Phenotypic and genotypic susceptibility testing of *Mycobacterium tuberculosis* cultures from Tshwane Metropolitan

By

OLUWASEGUN OLAYINKA ATANDA

Submitted in partial fulfilment of the requirements for the degree

Master of Science (Medical Microbiology)

Department of Medical Microbiology
Faculty of Health Sciences
University of Pretoria
Pretoria
South Africa

2015

STATEMENT OF ORIGINAL AUTHORSHIP

I declare that the dissertation, which I hereby submit for the degree MSc (Medical Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution. I further declare that all sources cited or quoted are specified and recognised by means of an inclusive list of references.

SIGNATURE: ..... DATE: .....

"TB is the child of poverty - and also its parent and provider",

Archbishop Desmond Tutu, in this quote

encapsulated the link between tuberculosis (TB) and poverty!

ACKNOWLEDGEMENTS

As a token of gratitude and appreciation, I would like to thank and acknowledge:

Almighty GOD for granting me the strength and wisdom to complete this project. It would not have been possible without his grace.

My supervisors Prof NM Mbelle and Dr MR Lekalakala for the academic inputs they have invested in me. *'A candle loses nothing by lighting another candle'*. This is how I appreciate the relationship I have enjoyed with you during the last two years. Thank you for all the time and effort you have put into my scientific development for which I am truly grateful.

Mr Lesibana Malinga of the TB unit, Medical Research Council, Pretoria for your assistance and encouragement during the course of this research project.

My wife Aanuoluwapo Atanda and daughter Toluwani Atanda, who have been amazingly supportive and understanding during my MSc journey. Thank you so much for pushing me when I needed a shove and pulling me back when I needed a break. You lightened the burden right from the start and carried it until the end. I can only try to repay my debt to you.

My colleagues in the department for all the wonderful times spent both inside and outside the laboratory. Your support and friendship has made this period of my life unforgettable.

Mrs Buki Onwuegbuna and all other staff of National Health Laboratory Service, Department of Medical Microbiology, University of Pretoria, for the technical and all other support rendered to me during the execution of this research program.

Last but not least my parents Mr and Mrs ED Atanda for bringing me up and for paying my fees during my school days. Thank you for teaching me the importance of education.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AC	Amplification control
ACP	Enoyl-acyl carrier protein
AFB	Acid-fast bacilli
A-Lys	Lysis buffer
A-NB	Neutralization buffer
AMI	Amikacin
AMTD	Amplified *Mycobacterium tuberculosis* direct
AMX-CLV	Amoxicillin-clavulanate
AP	Agar proportion
ATS	American Thoracic Society
CAP	Capreomycin
CC	Conjugate control
CFX	Ciprofloxacin
CFZ	Clofazimine
CLSI	Clinical Laboratory Standards Institute
CLR	Clarithromycin
CRI	Colorimetric redox indicator
CR	Complement receptors
CS	D-cycloserine
DOTS	Directly observed therapy short courses
DTH	Delayed Type Hypersensitivity
DST	Drug testing
ETH	Ethionamide
EMB	Ethambutol
FDA	Food and Drug Administration
FQ	Fluoroquinolone
GC	Growth control
GFX	Gatifloxacin
GU	Growth unit
IFN-γ	Gamma Interferon
INH	Isoniazid
KAN	Kanamycin
LAMP	Loop-mediated isothermal amplification
LFX	Levofloxacin
LPA	Line Probe Assay
LZD	Linezolid
MDR-TB	Multidrug-resistant tuberculosis
MEP-CLV	Meropenem-clavulanate
MFX	Moxifloxacin
MGIT	Mycobacteria growth indicator tube
MIC	Minimum inhibitory concentration
MODS	Microscopic 0bservation drug-susceptibility

MRC	Medical Research Council
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide
NCCLS	National Committee for Clinical Laboratory Standards
NHLS	National Health Laboratory Services
NR	Nitrate reduction assay
NTM	Nontuberculous mycobacteria
OFX	Ofloxacin
PAS	*Para*-amino salicylic acid
PCR	Polymerase chain reaction
PTA	Prothionamide
PZA	Pyrazinamide
QC	Quality control
RBU	Rifabutin
RIF	Rifampicin
RPE	Rifapentin
RRDR	Rifampicin resistance-determining region
RT-PCR	Real-time PCR
SIRE	Streptomycin, Isoniazid, Rifampin and Ethambutol
SLID	Second-line injectable drugs
SM	Streptomycin
SNPs	Single Nucleotide Polymorphisms
Sp-A	Surfactant protein A receptors
SRLs	Supranational Reference Laboratories
TAC	Thioacetazone
TB	Tuberculosis
TLA	Thin-layer agar
TST	Tuberculin skin test
TZD	Terizidone
VIO	Viomycin
WHO	World Health Organization
XDR-TB	Extensively drugresistant tuberculosis

CHAPTER 1

INTRODUCTION

Tuberculosis (TB) is a chronic airborne infectious disease caused by *Mycobacterium tuberculosis* and primarily affects the lungs. In 2012 the World Health Organisation (WHO) estimated 2 billion people, 8.3 new cases and 1.3 million deaths (WHO, 2013). South Africa has a particularly high burden of TB due to its high prevalence of HIV; having 948 out of 100,000 cases of infection and 500,000 new cases every year (WHO, 2010). TB transmission occurs when infectious people cough, sneeze, talk, laugh or spit droplets containing bacteria in the air (WHO, 2010). One of the most important factors influencing the current TB epidemic in resource-limited settings is poverty, which is closely related to malnutrition, overcrowding and lack of access to free or affordable health care services (Cegielski and McMurray, 2004). Countries with a high prevalence of tuberculosis are therefore often associated with a low socio-economic status. Furthermore, tuberculosis can occur at any stage in the immunosuppressive spectrum of HIV disease, with variable presentations particularly in high burden countries. Tuberculosis may therefore be the first presentation of HIV disease (Mukadi, Maher and Harries, 2001).

In recent years, multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) had devastating effects on populations of HIV-infected individuals in developing countries (Dorman and Chaisson, 2007). South Africa is one of the high burden multi-drug-resistant (MDR) TB countries (WHO, 2010). Multidrug-resistant TB (MDR-TB), defined by resistance to the two most potent first-line anti-TB agents (isoniazid and rifampicin), arises initially as a result of poorly implemented treatment programmes (Abdel Aziz, Wright, De Muynck and Laszlo., 2003). Worldwide TB control suffers from the lack of a totally effective vaccine; the current vaccine *Mycobacterium bovis* BCG does not prevent pulmonary TB in adults but is still useful since it protects infants from severe forms of disease (Chaisson and Churchyard, 2010). Extended treatment with multiple drugs is needed to effectively cure tuberculosis. The main reason for this requirement is the hydrophobic cell envelope surrounding members of the *Mycobacterium tuberculosis* complex (MTBC) that serves as a permeability barrier to many compounds, the sequestered, non-replicating subpopulation of TB that is affected by drugs only when the cells re-emerge from dormancy and the drug target

or drug-activating enzymes in TB that are altered by mutation and result in a population of drug-resistant cells (Parsons, Somoskovi, Urbanczik and Salfinger, 2004). In addition, active drug efflux pumps and degrading or inactivating enzymes and the genes that are associated with the functions have been disclosed in *M. tuberculosis* (Somoskovi, Parsons and and Salfinger, 2001).

Tuberculosis can be cured with rigorous treatment that lasts for six months – administration of improper treatment regimens by health care workers and failure to ensure that patients complete the whole course of treatment may result in the emergence of drug-resistant strains of TB. Efforts to treat patients with active disease and to control the spread of TB are complicated by resource constraints, coinfection with HIV, and the emergence of drug-resistant TB strains (WHO, 2010). The standard treatment regimen combines four first-line antibiotics, isoniazid, rifampicin, pyrazinamide and ethambutol which is necessary to kill actively replicating TB, inhibit the development of resistance and renders patients with tuberculosis non-contagious when they are properly administered (Somoskovi *et al.*, 2001). The irregular use of antitubercular drugs often results in re-occurrence of TB and the development of drug-resistant, multidrug-resistant, and extensively drug-resistant TB (Parsons *et al.*, 2004).

The directly observed treatment short course (DOTS) therapy using a standard short course of first-line antibiotics to combat resistance, as developed and promoted by WHO is widely endorsed by national TB programmes. Several studies report high cure rates using DOTS against drug-susceptible TB (WHO, 2005). However, treatment success was below average in the African region (72%), which can be partly attributed to HIV coinfection and in the European region (75%) partly due to drug resistance (Espinal *et al.*, 2000). Treatment strategies that include the use of second-line drugs in the directly observed treatment of MDR-TB (DOTS-Plus restorative strategies to combat resistance) can achieve cure rates nearly as high as those for drug-sensitive TB treated with first-line drugs (Farmer and Kim, 1998). Strategies for using second-line drugs (regimen includes two or more drugs to which the isolate is susceptible including one drug given parenterally for six months or more) fall into two broad categories: those using standardised regimens formulated for particular geographic areas based on drug resistance profiles of a sample of cases and those using individualised regimens selected on the basis of individual drug susceptibility testing (DST) (Farmer and

Kim, 1998). Although second-line therapy yields higher cure rates for MDR-TB, it is more expensive than first-line therapy and requires longer treatment duration (Gupta Raviglone and Espinal, 2001).Treatment of MDR-TB requires administration of second-line drugs for at least 18–24 months (Nathanson, Lambregts-van Weezenbeek, Rich, Gupta, Bayona, Blondal, Caminero , Cegielski, Danilovits, Espinal, Hollo, Jaramillo, Leimane, Mitnick, Mukherjee, Nunn, Pasechnikov, Tupasi, Wells, Raviglione, 2006). Therefore, rapid detection of resistance to second-line drugs is essential for patient management and effective public health interventions (Ahmad and Mokaddas, 2009). Conventional drug susceptibility testing is performed in two steps beginning with a primary culture followed by first-line drug testing and proceeding to second-line testing where multidrug resistance is to be determined (Hillemann, Rusch-Gerdes and Richter, 2009).

For years, conventional DST on solid egg- or agar-based media have been the standard technology and is still utilised in many countries worldwide. The other methodologies include the agar proportion method, the absolute concentration method and the resistance ratio method for egg-based Lowenstein-Jensen medium. Data using the proportion methods on egg- and agar-based media have provided the most comprehensive published evidence and regarded as the reference methods in many countries (CLSI). These methods are based on the estimation of growth or not of a *Mycobacterium tuberculosis* strain in the presence of a single 'critical concentration' of one drug. The critical concentration of an antituberculous drug represents the lowest concentration of the drug in the medium that indicates clinically relevant resistance if growth is observed (Heifets, 1991). The main disadvantage of conventional DST is the long turnaround time with 3 to 4 weeks of incubation needed until the results are obtained. To overcome this drawback, numerous new techniques have become available with the aim of more rapid detection of resistance (WHO, 2004).

There are two different strategies for determining drug resistance, the phenotypic and genotypic methods. The phenotypic methods which are based on the determination of growth or inhibition of growth in the presence of antibiotics have been the most commonly used (WHO, 2001). New faster DST methods have been introduced in recent years. With insights into mycobacterial genomes, molecular techniques have recently been established to detect

gene mutations that are known to be associated with resistance to certain antibiotics (Kim and Espinal, 2004).

Newer phenotypic techniques aim for a more rapid detection of growth by using the metabolic activities of growing bacteria. Rapid liquid culture-based techniques have been established that can detect growth-dependent changes such as CO_2 production (BACTEC 460 and MB/BacT) or oxygen consumption (Mycobacteria Growth Indicator Tube [MGIT] and VersaTREK) (Tenover, Crawford, Huebner, Geiter, Horsburgh and Good, 1993). Other techniques rely on the ability of living cells to convert an oxidation-reduction indicator dye into the reduced state (reazurin and tetrazolium bromide) or on the very specific property of *Mycobacterium tuberculosis* to reduce nitrate to nitrite (Ängeby, Werngren, Toro, Hedström, Petrini B, and Hoffner, 2003). A more rapid detection of growth can also be achieved by microscopic observation of liquid cultures in tissue-culture plates (microscopic-observation drug-susceptibility [MODS] assay). Since myco-bacteriophages are able to only replicate in living cells, phage-based tests have also been developed for reducing the time to get a DST result (Bergmann and Woods, 1998). These techniques should only be carried out by highly experienced personnel who are trained in the preparation of the appropriate drug dilutions and inoculums as well as the assessment of susceptibility or resistance. Furthermore appropriate internal quality control processes are required to ensure correctness of the results. In general, standardisation of these techniques is difficult because the efficiency of the drugs alters in different media and with different methods (Ruiz, Zerolo and Casal, 2000).

The BACTEC MGIT 960 system (Becton Dickinson Microbiology System, Sparks, MD, USA) is an automated, continuously monitoring liquid system. It is based on fluorometric technology for the detection of oxygen consumption owing to bacterial growth in the tubes. It offers the possibility of performing DST using prepared kits which are available for susceptibility testing of INH, RMP, EMB, SM, and pyrazinamide (PZA). The test is based on the detection of growth in the antibiotic-containing media compared with an antibiotic-free control tube which is inoculated with a 1:100 dilution of the TB strain or a 1:10 dilution in case of PZA testing (Ruiz *et al.*, 2000).

Recently a new second-line DNA strip assay GenoType®MTBDRs*l* (Hain Lifescience, Nehren, Germany) was developed to detect resistance to ethambutol (EMB), fluoroquinolones

and injectable aminoglycosides/cyclic peptides by focusing on the most prevalent *gyr*A, *rrs*, and *emb*B mutations (Brossier, Veziris, Aubry, Jarlier and Wougakoff, 2010). This technology is based on polymerase chain reaction (PCR) in combination with reverse hybridisation. The omission of a wild-type band and/or the appearance of a band representing a mutation are indicative of resistance (Van Ingen, Simons, de Zwaan, van der Laan, Kamst-van Agterveld, Boeree, van Soolingen, 2010). This assay has been evaluated for *Mycobacterium tuberculosis* cultures and specimens by comparing it with DNA sequencing and/or conventional susceptibility testing in liquid and solid media; however it has not been compared with a rapid automated system such as MGIT 960 (Lacoma, Garcia-sierra, Prat, Maldonado, Ruiz-Manzano, Haba, Gavin, Samper, Ausina and Dominguez, 2012).

AIM

The aim of this study is to access the ability of the GenoType$^{(R)}$MTBDR DNA strip and the Bactec MGIT 960 assay to detect resistance to first-line and second-line drugs in multidrug-resistant *Mycobacteria tuberculosis* (MDR-TB) when compared to the agar-proportion method.

OBJECTIVE

To compare the efficiency of the GenoType$^{(R)}$MTBDR DNA strip assay and the MGIT 960 system for the detection of resistance to first and second-line drugs and to review the use of the agar-proportion method as the gold standard.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Tuberculosis is a medical, social and economic disaster of immense magnitude and has received substantial attention in recent years from the general public and scientific communities (Sharma and Mohan, 2006). In 2012, WHO estimated 2 billion people, 8.3 new cases and 1.3 million deaths (WHO, 2013). South Africa has a particularly high burden of TB due to its high prevalence of HIV having 948 out of 100,000 cases and 500,000 new cases every year (WHO, 2010) (Table 2.1). The control of tuberculosis (TB) remains one of the most serious challenges to global health (WHO, 2007). Another new and potentially devastating threat to TB control is the emergence of strains that cannot be cured by a standard antituberculosis drug regimen (WHO, 2013). Drug resistant tuberculosis generally arises through the selection of mutated strains by inadequate chemotherapy (Sharma and Mohan, 2006). Resistance to at least two major antitubercular drugs, Isoniazid and Rifampicin, has been termed multidrug-resistant tuberculosis (Dye and Williams, 2009).

Currently, tuberculosis is potentially a devastating threat worldwide due to emergence of drug resistant strains, which hamper management and control (Prasad, 2005). The modern, standard short-course therapy for TB recommended by the World Health Organization is based on a four-drug regimen that relies on direct observation of patient compliance to ensure effective treatment (Chan and Iseman, 2002). The rapid spread of drug resistance, especially multi-drug resistant tuberculosis (MDR-TB) and currently extensively drug resistant tuberculosis (XDR-TB), both in new and previously treated cases, adds urgency to the need for decisive action for control measures (WHO, 2006). Resistant to at least two major antituberculosis drugs Isoniazid and Rifampicin with or without resistance to other antiTB drugs has been termed MDR-TB (Sharma and Mohan, 2004). MDR-TB is more difficult to treat than drug-susceptible TB, requiring the use of less effective second-line antitubercular drugs which are often associated with major side effects (Sharma and Mohan, 2006).

