence of INH resistance in this study population is still expected, for the same reasons explained above. Although the sensitivity is also 100.0%, its 95% confidence interval is wider than smear-positive specimens indicated below. This is emphasized with the lower PPV of 94.4% in smear-negative specimens, compared to 97.1% in smear-positive specimens, stated below.

3.4.2.2.3 Isoniazid: microscopy smear-positive specimens

Table 3.6 (c) The 2x2 tables used for the calculations for INH resistance/susceptibility

	MGIT (resistant)	MGIT (sensitive)	
LPA (resistant)	33	1	34
LPA (sensitive)	1	0	1
	34	1	35

Prevalence (%) 95% CI	Sensitivity (%) 95% CI	PPV (%) 95% CI
97.1 (85.1-99.9)	97.1 (84.7-99.9)	97.1 (84.7-99.9)

Thirty-seven out of 67 MGIT cultures had an INH DST with a smear-positive result. However, 2/37 did not have a corresponding direct INH LPA, equating to 35 INH direct LPAs. The sensitivity and PPV of the MTBDR*plus* (*version 1*) assay to detect INH resistance from direct smear-positive is describe in comparison to direct, smear-negative specimens are depicted above.

3.4.2.2.4 Isoniazid: culture isolates

Table 3.6 (d) The 2x2 tables used for the calculations for INH resistance/susceptibility

	MGIT (Resistant)	MGIT (sensitive)	
LPA (Resistant)	52	3	55
LPA (Sensitive)	3	0	3
	55	3	58

Prevalence (%) 95% CI	Sensitivity (%) 95% CI	PPV (%) 95% CI
94.8 (85.6-98.9)	94.5 (84.9-98.9)	94.5 (84.9-98.9)

Sixty-seven MGIT cultures had an INH DST result, however, 9/67 did not have a corresponding ‘culture’ INH LPA, resulting in 58 evaluable INH ‘culture’ LPAs.

3.4.3 GenoType® MTBDRs*l*

3.4.3.1 Fluoroquinolones (FLQ)

3.4.3.1.1 Ofloxacin: direct LPAs

Table 3.7 (a) The 2x2 tables used for the calculations for OFX resistance/susceptibility

	MGIT (resistant)	MGIT (sensitive)	
LPA (resistant)	10	0	10
LPA (sensitive)	1	45	46
	11	45	56

Prevalence (%) 95% CI	Sensitivity (%) 95% CI	Specificity (%) 95% CI	PPV (%) 95% CI	NPV (%) 95% CI
19.6 (10.2-32.4)	90.9 (58.7-99.8)	100.0.0 (92.1-100.0.0)	100.0.0 (69.2-100.0.0)	97.8 (88.5-99.9)

Of the 67 MGIT cultures, 11 did not have a corresponding 'direct' FLQ LPA result, resulting in a total 56 'direct' FLQ LPA results.

From overall direct smear microscopies, the prevalence of pre-XDR-TB (FLQ) within this study population was 19.6%, however, the ability of the MTBDRs*l* assay to detect FLQ resistance from direct smears was good as evidence by a high sensitivity and specificity of 90.9% and 100.0%, respectively. Also, the ability of the LPA to predict pre-XDR-TB (FLQ) was 100.0% (PPV), indicating that this LPA is an effective rapid diagnostic tool for the detection of FLQ resistance, within a MDR-TB patient population, from direct specimens, without having to rely on culture-positive specimens, reducing the TAT of the diagnosis of pre-XDR-TB (FLQ) from 4-6 weeks, to approximately 2-7 days, according to the present study, when performed within a high volume, public laboratory.

The prevalence of pre-XDR-TB (FLQ) in patients admitted to a TB drug-resistant hospital is expected to be higher than within the general community. It can therefore be understood that the MTBDR*sl* would be an effective 'secondary' screening tool for pre-XDR-TB within a community or hospital, only once an initial diagnosis of MDR-TB has been conducted by other assays, e.g. MTBDR*plus* (*version 1*) or GXP assays.

3.4.3.1.2 Ofloxacin: microscopy smear-negative specimens

Table 3.7 (b) The 2x2 tables used for the calculations for OFX resistance/susceptibility

	MGIT (resistant)	**MGIT (sensitive)**	
LPA (resistant)	1	0	1
LPA (sensitive)	1	17	18
	2	17	19

Prevalence (%) 95% CI	**Sensitivity (%) 95% CI**	**Specificity (%) 95% CI**	**PPV (%) 95% CI**	**NPV (%) 95% CI**
10.5 (1.3-33.1)	50.0 (1.3-98.7)	100.0.0 (80.5-100.0.0)	100.0.0 (2.5-100.0.0)	94.4 (72.7-99.9)

Twenty-eight of the 67 MGIT cultures had an ofloxacin DST with a smear-negative result, of which nine did not have a corresponding 'direct' FLQ result, equating to 19 evaluable 'direct' FLQ LPA results.

The sensitivity of the LPA from direct smear-negative specimens is 50.0%, whereas the specificity is 100.0%.This demonstrates that, although the LPA is not reliable for the detection of resistance to FLQ from direct smear-negative specimens, the LPA is reliable in detecting FLQ sensitivity this is re-confirmed with the NPV of 94.4%. The outcome has not been validated on both the MTBDR*plus*

(version 1) and MTBDRs*l* assays. However, the results are useful to clinicians when determining whether or not to include FLQ in the treatment regimen of their patients, especially in a population with an 80.0% prevalence of HIV co-infection. These patients generally present with paucibacillary TB which equates to a high percentage of smear-negative specimens.

3.4.3.1.3 Ofloxacin: microscopy smear-positive specimens

Table 3.7 (c) The 2x2 tables used for the calculations for OFX resistance/susceptibility

	MGIT (resistant)	**MGIT (sensitive)**	
LPA (resistant)	9	0	9
LPA (sensitive)	0	26	26
	9	26	35

Prevalence (%) 95% CI	**Sensitivity (%) 95% CI**	**Specificity (%) 95% CI**	**PPV (%) 95% CI**	**NPV (%) 95% CI**
25.7 (12.5-43.3)	100.0.0 (66.4-100.0.0)	100.0.0 (86.8-100.0.0)	100.0.0 (66.4-100.0.0)	100.0.0 (86.8-100.0.0)

Thirty-seven of the 67 MGIT cultures had an ofloxacin DST with a smear-positive result, of which 2 had no corresponding ‘direct’ FLQ LPA result, equating to 35 evaluable ‘direct’ FLQ, smear-positive LPA results.

The MTBDRs*l* assay is accurate in detecting FLQ resistance when applied to direct smear-positive specimens, according to the sensitivity and specificity of 100.0%each. The ability of the LPA to detect both resistance and sensitivity is also accurate according to the PPV and NPV of 100.0%, respectively. This re-enforces the comment made in ‘overall direct smear’ section for ofloxacin stating that the MTBDRs*l* tool would be valuable as an immediate follow-on diagnostic tool after the initial diagnosis of MDR-TB within a patient/s.

3.4.3.1.4 Ofloxacin: culture isolates

Table 3.7 (d) The 2x2 tables used for the calculations for OFX resistance/susceptibility

	MGIT (Resistant)	MGIT (sensitive)	
LPA (Resistant)	13	2	15
LPA (Sensitive)	0	43	43
	13	45	58

Prevalence (%) 95% CI	Sensitivity (%) 95% CI	Specificity (%) 95% CI	PPV (%) 95% CI	NPV (%) 95% CI
22.4 (12.5-35.3)	100.0.0 (75.3-100.0.0)	95.6 (84.9-99.5)	86.7 (59.5-98.3)	100.0.0 (91.8-100.0.0)

All 67 of the MGIT cultures had an ofloxacin DST result. However, 9/67 assays were not performed as isolates were erroneously discarded by the NHLS before an LPA could be performed. This equated to 58 evaluable 'culture' FLQ LPA results.