Table 2.1: The 22 high burden countries with 80% of the tuberculosis cases worldwide (WHO, 2009)

Country	Burden of TB incidence (no. of cases/100,000 individuals/year)	Global rank (by estimated cases)
Afghanistan	333	21
Bangladesh	246	5
Brazil	62	15
Cambodia	508	23
China	102	2
Democratic Republic of Congo	369	11
Ethiopia	356	7
India	168	1
Indonesia	285	3
Kenya	610	10
Mozambique	431	19
Myanmar	171	20
Nigeria	293	4
Pakistan	181	6
Philippines	296	9
Russian Federation	110	11
South Africa	948	5
Thailand	142	17
Uganda	411	16
United Republic of Tanzania	371	14
Vietnam	178	13
Zimbabwe	569	19

2.2 General characteristics of mycobacteria

Lehmann and Neumann first introduced the genus *Mycobacterium* into the scientific literature in 1896. The subsequent history of the genus has been profoundly influenced by the fact that only very few of the more than over 100 currently recognised species have been a devastating cause of human disease and suffering, above all *Mycobacterium tuberculosis* (Wayne, 1984).

The genus *Mycobacterium* is the only genus in the family of the Mycobacteriaceae and is related to other mycolic acid-containing genera. All *Mycobacteria* are aerobic (though some species are able to grow under a reduced oxygen atmosphere), nonspore-forming, nonmotile, slightly curved or straight rods (0.2 to 0.6 μm by 1.0 to 10 μm) which may branch (Draper and Daffe, 2005).

The most prominent feature of *Mycobacteria* that is uniformly present and distinctive to the genus is the lipid-rich cell envelope (Kremer and Besra, 2005). Different species have different temperatures of growth with a range of <30-45 $^{\circ}$C (Pfyffer, 2007). They can either grow slowly (require 7 days for growth) or rapidly (requiring less than 7 days for growth) when subcultured on Löwenstein-Jensen media (Brown-Elliot and Wallace, 2007). They belong to two groups: slow growers (further divided into photochromogens, scotochromogens, nonphotochromogens) and rapid growers (Vincent and Gutierrez, 2007).

2.3 Classification of mycobacteria

Mycobacteria can be classified into several major groups for purpose of diagnosis and treatment: *M. tuberculosis complex*, which can cause tuberculosis, *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*; *M. leprae*, which causes Hansen's disease or leprosy, nontuberculous mycobacteria (NTM) are all the other mycobacteria, which can cause pulmonary disease resembling tuberculosis, lymphadenitis, skin disease, or disseminated disease (Pfyffer, 2007).

There are well over 100 species of mycobacteria but most common species include: *M. avium* Complex (MAC), *M. kansasii*, *M. haemophilum*, *M. xenopi*, *M. malmoense*, *M. asiaticum*, *M. simiae*, *M. szulgai*,*M. marinum*, *M. ulcerans*, *M. genavense* (slow growers) and *M. fortuitum*, *M. chelonae*, *M. abscessus* (rapid growers) (Katoch, 2004).

2.4 Virulence factors of *M. tuberculosis*

The virulence of *Mycobacterium tuberculosis* is extraordinarily complicated and multifaceted (Barnes, 2000). Although the organism apparently does not produce any toxins, it possesses a huge repertoire of structural and physiological properties that have been recognised for their contribution to mycobacterium virulence and to pathology of tuberculosis (Beaucher *et al,* 2002). Some of the general properties of *Mycobacterium tuberculosis* that renders it virulent are:

Special mechanisms for cell entry. The tubercle bacillus can bind directly to mannose receptors on macrophages via the cell wall associated mannosylated glycolipid, LAM or indirectly via certain complement receptors or Fc receptors (Banu *et al,* 2002).

Intracellular growth, MTB can grow intracellularly. This is an effective means of evading the immune system. Once MTB is phagocytosed, it can inhibit phagosome-lysosome fusion by secretion of a protein that modifies the phagosome membrane (Beaucher *et al,* 2002).

Detoxification of oxygen radicals. MTB interferes with the toxic effects of reactive oxygen intermediates produced in the process of phagocytosis by three mechanisms:

1. Compounds including glycolipids, sulfatides and LAM down regulate the oxidative cytotoxic mechanism.
2. Macrophage uptake via complement receptors may bypass the activation of a respiratory burst.
3. The oxidative burst may be counteracted by production of catalase and superoxide dismutase enzymes (Bardarov *et al,* 2002).

Antigen 85 complex. This complex is composed of a group of proteins secreted by MTB that are known to bind fibronectin. These proteins may aid in walling off the bacteria from the immune system and may facilitate tubercle formation (Beaucher *et al.,* 2002).

Slow generation time. Because of MTB's slow generation time, the immune system may not readily recognise the bacteria or may not be triggered sufficiently to eliminate them. Many other chronic diseases are caused by bacteria with slow generation times, for example, slow-growing *M. leprae* causes leprosy, *Treponema pallidum* causes syphilis, and *Borrelia burgdorferi* causes Lyme disease (Beaucher *et al.*, 2002).

High lipid concentration in cell wall. This accounts for impermeability and resistance to antimicrobial agents, resistance to killing by acidic and alkaline compounds in both the

intracellular and extracellular environment, and resistance to osmotic lysis via complement deposition and attack by lysozyme (Beaucher *et al.,* 2002).

Cord factor. Cord factor (trehalose 6, 6' dimycolate) is a glycolipid found in the cell walls of mycobacteria, which causes the cells to grow in serpentine cords. It is primarily associated with virulent strains of MTB. It is known to be toxic to mammalian cells and to be an inhibitor of PMN migration. Its exact role in MTB virulence is unclear, although it has been shown to induce granulomatous reactions identical to those seen in TB (Beaucher *et al.,* 2002).

Adherence. The specific bacterial adhesins involved in the complex interaction between *M. tuberculosis* and the human host are largely unknown. Nevertheless, a few potential adherence factors have been considered, including the heparin-binding hemagglutin (**HbhA**), a fibronectin-binding protein, and a polymorphic acidic, glycine-rich protein, called **PE-PGRS**. HbhA is a surface-exposed protein that is involved in binding *Mycobacterium tuberculosis* to epithelial cells but not to phagocytes. It could be involved in extrapulmonary spread after the initial long-term colonisation of the host. Fibronectin-binding proteins (**FbpA**), first identified as the α-antigen (**Antigen 85 complex**), can bind to the extracellular matrix protein fibronectin in vitro. This property may represent a mechanism of tissue colonisation. The surface-exposed PE-PGRS proteins found in *M. tuberculosis* and *Mycobacterium bovis* also show fibronectin-binding properties (Beaucher *et al*, 2002).

2.5 Pathogenesis of *Mycobacterium tuberculosis*

Primary Infection. *Mycobacterium tuberculosis* is described as an intracellular pathogen whose success relies on avoiding the killing mechanisms of professional phagocytes (Akira *et al.*, 2006). Primary tuberculosis is the initial infection in which inhaled droplet nuclei containing tubercle bacilli are deposited in the peripheral respiratory alveoli, most frequently those of the well-ventilated middle and lower lobes (Harding and Boom, 2010). In these lobes they are recognizsed by alveolar macrophage complement receptors (CR1, CR3, CR4) and phagocytosed (El-Etr and Cirillo, 2001). This process causes a two-front battle with the macrophage, which may be resolved in days or last for years (Akira *et al.*, 2006). The first is with the phagosome/lysosome digestive mechanisms of the macrophage. In this process, MTB is able to interfere with the acidification of the phagosome, which renders the lysosomal

enzymes (which require acidic pH) less effective. This allows the organisms to multiply freely in the cytoplasm of the nonactivated macrophage (Kenneth *et al.*, 2010). The second process is the triggering of T_H1 immune responses, beginning with digestion and surface presentation of mycobacterial components and ending with cytokine activation of the macrophages (Kenneth *et al.*, 2010). The short- and long-term outcomes of the infection depend on the ability of the macrophage activation process to overcome the intracellular edge that MTB has as a result of its ability to block acidification of the phagosome (Kenneth *et al.*, 2010).

In the early stages of infection, MTB-laden macrophages are transported through lymphatic channels to the lymph nodes draining the infected site. From there, a low-level bacteremia disseminates the bacteria to a number of tissues, including the liver, spleen, kidney, bone, brain, meninges, and apices or other parts of the lung. Although the primary site of infection and enlarged lymph nodes can often be detected radiologically (Hernandez-Pando *et al.*, 2000),the primary evidence for their existence is reactivation at nonpulmonary sites later in life. Tuberculous meningitis is the most serious of these (Akira *et al.*, 2006). In the primary lesion as MTB cells multiply, macrophages and dendritic cells release cytokines (tumor necrosis factor, interleukin 12, interferon gamma [IFN-γ]), which attract T cells and other inflammatory cells to the site (Tufariello *et al.*, 2003). The recruited CD4 T cells initiate the T_H1-type immune response over the following 3 to 9 weeks in which IFN-γ is the primary activator of macrophages (Tufariello *et al.*, 2003). During these weeks as the bacteria multiply, they may generate quantities of mycobacterial proteins exceeding thresholds required to also trigger a DTH response. The DTH with its phagocytes, fluid, and release of digestive enzymes adds a destructive component to the process and is the sole known source of injury in tuberculosis. If the T_H1 process is effective, the source of DTH stimulation wanes and the disease resolves. The mycobacterial protein-specific DTH sensitisation remains and its elicitation is the basis of the tuberculin skin test (Tufariello *et al.*, 2003).

The mixture of the T_H1 immune and DTH responses is manifest in a microscopic structure called a granuloma which is composed of lymphocytes, macrophages, epithelioid cells (activated macrophages), fibroblasts, and multinucleated giant cells all in an organised pattern (Kenneth *et al*, 2010). As the granuloma grows, the destructive nature of the hypersensitivity component leads to necrosis usually in the centre of the lesion. This is termed caseous necrosis because of the cheesy, semisolid character of material at the centre of gross lesions,

but the terms fits the smooth glassy appearance of microscopic granulomas as well (Kenneth *et al.*, 2010). Primary infections are handled well once the immune response halts the intracellular growth of MTB (Kenneth *et al.*, 2010). Bacterial multiplication ceases, the lesions heal by fibrosis, and the organisms appear to slowly die. This sequence occurs in infections with multiple other infectious agents for which it is the end of the story (El-Etr and Cirillo, 2001). In tuberculosis some of the organisms, when faced with oxygen and nutrient deprivation, enter a prolonged dormant state rather than dying (El-Etr and Cirillo, 2001). Specific factors facilitating this change are not known but the waxy nature of the MTB cell wall must aid survival under these conditions as it does in the environment (Akira *et al.*, 2003). These organisms in the lung and elsewhere lay waiting for reactivation months, years, or decades later. For most persons who undergo a primary infection this never happens, either because of the complete killing of the original population or the failure of factors favouring reactivation to materialise (El-Etr and Cirillo, 2001).

Secondary infection: Although mycobacterial factors have been identified (resuscitation-promoting factor), little is known of the mechanisms of reactivation of these dormant foci. It has generally been attributed to some selective waning of immunity. The new foci are usually located in body areas of relatively high oxygen tension that would favour growth of the aerobe MTB. The apex of the lung is the most common, with spreading, coalescing granulomas, and large areas of caseous necrosis. Necrosis often involves the wall of a small bronchus from which the necrotic material is discharged, resulting in a pulmonary cavity and bronchial spread. Frequently, small blood vessels are also eroded. In all of this the basis for the highly destructive nature of MTB is largely unknown. It produces no exotoxins, and both the intact cell and cellular components are remarkably innocuous to humans and experimental animals not previously sensitised to tuberculin. It appears that with the failure of the host to control growth of MTB the rising load of mycobacterial protein stimulates a progressively autodestructive DTH response (Kenneth *et al.*, 2010).

2.6 Diagnosis of TB

Laboratory diagnosis of active TB is based on smear microscopy assessment of acid-fast bacilli (AFB), growth of *M. tuberculosis* in solid or liquid culture and detection of *M. tuberculosis* nucleic acid in clinical specimens. These methods vary in cost, turnaround time and laboratory infrastructure requirements (Drobniewski, Eltringham, Graham, Magee, Smith

and Watt, 2012). Smear microscopy using Ziehl-Neelsen staining is a rapid test but is not sensitive (34-80% of positive cultures) and is often negative in HIV-coinfected pulmonary TB patients due to paucibacillary load (Palomino, 2012).

Definitive diagnosis of active TB is based on the culture of *M. tuberculosis* (regarded as the gold standard), especially in smear-negative specimens, and enables species-specific identification and DST to guide therapy (Palomino Martin, Von Groll and Portaels, 2008). Culture on a solid (Lowestein-Jensen) medium is time consuming, requiring 4-6 weeks for detectable growth and an additional 2-3 weeks for species-specific identification. Liquid (Middlebrook 7H9) media supports more rapid growth (Palomino, 2012). Fully automated liquid culture systems such as the MGIT 960 culture system allow for the recovery of mycobacteria from clinical specimens within 14days; however additional time is needed for the identification of *M. tuberculosis* (Palominoet al., 2008). The Accuprobe assay can identify *M. tuberculosis* and five common non-tuberculous mycobacteria in 2 hours in culture-positive tubes. Reverse hybridisation-based line probe assays also identify *M. tuberculosis* and several NTM species in both culture isolates and clinical specimens (Drobniewski *et al.*, 2012). Simple, rapid and relatively inexpensive oligochromatographic/immunochromatographic assays have also been developed for the detection of *M. tuberculosis* and NTM species in culture-positive samples (Palomino, 2012).

Molecular methods, typically involving PCR/real-time PCR, have also been developed. These assays are mainly recommended for the identification of *M. tuberculosis* in sputum smear-positive specimens. Commercial tests perform better than in-house developed tests and some have been approved by the Food and Drug Administration (FDA) of the USA and the WHO for routine diagnosis of TB (Palomino, 2012). These tests include the Cobas TaqMan MTB test, Amplified Mycobacterium Tuberculosis Direct (AMTD) test, BDProbeTec ET *Mycobacterium tuberculosis* complex direct detection assay and GeneXpert MTB/RIF assay (Drobniewski *et al.*, 2012). The pooled sensitivity of these tests for smear-positive and smear-negative respiratory samples is ~95 % and ~70 % respectively while their performance in extrapulmonary specimens is usually much lower (Drobniewski *et al.*, 2012).

The GeneXpert MTB/RIF assay is a point-of-care TB diagnostic test (Boehme, Nabeta, Hillemann, Nicol, Henai and Krapp., 2010). Although originally intended for respiratory

specimens, it has also performed well for a variety of non-respiratory samples. GeneXpert MTB/RIF has detected 98-100% of smear-positive and 57-83% of smear negative pulmonary TB samples. A sensitivity of 53-95% has been reported for extrapulmonary specimens (Chang, Lu, Wang, Zhang, Jia and Li, 2012). The WHO has strongly recommended the GeneXpert MTB/RIF assay wherever possible, particularly for HIV-co-infected individuals (WHO, 2012).

2.7 Treatment of TB

Mycobacteria are inherently resistant to many antimicrobial agents based on the unusually impermeable nature of their lipid-rich cell wall. However, several antimicrobics have been shown to be effective in the treatment of MTB infection. The term *first-line* is used to describe the primary drugs of choice (isoniazid, ethambutol, rifampicin, pyrazinamide, streptomycin) that have long clinical experience to back up their efficacy and to manage their side effects. Second-line agents are less preferred and reserved for use when there is resistance to the first-line agents (Nafees, James and Lawrence, 2010)

Prolonged multidrug therapy for TB is required because *M. tuberculosis* grows very slowly and has an increased capacity for dormancy (low metabolic activity). Successful treatment (conversion of sputum from culture-positive to culture-negative within 2 months) depends on the duration and combination of drugs prescribed the patient's adherence to treatment and adverse drug reactions (Blumberg, 2003). The preferred treatment regimen for pulmonary TB caused by drug-susceptible *M. tuberculosis* in both HIV-coinfected and HIV-seronegative adults which is endorsed by the American Thoracic Society (ATS) includes daily therapy or therapy 5 days per week (when drugs are administered under directly observed therapy (DOT) with INH, RIF, PZA and EMB for 2 months in the initial phase of treatment. The 4-month continuation phase of treatment includes daily therapy or therapy 5 days per week (when drugs are given under DOT) with INH and RIF (Blumberg, 2003)

However, patients with cavitations on their initial chest radiograph and/or with positive cultures after 2 months of therapy receive a 7-month continuation phase of treatment with either daily or twice weekly therapy with INH and RIF. The 6-month treatment course is also recommended for extrapulmonary TB involving any site except meninges in which case a 9-

to 12- month regimen is recommended (Blumberg, 2003). Treatment of HIV-TB coinfected patients is complicated by drug-drug interactions and may require substitution of RIF with other rifamycins. Other common co-morbidities such as diabetes may also complicate treatment (Jimenez-Corona, Cruz-Hervert, Garcia-Garcia, Ferreyra-Reyes, Delgado-Sanches, Bobadilla-Del-Valle, 2013). Treatment of drug-susceptible TB is possible in ≥95 % of disease cases; however supervised therapy for ≥6 months is challenging (Blumberg, 2003).