The sensitivity, specificity, PPV and NPV of MTBDR*sl* in the detection of FLQ resistance in culture-positive isolates is 100.0%, 95.6%, 95.7% and 100.0%, respectively. Hence, this LPA is highly accurate in determining fluoroquinolone resistance (e.g. ofloxacin) from TB DNA extracted from MGIT positive isolates.

3.4.3.3 Aminoglycosides (AG): Kanamycin

3.4.3.3.1 Kanamycin: direct LPAs

Table 3.8 (a) The 2x2 tables used for the calculations for KAN resistance/susceptibility

	MGIT (resistant)	MGIT (sensitive)	
LPA (resistant)	3	2	5
LPA (sensitive)	4	45	49
	7	47	54

Prevalence (%) 95% CI	Sensitivity (%) 95% CI	Specificity (%) 95% CI	PPV (%) 95% CI	NPV (%) 95% CI
13.0 (5.4-24.9)	42.9 (9.9-81.6)	95.7 (85.5-99.5)	60.0 (14.7-94.7)	91.8 (80.4-97.7)

Sixty-seven of the MGIT culture isolates had a kanamycin DST result, of which 13 did not have a corresponding ‘direct’ AG/CP LPA result, equating to 54 evaluable ‘direct’ AG/CP LPA results (capreomycin resistance was not tested by phenotypic methods).

From the overall direct smear-positive and negative specimens, the MTBDR*s*/ performs poorly in the detection of aminoglycoside/capreomycin (AG/CP) resistance (e.g. kanamycin). This is demonstrated by the sensitivity and PPV indices of 42.9% and 60.0%, respectively. However, the ability for the LPA to confirm AG/CP sensitivity is high according to the specificity and NPV indices of 95.7% and 91.8%, respectively.

3.4.3.3.2 Kanamycin: microscopy smear-negative specimens

Table 3.8 (b) The 2x2 tables used for the calculations for OFX resistance/susceptibility

	MGIT (Resistant)	MGIT (sensitive)	
LPA (Resistant)	1	1	2
LPA (Sensitive)	2	13	15
	3	14	17

Prevalence (%) 95% CI	Sensitivity (%) 95% CI	Specificity (%) 95% CI	PPV (%) 95% CI	NPV (%) 95% CI
17.6 (3.8-43.4)	33.3 (0.8-90.6)	92.9 (66.1-99.8)	50.0 (1.3-98.7)	86.7 (59.5-98.3)

Twenty-eight of the MGIT cultures had a kanamycin DST with a smear-negative result. However, 11/28 did not have a corresponding ‘direct’ LPA, thus equating to 17 evaluable ‘direct’ AG/CP LPA results.

The MTBDR*s*/ assay is poor at detecting AG/CP resistance from direct smear-negative specimens according to the sensitivity and PPV indices of 33.3% and 50.0%, respectively.

3.4.3.3.3 Kanamycin: microscopy smear-positive specimens

Table 3.8 (c) The 2x2 tables used for the calculations for OFX resistance/susceptibility

	MGIT (Resistant)	MGIT (sensitive)	
LPA (Resistant)	2	1	3
LPA (Sensitive)	2	30	32
	4	31	35

Prevalence (%) 95% CI	Sensitivity (%) 95% CI	Specificity (%) 95% CI	PPV (%) 95% CI	NPV (%) 95% CI
11.4 (3.2-26.7)	50.0 (6.8-93.2)	96.8 (83.3-99.9)	66.7 (9.4-99.2)	93.8 (79.2-99.2)

Thirty-seven of the MGIT cultures had a kanamycin DST with a smear-positive result. However, 2/37 did not have a corresponding 'direct' AG/CP LPA result, thus equating to 35 evaluable 'direct' AG/CP LPA results.

3.4.3.3.4 Kanamycin: culture isolates

Table 3.8 (d) The 2x2 tables used for the calculations for OFX resistance/susceptibility

	MGIT (resistant)	MGIT (sensitive)	
LPA (resistant)	3	0	3
LPA (sensitive)	4	51	55
	7	51	58

Prevalence (%) 95% CI	Sensitivity (%) 95% CI	Specificity (%) 95% CI	PPV (%) 95% CI	NPV (%) 95% CI
12.1 (5.0-23.3)	42.9 (9.9-81.6)	100.0.0 (93.0-100.0.0)	100.0.0 (29.2-100.0.0)	92.7 (82.4-98.0)

Sixty-seven MGIT cultures had a kanamycin DST result, of which nine did not have a corresponding 'culture' AG/CP LPA result, thus equating to 58 evaluable 'culture' AG/CP LPA results.

Overall, the MTBDR*sl* is poor at detecting AG/CP resistance from either direct smear specimens or MGIT culture isolates. However, from culture isolates, AG/CP sensitivity is assured by the specificity and NPV indices of 100.0% and 92.7%, respectively.

3.4.4 Summary: Direct LPAs

The specificity and NPV indices for the MTBDR*plus* (*version 1*) assay were assumed to be 0.00% for each, as a drug-sensitive control group was not used in this study. However, specificity and NPV indices were included for ofloxacin and

kanamycin, as the MDR-TB patients were considered the control group for the MTBDR*sl* assay.

3.4.4.1 Sensitivity: MTBDRplus (version 1)

3.4.4.1.1 Microscopy smear-negatives (n=36)

Comment: MTBDR*plus (version 1)* has not been validated for microscopy smear-negatives.

On direct smear, negative specimens, the sensitivities for RIF and INH were 94.1% (95% CI: 71.3-99.9%) and 100.0% (95% CI: 80.5-100.0%), respectively. However, it must be taken into consideration that about 25.0% of the LPAs were not interpretable, reconfirming that the MTBDR*plus (version 1)* is not validated for smear negative specimens.

3.4.4.1.2 Microscopy smear-positives (n=70)

On direct smear-positive specimens, the sensitivities of the MTBDR*plus* (*version 1*) LPAs were 94.3% (95% CI: 80.8-99.3%) and 97.1% (95% CI: 85.1-99.9%) for RIF and INH, respectively.

3.4.4.2 Sensitivity and specificity: MTBDRsl

3.4.4.2.1 Microscopy smear-negatives (n=36)

Comment: MTBDR*sl* not validated for microscopy smear-negatives.

The sensitivity and specificity of the MTBDR*sl* LPAs on microscopy smear-negative specimens were 50.0% (95% CI: 1.3-98.7%) and 100.0% (95% CI: 80.5-100.0%), respectively for ofloxacin, and, 33.3% (95% CI: 0.8-90.6%) and 92.9% (95% CI: 66.1-99.8%), respectively for kanamycin.

3.4.4.2.2 Microscopy smear-positives (n=70)

On direct microscopy smear-positive specimens, the sensitivity and specificity were 100.0% (95% CI: 66.4-100.0%) and 100.0% (95% CI: 86.8-100.0%), respectively for ofloxacin, and, 50.0% (95% CI: 6.8-93.2%) and 96.8% (95% CI: 83.3-99.9%), respectively for kanamycin.

3.4.4.3 Positive and negative predictive values

3.4.4.3.1 Microscopy smear-negatives

The positive predictive value (PPV) for RIF and INH were 94.1% (95% CI: 71.3-99.9%) and 94.4% (95% CI: 72.2-99.9%), respectively. The PPV and NPV for ofloxacin and kanamycin were 100.0% (95% CI: 2.5-100.0%), 94.4% (95% CI: 72.7-99.9%) and 50.0% (95% CI: 1.3-98.7%), 86.7 (95% CI: 59.5-98.3%), respectively.

3.4.4.3.2 Microscopy smear-positives

The positive predictive value (PPV) for the *MTBDRplus (version 1)* assay was 94.3% (95% CI: 80.8-99.3%) for RIF and 97.1% (95% CI: 84.7-99.9%) for INH. The PPV and NPV for ofloxacin was 100.0% (95% CI: 66.4-100.0%) and 100.0% (95% CI: 86.8-100.0%), respectively, for kanamycin the PPV and NPV were 66.7% (95% CI: 9.4-99.2%) and 93.8% (95% CI: 79.2-99.2%), respectively.

3.4.5 Summary: culture LPAs

3.4.5.1 Sensitivity and specificity

The sensitivity of the *MTBDRplus (version 1)* and MTBDR*sl* tests were 98.2% (95% CI: 90.6-100.0%), 94.5% (95% CI: 84.4-98.6%) for RIF and INH, respectively

and 100.0% (95% CI: 75.3-100.0%) and 42.9% (95% CI: 9.9-81.6%) for ofloxacin and kanamycin, respectively.

The specificity of ofloxacin and kanamycin were 95.6% (95% CI: 84.9-99.5%) and 100.0% (95% CI: 93.0-100.0%), respectively.

3.4.5.2 Positive and negative predictive values

The PPV for the *MTBDRplus (version 1)* was 98.2% (95% CI: 90.6-100.0%) and 94.5% (95% CI: 84.9-98.9%) for RIF and INH, respectively. The PPV and NPV for ofloxacin and kanamycin were 86.7% (95% CI: 59.5-98.3%), 100.0% (95% CI: 91.8-100.0%) and 100.0% (95% CI: 29.2-100.0%), 92.7% (95% CI: 82.4-98.0%), respectively.

3.5 Limitations to the study

Limitations to the study included:

- The predictive indices of the direct smear-positive and smear-negative LPA results should be considered with reservation, as the numbers in this study are small
- Study population only included previously diagnosed MDR-TB patients from a peripheral clinic either by MGIT drug susceptibility test (DST) or rifampicin resistance by the GenoType® MTBDR*plus* (*version 1*) assay
- No TB drug-sensitive specimens were collected within the study population mentioned above. Thus no ‘sensitive’ control group was incorporated into the study. Hence, the specificities and negative predictive values could not be determined

- All of the GenoType® *MTBDRplus (version 1)* and MTBDR*sl* assays were performed by Lynsey E. Isherwood, limiting the manpower of the study procedures. This, in turn, lengthened the turn-around-time (TAT) of the LPAs from two days to approximately seven days (refer to next bullet point below)
- Decontaminated sputum specimens (pellets) and MGIT isolates were collected once a week (on Tuesday of each week). Specimens could only be collected once the phenotypic results of each had been recorded onto the NHLS DISA system (electronic data capture system from which gold standard results are collected, stored and disseminated when required)
- Only 20/40 contaminated isolates were subjected to the LPAs, the other 20 were erroneously discarded. Although no gold standard MGIT DSTs were available for comparison, interpretable results were obtained and were thus included for interest.

Table 3.9 Summary of prevalence of MDR-TB, as well as the sensitivity and positive predictive value (PPV) of the GenoType® MTBDR*plus* (*version 1*)assay, compared to phenotypic MGIT 960 drug susceptibility tests.

GenoType® MTBDR*plus* (*version 1*)

Specimen type	n	Prevalence (%) 95% CI	Sensitivity (%) 95% CI	PPV (%) 95% CI
RIF overall direct smears	56	94.6 (85.1-98.1)	98.1 (89.9-100.0)	94.5 (84.9-98.9)
*RIF microscopy smear-negatives	18	94.4 (72.7-99.9)	94.1 (71.3-99.9)	94.1 (71.3-99.9)
*RIF microscopy smear-positives	35	94.3 (80.8-99.3)	100.0 (89.4-100.0)	94.3 (80.8-99.3)
RIF culture isolates	58	98.3 (90.8-100.0)	98.2 (90.6-100.0)	98.2 (90.6-100.0)
INH overall direct smears	55	96.4 (87.5-99.6)	98.1 (89.9-100.0)	96.3 (87.3-99.5)
*INH microscopy smear-negatives	18	94.4 (72.7-99.9)	100.0 (80.5-100.0)	94.4 (72.7-99.9)
*INH microscopy smear-positives	35	97.1 (85.1-99.9)	97.1 (84.7-99.9)	97.1 (84.7-99.9)
INH culture isolates	58	94.8 (85.6-98.9)	94.5 (84.9-98.9)	94.5 (84.9-98.9)

*The predictive indices of the direct smear-positive and smear-negative LPA results should be considered with reservation, as the numbers in this study are small.

Table 3.10 Summary of prevalence of XDR-TB, as well as the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the GenoType® MTBDR*sl* assay, compared to phenotypic MGIT 960 drug susceptibilitytests.

GenoType® MTBDR*sl*

Specimen type	n	Prevalence (%)	Sensitivity (%) 95% CI	Specificity (%) 95% CI	PPV (%) 95% CI	NPV (%) 95% CI
ofloxacin overall direct smears	56	19.6 (10.2-32.4)	90.9 (58.7-99.8)	100.0 (92.1-100.0)	100.0 (69.2-100.0)	97.8 (88.5-99.9)
*ofloxacin microscopy smear-negatives	19	10.5 (1.3-33.1)	50.0 (1.3-98.7)	100.0 (80.5-100.0)	100.0 (2.5-100.0)	94.4 (72.7-99.9)
*ofloxacin microscopy smear-positives	35	25.7 (12.5-43.3)	100.0 (66.4-100.0)	100.0 (86.8-100.0)	100.0 (66.4-100.0)	100.0 (86.8-100.0)
ofloxacin culture isolates	58	22.4 (12.5-35.3)	100.0 (75.3-100.0)	95.6 (84.9-99.5)	86.7 (59.5-98.3)	100.0 (91.8-100.0)
kanamycin overall direct smears	54	13.0 (5.4-24.9)	42.9 (9.9-81.6)	95.7 (85.5-99.5)	60.0 (14.7-94.7)	91.8 (80.4-97.7)
*kanamycin microscopy smear-negatives	17	17.6 (3.8-43.4)	33.3 (0.8-90.6)	92.9 (66.1-99.8)	50.0 (1.3-98.7)	86.7 (59.5-98.3)
*kanamycin microscopy smear-positives	35	11.4 (3.2-26.7)	50.0 (6.8-93.2)	96.8 (83.3-99.9)	66.7 (9.4-99.2)	93.8 (79.2-99.2)
kanamycin culture isolates	58	12.1 (5.0-23.3)	42.9 (9.9-81.6)	100.0 (93.0-100.0)	100.0 (29.2-100.0)	92.7 (82.4-98.0)

*The predictive indices of the direct smear-positive and smear-negative LPA results should be considered with reservation, as the numbers in this study are small.

Chapter 4

4.0 Discussion

4.1 Introduction

“For 2 weeks in January, India coughed and the rest of the world paid attention”. This was India’s first outbreak of ‘totally drug-resistant tuberculosis’ (TDR-TB) in January 2012, a term loosely used to described a strain of TB resistant to all second-line TB drugs (101).In a report by the World Health Organisation (WHO), they clearly state that the emergence of TDR-TB is a wake-up call for all Ministries of Health (102).

"Urgent action is needed", says Dr Josè A Caminero, lead author of The Union's Guidelines for the Clinical and Operational Management of Multidrug-Resistant Tuberculosis. "But the first important message that we want to send to both patients and everyone tasked with managing TB patients is that, with good clinical and operational case management, all forms of drug-resistant TB (DR-TB) have the potential for cure, including those cases with a very extensive pattern of resistance" (103).

Dr Josè A Caminero gave this statement on World TB Day on 24 March 2013 on behalf of International Union Against Tuberculosis and Lung Disease (The Union). Based on The Union’s 93 years of field-work, they are certain that M/XDR-TB can be prevented if national TB programmes built-in solid policies for the management of drug-sensitive TB.