2.8 Evolution of drugresistant TB

The discovery of anti-TB drugs in the 1940s followed by combination chemotherapy made TB a curable disease. In the developed countries, effective treatment and surveillance reduced tuberculosis dramatically with high hopes of total eradication (Raviglione *et al.*., 1992). However, in the 1980s, it was realised that tuberculosis had not only ceased to decline in the developed countries, notably the USA, but was actually increasing in underdeveloped countries, particularly in major cities (Raviglione *et* al., 1995). TB patient intolerance to one or more drugs and non-adherence to treatment often result in much lower cure rates and the evolution of drug-resistant strains of *M. tuberculosis* (Mitchison and Davies, 2012). Resistance of *M. tuberculosis* to antiTB drugs is caused by chromosomal mutations occurring at a frequency of $10^{-3} – 10^{-9}$ in genes encoding drug targets (Blumberg, 2003). The probability of two independent mutations conferring resistance to two antiTB drugs in a single bacillus is much lower ($10^{-12} – 10^{-15}$); most TB patients do not contain any such strains if they have never been exposed to antiTB drugs previously (Mitchison and Davies, 2012).

However, during inappropriate therapy, only drug-susceptible strains are killed, while drug resistant strains survive and multiply (WHO, 2010). Sequential accumulation of mutations results in the evolution of MDR-TB strains (Mitchison and Davies, 2012). Effective treatment of MDR-TB is more difficult than that of drug-susceptible TB and often results in treatment failure particularly among HIV-coinfected patients (Orenstein, Basu, Shah, Andrews and Friedland, 2009). Incomplete treatment of MDR-TB patients further amplifies drug resistance in *M. tuberculosis* strains and leads to the emergence of XDR-TB (Ahmad and Mokaddas, 2012).

2.9 Anti-TB drugs: mechanism of action and molecular basis of resistance

The emergence of MDR-TB has resulted in the development of novel methods for rapid diagnosis and effective treatment strategies with several old and new anti-TB agents (Gandhi, Andrews, Brust, Montreuil, Weissman and Heo, 2012). Anti-TB drugs are now recognized as first-line (most effective), second-line (less effective, more toxic) and third-line (reinforcing) agents based on their efficacy and tolerability (Mitchison and Davies, 2012).

First-line drugs (INH, RIF, EMB and PZA) are highly effective, relatively less toxic than other anti-TB drugs and mostly bactericidal oral agents suitable for combination therapy (Blumberg, 2003). SM is used as a second-line agent because of its intramuscular administration, due to the requirement for frequent patient visits to health care facilities, higher rates of resistance among clinical *M. tuberculosis* isolates and the availability of more effective anti-TB drugs. Other rifamycins (rifabutin (RBU) and rifapentine (RPE)) although more expensive may be used in place of RIF in selected patients populations (Blumberg, Burnam, Chaisson, Daley, Etkind and Friedman, 2003).

Second-line agents have been further divided into three different groups based on their mode of action, route of entry and potency (Gandhi *et al.*, 2012). The first group includes injectable aminoglycosides (Streptomycin, SM, Kanamycin, KAN and Amikacin, AMI) and cyclic polypeptides (Capreomycin, CAP; and Viomycin, VIO) (Blumberg, 2003). The second group fluoroquinolones (FQs) include ofloxacin (OFX), levofloxacin (LFX), moxifloxacin (MFX) and gatifloxacin (GFX). Higher doses of older FQs namely OFX and LFX have been safely administered. GFX and MFX at regular doses and LFX at higher doses are bactericidal (Johnson, Hadad DJ, Boom WH, Daley CL, Peloquin CA and Eisenach KD, 2006). The third group includes mainly bacteriostatic, less efficacious, expensive and more toxic oral agents such as ethionamide (ETH), prothionamide (PTA), D-cycloserine (CS), terizidone (TZD) and *para*-amino salicylic acid (PAS) that are used mainly for the treatment of MDR-TB and XDR-TB based on the susceptibility of *M. tuberculosis* strains (Gandhi *et al.*, 2012).

Third-line reinforcing agents such as linezolid (LZD) at the full or half dose (to reduce toxicity), amoxicillin-clavulanate (AMX-CLV), meropenem-clavulanate (MEP-CLV), clofazimine (CFZ) and thiacetazone (TAC) have been used occasionally for the treatment of MDR-TB and XDR-TB but are not recommended for routine use due to variable efficacy and

serious side effects (Cox and Ford, 2012). Thiacetazone is highly toxic and is contraindicated for HIV-coinfected TB patients (Dooley, Obuku, Durakovic, Belitsky, Mitnic and, Neuremberger, 2013).

Table 2.2 Anti-TB drugs, action and genes affected by resistant mutations (Bwanga, Hoffner, Haile, Joloba, 2009)

Drug	**Mechanism of action**	**Genes affected by resistant mutations**
First line drugs (Oral)		
Isoniazid (INH)	Inhibit mycolic acid synthesis	*Kat*G, *inh*A, *oxy*R
Rifampicin (RIF)	Binds to RNA polymerase inhibiting RNA synthesis	*rpo*B
Pyrazinamide (PZA)	Activated to pyrazinoic acid, which is bactericidal	*pnc*A
Second line drugs		
a. Injectable drugs		
Streptomycin (SM)	Binds to ribosomal proteins and inhibits protein synthesis	*rrs*, *rpsl*
Amikacin (AMI)	Disrupts ribosomal function and inhibits protein synthesis	*rrs*, *rpsl*
Kanamycin (KAN)	Binds to 30S ribosomal subunit, inhibits protein synthesis	*rrs*, *rpsl*, *eis*
Capreomycin (CAP)	Similar to aminoglycosides	*rrs*, *rpsl*
b. Fluoroquinolones		
Ciprofloxacin (CFX)	Disrupts the DNA-DNA gyrase complex blocking DNA synthesis	*gyrA*, *gyrB*
Ofloxacin (OFX)		
Moxifloxacin (MFX)		
Gatifloxacin (GFX)		
Levofloxacin (LFX)		
c. Oral bacteriostatic anti-TB agents (second-line)		
Cycloserine (CS)	Inhibits cell wall synthesis	-
Ethionamide (ETH)	Inhibits oxygen dependent mycolic acid synthesis	-
P-aminosalicylic acid (PAS)	Disrupts folic acid metabolism.	-
Rifabutin (RBU)	Binds to RNA polymerase, Inhibits RNA synthesis	*rpoB*
Thioacetazone (TAC)		
d. Anti-TB agents with unclear efficacy		
Clofazimine (CFZ)	-	-
Amoxicillin/ Clavulanate (AMX-CLV)	-	-
Clarithromycin (CLR)	-	-
Linezolid (LZD)	-	-

2.9.1 Isoniazid

Hans Meyer and Josef Mally first synthesised isonicotinic acid hydrazide from ethyl isonicotinate and hydrazine in 1912 as part of their research work at German Charles University in Prague (Meyer and Mally, 1912). Isoniazid (INH), a pro-drug, has potent activity against actively dividing *M. tuberculosis.* INH is activated by the *KatG*-encoded catalase-peroxidase (Zhang, Heym, Allen Young and Cole, 1992). Activated INH mainly targets the NADH specific enoyl-acyl carrier protein (ACP) reductase (*inhA*) which is involved in mycolic acid synthesis. Depletion of mycolic acids results in bacterial killing (Almeida Da Silva and Palomino, 2011). The molecular basis of resistance to INH is complex, as resistance-conferring mutations are found in several genes (Zhang *et al.*, 1992). High-level resistance to INH is mainly due to mutations within the *KatG* gene, while mutations in the *inhA* regulatory region confer low-level resistance to INH in *M. tuberculosis* isolates (Almeida Da Silva and Palomino, 2011).

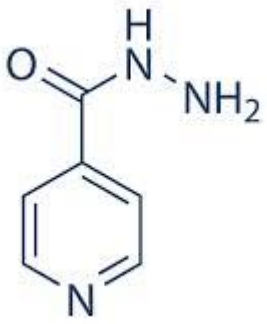


Figure 2.1 Chemical structure of Isoniazid (Bwanga *et al.*, 2009)

KatG mutations occur frequently (~50- 95%), particularly at codon 315 (*katG*315) in INH-resistant *M. tuberculosis* strains (Ahmad and Mokaddas, 2004). The frequency of mutations in the *inhA* regulatory region also varies in INH-resistant strains (Al-Mutairi, *et al.*, 2011). Mutation in other genes (*acp*M, *ahp*C and *Kas*A) and the induction of efflux pumps are also involved, but have a minor role in INH resistance (Almeida Da Silva and Palomino, 2011).

2.9.2 Rifampicin

In 1957, Prof. Piero Sensi and colleagues at the Dow-Lepetit Research Laboratories in Milan, Italy discovered a new bacterium *Nocardia mediterranei* (formerly *Streptomyces* mediterranei) in a sample of soil from a pine wood on the French Riviera (Sensi, 1983). This new species appeared immediately of great scientific interest since it was naturally producing a new class of molecules with antibiotic activity. These molecules were named "Rifamycins", in memory of the then popular French crime story Rifi, describing a jewel heist and rival gangs (Aronson, 1999). Several Rifamycins were characterised but subsequent studies leading to highly active derivatives were performed on Rifamycin B that was itself practically inactive. After two years of attempts to obtain more stable semi-synthetic products, in 1959 a new molecule with high efficacy and good tolerability was produced and was named "rifampicin" (Sensi, 1983). Thus, Rifampicin is a semisynthetic bactericidal antibiotic drug of rifamycin group and was introduced for clinical use in 1967 as a major addition to the cocktail drug treatment of tuberculosis and meningitis, along with isoniazid, ethambutol, pyrazinamide and streptomycin (Long, 1992).

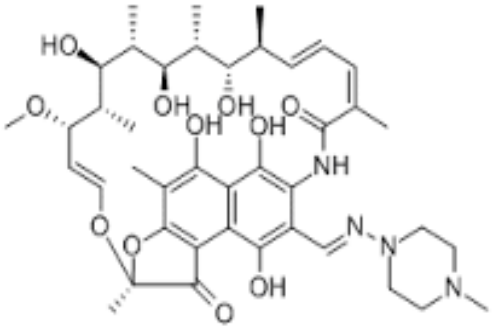


Figure 2.2 Chemical structure of Rifampicin (Bwanga *et al*., 2009)

Rifampicin acts by binding to the beta subunit of DNA-dependent RNA polymerase. During transcription, DNA enters through the jaw side of the RNA polymerase and both the DNA and the new RNA strands get out at the exit channel (Klug, 2001). Rifampicin binds to the exit end of the RNA polymerase in bacterial cells and directly blocks the channel of the elongating RNA when the transcript becomes 2 to 3 nucleotides long (Campbell, Korzheva, Mustaev, Murakami, Satish and Goldfarb, 2001). This inhibits transcription of DNA to RNA and subsequent translation to proteins (Klug, 2001). The human RNA polymerase variant is not affected by rifampicin even at 10 times the inhibitory concentration in mycobacteria (Wehrli, 1983). The rifampicin-RNA polymerase complex in mycobacteria is extremely stable yet

experiments have shown that this is not due to any form of covalent linkage (Telenti, 1993). It is hypothesised that hydrogen bonds and π-π bond interactions between naphthoquinone and the aromatic amino acids (phenylalanine, tryptophan, and tyrosine) are the major stabilisers (Rifampicin, 2010).

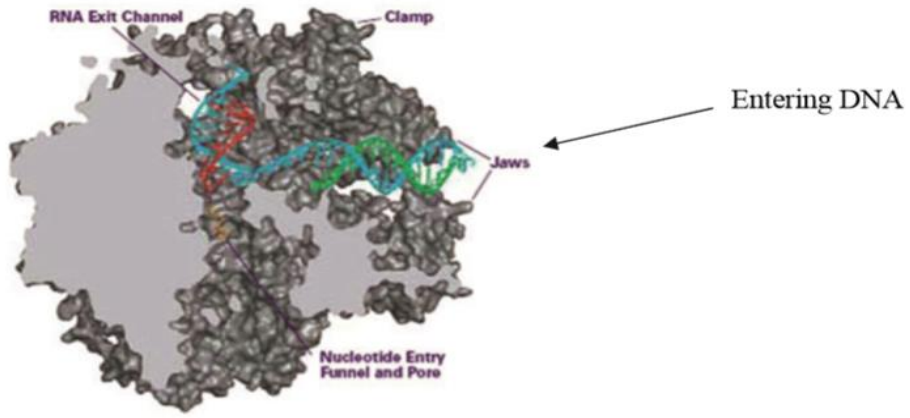


Figure 2.3 Crystal structure of the RNA polymerase enzyme (Bwanga *et al*., 2009)

Mono-resistance to RIF is rare except in patients with HIV-coinfection or other underlying disease because nearly 85-90% of RIF-resistant *M. tuberculosis* isolates are also resistant to INH. Resistance to RIF is regarded as a surrogate marker for MDR-TB (Almeida Da Silva and Palomino, 2011). Approximately 90-95% of RIF-resistant *M. tuberculosis* isolates contain mutations within an 81-bp rifampicin resistance-determining region (RRDR). Resistance in 5-10% of isolates is due to mutations in the N-terminal (codon V146) or cluster II regions of the *rpoB* gene or other genes (Al-Mutairi, Achmad and Mokaddas., 2011). Compensatory mutations that improve competitive fitness occur in other RNA polymerase (*rpoA*, *rpoC*) genes in RIF-resistant *M. tuberculosis* strains (Comas, Borrell, Roetzer, Rose, Mall and Kato-Maeda, 2011). While cross-resistance between RIF and RPE is common, ~12-20% of RIF-resistant strains remain susceptible to RBU, making RBU a useful drug for the treatment of some patients (Tan, Hu, Zhao, Cai, Luo and Zou., 2012).

2.9.3 Pyrazinamide

Pyrazinamide (PZA), a pro-drug is highly effective against semi-dormant bacilli in an acidic environment (such as the phagosome) (Mitchison and Davies, 2012). PZA is activated by pyrazinamidase (*PncA*) to form pyrazinoic acid which inactivates a vital step in fatty acid

synthesis (Scorpio and Zhang, 1996). PZA also inhibits protein translation and the ribosome-sparing process of translation by binding to ribosomal protein S1 (*RpsA*) in *M. tuberculosis*. Most (68-95%) PZA-resistant *M. tuberculosis* strains contain mutations in *pncA* or *rpsA* (Shi, Zhang, Jiang, Yuan, Lee and Barry, 2011).

2.9.4 Ethambutol

Ethambutol (EMB) inhibits the synthesis and polymerization of cell wall arabinan which leads to the accumulation of free mycolic acids and incomplete cell wall assembly. EMB mainly interacts with membrane-associated arabinosyltransferases encoded by three contiguous genes (*embCAB* operon) as an arabinose analog (Telenti, Sreevatsan, Bernasconi, Stock-bauer and Wieles, 1997). The molecular basis or resistance to EMB is not completely defined. Resistance-conferring mutations in three *emb* genes have been identified in *M. tuberculosis* strains and mutations in *embB* occur most frequently (20-89%) particularly at *embB* codon 306 (*embB306*), *embB406* and *embB497* (Ahmad, Jaber and Mokaddas, 2007). Some EMB-resistant *M. tuberculosis* strains do not contain a mutation in any of the genes currently implicated in EMB resistance (Telenti *et al.*, 1997).

2.9.5 Streptomycin

Streptomycin (SM) is now used as a second-line drug for treating patients who have failed therapy or have MDR-TB only if the *M. tuberculosis* strain is susceptible to SM (Blumberg *et al.*, 2003). SM inhibits protein synthesis by binding to a ribosomal protein (RpsL) and 16S rRNA (Almeida Da Silva and Palomino, 2011). High-level resistance mainly involves missense mutation in *rpsL* codon 43 (*rpsL43*) and *rpsL88*, while mutations in the *rrs* gene cause low-level resistance (Sreevatsan, Pan, Stockbauer, Williams, Kreiswort and Musser, 1996). Other targets causing low-level resistance to SM in *M. tuberculosis* include the *gidB* gene which encodes 7-methylguanosine methyltransferase and efflux pumps (Spies, Ribeiro, Ramos, Ribeiro, Martin and Palomino, 2011).