It can therefore be concluded that, the rapid diagnosis of drug-resistant TB may not necessarily be the weakest link, but rather the lack of solid national TB control

programs. Hence, a successful national roll-out and sustainability of rapid LPA diagnostics must be backed by a strong health system in order to prevent the collapse thereof.

An important step of the Global Plan to Stop TB and the World Health Organisation's (WHO's) new global Stop TB Strategy, is to develop new tools for use in tuberculosis (TB) control programmes (104). WHO aims to eliminate TB by 2050, however, this goal will not be met until new approaches to TB diagnostics, medical management, infection control and preventive measures, including vaccine development and use, are investigated and implemented (105).

In recent years, South Africa witnessed the emergence of XDR-TB with many of the cases occurring as a result of transmission (primary XDR-TB cases). These cases arose from a considerable pool of MDR-TB and occurred amid the high TB/HIV co-infection burden in South Africa (104) (105). There is therefore an urgency for policy makers, including the WHO to emphasise and prioritize the need to identify and endorse tools that rapidly diagnose XDR-TB: one of these tools for implementation is the GenoType® MTBDR*sl* assay. The WHO Expert Group endorsed the MTBDR*plus (version 1)* and MTBDR*sl* assays in May 2008 and February 2013, respectively (63) (106).

4.2 M/XDR-TB in South Africa

South Africa is a resource-limited, high HIV-prevalence country. TB is the leading cause of death among HIV-infected patients in South Africa. The WHO reported in 2009 that 71.0% of TB patients in South Africa are HIV positive (107). The epidemics of HIV and TB in South Africa are closely related. With the implementation of anti-retroviral therapy, the mortality rates in HIV/TB co-infected

patients have improved, but drug-resistant TB has emerged as a major cause of death (39).

The emergence and spread of MDR-TB and XDR-TB, collectively known as M/XDR-TB, in South Africa is hampering efforts for the control and management of TB. This is threatening WHO's target of diagnosing and treating 80% of estimated M/XDR-TB by 2015 (108). South Africa reports the highest number of cases of MDR-TB and XDR-TB in the Sub-Saharan region (107).

In 2010, the National Health Laboratory Service (NHLS) of South Africa diagnosed 7,386 MDR-TB and 741 XDR-TB cases indicating that approximately 10.0% of drug-resistant cases in South Africa are XDR-TB (76). This is a cause for concern as these are laboratory-based figures and the actual XDR-TB burden may be much higher due to standardised treatment of MDR-TB, which may not be appropriate in individual cases, delayed testing for resistance to second-line drugs, lack of efficient contact tracing systems and correct treatment implementation.

Fluoroquinolones (FLQs) and aminoglycosides (AG) together with other drugs such as ethionamide, cycloserine and para-amino salicylic acid (PAS) are second-line drugs used to treat MDR-TB. The treatment of XDR-TB as is the case with MDR-TB is expensive and more toxic, with longer treatment periods, compared to the treatment of drug-susceptible TB (57). Management of XDR-TB is even more challenging than that of MDR-TB. It requires appropriate drug selection for treatment which is more complex with fewer drug options than is the case for MDR-TB while implementation of realistic infection control measures is difficult to institute and maintain. Furthermore, in South Africa by far the majority of cases occur in HIV-infected patients who require antiretroviral treatment (10).

With the introduction of more sensitive and rapid first-line diagnostic tests for TB, such as Hain Lifescience line-probe assay (LPA) GenoType® MTBDR*plus (version 2),* the Cepheid Xpert® MTB/RIF (GeneXpert-GXP) (Cepheid, Sunnyvale, CA), and the increased access to TB culture and sensitivity testing, there is an increase in the detection of MDR-TB, however, further increased recognition of MDR-TB and XDR-TB is still needed.

4.3 Implications of delayed XDR-TB diagnosis within South African hospitals

Phenotypic MGIT 960 culture and drug susceptibility tests (DSTs) are cumbersome and expensive. They also take between 4 to 6 weeks to yield a definitive result, thus increasing the risk of nosocomial pre-XDR-TB and XDR-TB transmission involving patients and health care workers (HCWs) in a MDR/XDR-TB setting. The GenoType MTBDR*plus (version 1)* and MTBDR*sl* line-probe assays (LPAs) on the other hand have a turn-around-time (TAT) of 2 days (109) (65).

In 2007, Andrews, *et al.* noted that M/XDR-TB can be involved in explosive nosocomial outbreaks in HIV-infected populations in dedicated M/XDR-TB hospitals. The authors suggested that reduced transmission of M/XDR-TB amongst patients and health care workers can be accomplished by introducing a rapid DST such as genotypic LPAs (44).

A retrospective study of the case records of 334 XDR-TB patients, within the Western and Eastern Cape provinces of South Africa, from January 1996 to February 2008 Jarand, *et al.* determined that 10 of these patients were health care workers (HCWs) and that the overall mortality in these HCW patients was

40% (110). This study confirmed that XDR-TB amongst South African HCW is of concern to the TB/HIV treatment policy-makers. A limitation of this study was that the focal point of infection of these HCWs could not be identified i.e. whether the infections were acquired nosocomial or was community-based.

A startling discovery of M/XDR-TB nosocomial infections amongst HCWs was uncovered in a study conducted by O'Donnell, *et al.* in Kwa-Zulu Natal, South Africa. In this study, HCWs were found to have a five to six-fold increased rate of hospital admission for M/XDR-TB, as compared to non-HCWs (111).

4.4 Study-related challenges

The MTBDR*plus (version 1)* and MTBDR*sl* LPAs were evaluated against the gold standard MGIT 960 culture and DST. There were 2 notable problems encountered with the culture and DST conducted at the Mycobacteriology Referral Laboratory of the National Health Laboratory Services (NHLS) involving the 150: the high rate of contamination of cultures 26.7% (n=40) and the negative cultures 20.7% (n=31) on sputa from patients that were initially diagnosed as MDR-TB based on culture and DST results received at clinics before the patients were admitted to Sizwe Hospital. As a result of these two problems, 47.3% (n=71) patients, all labeled as MDR-TB, had an unknown drug resistance status requiring further investigation. Some of these may well have been pre-XDR-or even XDR-TB patients presenting the risk of transmission of highly resistant strains and consequent morbidity and mortality.

Of the 40 contaminated culture isolates 50.0% (n=20) were subjected to both the MTBDR*plus (version 1)* and MTBDR*sl* assays (LPAs). Unfortunately, the other 50% of the contaminated cultures were erroneously discarded before they could

be tested by LPA. It was however concluded from the 20 contaminated cultures tested that interpretable LPA results could be obtained from 100.0% of these cultures: The LPAs indicated that; 70.0% (n=14) were MDR-TB; 15.0% (n=3) were pre-XDR (FLQ), 10% (n=2) were XDR-TB and one was positive for MTB, but fully susceptible. In the face of high contamination rates of MGIT cultures, the study clearly demonstrates the value of processing contaminated cultures with the MTBDR*sl* assay.

The 21.0% (n=31) negative cultures at admission are of concern because it reflects numerous clinical, diagnostic and laboratory errors that may be occurring. These factors include; poor sputum quality, misdiagnosis at peripheral clinics (i.e. patients have drug-sensitive, rather than drug-resistant, strains of TB) and transcription errors of results onto the electronic database at the laboratories. Interpretable LPA results from culture-negative isolates are not possible.

4.5 Overall performance of MTBDRplus (version 1) assay

The MTBDR*plus (version 1)* assay performed well from both direct smear-negative and smear-positive specimens. The sensitivity for RIF and INH from smear-negative specimens was 94.1% and 100.0%, respectively, whereas the positive predictive value (PPV) was 94.1% and 94.3%, respectively. In a study conducted by Barnard, *et al.* within a high-volume public health laboratory in South Africa, there was a high proportion of interpretable results (80.0%) from sputum-smear-negative specimens, however, as only 25.0% of the smear-negative specimens were culture-positive, the overall yield was low (14.0-16.0%). At that time, the LPA was being utilized in South Africa as a means of the diagnosis of TB (112).