2.9.6 Kanamycin, amikacin and cyclic polypeptides

Other aminoglycosides such as KAN and AMI and cyclic polypeptides such as CAP and VIO also inhibit protein synthesis in *M. tuberculosis* and are used as bactericidal second-line drugs

(Almeida Da Silva and Palomino, 2011). Resistance to KAN and AMI is mainly associated with mutations in the *rrs* gene while mutations in the *rrs* gene and rRNA methyltransferase (TlyA) confer resistance to CAP and/or VIO in *M. tuberculosis* (Georghiou Magana, Garfein, Catanzaro,Catanzaro and Rodwell, 2012).

2.9.7 Fluoroquinolones

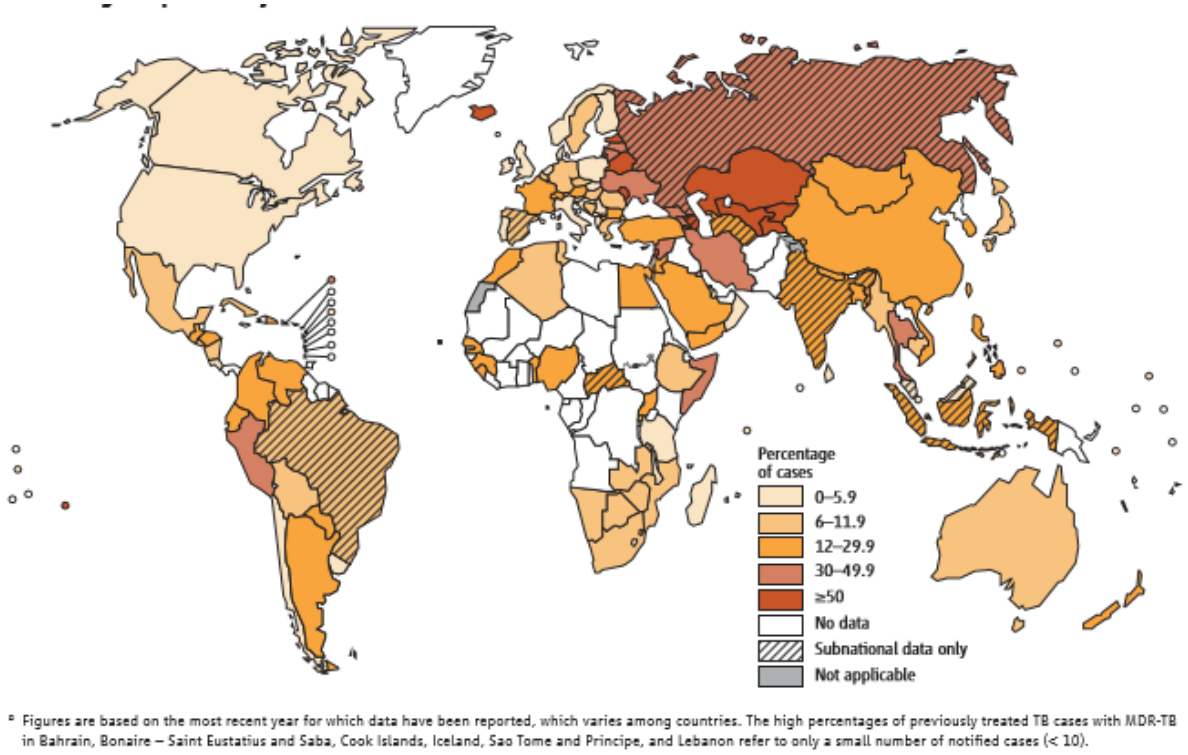
Fluoroquinolones (FQs) include ofloxacin (OFX), levofloxacin (LFX), moxifloxacin (MFX) and gatifloxacin (GFX). OFX is bacteriostatic while LFX (at high doses), GFX and MFX are bactericidal and suitable agents for combination therapy (Johnson, Hadad, Boom, Peloquin and Eisenach, 2006). FQs inhibit DNA gyrase (a type II topoisomerase) which consists of two A and two B subunits (encoded by the *gyrA* and *gyrB* genes respectively) and cause cell death due to inhibition of DNA replication and repair (Rieder, 2009). Mutations in small regions of the *gyrA* (mostly codon 90 or 94) and *gyrB* genes or changes in drug efflux pumps confer resistance to FQs in *M. tuberculosis* isolates (Sirgel, Warren, Streicher, Victor, van Helden and Böttger, 2012). Cross-resistance between different FQs is not 100% as some of OFX-resistant strains are susceptible to MFX and vice versa (Rieder, 2009).

2.10 Development of MDR-TB

Global efforts to control the TB pandemic have been undermined by the emergence and spread of strains that are resistant to the commonly used first-line anti-TB drugs isoniazid, rifampicin, ethambutol, and pyrazinamide. Strains resistant to at least isoniazid and rifampicin, the two most effective TB drugs are termed *multidrug-resistant* (Center for Disease Control [CDC], 2006). MDR-TB treatment is rather complicated as it requires second-line drugs some of which are only injectables, are less effective, more toxic and more expensive than the first-line agents (WHO, 2006). Treatment lasts for 18-24 months but only around 50%- 60% of MDR-TB patients will be cured compared with 95%–97% cure rate for patients with drug-susceptible strains treated with first-line agents (Goble Iseman, Madsen, Waite, Ackerson and Horsburgh., 1993; American Thoracic Society, 2003). The recent emergency of extensively drug resistant tuberculosis (XDR-TB) defined as MDR-TB strains with resistance to a fluoroquinolone and to at least one injectable second-line drug (kanamycin, amikacin, or capreomycin) has further complicated the problem of MDR-TB (CDC, 2006). A study in South Africa found a mortality rate from XDR-TB of 90% among the HIV-infected patients due to lack of treatment options (Gandhi *et al*., 2006).

2.11 Epidemiology of MDR-TB

The Global Project on Anti-Tuberculosis Drug Resistance Surveillance has been gathering data since 1994. The latest data indicates that every region of the world has reported MDR-TB, as shown in figure 2.1 (WHO, 2013)



[a] Figures are based on the most recent year for which data have been reported, which varies among countries. The high percentages of previously treated TB cases with MDR-TB in Bahrain, Bonaire – Saint Eustatius and Saba, Cook Islands, Iceland, Sao Tome and Principe, and Lebanon refer to only a small number of notified cases (< 10).

Figure 2.4 Proportion of MDR-TB among previously treated TB cases, 1994- 2012

Source: World Health Organization

2.11.1 MDR-TB Prevalence

The number of prevalent cases of MDR-TB in many parts of the world is estimated to be much higher than the number of incident case arising annually. Globally, the median prevalence of MDR-TB is reported to be 3% among the new and 15% among the re-treatment cases (WHO, 2008). Countries and territories in Eastern Europe such as Tajikistan, Uzbekistan and parts of China have the highest MDR-TB prevalence – up to 15% among the new and 60% among the previously treated cases (Wright, Van Deun, Falzon, Gerdes and Feldman., 2009). In sub-Saharan Africa inadequacy of laboratory services makes it difficult to estimate the actual burden of MDR-TB. In South Africa, the WHO surveillance reported a prevalence of 1.8 % among the new and 6.7 % among the previously treated cases (WHO, 2013).

2.11.2 MDR-TB incidence

The estimated global number of incident MDR-TB cases among new and relapsed TB cases in 2008 was 440 000 (95% CI: 390 000–510 000) (WHO, 2010). Based on incident MDR cases, the WHO and the Stop TB Partnership identified the 27 high MDR-TB-burden countries responsible for 85% of the global estimated burden of MDR-TB. The countries referred to were member states estimated by WHO in 2008 to have had at least 4000 MDR-TB cases arising annually and/or at least 10% of newly registered TB cases with MDR-TB (WHO, 2010). The countries are Armenia, Azerbaijan, Bangladesh, Belarus, Bulgaria, China, Democratic Republic of the Congo, Estonia, Ethiopia, Georgia, India, Indonesia, Kazakhstan, Kyrgyzstan, Latvia, Lithuania, Myanmar, Nigeria, Pakistan, Philippines, Republic of Moldova, Russian Federation, South Africa, Tajikistan, Ukraine, Uzbekistan and Vietnam. China and India account for almost 50% of the estimated global number of incident MDR-TB cases (WHO, 2010).

Due to the limitations in susceptibility testing in many countries, it is believed that the true magnitude of the MDR-TB problem in the world is larger than is currently known (2008). According to the Stop TB Partnership's Global Plan to Stop TB, 2006–2015, an estimated 1.3 million MDR-TB cases will need to be treated in the 27 highest MDR-TB burden countries between 2010 and 2015 alone, at an estimated total cost of US$ 16.2 billion.

2.11.3 Risk factors for MDR-TB

2.11.3.1 Genetic factors

There is some evidence to postulate host genetic predisposition as the basis for the development of MDR-TB (Sharma, Turaga and Balamurugan, 2003). The accumulation of changes in the genomic content, occurring through gene acquisition and loss are the major underlying events in the emergence of fit and successful strain variants in the *Mycobacterium tuberculosis* complex (Kato-Maeda, Bifani and Krieswirth, 2001). Spontaneous chromosomally borne mutations occurring in *Mycobacterium tuberculosis* at a predictable rate are thought to confer resistance to anti-TB drugs (Sharma and Mohan, 2004).

2.11.3.2 Factors related to previous antituberculosis treatment

Incomplete and inadequate treatment: MDR-TB develops due to errors in TB management such as the use of single drug to treat TB, the addition of a single drug to a failing regimen, the failure to identify pre-existing resistance, the initiation of an inadequate regimen using first-line antitubercular drugs and variations in bioavailability of anti-TB drugs which all predispose the patient to the development of MDR-TB (Sharma and Mohan, 2003). Shortage of drugs has been one of the most common reasons for the inadequacy of the initial anti-TB regimen, especially in resource poor settings (Mwinga, 2001). Other major issues significantly contributing to the higher complexity of the treatment of MDR-TB is the increased cost of treatment (Chan and Iseman, 2002).

Inadequate treatment adherence: Non-adherence to prescribed treatment is often underestimated by the physician and is difficult to predict. Certain factors such as psychiatric illness, alcoholism, drug addiction and homelessness do predict non-adherence to treatment (Sharma and Mohan, 2004). Poor compliance with treatment is also an important factor in the development of acquired drug resistance (Jacaban, 1994). Reasons for default may include change of location, symptom relief, adverse drug reactions and inability to afford treatment (Johnson *et al.*, 2003). MDR-TB requires a two- to fourfold longer period of treatment compared with drug susceptible TB (Chan and Iseman, 2002). The shortest treatment course so far validated for drug susceptible TB lasts six months (Chan and Iseman, 2002). Most of the problems from which drug resistance originates are related to length of treatment (especially considering tolerability). The longer time that is required to treat MDR-TB clearly implies an additional risk of poor treatment adherence and consequently of treatment failure (Drobniewski and Balabanova, 2002).

2.11.3.3 HIV infection

Upcoming evidence suggests a possible association between HIV and MDR-TB. MDR-TB has been widely documented in nosocomial and other congregate settings among people living with HIV (Gandhi, 2006). The fourth report issued by the WHO (2012) on anti-tuberculosis drug resistance indicated a significant association between HIV- positive status and MDR-TB in Latvia and Donetsk Oblast of Ukraine. Furthermore, in Lithuania HIV-

positive TB patients had an 8.4 (95% CI: 2.7– 28.2) times higher odds of harbouring MDR-TB strains than TB patients for whom the HIV status was unknown (WHO, 2010). Lastly, preliminary results of a survey conducted in Mozambique in 2007 have also found a significant association between HIV and MDR-TB (WHO, 2010).

2.11.3.4 Gender

While males predominate among TB cases in the world, an association between gender and MDR-TB has been controversial. Studies in South Africa, Australia, the Netherlands and the United States of America have reported slightly higher odds ratios among females than males, while other studies fail to find such associations (WHO, 2010). In general, it appears that the overall risk of harbouring MDR-TB strains is not influenced by gender.

Other factors: Some other factors also play important role in the development of MDR-TB such as poor administrative control on purchase and distribution of drugs with no proper mechanism on quality control and bioavailability tests (Prasad, 2005).

2.12 Diagnosis of drug-resistant TB, MDR-TB and XDR-TB

2.12.1 Phenotypic susceptibility testing techniques for *M. tuberculosis*

Conventional DST methods detect *M. tuberculosis* growth in the presence of antibiotics by one of three methods (absolute concentration, resistance ratio or proportion method) on solid (Lowenstein Jensen) medium but they require 2-4 weeks to obtain results from primary culture. The broth-based fully automated culture systems (such as the Bactec MGIT 960 TB system) are now routinely used for first- and second-line anti-TB DST of *M. tuberculosis* and report results within 5-8 days from a primary culture (Palomino *et al.*, 2008). Direct DST with smear-positive specimens has also been recently reported for INH and RIF with the Bactec MGIT 960 system which reliably detects MDR-TB cases within 8-10 days (Siddiqi, Ahmed, Asif, Behera Javaid and Jani , 2012).

Simple, inexpensive non-commercial phenotypic methods have also been developed for DST of *M. tuberculosis* in resource-poor settings (Moore and Shah, 2011). These methods include the microscopic observation drug susceptibility (MODS) test, thin layer agar (TLA) method,

nitrate reductase (NR) assay, colorimetric redox indicator (CRI) methods and phage-based assays (Palomino *et al.*, 2008). The MODS, NR and CRI are the only methods endorsed by WHO (WHO, 2012).

Microscopic-observation drug-susceptibility assay. The MODS assay is a liquid culture method based on the microscopic detection of characteristic *M. tuberculosis* morphology (cording), performed in 24-well plates (Palomino *et al.*, 2008; Moore, Mendoza and Gilman., 2004). It can be performed from cultures and directly from decontaminated sputum samples by inoculating into Middlebrook 7H9 broth containing first-line anti-TB drugs and the cording growth of *M. tuberculosis* is detected by an inverted microscope. A large number of samples can be tested and results are available within 7-9 days and exhibit ≥95 % agreement with conventional DST for INH, RIF and MDR-TB. The TLA method is a modification of the MODS assay for the detection of MDR-TB and XDR-TB in cultures (Moore and Shah, 2011). One of the major advantages of using the MODS technique is the low material and running costs for the test, although an inverted light microscope is not available in all laboratories. Tests in several laboratories have demonstrated high concordance for INH (97%), RMP (100%) and fluoroquinolones (100%) compared with reference standard techniques (Moore, Evans and Gilman., 2006; Devasia, Blackman and May, 2009) but lower concordance for EMB (95%) and SM (92%) (Moore *et al.*, 2006). The technique is rapid; in general, no more than 7 days are needed for obtaining DST results to analyse the strains. Time to results for the direct analysis of sputum samples ranges from 15 to 29 days (Bwanga *et al.*, 2009)

The MODS technique also has some limitations. Reading of the plates has to be performed on a daily basis and is therefore laborious and time-consuming. Furthermore, no unambiguous identification of *M. tuberculosis* is performed. The typical cording formation can sometimes also been seen in nontuberculuos mycobacteria species (e.g., *Mycobacterium kansasii*). Another topic is the sealing technology in ziplock$^{(R)}$ plastic bags. The tightness of these bags has to be assured. Normally the bags are not opened throughout the whole procedure. As consequence, subsequent analysis can only be performed after opening of the bags and the plates, which presents a safety risk. Although MODS technology is labour intensive, once standardised, it may be a cost-effective alternative in high burden developing countries (Moore *et al.*, 2006).

Nitrate reduction assay. The nitrate reduction assay (NR) is an inexpensive alternative method for the rapid and accurate detection of resistance to all first-line drugs in culture isolates based on the ability of *M. tuberculosis* to reduce nitrate to nitrite, which can easily be detected with specific reagents producing a colour change. It utilises the detection of nitrate reduction as an indication of growth and therefore results also can be obtained faster than by visual detection of colonies. This technique has also been established as a low-cost, easy-to-perform DST assay for low-income countries (Moore and Shah, 2011). NR has the advantage of being performed on the classical Löwenstein–Jensen medium with the well-known critical concentrations for the antibiotics. Although some standardisation still seems to be necessary, mainly for application directly on sputum samples, preliminary results appear promising and show a good sensitivity and specificity. For the NRA technology, tight tubes are used, therefore safe handling is ensured. NRA technology is also (as MODS) a promising, highly cost-effective technology for high-burden developing countries (Musa, Ambroggi, Sout and Angeby, 2005; Affolabi, Odoun and Sanoussi, 2008; Shikama Ferro e Silva and Villela, 2009)

Phage-based assays. These assays use mycobacteriophages (viruses that efficiently and specifically infect mycobacteria) to detect the presence or growth of *M. tuberculosis* by using a phage amplification assay or the more sensitive luciferase reporter mycobacteriophages in the absence or presence of anti-TB drug(s). Viable *M. tuberculosis* growing in the presence of anti-TB drugs emits light in the presence of luciferin and is identified as a drug-resistant strain (Lew, Pai, Oxlade, Martin and Menzies, 2008). One commercial phage assay is available (FASTPlaque™, Biotech Labs Ltd). The assay includes the *M. tuberculosis*-specific phage D29, whose replication can be determined by counting the viral particles on fast-growing *Mycobacterium smegmatis* (Wilson, al-Suwaidi, McNerney, Porter and Drobniewski., 1997). After overnight incubation, the clear plaques are visible on indicator plates harbouring the turbid lawn of *M. smegmatis*. An alternative system is the luciferase reporter phage assay which uses recombinant mycobacteriophages carrying the firefly luciferase gene whose activity can be detected by luminescence (Jacobs Barletta and Udani, 1993). The assay is based on the fact that replication of the phage is dependent on viable mycobacterial cells. In the presence of a drug, the phages are only able to replicate if a drug-resistant strain is present. Visualisation of the amplified phages can be performed according to the respective test, either by counting the plaques or by determination of the light emission.

Most evaluation studies using phage assays are restricted to the detection of RMP resistance in culture isolates (Pai, Kalantri, Pascopella, Riley and Reingold, 2005).

Etest (bioMerieux) is a predefined, stable gradient of 15 antibiotic concentrations on a plastic strip and is used for MIC determination for a variety of antibiotics (Hausdorfer, Sompek, Allerberger, Dierich and Rüsch-Gerdes., 1998). Owing to their well-known use for other bacteria and the possibility of obtaining rapid results without additional equipment, Etest strips have also been tried for DST of *M. tuberculosis* (Freixo, Caldas and Said, 2004). Good overall agreement with conventional DST for RMP and INH has also been reported for this method. Again, EMB and SM testing gave the most discrepancies. Results could be obtained within 5–15 days. Although these test results seem rather promising, a major concern of this methodology is the associated biosafety risks. A high amount of TB bacteria grow on normal culture plates, which cannot be adequately closed. Closing those plates using adhesive tapes is laborious and not applicable for routine use (Freixo *et al*., 2004)

Growth on membranes (Anopore) A new method uses Anopore strips, an inert nanoporous ceramic material. Placed upon appropriate media, it supports growth of *M. tuberculosis* with and without drugs. After 3 days, the resulting microcolonies are heat-killed, stained and imaged directly on the membrane by fluorescence and electron microscopy. The method has recently been evaluated for RMP and INH testing (Ingham, Ayad, Nolsen and Mulder, 2008)

Thin-layer agar Microcolonies of *M. tuberculosis* grown in 7H10 Middlebrook thin layer agar (TLA) can be seen under a microscope (10× objective). The TLA method is more rapid than DST on solid media, but slower than on liquid media. The method is inexpensive and simple to perform without highly sophisticated equipment, and is an alternative method in high burden countries with low resources. Nevertheless, the method can only be used if the laboratories are able to work under safe conditions (Robledo, Mejia, Paniagua, Martin and Guzmán, 2008)

2.12.2 Genotypic-based method

Using molecular methods in the detection of genetic mutations that are associated with resistance to certain antibiotics has recently become more established than before. They offer several advantages such as faster turnaround times and the possibility of omitting the cultures. A prerequisite is the knowledge of specific molecular changes that are undoubtedly associated with resistance. The development of drug resistance in *M. tuberculosis* complex isolates is exclusively the result of random genetic mutations in particular genes (Zhang and Telenti, 2000; Maus, Plikaytis and Shinnick., 2005a). Resistance to antituberculosis agents develops by sequential acquisition of mutations in target genes owing to the natural mutation rate of genomic DNA. Thus far, no single pleiotropic mutation has been found to cause the MDR phenotype, which is also found to be caused by sequential accumulation of mutations in different genes (Maus *et al*., 2005a)

Telenti and colleagues determined the site of mutations that resulted in RMP resistance in *M. tuberculosis* as the *rpo*B gene, which codes for the β-subunit of the DNA-dependent RNA polymerase (Telenti *et al*., 1993). They showed that almost all RMP-resistant isolates had mutations in a well-defined 81-bp hotspot region of *rpo*B. Subsequently, further clinical studies revealed that mutations are found in this region in 95–99% of resistant isolates (Ling *et al*., 2008a). INH is a prodrug that needs to be activated by the *M. tuberculosis* catalase-peroxidase enzyme (KatG) to its active form. The active drug inhibits the enoylreductase InhA, an enzyme of the bacterial fatty acid biosynthesis (Vilcheze and Jacobs, 2007). Resistance develops due to point mutations in the *kat*G gene, resulting in failure to activate the prodrug (Zhang and Telenti, 2000). In addition to mutations in *kat*G, mutations in the enoylreductase InhA-coding gene, *inh*A may contribute to INH resistance due to overexpression or alteration of the drug target (Piatek, Telenti and Murray, 2000). Mutations within the *oxy*R–*ahp*C intergenic region, resulting in increased expression of alkyl hydroperoxidase are considered to compensate for the loss of KatG function (Sherman, Mduli and Hickey, 1996). In addition to mutations in *katG*, *inhA* and *oxyR–ahpC*, mutations in *ndh* and *kasA* were found in some cases as markers of INH resistance, although the molecular mechanisms involved remain speculative (Lee, Teo and Wong, 2001).

Ethambutol is an inhibitor of EmbB; an arabinosyltransferase encoded by the *emb*B gene, and inhibits the synthesis of arabinogalactan, a major component of the mycobacterial cell wall. Although the precise mechanism of inhibition is not known, it was suggested to be the

primary target of EMB (Plinke, Rüsch-Gerdes and Niemann, 2006). In up to 65% of EMB-resistant clinical isolates of *M. tuberculosis*, point mutations in the *embCAB* operon were identified, and mutations at codon 306 of *embB* occur most frequently (Hillemann *et al*., 2009). Pyrazinamide, an analog of nicotinamide, is only active at an acidic pH, which often leads to difficulties in PZA susceptibility testing. PZA is a prodrug of pyrazinoic acid that is converted to its active form by the mycobacterial nicotinamidase/pyrazinamidase (PZase) (Zhang and Telenti, 2000). Defective PZase activity due to *pnc*A mutations scattered over the whole gene are found to be responsible for the majority of PZA-resistant clinical isolates tested so far (Scorpio and Zhang, 1996).

Although chemically classified into different groups, amikacin, kanamycin and capreomycin are all inhibitors of protein biosynthesis. Cross-resistance has repeatedly been demonstrated (Maus *et al*., 2005). The association of particular *rrs* (16S rRNA gene) mutations with resistance to these drugs has been described. Mutations A1401G, C1402T and G1484T in the *rrs* gene have been linked to amikacin, kanamycin and capreomycin resistance, each of them being responsible for a specific resistance pattern. Mutations G1484T and A1401G were found to cause high level resistance to all drugs, whereas C1402T causes resistance to only kanamycin and capreomycin. Recently, it was demonstrated that mutations in the *tlyA* gene, encoding a putative rRNA methyltransferase, confer resistance to capreomycin (Maus *et al*., 2005b). The main target of fluoroquinolones in *M. tuberculosis* is the DNA gyrase, encoded by *gyr*A and *gyr*B (Takiff, Salazar and Guerrero., 1994). Mutations in the 'quinolone resistance-determining-region' in *gyrA* have been most frequently associated with resistance, often carrying mutations in codons 90, 91 and 94 (Zhang and Telenti, 2000). Regarding ethionamide, as it is a structural analog of INH, it was suggested that it has the same cellular target, the InhA enzyme (Banerjee, Dubnau and Quernard, 1994). In analogy to this, *inhA* gene mutations are assumed to cause resistance to ethionamide (Morlock, Metchock, Sikes, Crawford and Cooksey, 2003). Mutations in the gene encoding monooxygenase (*ethA*), the activating enzyme for ethionamide (a prodrug), were also found.

Based on this knowledge, various molecular assays have been established, which allow for the prediction of drug resistance in clinical *M. tuberculosis* complex isolates within one working day. Generally, DNA sequencing-based approaches are considered to be the reference assays for the detection of mutations, providing the highest level of information. It can be performed by both manual and automated procedures. Automated DNA sequencing is widely used to

search for mutations associated with resistance, for example, by analysing mutations in the *rpo*B, *gyr*A, *gyr*B *and* *pnc*A genes (Mestdagh, Fonteyne and Realini, 1999). Its application in routine use is often found to be too cumbersome, and extensive equipment is needed. Nevertheless, in the case of unknown or new mutations, or discrepancies between phenotypic and genetic-based results, DNA sequencing should be applied (Maus, 2005b).

Several other genotypic methods have also been developed and are in use, such as PCR-single strand conformation polymorphism (SSCP) analysis, multiplex allele-specific (MAS) PCR heteroduplex formation, hybridisation assays, DNA microarrays or high-density oligonucleotide arrays, PCR-restriction fragment length polymorphism analysis (PRA) and real-time PCR techniques. At present, a variety of real-time PCR instruments are available, together with several fluorescence formats for correlating the amount of PCR product with fluorescence signals. All real-time systems have the advantage of running the reaction as a closed system and therefore diminishing the chances of contamination. Only recently, a new real-time based PCR system was developed for the direct identification of *M. tuberculosis* complex bacteria with simultaneous detection of RMP resistance from specimens (Xpert® MTB/RIF, Cepheid). The system has the additional feature of being fully automated from DNA extraction to the PCR and post-PCR analysis. For this reason, this assay is of especially great value, whether or not the safety standards for culturing mycobacteria have been realised in the laboratory (Park, Song and Song, 2006).

SEQUENCING: DNA sequencing remains the gold standard in genetic-based DST methods since it can reveal the complete genetic profile of the region targeted by the anti-TB drug (Richter,Rusch-Gerdes and Hillemann, 2009). Not only can sequencing be used for species identification (by analysis of 16S rRNA) and searching for known resistance-causing mutations but it can also be used to screen for novel SNPs that may be associated with drug resistance (Tortoli, 2003). However, the major drawbacks of sequencing are the costs associated with the test, the technical skill required to operate the expensive equipment and the unavailability of sequencing facilities in most resource-poor settings where the burden of TB is high (Richter *et al*., 2009).

2.12.3 Commercial genotypic-based assays

Three commercial reserve hybridisation-based line probe assays have also been developed.

INNO-LiPA Rif. TB detects *M. tuberculosis* and its resistance to RIF but it can also predict the MDR status of ~85 -90 % of *M. tuberculosis* strains (Drobniewski *et al.*, 2012).

Genotype MTBDR*plus* detects RIF and INH resistance (MDR-TB strains) in culture isolates and sputum samples targeting *rpo*B, *kat*G, and *inh*A genes (Al-Mutairi *et al.*, 2011). The sensitivity for the detection of *M. tuberculosis* in smear- positive samples is 78- 98 % but it is lower for smear-negative pulmonary and extrapulmonary specimens (Drobniewski *et al.*, 2012). The sensitivity for RIF + INH resistance (MDR-TB) detection in culture isolates and smear- positive sputum samples has been reported as ~85- 90% (Mironova *et al.*, 2012)

Genotype MTBDR*sl* detects resistance to FQs, injectable drugs (KAN/AMI/CAP) and EMB in culture isolates and clinical specimens (Drobniewski *et al.*, 2012). Pooled sensitivities of genotype MTBDR*sl* for the detection of resistance to FQs, KAN, AMI and CAP were reported as 85%, 83%, 90% and 87% respectively (Lacoma *et al.*, 2012). Genotype MTBDR*sl* and genotype MTBDR*plus* together detect XDR –TB strains reducing the time to diagnosis of XDR –TB in high – prevalence settings (Barnard, Warren, Van Pittius, van Helden, Bosman, Streicher., 2012).

Molecular beacons detect *M. tuberculosis* complex and associated RIF resistance directly from sputum samples using ultrasensitive semi-nested PCR (Helb, Jones and Story, 2009). Initial study results indicated that the method had a detection limit of 4.5 bacilli or 131 CFU/ml (in clinical specimens) per reaction and a turnaround time of less than 2h (Piatek *et al*., 2000). The system is fully automated in such a way that sample decontamination, PCR and real-time analysis occurs within the apparatus (Raja, Ching and Xi, 2005). The only manual step consists of adding bactericidal buffer to the specimen and transferring the mixture to the cartridge (Boehme *et al*., 2010).

Loop-mediated isothermal amplification is a rapid (< 90 min), highly specific and sensitive technique which requires minimal infrastructure, laboratory skills and relies on auto-cycling strand displacement DNA amplification which is performed at a uniform temperature by the enzyme *Bst* polymerase (Zhu, Zhang and Zhao, 2009). LAMP-based assays targets areas specific to mycobacterium including the *gyr*B (Iwamoto, Sonobe, Hayashi, 2003), *rrs*

(Pandey, Poudel and Yoda, 2008) and *rim*M (Zhu, Zhang and Zhao, 2009) genes encoding 16S rRNA-processing protein.

2.12.4 Immunological methods

Microbiological methods represent the most direct indication of disease due to *M. tuberculosis* and although culture methods and the genotypic-based assays often perform with high specificity they often have poor sensitivity (Sarmiento, Weigle, Alexander, Weber and Miller, 2003). This is evident in paucibacillary disease, including smear negative and extra-pulmonary TB (Ling *et al*., 2008, Reid and Shah, 2009). This method measures nonspecific mediators of inflammation secreted by innate and adaptive immune cells, aspects of the T-cell mediated immune response to *M. tuberculosis* antigens or the detection of specific antibodies against these antigens by serological tests (Dinnes, Deeks and Kunst, 2009).

Tuberculin skin test, also called Mantoux skin test, has been used for the diagnosis of tuberculosis for more than a century. Despite the numbers of logistic and performance problems and poor specificity, TST is still performed as a routine diagnostic method. The purified protein derivative (PPD) antigens that are used for TST are highly homologous to antigens of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) vaccine and nontuberculosis mycobacteria (NTM) antigens. These and other factors may lead to false positive and false negative TST results. Although other antigens have been evaluated as skin test reagents, e.g. molybdopterin-64 (MPT-64) and molybdopterin-59 (MPT-59), none of them proved superior to the tuberculin skin test (Wilcke, Jensen, Ravn, Andersen and Haslov, 1996).

Interferon gamma-releasing Assay (IGRA) IFN-γ is a cytokine that plays a critical role in resistance to *Mycobacterium tuberculosis* infection; MTB-infected individuals respond to MTB antigen stimulation by releasing increased amounts of this cytokine from effector memory cells. Methods based on measuring the IFN-γ production by antigen stimulated human T lymphocytes have been developed. The enzyme-linked immunospot assay (ELISpot) (T-SPOT®.TB, Oxford Immunotec, Oxford, UK) and the enzyme-linked immunosorbent assay (ELISA) (QuantiFERON-TB Gold In-Tube, QFT-GIT, Cellestis, Carnegie, Australia) are two new blood tests. Both IGRAs have high sensitivity and specificity, for QFT-GIT 81% - 92.6% and 98.8% - 99.2% and for T-SPOT®.TB 87.5% - 95.6% and 86.3% - 99.9% respectively (Harada, Higuchi, Yoshiyama, Kawabe, Fujita, Sasaki,

Horiba, Mitarai, Yonemaru, Ogata, Ariga, Kurashima, Wada, Takamori, Yamagishi, Suzuki, Mori and Ishikawa., 2008; Diel, Loddenkemper and Nienhaus, 2010; Oxford Immunotec, 2011).

2.13 Treatment of drugresistant TB, MDR TB AND XDR- TB

Monodrug-resistant TB is caused by a *Mycobacterium tuberculosis* strain that grows in vitro in the presence of one anti-TB drug. Polydrug-resistant TB is defined as resistance to various combinations of first-line drugs, excluding INH + RIF (Blumberg, Burnam, Chaisson, Daley , Etkind and Friedman, 2003). The resistance profile of an *M. tuberculosis* strain is the most important strain characteristic, as treatment regimens and durations are devised according to the profile (Furin, 2007). Monodrug resistance of *M. tuberculosis* to SM is common but rare for EMB. Treatment duration/failure for SM or EMB-resistant TB is the same as that for susceptible TB, as SM is no longer used as a first-line drug while EMB is used only during the initiation phase and is easily replaced by oral FQs (Mitchison and Davies, 2012). Treatment of INH-resistant TB with a daily 9-month regimen of RIF, PZA and EMB in low-TB-incidence countries is favourable (Mitchison and Davies, 2012). For other settings, daily therapy for >6 months with regimens containing RIF, other effective drugs and KAN/AMI/CAP yields a better prognosis (Menzies, Benedetti, Paydar, Royce, Madhukar and Burman, 2009).