Both studies confer the reliability of the MTBDR*plus (version 1)* assay in the rapid screening of MDR-TB from direct smear-negative specimens, culture-positive specimens. However, one must bear in mind a factor that contributes towards the reliability of the MTBDR*plus (version 1)* for the wide scale screening MDR-TB. That is, it depends on the feasibility from the laboratory and its staff's point-of-view.

As South Africa is faced with a high rate of paucibacillary TB, due to the 80.0% HIV/TB co-infection population, obtaining a reliable result direct from sputum smear-negative specimens within 2 days, is beneficial to both the patient and the clinicians.

The sensitivity for RIF and INH from direct smear-positive specimens in this study was 100.0% and 97.1%, respectively, whereas Barnard, *et al.* demonstrated sensitivities of 98.9% for RIF and 94.2% for INH. In Germany, Hillemann, *et al.* concluded sensitivities of 98.1% and 90.2% for RIF and INH, respectively (112) (109). Therefore, this study demonstrated good correlation between Barnard, *et al.* and Hillemann, *et al.* for direct sputum smear-positive specimens.

4.6 Current status of the MTBDRplus (version 1) assay

An Expert Group was convened by the World Health Organisation (WHO) and the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), in March 2008. This Expert Group recommended that the MTBDR*plus (version 1)* not be tested on direct smear-negative specimens, but only from direct smear-positive specimens, or from isolates grown from either smear-positive or negative microscopy results (113).

At the time of this study, the MTBDR*plus (version 1)* had already been validated in 75 countries, including South Africa; using it as it routine LPA for the screening of MDR-TB from direct smear-positive specimens, or from culture-positive, smear-positive or negative specimens. The WHO never recommended the MTBDR*plus (version 1)* for smear-negative specimens, and thus posed a problem in a setting with a high prevalence of HIV/TB co-infection, where paucibacillary TB presents with smear-negative results.

Shortly after this study was completed, Hain Lifescience (GmbH) launched a *version 2* of the MTBDRplus assay. In a study conducted by Barnard, et al (2012), *version 2* proved to be sensitive (73.1%) and highly specific (100.0%) on culture positive samples. The presence of *Mycobacterium tuberculosis* (MTB) in smear-negative specimens, was dependent on bacillary load i.e. the presence of MTB was found in 57-58% of the smear-negative specimens. However, the sensitivity and specificity of RIF on smear-negative specimens, was 100.0% (114). This then suggests that the *version 2* is an accurate screening tool for detecting MDR-TB, in a high paucibacillary TB setting. Other benefits of the MTBDR*plus version 2* compared to its predecessor include:

- Less labour intensive
- Turn-around-time is reduced from 2 days to 5 hours
- Improved sensitivity (10-100.0 cells/ml)
- The test comes with an easy-to-use amplification mix including an optimized Hain *Taq* DNA polymerase. Hence, no sub-standard *Taq* can be utilised (before then, laboratories utilised *Taq* according to their standard operating procedures)

- The DNA isolation protocol has shifted to the GenoLyse procedure (reduces DNA extraction by 30 minutes)
- The banding patterns on the DNA strips are clearer

In many laboratories across South Africa, the MTBDR*plus* (*version 2)* has routinely replaced *version 1*. However, the implementation *version 2* is at the discretion of the management of each laboratory.

4.7 Overall performance of MTBDRsl assay

This study demonstrated that the sensitivity and specificity for the detection for ofloxacin resistance was 90.9% and 100.0%, for the overall direct testing. However, the sensitivity and specificity for kanamycin was 42.9% and 95.7%. This indicates that the MTBDR*sl* assay, on direct specimen testing, is reliable for the detection of ofloxacin resistance, whereas resistance detection of kanamycin is poor.

The same could be said for the MGIT culture-positive isolates used to test the MTBDR*sl* assay; the sensitivity and specificity for ofloxacin was 100.0% and 95.6%, whereas the sensitivity and specificity for kanamycin was 42.9% and 100.0%, respectively.

A study on the feasibility of using the MTBDR*sl* for routine screening of resistance to second-line drugs was first published by Hillemann, *et al.* in 2009 (65). The primary aim of the study was to determine the sensitivity and specificity of the new assay for the detection of FLQ, AM, CAP and EMB resistance. The sensitivity of the MTBDR*sl* assay for FLQ, AM, CAP and EMB were 90.2%, 83.3, 86.8 and

59.0%, respectively. The specificity was 99.1% for CAP and 100.0% for FLQ, AM and EMB.

Said, *et al.* evaluated the MTBDR*sl* assay within the diagnostic microbiology laboratory at Tshwane Academic Division of the NHLS at the Pretoria Hospital in Pretoria, South Africa. In this study, the MTBDR*sl* assay was evaluated on 342 MDR-TB sputum specimens, comparing the findings of this method against drug susceptibility test results of the agar proportion method for OFX, KAN, CAP and EMB.

Good correlation between the LPA and agar proportion method was achieved, however, the MTBDR*sl* assay in this study demonstrated a lower overall sensitivity than in other studies and this may be due to the non-inclusion of other important gene loci that may be more common in the study population concerned on the DNA strip that encode resistance i.e. *tlyA* for CAP resistance and *eis* for low-level KAN resistance. Sensitivity and specificity for the LPA were 70.3% and 97.7% respectively for OFX, 25.0% and 98.7% for KAN, 21.2% and 98.7% for CAP and 56.3% and 56.0% for EMB (96).

In another study conducted by Barnard, *et al.* at a high-throughput TB diagnostic laboratory in Cape Town, South Africa; it was demonstrated that the time to diagnosis of XDR-TB improved with the implementation of the MTBDR*sl* assay. The turn-around-time (TAT) was reduced by 93.3 % ($P<0.001$), as compared to phenotypic results. TAT was calculated from the date that the specimen was received, to the date in which the second-line DST result was released. The TAT time of less than 7 days was concluded within this high-volume public health

laboratory. This TAT included time of specimen collection, transport, processing and reporting (115).

4.8 Current status of the MTBDRsl assay

Before the WHO endorsement of the MTBDRs*l* assay in February 2013, WHO had recommended more large-scale studies be conducted before the MTBDRs*l* assay could utilised as a routine second-line assay: this recommendation was made at the WHO Expert Group Meeting in 2012 (60). Hence more studies were conducted, within different epidemiological locations, and the results of which were presented to the WHO Expert Group Meeting in Geneva, Switzerland in February 2013 (106). A summary of the findings were as follows:

4.8.1 WHO Expert Group recommendations for molecular LPAs in the detection of second-line anti-TB drug resistance: February 2013

Taking into account the objectives of this study, a few WHO recommendations are applicable and promote supporting evidence in the importance of screening for XDR-TB within a dedicated drug-resistant TB facility. The recommendations are:

1. Conventional culture and DST cannot be replaced by the MTBDRs*l* assay
2. All injectables and FLQ should routinely be tested in specimens from confirmed MDR-TB patients, to screen for XDR-TB
3. The Genotype MTBDRs*l* may be used as a rule-in test for XDR-TB but cannot be used to define XDR-TB for surveillance purposes
4. Molecular DST offers promise: however, only a few mutations conferring resistance have been described for most second-line drugs and testing is technically demanding and expensive

5. Prevention through strong basic TB care and proper management of drug-resistant TB still remain of the utmost importance

4.9 MTBDRsl implementation: Sizwe Tropical Disease Hospital

Once MDR-TB patients are admitted to Sizwe Hospital, it is imperative to not only confirm their MDR-TB status upon admission, but to also investigate their resistance patterns to second-line drugs (FLQ, AM/CAP and EMB) as soon as possible in order to decrease morbidity and mortality within this setting.