Monodrug-resistant strains of *M. tuberculosis* to PZA have also been described and are associated with a poorer clinical outcome despite a 9-month treatment with RIF-containing regimens (Yee, Menzies and Brassard, 2012). Monoresistance to RIF, the most effective drug against non-replicating dormant bacilli occurs rarely (except in HIV-coinfected patients) and is associated with poorer outcomes and a higher relapse rates even with the use of 4-drug regimens for >9 months, but outcomes improve with 12-month therapy (Lew *et al.*, 2008). Treatment of infections involving polydrug-resistant (INH + EMB, INH + PZA, EMB + PZA or INH + EMB + PZA) *M. tuberculosis* strains requires careful clinical evaluation but can be managed with extended treatment (~18 months) using regimens containing first- line drug(s), FQs, KAN/AMI/CAP and some second-line agents (Lew *et al.*, 2008).

Treatment of MDR-TB is lengthy, expensive, toxic and associated with higher rates of clinical failure and disease relapse. Successful management of MDR-TB requires DST for first- and

second-line drugs and monitoring of patients for bacteriological (sputum smear and culture) and radiological improvement and adverse drug reactions (Orenstein *et al.*, 2009). In developing countries, treatment of MDR-TB is difficult due to delayed diagnosis, inadequate DST facilities and the lack of adequate supplies of second-line drugs. Two basic treatment approaches are used: either a standardised treatment regimen design based on representative drug-resistance surveillance data or individualised regimens tailored on a previous history of anti-TB treatment and DST results (Caminero *et al.*, 2010).

Treatment recommendations for MDR-TB include an intensive treatment phase of 6-8 months (4 months after culture conversion) with 5-6 effective drugs that preferably include PZA, a FQ (LFX/GFX/MFX), and injectable agent (KAN/AMI/CAP/VIO) and 2-3 other second-line drugs (Orenstein *et al.*, 2009). EMB and high-dose INH may also be included as additional drugs in some cases. The injectable agent may be discontinued after 8 months; however a continuation phase with 4 effective drugs should last for ≥20 months (18 months after culture conversion) (Caminero, Sotgiu, Zumla and Migliori , 2010). Adverse reactions occur frequently and pose a challenge in MDR-TB treatment (Van Deun, Maug, Salim, Das, Sarker, Daru and Rieder, 2010). A once-monthly sputum-smear examination, culture and DST predict response to treatment and should be carried out until sputum culture-negative status has been achieved (Ahmad and Mokaddas, 2012). Surgery could also be considered in specific cases when an insufficient number of second-line drugs are available, the patient has localised lesions and can tolerate surgery (Gegia, Kalandadze, Kempker, Magee and Blumberg, 2012).

Extensively drug resistant (XDR) is defined as TB with resistance to at least isoniazid and rifampin plus a fluoroquinolone (e.g. monofloxacin, ofloxacin, levofloxacin, sparfloxacin, gatifloxacin, ciprofloxacin) and one of three injectable second-line drugs (capreomycin, kanamycin, and amikacin). Pre-extensively drug resistant (Pre-XDR) is defined as disease caused by a TB strain resistant to isoniazid and rifampin and either a fluoroquinolone or a second-line injectable drug, but not both (WHO, 2008). Furthermore, while the principles of treatment are the same, successful treatment of XDR-TB is more difficult than that of MDR-TB even in developed countries (Gandhi *et al.*, 2012). In resource-poor countries, XDR-TB is generally an untreatable disease mainly due to the non-availability of drugs effective in killing semi-dormant/dormant bacilli. Thus, efforts should be made to appropriately manage MDR-

TB patients to avoid XDR-TB (Jacobson, Tierney, Jeon, Mitnick and Murray, 2010). The addition of PZA, EMB and/or SM (if still effective) to multidrug regimens improves the prognosis of XDR-TB. The treatment of XDR-TB should include an injectable agent and a FQ based on an actual DST profile or previous treatment history (Jacobson *et al.*, 2010). Other experimental drugs have also been used occasionally with varying degrees of success (Amaral and Viveiros, 2012). Surgical resection may lower the bacterial burden and improve the clinical outcome for pulmonary TB patients with localised disease and limited lung tissue damage (Gegia *et al.*, 2012). Patients who fail to undergo sputum culture conversion within 6 months of starting treatment with several anti-TB drugs are more likely to fail therapy (Ahuja, Ashkin, Avendano, Banerjee, Bauer and Bayona., 2012).

CHAPTER 3

COMPARISON BETWEEN THE BACTEC MGIT 960 SYSTEM ASSAY AND GENOTYPE MTBDR DNA STRIP ASSAY FOR SUSCEPTIBILITY TESTING OF FIRST- AND SECOND-LINE ANTI-TUBERCULOSIS DRUGS

Abstract

BACKGROUND The increase in incidence of multidrug-resistant tuberculosis (MDR-TB) and the emergence of extensively drug-resistant tuberculosis (XDR-TB) have brought tremendous challenges to combat tuberculosis. Susceptibility testing of the causative agent *Mycobacterium tuberculosis* is critical for control of the disease. Phenotypic methods are labour intensive and time-consuming, this creates an urgent need to explore other methodologies such as the molecular assays that are easier to perform with a rapid turn-around time. **OBJECTIVE** To compare the performance of Line probe assay (Genotype$^{(R)}$MTBDR*plus* V2.0 and Genotype$^{(R)}$MTBDR*sl* V2.0) assay to BACTEC MGIT 960 system for the detection of resistance to first and second-line drugs. **METHODS** One hundred (100) consecutive non-repeat *Mycobacterium tuberculosis* cultures resistant to either isoniazid or rifampicin or both by BACTEC MGIT 960 system were collected from the medical microbiology diagnostic laboratory (NHLS). Isolates were sub-cultured into liquid media (BACTEC MGIT 960). All viable cultures were processed with Genotype$^{(R)}$MTBDR*plus and* Genotype$^{(R)}$MTBDR*sl* assay and subjected to susceptibility for Isoniazid (INH), Rifampicin (RIF), Kanamycin(KAN) ,Ofloxacin(OFX) and Ethambutol (ETH). **RESULTS** Of the 100 collected cultures, only 97 and 90 were viable after sub-culture for Genotype$^{(R)}$MTBDR*plus and* Genotype$^{(R)}$MTBDR*sl* assay respectively. Genotype$^{(R)}$MTBDR*plus* detected 40/97 (41%) MDRs, the rest were mono-resistant (14/97 INH mono-resistant and 43/97 RIF mono-resistant). Fifty three MDR-TB isolates were detected by BACTEC MGIT 960 system. For second-line drugs, BACTEC MGIT 960 system showed 26/90 (28.8%), 9/90 (10%) and 3/90 (3.3%) resistance to Ethambutol, Ofloxacin and Kanamycin respectively while Genotype$^{(R)}$MTBDR*sl* assay detected 32/90 (35.5%), 16/90 (17.7%) and 5/90 (5.5%) resistance to Ethambutol, Fluoroquinolones and Aminoglycosides respectively. Genotype$^{(R)}$MTBDR*sl* also showed good concordance by detecting 5.5 % (5/90) XDR-TB compared to 3.3 % (3/90) detected by MGIT 960 system. Compared with BACTEC MGIT 960 system, the sensitivity and negative predictive value was

100% for all the drugs except ethambutol (63% 85 % respectively). The specificity, positive predictive values were 58 % and 87 % for RIF, 93 % and 94 % for INH, 91 % and 56 % for OFX, 97 % and 60 % for RIF, 89 and 71 % for EMB. **CONCLUSION** Line probe assay (Genotype(R)MTBDR*plus and* Genotype(R)MTBDR*sl*) showed an excellent agreement in detection of INH resistant, good agreement for RIF, OFX, KAN and moderate agreement for EMB resistance. This data suggest that even though Line probe assay is a rapid (turnaround time) and sensitive rest for the detection of MDR and XDR-TB, conventional DST should always be carried out before making conclusive test results.

KEYWORDS: Multidrug-resistance, *Mycobacterium tuberculosis*, Line-probe assay, MGIT 960 system, Isoniazid, Rifampicin, Ethambutol, Ofloxacin, Kanamycin.

3.1 Introduction

Tuberculosis remains a worldwide healthcare concern and has been characterised as an epidemic by the World Health Organization (WHO, 2010). The distribution of TB in different geographic regions is characterised by the prevalence of different MTB strains with varied virulence and drug resistance (Dou, Tseng and Lin., 2008). Instead of being eradicated, drug-resistant strains have evolved and have been documented in every country surveyed (WHO, 2000). Despite global attempts at TB control, the worldwide disease incidence rate was 139 per 100,000 of the population in 2008, with the incidence rate in Africa reaching 350 per 100,000 in the same year (WHO, 2011). A crucial aspect of any TB control program is the ability to determine where transmission occurs in order to prevent further spread of infection and prevent active disease by identifying newly infected people and providing them with preventive therapy (Kulaga, Behr and Schwartzmank, 1999). The dynamics of the TB epidemic are greatly determined by how soon cases are diagnosed, impacting the duration of infectiousness (Wood, Middelkoop and Myer, 2007).

A recent prospective study in South Africa showed that 17% of 367 TB cases diagnosed on smear results never started treatment owing to incomplete sputum sample collection, problem with sample transport and poor record-keeping of the samples taken and their subsequent results (Botha, Den Boon and Lawrence, 2008). This illustrates that the key to effective combat of TB remains comprehensive case finding and reporting (Botha *et al*., 2008). Once a strain of *Mycobacterium tuberculosis* develops resistance to isoniazid and rifampicin, it is defined as multidrug-resistant tuberculosis (MDR-TB) (Becerra Freeman and Bayona , 2000). The increase of MDR-TB incidence and the emergence of XDR-TB have brought tremendous challenges to combatting tuberculosis (WHO, 2008). Despite the use of effective chemotherapy during the past 50 years, drug-resistant TB is increasingly a worldwide problem, complicating TB treatment (Espinal *et al*., 2000). More recently, extensively drug-resistant TB (XDR-TB) has been recognised as an even more severe public health problem (Shah, Wright and Bai, 2007).

The chemotherapy of MDR-TB relies upon residual first-line drugs appropriately combined with additional second-line drugs based on the outcome of drug susceptibility testing (Di Perri and Bonora, 2004). However, globally, up to 96 % of incident MDR-TB cases are not being diagnosed and treated according to international guidelines (WHO, 2011). Molecular genotyping is an important tool for understanding TB epidemiology, as it determines

transmission rates and identifies predominant genotypes among the *M. tuberculosis* isolates and strains with an enhanced capacity to spread in association with outbreaks and drug resistance (Puustinen, Marjamaki and Rastogi, 2003; Ahmed and Hasnain, 2004).

BACTEC MGIT 960 system is a rapid phenotypic DST method based on liquid media and has been applied for DST to first-line drugs in many studies (Bemer, Palicova, Rusch-Gerdes , Drugeon and Pfyffer, 2002; Espasa, Salvado, Vincente, Tudo and Alcaide, 2012; Huang Tu, Lee, Huang and Liu,, 2002; Johansen, Thomsen, Marjamaki, Sosnovskaja and Lundgren, 2004; Kobayashi, Abe and Mitarai., 2006; Maniati, Costopoulos, Gitti, Nicolaou and Petinaki, 2004; Kontos Nicolaou, Costopoulos, Gitti, Petinaki and Maniati., 2003; Kruuner, Yates and Drobniewski, , 2006; Tomita, Takeno, Suzuki, Sakatani, Kinoshita, Kobayashi., 2004). It automatically reports the drug susceptibilities according to the predefined algorithms in 4-13 days after inoculation (Piersimoni, Olivieri, Benacchio and Scarparo, 2006). It has also been used for testing *M. tuberculosis* against second-line drugs and establishing the second-line drugs critical concentrations and guidelines of BACTEC MGIT 960 system (Lin, Desmond, Bonato, Gross, Siddiqi, 2009; Rodrigues, Jani, Shena, Thakkar, Siddiqi, and Mehta, 2008; Rusch-Gerdes, Pfyffer GE, Casal M, Chadwick M, Siddiqi, 2006).

The world Health Organization have endorsed the use of the molecular test GenoType® MTBDR*plus* (Hain Lifescience, Nehren, Germany) for rapid detection of high-risk MDR-TB cases (Akpaka *et al*., 2008). GenoType MTBDR*plus* test is a PCR-based amplification and reverse blotting assay that employs specific probes hybridized to nitrocellulose strips to detect RIF and INH resistance (Hillemann *et al*., 2007). The assay detects mutations in the *rpo*B gene for RIF resistance, in the *katG* gene for high-level INH resistance and in the *inhA* regulatory region gene for low-level INH resistance (Causse *et al*., 2008). GenoType®MTBDR*sl* (Hain Lifescience, Nehren, Germany) is the only rapid test that detects resistance to second-line fluoroquinolone (FQ) and second-line injectable drugs (SLID) as well as detecting XDR-TB. MTBDR*sl* can be performed on TB bacteria grown from sputum, which is called indirect testing and can take a long time, or can be performed immediately on sputum, which is called direct testing (Theron *et al*., 2014). Drug susceptibility testing by the agar proportion method as described by Canetti, Wallace, Khomenko, Mahler, Menon, Mitchison, Rist and Smelev, (1969), Kent and Kubica (1985) and Abdel Aziz (2003) determines the percentage of growth (number of colonies) of a defined inoculum on a drug-free control medium versus growth on culture media containing the critical concentration on

an anti-TB drug. The aim of this study was to compare the performance of the Genotype MTDR DNA strip V2.0 assay and BACTEC MGIT 960 system for the detection of resistance to first- and second-line drugs. The agar proportion method was taken as the reference standard.

3.2 Materials and methods

3.2.1 Study design and location

This study was a descriptive study comparing the performance of the BACTEC MGIT 960 system (Becton Dickinson Microbiology System, Sparks, MD, USA) and the DNA strip assay GenoType®MTBDR (Hain Lifescience, Nehren,Germany) for susceptibility testing of the first- and second-line anti-TB drugs. The experimental work was divided into two components: the collection of MDR-TB cultures was done in the NHLS Tshwane Academic Division TB laboratory in the Department of Medical Microbiology University of Pretoria. All susceptibility testing was done at the TB Centre located at MRC Pretoria due to the availability of the required Level 3 biosafety cabinet for processing of MDR-TB cultures.

3.2.2 *Mycobacterium tuberculosis* culture

One hundred (100) consecutive nonrepeat *Mycobacterium tuberculosis* cultures resistant to either isoniazid or rifampicin or both by BACTEC MGIT 960 system were collected from the medical microbiology diagnostic laboratory (NHLS). The *Mycobacterium tuberculosis* cultures were confirmed with the Ziehl-Neelsen staining method (Appendix I).

3.2.3 MGIT 960 system assay

BACTEC MGIT 960 TB cultures were first inoculated into MGIT 960 vials and cultured into the BACTEC MGIT 960 instrument (Becton Dickinson Microbiology System, Sparks, MD, USA) until it was flagged positive. The test was based on growth of the *Mycobacterium tuberculosis* isolate in a drug-containing tube compared to a drug-free tube (Growth Control). Susceptibility testing to OFX, KAN, SM, INH, RIF and EMB using the BACTEC MGIT 960 system was performed. The drugs were obtained as BACTEC MGIT 960 SIRE kit from Becton Dickinson Microbiology System, Sparks, MD, USA as lyophilized vials of streptomycin, isoniazid, rifampicin, ethambutol and eight vials of SIRE supplement. The second-line drugs,OFX and KAN, were obtained in a chemically pure form from Sigma and

prepared according to the standard procedures described by Rodrigues (Rodrigues *et al*., 2008) and Rusch-Gerdes (Rusch-Gerdes *et al*., 2006). Each of the lyophilised drug vials was reconstituted with 4 ml of deionised water to make a stock solution and final concentration as described in Table 3.1. Final drug concentrations used for second-line were 2.5 µg/ml for KAN and 2.0 µg/ml for OFX.

Table 3.1: Concentration, volume and final concentration of first-line drugs used for MGIT 960 system susceptibility testing (Siddiqi and Rusch-Gerdes, 2006)

Drug	Concentration of drug after reconstitution *	Volume added to MGIT tube	Final concentration in MGIT tube
STR	83 µg/ml	100 ml	1.0 µl/ml
INH	8.3 µg/ml	100 ml	0.1 µl/ml
RIF	83 µg/ml	100 ml	1.0 µl/ml
EMB	415 /ml	100 ml	5.0 µl/ml

*The drugs were reconstituted using 4 ml sterile deionised water to achieve the indicated concentration

The day a MGIT tube flagged positive by the instrument was considered as Day 0 after which each positive tube was incubated for one more day before being used for the susceptibility testing at Day 1. 5 MGIT tubes were labelled for each test culture as GC (growth control), SM, INH, RIF and EMB for the first-line drugs while 3 MGIT tubes were labelled as GC, OFX and KAN for second-line drugs respectively. 0.8 ml of BACTEC 960 SIRE supplement was aseptically added to each of the MGIT tubes after which 100 µl of properly reconstituted drugs were aseptically added to each labelled MGIT tube apart from the GC tube. 500 µl of the well-mixed Day 1 culture was aseptically added into each of the drug containing tubes using a pipette. For the GC, the test culture suspension was first diluted by adding 0.1 ml of the test culture suspension to 10 ml of sterile saline (1:100); 500 µl of the diluted suspension was added in to the growth control tube. All the caps were tightened and mixed thoroughly by inverting several times. All inoculated drug-containing and GC tubes were placed in the DST set carrier and entered into the MGIT 960 instrument using the DST entry feature. The instrument flagged the DST set complete when the growth control reached a growth unit (GU) value of 400. At that point, the GU values of drug-containing tubes were retrieved from the instrument by printing out the DST set report but interpreted manually for the second-line

drugs. All the BACTEC MGIT 960 test methods were carried out in the biosafety cabinet level 3.

3.2.4 GenoType®MTBDR DNA strip assay V2.0

The GenoType®MTBDR*plus* and GenoType®MTBDR*sl* assays were performed respectively according to the instructions provided by the manufacturer (Hain Lifescience, Nehren, Germany). Firstly, Genolyse$^{(R)}$ Kit (Hain Lifescience, Germany) was used for crude DNA extraction from 1 ml of the culture samples which was centrifuged (Eppendorf Centrifuge 5417C) for 15 minutes at 12,000 rpm, the supernatant was then discarded and the pellet was resuspended in 100 µl of lyses buffer (A-LYS). The resuspended pellet was incubated at 95 ^{o}C for 5 minutes and 100 µl neutralization buffer (A-NB) was added and vortexed. It was centrifuged for 5 minutes at 14,000 rpm to get the DNA to be used for amplification which is the next step after DNA extraction. Furthermore, before amplification, a master mix containing AM-A and AM-B was prepared and mixed carefully according to the manufacturer's master mix preparation table; 5 µl of the extracted DNA was used for amplification, which was performed in an automated thermocycler (BIOER Life Express) according to the following protocol: 15 minutes of denaturation at 95^{o}C, followed by 10 cycles comprising 30 seconds at 95^{o}C and 120 seconds at 65^{o}C; an additional 20 cycles comprising 25 seconds at 95^{o}C, 40 seconds at 50^{o}C and 40 seconds at 70^{o}C and a final extension at 70^{o}C for 8 minutes. Hybridisation and detection were performed in an automated washing and shaking device (GT-blot 48; Hain Lifescience, Nehren, Germany). After the final wash in the hybridisation process, the strips were air dried and pasted on the evaluation sheet provided with the kit; the strips were pasted by aligning the bands CC and AC with the respective lines on the sheet.

The GenoType®MTBDR*plus* assay strips contain 27 reaction zones; 21 of them are probes for mutations and six are control probes for verification of test procedures. The six control probes include a conjugate control, amplification control, a Mycobacterium tuberculosis complex-specific control (TUB), an *rpoB* amplification control, a *Kat*G amplification control and an *inh*A amplification control. For the detection of RIF resistance, the probes cover the *rpo*B gene while the INH resistance specific probes cover positions in *Kat*G and *inh*A genes, as shown in Fig. 4.1. Furthermore, GenoType®MTBDR*sl* V1.0 and GenoType®MTBDR*sl* V2.0 assay strips were used in this study for second-line drugs. The GenoType®MTBDR*sl*

V2.0 assay strip contains 27 reaction zones; 20 of them are probes for mutations and seven are control probes for verification of test procedures. The seven control probes include a conjugate control, amplification control, a *Mycobacterium tuberculosis* complex-specific control (TUB), a *gyr*A and *gyr*B amplification controls for detection of resistance to fluoroquinolones, *rrs* and *eis* amplification controls for the detection of resistance to aminoglycosides as shown in Fig. 4.2. The absence of at least one of the wild-type bands or the presence of bands indicating a mutation in each drug resistance-related gene implies that the tested culture sample is resistant to the respective anti-TB drugs. When all the wild-type probes of a gene stain positive and there is no detectable mutation within the region examined, the sample is susceptible to the respective drug. All the expected control bands appeared correctly. GenoType®MTBDR*sl* V1.0 assay strip contains 22 reaction zones; 16 are probes for mutations while six are control probes for verification of test procedures which includes a conjugate control, amplification control, *Mycobacterium tuberculosis* complex-specific control (TUB), a *gyr*A amplification control for detection of resistance to FQ, *rrs* amplification control for the detection of aminoglycosides resistance and *emb*B amplification control for the detection of resistance to ethambutol.

3.2.5 Agar proportion method

Drug susceptibility testing by agar proportion method was done on Microplate (LasecSA) from Lowenstein–Jensen medium (Appendix II) culture.

Preparation of bacterial suspension. A representative sample of 5–10 mg was taken from the primary culture (Lowenstein–Jensen medium) with a loop and placed in a sterile, small, thick-walled screw-capped glass tube containing 5-7 sterile glass beads (approximately 3 mm in diameter). The loop was gently shaken over the beads; 5 ml of distilled water was added slowly under continuous shaking. The tube was left to stand for 15 minutes to allow the larger aggregates of bacterial to settle. A homogenous upper part of the supernatant was aseptically transferred into another tube with similar dimensions to McFarland standard No. 1 (Appendix II) for visual comparison with the standard. The turbidity of each bacterial suspension was adjusted to match the McFarland standard No. 1 (Appendix III). A bacterial suspension that appeared too turbid was diluted with sterile distilled water but an insufficiently turbid suspension was left to settle and some of the supernatant was discarded to concentrate cells; turbidity was adjusted by adding a few drops of distilled water.

Inoculation. The objective of the technique was to achieve a growth of 30 – 100 colonies on the growth control (drug-free) medium using the most dilute suspension for inoculation. Unlike Canetti *et al*. (1965) who originally based their method on three concentrations per drug, eight concentrations per drug were used in this method; each microplate (LasecSA) used had eight concentrations per drug and two control wells for growth control (drug-free) (Figure 3.1). All sets of the microplate was labelled properly with an identification number and inoculation was performed with pipettes by delivering 0.1 ml into each of the drug-free and drug-containing wells of each microplate. Furthermore, each of the inoculated microplates was sealed with paraffin after which it was incubated at 36 ± 1^{o}C. The inoculated plates were examined for contamination after one week of incubation and for DST interpretation it was after four and six weeks of incubation. The incubated plates were read after four weeks and six weeks for provisional results and definitive interpretation.

Drug susceptibility was read by visual comparison of the drug containing media (1:1 bacterial suspensions) with the drug-free control on which 1:100 bacterial suspensions were inoculated. The growth was evaluated according to the proportional method by comparing the 1:100 diluted controls to the drug-containing wells. The strain was reported susceptible (S) if there was clearly more growth in the 1:100 diluted control than in the drug-containing well with the critical concentration and resistant (R) if there was more growth in the drug-containing well than in the 1:100 control. All the above methods were carried out in the bio safety cabinet 3.

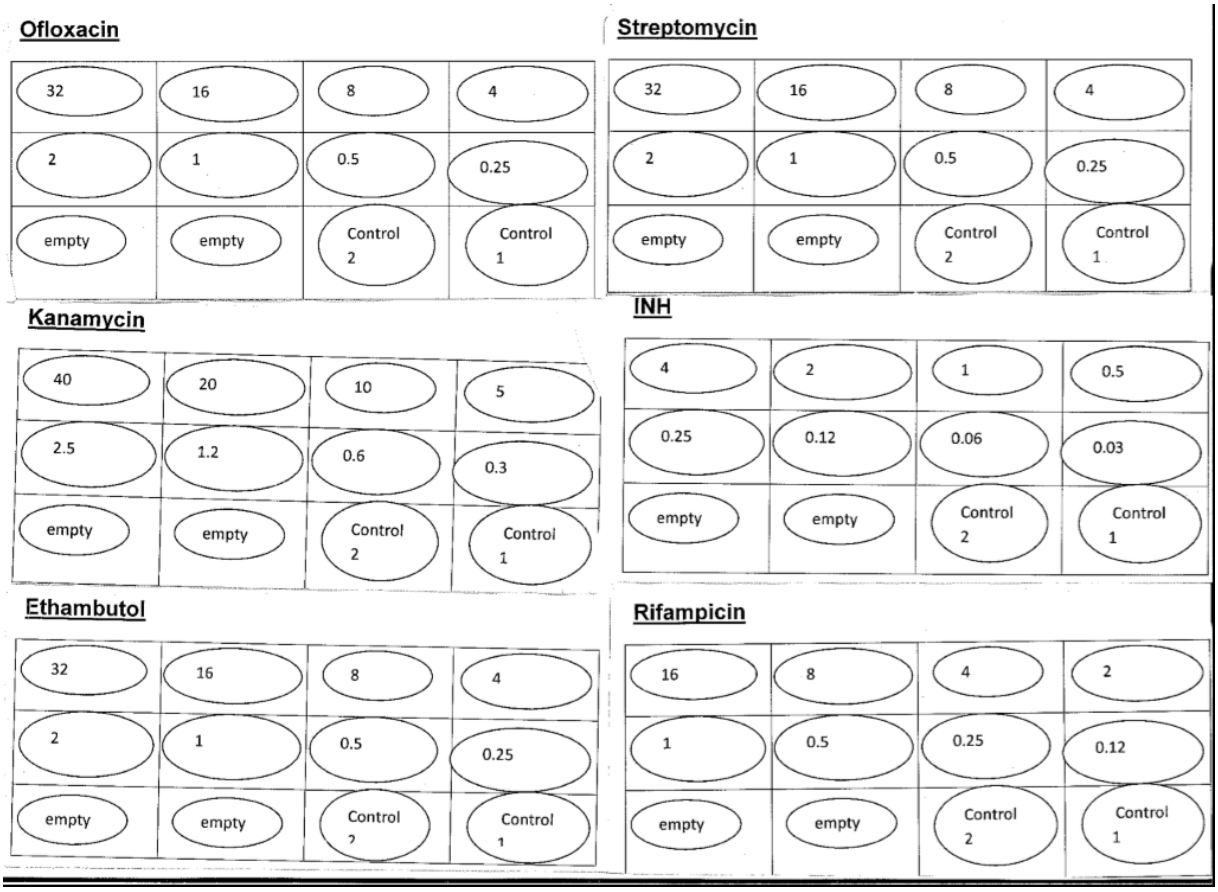


Figure 3.1: Outline of the template used for drug concentrations and controls in the 12-well plate. The template outlining the distribution and concentrations (µg/ml) of the drugs used is shown.

Quality control

M. tuberculosis H37Rv (ATCC 25177) was used as a quality control by all the methods and was tested with each batch of DST. This QC strain is susceptible to the first- and second-line drugs tested in this study. There was not a single incidence where H37Rv failed to give the expected results.

Ethical approval

Ethical approval was obtained from the Student Ethics Committee of the Faculty of Health Sciences, University of Pretoria with protocol number **318/2013** (Appendix IV) and preceded experimental work.

Statistical analysis

All results were expressed in percentages. The agreement, sensitivity, specificity, positive and negative predictive values of LPA compared to BACTEC MGIT 960 system were calculated for INH, RIF, EMB, OFX and KAN. Agreement between the two methods was assessed using the kappa statistic. The kappa value was interpreted as follows: <0.2, poor; 0.21- 0.4, fair; 0.41- 0.6, moderate; 0.61- 0.8, good and ≥ 0.81 excellent (Altman, 1999).

CHAPTER 4

RESULTS

A total of 100 *Mycobacterium tuberculosis* cultures were used for this study of which 97 were viable after subculture. The results of the routine DST using the BACTEC MGIT 960 system were compared to the results obtained by GenoType(R)MTBDR*plus* V2.0 assay. The results are summarizsed in Table 4.2. The GenoType(R)MTBDR*plus* V2.0 assay detected 40/97 (41%) multidrug-resistance and 57/97 (58.7%) mono-resistance whereby 43/57 (75%) were RIF mono-resistant and 14/57 (25%) were INH mono-resistant. Fifty-three multidrug-resistant TB cultures were detected by the BACTEC MGIT 960 system from which 15 (28.3%) were resistant to all first-line drugs tested (SIRE). The sensitivity, specificity, positive and negative predictive values for detection of RIF resistance by Genotype(R)MTBDR*plus* was found to be 100, 58, 87 and 100 per cent respectively while for INH was 100, 93, 94 and 100 per cent respectively. There were three and ten discrepant results for INH and RIF respectively between BACTEC MGIT 960 system and GenoType(R)MTBDR*plus* V2.0 assay. The time to report results of positive cultures using BACTEC MGIT 960 system is shown in Table 4.1. The majority of results were reported within 8- 14 days, with an overall average of 11 days while with GenoType(R)MTBDR*plus* V2.0 assay turnaround time was two days. The mutation patterns of RIF and INH produced by the Genotype(R)MTBDR*plus* are shown in Figure 4.1. Specific mutation was detected in 83 of 97 (85.5%) RIF resistant isolates. Of these, 46 had mutation in codon S531L, 27 in D516V, 7 in H526Y and 3 in H526D.

INH resistance was detected by probes of two genes; *kat*G and *inh*A. There were mutations in 54 of 97 (55.6 %) resistant isolates. Specific mutations in codon S315T1 of *kat*G gene was found in 32 isolates. Mutations in *inh*A gene occurred in 22 of 54 (40 %) INH resistant isolates of which 14 had mutation in codon C15T and eight in T8A (Table 4.4).

Table 4.1: **Time to complete drug susceptibility testing results with MGIT 960 systems**

	No. of specimens which turned positive after days. Total 97 (%)	**Average time to detect (No. of days)**
3 – 7 days	14 (14)	5
8 – 14 days	68 (70)	11
15 – 21 days	15 (16)	18

Table 4.2: Summary of results of Rifampicin and Isoniazid resistance by Genotype(R)MTBDR*plus* compared to the BACTEC MGIT 960 System

	GenoType®MTBDR*plus* V2.0(%)	**BACTEC MGIT 960 SYSTEM(%)**
MDR	40/97(41)	53/97(55)
RIF mono-resistance	43/57(75)	33/44(75)
INH mono-resistance	14/57(25)	11/44(25)

INH = Isoniazid, RIF = Rifampicin, MDR = Multidrug resistance

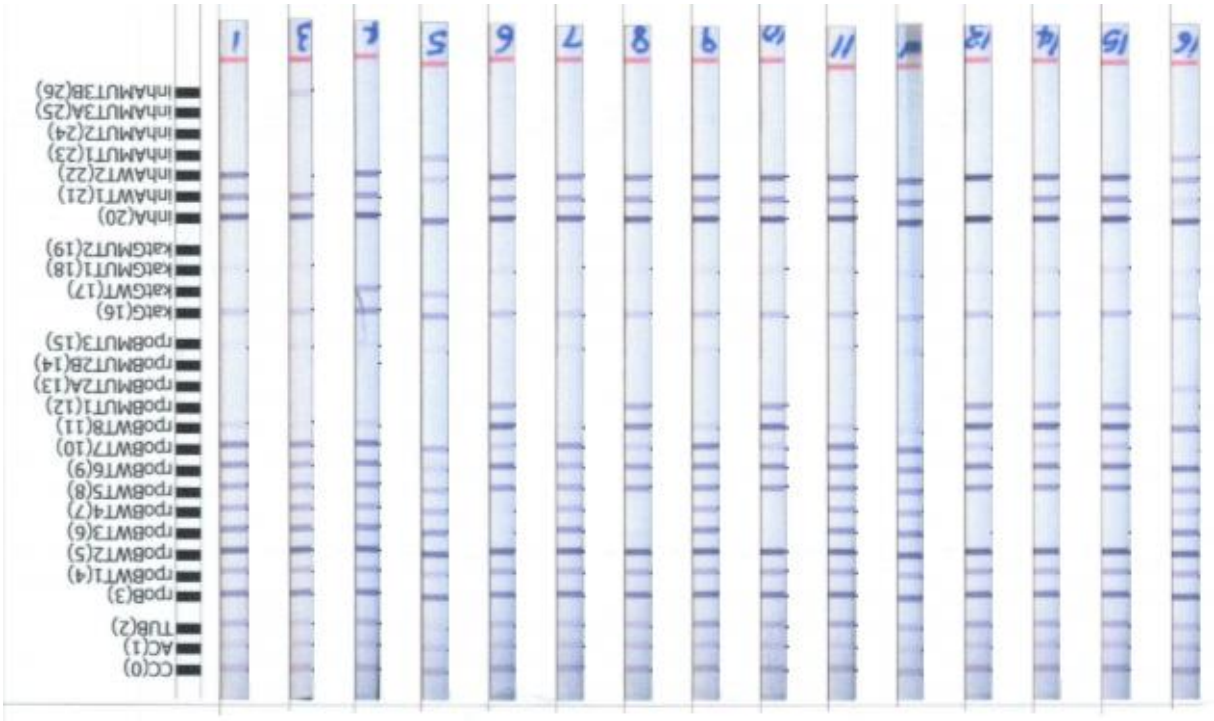


Figure 4.1: Representative DNA patterns obtained by the MTBDR*plus* assay. Lane 4 showed RIF and INH susceptible patterns while lanes 3, 5,6,7,8, 10, 12, 13, 14, 15 and 16 showed RIF and INH resistant patterns; lanes 1, 9 and 11 showed susceptibility to RIF and resistant patterns to INH.