This study has shown that the MTBDR*sl* assays is useful for the rapid screening of resistance and sensitivity to FLQ (good rule-in assay), as well confirmatory sensitivity to AM/CAP within this MDR-TB, high HIV-prevalent cohort. Unfortunately, the MTBDR*sl* assay is not accurate in the detection of AG/CP resistance.

In 2011, 572 MDR TB cases, with 5.41% (n=31) of these being XDR-TB, were admitted to Sizwe Hospital. These statistics suggest that based on the fact that XDR- TB (FLQ) is a precursor to XDR-TB; the incidence of XDR-TB is indeed on the rise.

The prevalence of pre-XDR-TB (FLQ) in patients admitted to a TB drug-resistant hospital is higher than within the general community. It is thus expected to have a higher PPV, as the incidence of pre-XDR-TB increases within a specific population. It can therefore be understood that the MTBDR*sl* would not be an effective screening tool for pre-XDR TB at a peripheral clinic, but rather as a 'second' screening tool only once MDR-TB has been confirmed through other rapids diagnostics such as the MTBDR*plus* or GeneXpert assays.

In other words, by screening for pre-XDR TB (FLQ) at admission to Sizwe Hospital from direct smear specimens, clinicians are able to rapidly identify a patient's potential resistance to FLQ, with confidence and prescribe a drug regimen accordingly. This would contribute to the decrease in mortality, morbidity and nosocomial incidences.

The roll-out of the GeneXpert throughout South Africa has been rapid from 2011 until present. Thus, if directly followed on from the GeneXpert MTB/RIF positive results, the MTBDR*sl* assay may complement the screening of MDR-TB, by effectively indicating resistance to FLQ; which could be implied as a precursor for XDR-TB or total drug-resistant TB (TDR-TB).

The MTBDR*sl* assay demonstrated that it is an extremely useful rapid screening tool for pre-XDR TB or XDR-TB at admission to a TB-resistant hospital, where the initial diagnosis of MDR-TB is already confirmed. This tool provided clinicians with preliminary data on the resistance status of a patient's strain which could be used as a guide for initial treatment. Such early evidence-based treatment could prevent transmission of XDR-TB by inappropriate administration of drugs to strains of TB that are potentially resistant to them.

When consulting with the clinicians towards the end of this study, they conferred that, although this study provided them with rapid and accurate FLQ resistance detection at admission, the first prize would be to know the resistance patterns of individual drugs: not only for classes of drugs (i.e. FLQ and/or AG and CP). This detailed knowledge would be valuable in the prescribing of individualised treatment regimens, where the choices of second-line drugs are limited.

Chapter 5

5.0 Conclusion and recommendations

As the GenoType ® MTBDR*plus (version 1)* is routinely used in South Africa, this study suggests that the additional implementation of the MTBDR*sl* assay, on admission to a TB drug-resistant facility in South Africa, will be a valuable screening tool for XDR-TB. This would help curb the high morbidity and mortality rates, and increase cure rates by enabling clinicians to administer correct, individualised treatment regimens.

The value of the implementation of genotypic LPAs, together with clinical assessments for rapid screening of XDR-TG is schematically shown in figure 5.1.

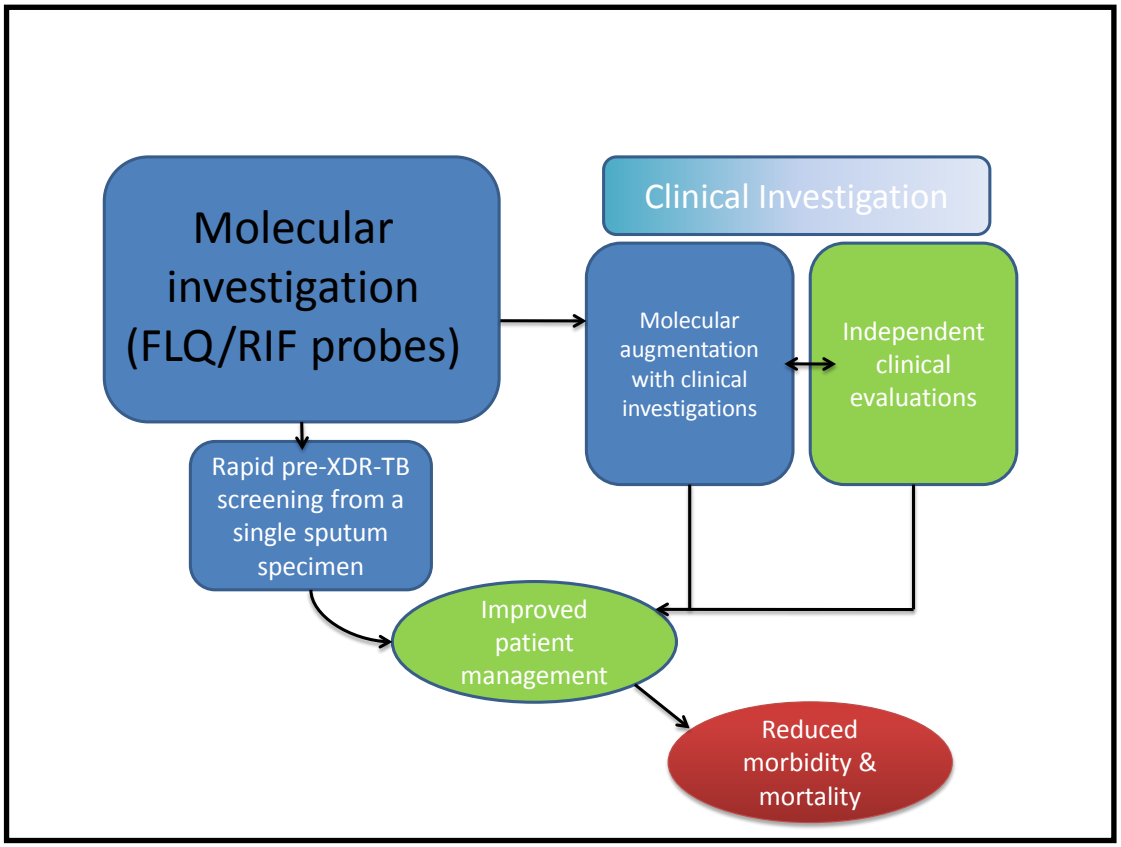


Figure 5.1 Schematic depiction of the value of the implementation of genotypic LPAs, together with clinical assessments, for the rapid screening of pre-XDR-TB (Source: Lynsey E. Isherwood).

The MTBDR*sl* assay would have to run parallel to the phenotypic MGIT culture and DST for conclusive results. However, if the patient is not clinically well at admission, the MTBDR*sl* assay may be used as a confirmatory diagnosis for second-line resistance to FLQ and/or AM/CP.

The MTBDR*sl* assay will also be an effective 'secondary' screening tool, followed-on from GXP MTB/RIF positive results. The LPA can provide clinicians with an accurate pre-XDR TB (FLQ) diagnosis, even from smear-negative specimens, which is advantageous in a setting with a high rate of paucibacillary TB.

FLQ resistance can be used as a marker for the early detection of XDR-TB and is critical in the tailoring of second-line treatment regimens.

This study demonstrated that the MTBDR*sl* assay is a good 'rule-in' assay for the detection of ofloxacin resistance (i.e. if the result shows resistance, then the isolate is probably resistant) and 'rule-out' assay for the detection of kanamycin resistance (a "susceptible" result on LPA would not reliably exclude kanamycin resistance).

The implementation of both the MTBDR*plus (version 1)* and MTBDR*sl* assays provide a quick and effective tool for the diagnosis of M/XDR-TB. However, performance of these LPAs is limited due to the complex infrastructure and highly-skilled personnel that is needed. Ultimately, a point-of-care assay is required for the diagnosis of XDR-TB.

South Africa has courageously and rapidly rolled out the GeneXpert (a rapid PCR assay that determines MDR-TB within 2 hours, using RIF as a marker). However, due to the increasing incidence of XDR-TB, a second, or improved version of GeneXpert, is urgently needed using the *gyrA* and *gyrB* gene probes (for FLQ resistance detection) as markers.

Incorporation of the *gryA* and *gyrB* gene probes in the GXP assay would alleviate the need for a 'secondary' screening tool for XDR-TB. The rapid detection (less than 2 hours) of FLQ resistance, together with MTB/RIF, from a single sputum specimen, in a single GXP cartridge would create a simple and effective diagnostics tool.

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Appendix A: Baseline data collection

Baseline Data Collection

Date: | | | | | | | | | |

D D M M Y Y Y Y

PID: | | | | / | | | |

TE Number: _____

Date of admission | | | | | | | | |

D D M M Y Y Y Y

Hospital number: | | | | | / | | |

Age | | | DOB | | | | | | | | |

D D M M Y Y Y Y

Ethnicity

- [ ] Black
- [ ] White
- [ ] Colored
- [ ] Asian
- [ ] Other

Country of Origin: _____

If not from South Africa, how long have you been living in South Africa for? Years Months

If South African, what is your province of origin: _____

Which province of South Africa have you been living in for the last 2 years? _____

Home Environment:

What kind of dwelling to you live in?

- ☐ House
- ☐ Hostel
- ☐ Suburbs
- ☐ Informal settlement
- ☐ Homeless
- ☐ Flat
- ☐ Other (please specify): _____

How many **adults** live with you? ☐☐

Are any of these adults currently taking TB medication? ☐ Yes ☐ No ☐ Unsure ☐ Do not want to answer

How many **children**, ≤ 16 years, live with you? ☐☐

Are any of these children currently taking TB medication? ☐ Yes ☐ No ☐ Unsure ☐ Do not want to answer

Employment:

Are you employed: ☐ Yes ☐ No

If so, what type of environment do you work in?

- ☐ Hospital
- ☐ Clinic
- ☐ Mining
- ☐ Construction / Building
- ☐ School
- ☐ University
- ☐ Office administration
- ☐ Farming
- ☐ Company hygiene / cleaning
- ☐ Domestic hygiene / cleaning
- ☐ Other (please specify) _____

HIV History (prior to admission)

Have you ever been tested for HIV? ☐ Yes ☐ No

If yes, date of test:

D	D	M	M	Y	Y	Y	Y

If yes, what was the result? ☐ positive ☐ negative

Thus, current HIV status ☐ positive ☐ negative ☐ unknown

ARV naïve at admission?: ☐ Yes ☐ No

CD4 count history (prior to admission)

Have you ever had a CD4 test done? ☐ Yes ☐ No

If yes, what was your value? _____ Date: D D M M Y Y Y Y

_____ Date: D D M M Y Y Y Y

Viral load history (prior to admission)

Have you ever had a viral load test done? ☐ Yes ☐ No

If yes, what was your value? _____ Date: D D M M Y Y Y Y

_____ Date: D D M M Y Y Y Y

TB history (prior to admission)

Have you had previous TB episodes? ☐ Yes ☐ No

Were you ever given injections? ☐ Yes ☐ No

Episodes of TB: Date diagnosed: D D M M Y Y Y Y

Treatment length Months **Completed Rx** Yes No Other (please specify):

Date diagnosed: D D M M Y Y Y Y

Treatment length Months Completed Rx Yes No

Date diagnosed: D D M M Y Y Y Y

Treatment length Months Completed Rx Yes No Other (please specify):

Are you currently on TB treatment? Yes No

Please 'tick' which anti-TB drugs you have previously taken:

INH RIF PZA Ethambutol Capreomycin

Streptomycin Kanamycin Ethionamide Ofloxacine Ciprofloxacin

PAS Other: (please state)

TB-hospital admission history (prior to current admission):

Have you ever been admitted into a TB-hospital? ☐ Yes ☐ No

If so, how many times have you been admitted? ☐

If so, where? First time: _____

Second time: _____

For how long? First time: ☐☐ **Days** ☐☐ **Months**

Second time: ☐☐ **Days** ☐☐ **Months**

Did you complete Rx? First time: ☐ Yes ☐ No

Second time: ☐ Yes ☐ No

Did you abscond? First time: ☐ Yes ☐ No

Second time: ☐ Yes ☐ No

Direct microscopy smear results (prior to admission):

☐ Yes ☐ No

		Stain type (if unknown, please state 'unk')			Smear Result				
Date	**Lab No.**	**Ziehl-Neelson (ZN)**	**Auramine**	**Fluo**	**Neg**	**Scanty**	**AFB+**	**AFB++**	**AFB+++**

Culture results (prior to admission):

☐ Yes ☐ No

Date	Lab No.	Pos/Neg	Organism	Strep	INH	RIF	Ethambutol		Ethionamide		Kanamycin		Ofloxacin	

S = Sensitive R = Resistant ND = Not Done

PCR results (prior to admission):

☐ Yes ☐ No

Date	Lab No.	Pos/Neg	Organism	INH	RIF

S = Sensitive R = Resistant ND = Not Done

Appendix B: Patient information and consent form

PARTICIPANT INFORMATION AND CONSENT FORM

Important Notice: Each participant must receive, read and/or understand this document before any study-related procedures can be performed.

Protocol Number: CHRU-GLPA01

Title:

'The evaluation of rapid screening of M/XDR-TB patients within a dedicated M/XDR-TB hospital in Gauteng, South Africa'.

Sponsor:

Clinical HIV Research Unit (CHRU), Wits Health Consortium, University of the Witwatersrand, Johannesburg, South Africa.

Principal Investigator (Scientific):

Ms Lynsey E. Isherwood, Clinical HIV Research Unit (CHRU), Wits Health Consortium, University of the Witwatersrand, Johannesburg, South Africa.

Principal Investigator (Clinical):

Dr Francesca M. Conradie, Clinical HIV Research Unit (CHRU), Wits Health Consortium, University of the Witwatersrand, Johannesburg, South Africa.

PARTICIPANT INFORMATION LEAFLET

Good day, my name is:

Surname:

First name:

I am a (insert designation):

The Principal Investigators on this study are Lynsey Isherwood and Francesca Conradie. Their telephone number is: (011) 531-4347.

I would like to invite you to consider participating in a research study called;'***The evaluation of rapid screening of M/XDR-TB patients within a dedicated M/XDR-TB hospital in Gauteng, South Africa'***

Before you agree to participate in the above study, it is important that you read and/or understand the following explanation of the purpose of the study, as well as your responsibility within the study. If you are *unable* to read, you will be advised to have an impartial witness to sit with you during the consent procedure. An impartial witness is someone who can listen and sign on your behalf; this person must not be bias by the study. A 'study' is a project that involves research.

The below information sheet is to inform you, in detail, about the study and to decide whether or not you would like to participate. Please note that participation on the study is completely voluntary and hence you may withdraw at *any* time. This decision will *not* adversely affect your ongoing treatment in any way.

If you have any questions at *any* time during and after the consent procedure, please feel free to present these questions to me. Thus, you should not sign consent unless all your questions have been answered to your satisfaction.

As you are within a clinical institution that is interested in your well-being, I ask that you be completely honest about your health history. If you agree to participate, and are satisfied with the study and understand your participation, you are asked to please sign and date the consent document at the end of the consent procedure. A copy of this information leaflet and the consent form will be given to you to keep. The original documents will be kept in a file at the research facility within Sizwe Hospital.

A. PURPOSE OF STUDY

You have been referred to Sizwe Hospital as you have a strain (type) of TB that is resistant to the only two drugs (known as first-line drugs) that are used to treat TB; namely Isoniazid (INH) and Rifampicin (RMP). Now, you will need to have further tests done to confirm which other drugs you may be resistant to. Thereare three tests that are performed as part of your standard of care within this hospital.These include:

1. **Sputum microscopy smear:** This is a 'reading' from the microscope to count the number of 'bacilli' in your sputum. Bacilli are the microscopic 'bugs' that cause the disease *Mycobacterium tuberculosis*. The results of these tests are available very quickly.
2. **Sputum TB-culture:** Your sputum sample is placed into a culture tube and the 'bugs' are left to grow under controlled conditions.
3. **ADrug Susceptibility Test (DST):** This is performed on your culture, after the 'bugs' have grown. This test then confirms which other drugs you are resistant to. It is at this point that the clinicians will decide if you have the multi-drug resistant (MDR) or extensively-drug resistant (XDR) strain of TB.

However, the results of test number 2 and 3 can take anything from 4 to 6 weeks to obtain a result.

This study will be testing a different type of TB drug-resistant test in the laboratory (this is the experimental part of the study). There are two new, rapid tests; namely the MTBDR*plus* and MTBDR*sl* tests. These are able to detect different levels of TB drug-resistance. These tests use the genes of the TB bug, together with a special machine in the laboratory, to detect TB drug-resistance.

These new tests are believed to obtain M/XDR-TB results within 2 days; and not 4 to 6 weeks as with the current tests. Although these tests have proven to work in other clinics and laboratories, they have not been used in a clinical setting such as Sizwe Hospital.

The *main* purposes of the study are as follows;

1. Calculate the specificity (how specific the new test is to your 'bug'), sensitivity (how sensitive the new test is to your 'bug') of the new rapid tests, when compared to thestandard of care in at Sizwe Hospital (i.e. tests 1, 2 and 3 above).
2. Measure the time difference (in days) between the result of the new rapid test and the results of tests 1, 2 and 3 above.
3. To analyze the value of providing the clinicians at Sizwe Hospital with the new rapid test results. This includes the separation of new and re-admitted patients to the appropriate drug-resistant ward.

B. STUDY PROCEDURES

Two early morning sputum samples are required as part of your standard of care within Sizwe Hospital. Early morning sputum samples are the best quality of samples. These sputum samples contain the TB 'bugs'. Thus, during the morning on the day after admission, these two sputum samples will be collected from you. For this study, we would like to use one of these sputum samples. The same sputum sample that is collected as part of your standard of care will be used for the new rapid tests. Thus, no extra sputum is required from you, unless the sample is contaminated (if the TB 'bugs' are not 'readable' in the laboratory). Please note that this study will not use your sputum sample for the new rapid tests, unless you have given consent to do so. Please note that your standard of care will not be affected by the simultaneous use the new rapid tests. In other words, the doctors will still receive your standard test results for your sputum microscopy smear, TB-culture and DST, and you will still receive the standard treatment as prescribed by the clinicians at Sizwe Hospital.

Only one sputum sample is required, and that is your 'admission' sample as described above. Please note that, for this study, no further sputum samples or tests are required at any other time-point during your treatment. The only other time that another sputum sample may be requested, is if the laboratory has a contaminated result. Should this happen, you will be notified and arrangements will be made to collect another sputum sample from you.

The investigators would also like to collect and analyse baseline data from you. A questionnaire will be used for this. You will only be required to complete the questionnaire once. This will only be done after you have consented to participate in the study. A copy of the data collection sheet will be given to you to look at, together with this consent form. All the data collected on this questionnaire will be referenced to your anonymous study number. Thus, *none* of the data used from this questionnaire, will be used to disclose who you are.

C. CONFIDENTIALITY

All the above information will be kept confidential and included into your study file/hospital file. Only those staff members involved in the study, as well as the clinicians at the hospital, will have access to your file. Your information will also be stored on a computer. You will be allocated a 'study number' on the computer. Thus your name will not be entered onto the data system. Also, no information that will disclose who you are will be used.

As this is a research site, we have the obligation to publish our results in medical journals and at relevant medical conferences. Please note, however, that all data collected for these publications and abstracts, will be done so with the strictest confidentiality. I stress again, that *no* information, that will identify you, will be used.

The University of the Witwatersrand Human Research Ethics Committee (HREC) may request an inspection of all documentation relating to this study. This may include your information. Thus, by agreeing to participate in the study, you authorize me to release your medical records to the HREC. Also, your personal doctor may want to peruse your medical records. Again by agreeing to participate, you authorize me to release your records to your doctor.

D. BENEFITS

Although the results of this study may not benefit you personally, the results of this study may be useful in diagnosing and treating drug-resistant strains of TB more rapidly in the future. Thus potential M/XDR-TB patients can be identified more rapidly and correct medication can be

administered quicker too. This should result in lowered morbidity and mortality rates in M/XDR-TB patients.

1. **Study withdrawal:** it must be clearly emphasized that participation is voluntary and that you may decide to discontinue from the study at any time. This decision will *not* adversely affect your ongoing treatment. However, I retain the right to withdraw you from the study, if it is in *your* best interest.
2. **New findings on the study:** The investigators will keep the academic institution & clinicians informed as to the progress of the study, which includes any new findings that occur.

E. RISKS

There will be no risk to participating in this study. All standard laboratory procedures will continue together with this study. Thus, you will be not excluded from standard care in the hospital. You will only have an extra two types of 'tests' done at the same, as your standard of care tests.

F. FINANCIAL ASPECTS OF THE STUDY

1. **Voluntary participation:** As participation is voluntary, no monies will be paid to you.
2. **Study funding:** This study will be completed self-funded through sponsors. Thus the cost of all study procedures, as a direct result of this research study, will be funded by the sponsors. Neither you nor your medical aid will be liable for any procedures directly related to this study.

G. ETHICS APPROVAL

This study has been submitted to theUniversity of the Witwatersrand, Human Research Ethics Committee (HREC) and has been granted written approval. This is an independent committee established to help protect the rights of research participants.

The study has been designed according to both the ICH Good Clinical Practice (GCP) guidelines (version 2002) as well as the South African Good Clinical Practice Guidelines (version 2006). These guidelines enforce the ethical application of clinical research on human participants. No members of staff, who are conducting the research project, have any personal financial gain by doing so. Hence, our actions will not be bias.

H. **ADDITIONAL SOURCE OF INFORAMTION**

If you are in need of any information with respect to your rights as a participant on this research project, or, if you are unhappy with the research project, you may contact the Chairperson of theUniversity of the Witwatersrand HREC, namely; Professor Cleaton-Jones. The telephone number is: (011) 717-2301.

Also, you may contact one of the principal investigators; namely Lynsey Isherwood or Dr Francesca Conradie on (011) 531-4347.

PATIENT CONSENT FORM

I, Surname:

First name:

Hereby understand the information given to me in the patient information leaflet and thus give permission for my sputum samples to be collected and used in this study. I also understand and grant permission for the collection of certain other information such as described in the Patient Information Leaflet.

I also understand and grant permission to the following:

1. A portion of my sputum sample will be stored at the laboratory until they are needed for the new rapid tests.
2. The data collected from this study will be published in medical journals and at relevant medical conferences, however, all data collected for these publications and abstracts will be done so with the strictest confidentiality and that no data, that reveals my identification, will be used.
3. The University of the Witwatersrand Human Research Ethics Committee (HREC) may inspect my files.
4. My own doctor to inspect my files.
5. If I do give consent now, I will be able to withdraw my consent at any stage of the study and that this decision will not discriminate me from my ongoing treatment. Thus, I will continue to receive treatment agreed to before consenting to the study.

_____ _____

Signature (Participant) Date

_____

Name of Witness (if applicable)

_____ _____

Witness' signature Date

_____

Investigator's name

_____ _____

Investigator signature Date