Second-line drug testing

Ninety of 100 *Mycobacterium tuberculosis* cultures were viable after subculture. The results of DST using the BACTEC MGIT 960 system were compared to those obtained by GenoType(R)MTBDR*sl* V2.0 assay (Table 4.3). The BACTEC MGIT 960 system showed 32/90 (35.5%), 9/90 (10%) and 3/90 (3.3%) resistance to Ethambutol, Ofloxacin and Kanamycin respectively while Genotype(R)MTDR*sl* V2.0 assay detected 32/90 (35.5%), 16/90 (17.7%) and 5/90 (5.5%) resistance to ethambutol, fluoroquinolones and aminoglycosides respectively. The sensitivity, specificity, positive and negative predictive values for detection of OFX resistance by Genotype(R)MTBDR*plus* was found to be 100, 91, 57 and 100 per cent respectively, KAN resistance was 100, 97, 60 and 100 per cent respectively while for EMB it was 63, 89, 71 and 85 respectively. Nine (16) of the FQ resistant isolates had *gyr*A A90V mutant probe hybridisation positivity (with loss of hybridisation band *gyr*A WT) while the five aminoglycoside resistant isolate had *rrs* A1401G and C1402T mutant probe hybridisation (Table 4.4). There was no mutant hybridisation for *gyr*B of FQ and *eis* of aminoglycoside. Hence, there was no discordance between GenoType(R)MTBDR*sl* V1.0 and

GenoType(R)MTBDR*sl* V2.0 assay for the detection of resistance between the fluoroquinolones and aminoglycosides.

Table 4.3: Summary of results of Ofloxacin and Kanamycin resistance by Genotype(R)MTBDR*sl* compared with BACTEC MGIT 960 System

	GenoType®MTBDR*plus* V2.0(%)	**BACTEC MGIT 960 SYSTEM(%)**
OFX-resistance	16/90(18)	9/90(10)
KAN-resistance	5/90(6)	3/90(3)
EMB-resistance	32/90(36)	32/90(36)
XDR	5	3

KAN = Kanamycin, OFX = Ofloxacin, XDR = Extensively Drug Resistance

Table 4.4: Mutation pattern of FQ (*gyr*A & *gyr*B), aminoglycoside (*rrs* & *eis*), RIF (*rpo*B), INH (*kat*G & *inh*A) obtained from Genotype(R)MTBDR*sl* V2.0 and Genotype(R)MTBDR*plus* V2.0 assay

Drug	Locus	Mutation	Frequency (no. of isolates)
OFX	*gyr*A	A90V	9
		S91P	1
		D94G	3
		D94A	1
	*gyr*B	WT	2
		-	-
KAN	*rrs*	A1401G	3
		C1402T	2
	eis	-	-
RIF	*rpo*B	S531L	46
		D516V	27
		H526Y	7
		H526D	3
INH	*kat*G	S315T1	32
	*inh*A	T8A	8
		C15T	14
EMB	*emb*B	M306V	15
		M306I	9

Table 4.5: Performance of LPA result when compared with MGIT 960 for RIF, INH, OFX, KAN and EMB

Drug	No of isolates	Both S	Both R	LPA R, MGIT S	LPA S, MGIT R	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)	Agreement (%)	K-Value
RIF	97	14	73	10	0	100	58	87	100	89	0.68
INH	97	43	51	3	0	100	93	94	100	96	0.93
OFX	90	74	9	7	0	100	91	56	100	92	0.68
KAN	90	85	3	2	0	100	97	60	100	97	0.74
EMB	90	58	17	7	10	63	89	71	85	82	0.54

S = Susceptible; R = Resistant; LPA = Line Probe Assay; RIF = Rifampicin; INH = Isoniazid; OFX = Ofloxacin; KAN = Kanamycin; EMB = Ethambutol

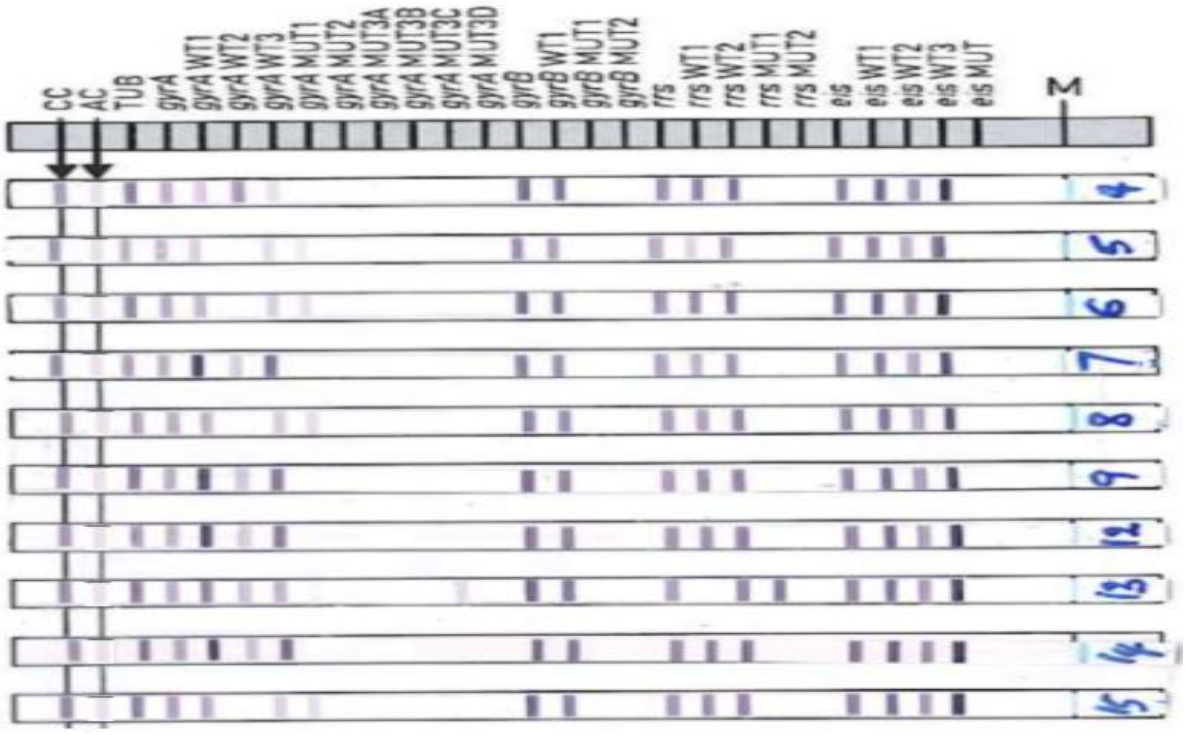


Figure 4.2: **Representative isolates of Genotype MTBDR*sl* V2.0 strips (Hain Lifescience, Nehren, Germany). (Lanes 4, 7, 9, 12, 14) *Mycobacterium tuberculosis* susceptible to fluoroquinolone and aminoglycoside. (Lanes 5, 6, 8 and 15). *M. tuberculosis* susceptible to aminoglycoside and shows *gyrA* A90V mutation. (Lane 13) showed *M. tuberculosis* with *gyrA* D94G mutation**

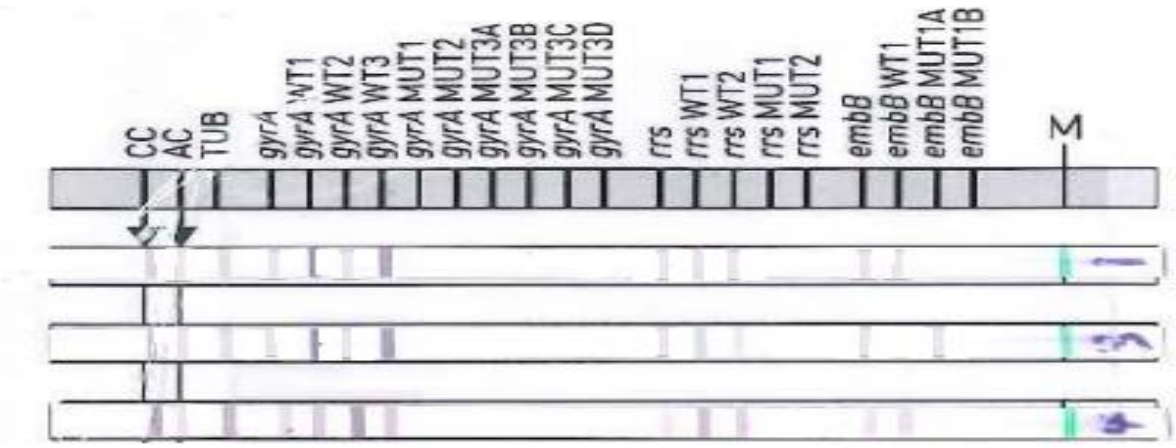


Figure 4.3: Representative DNA patterns obtained with GenoType(R)MTBDR*sl* V1.0 assay CC,AC,TUB-controls; *gyr*A-OFX DST, *rrs*-KAN DST, *emb*B-EMB DST

Resolution of discrepant results

There were overall 22 discrepancies for first- and second-line drugs (3 resistant to INH, 10 to RIF, 2 to KAN and 7 to OFX). Isolates with discrepant results were resolved by repeating the DST with the agar proportion method. Agar proportion confirmed the original results with the BACTEC MGIT 960 system while it confirmed the results with Genotype(R)MTBDR*plus* in 13 cases (3 false-resistant for INH and 10 false-resistant to RIF). Moreover, the other nine discrepancies remain unchanged (7 for OFX and 2 for KAN), with a high drug concentration, the discordance was overcome.

Turnaround time and agreement

After isolate growth in culture, the average turnaround time to DST result ranged from 21 to 2 days, in the following order from slowest to fastest: Agar proportion method, MGIT 960 system, Line probe assay. Figure 4.5 is a comparison of the turnaround time of DST results in days. The horizontal axis is the different DST methods (agar proportion, MGIT 960 system and Line probe assay) while vertically represents the time to DST result after culture in days. The mean turnaround time for agar proportion was 21 days, MGIT 960 was 11 days and LPA was 2days. Figure 4.6 is a dendrogram showing 96 % and 89 % agreement between INH and RIF respectively for the different methods (Table 4.5). In this study, susceptibility testing with MGIT 960 system for second-line drugs was done using different drug concentrations of 1.0 µg/ml and 2.0 µg/ml for OFX while 2.5 µg/ml and 5.0 µg/ml was used for KAN: thus, Figure 4.6 shows that

there is 100 % agreement between the different drug concentrations to both Kanamycin and Ofloxacin.

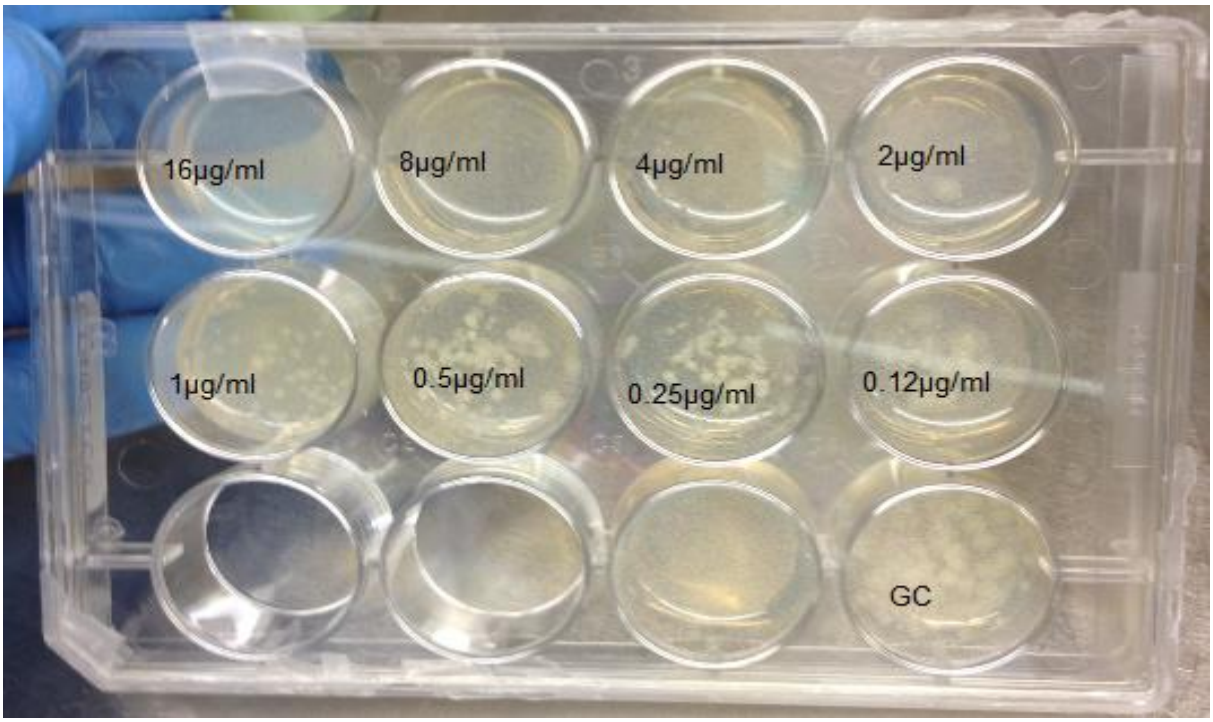


Figure 4.4: **Representative readout for drug susceptibility testing in the 12-well microplate (LasecSA).**

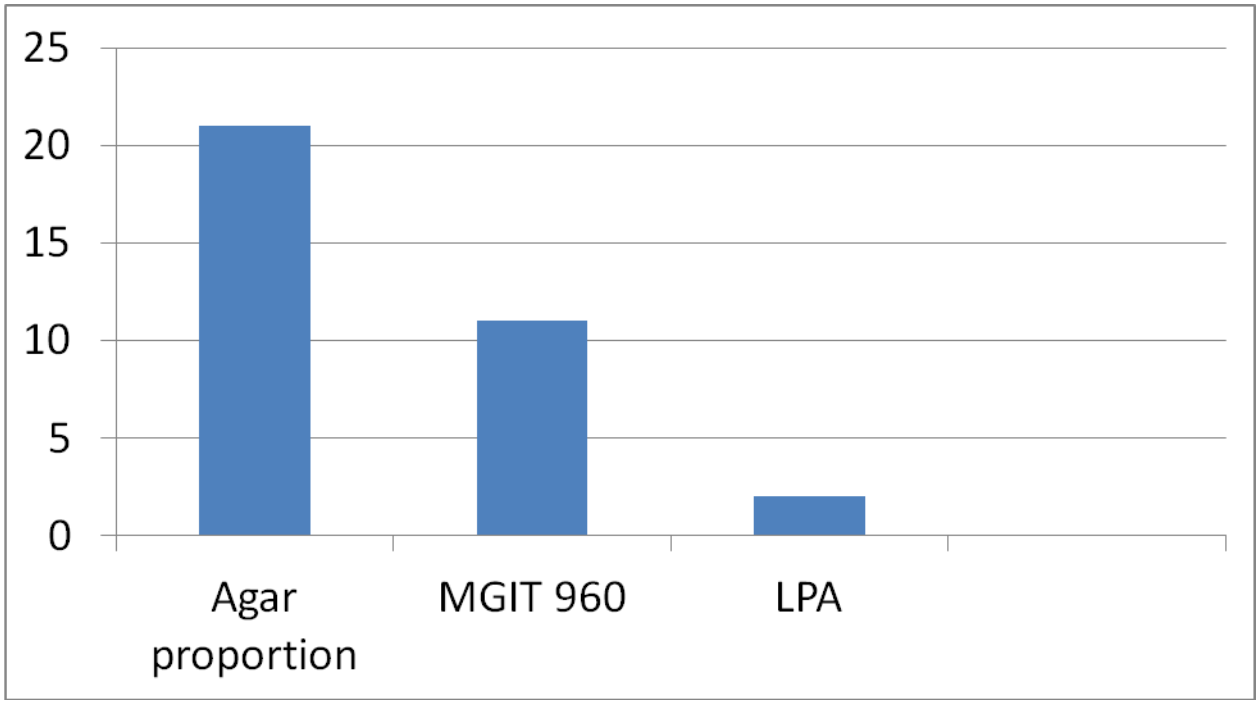


Figure 4.5 **A comparison of the turnaround times of DST methods**

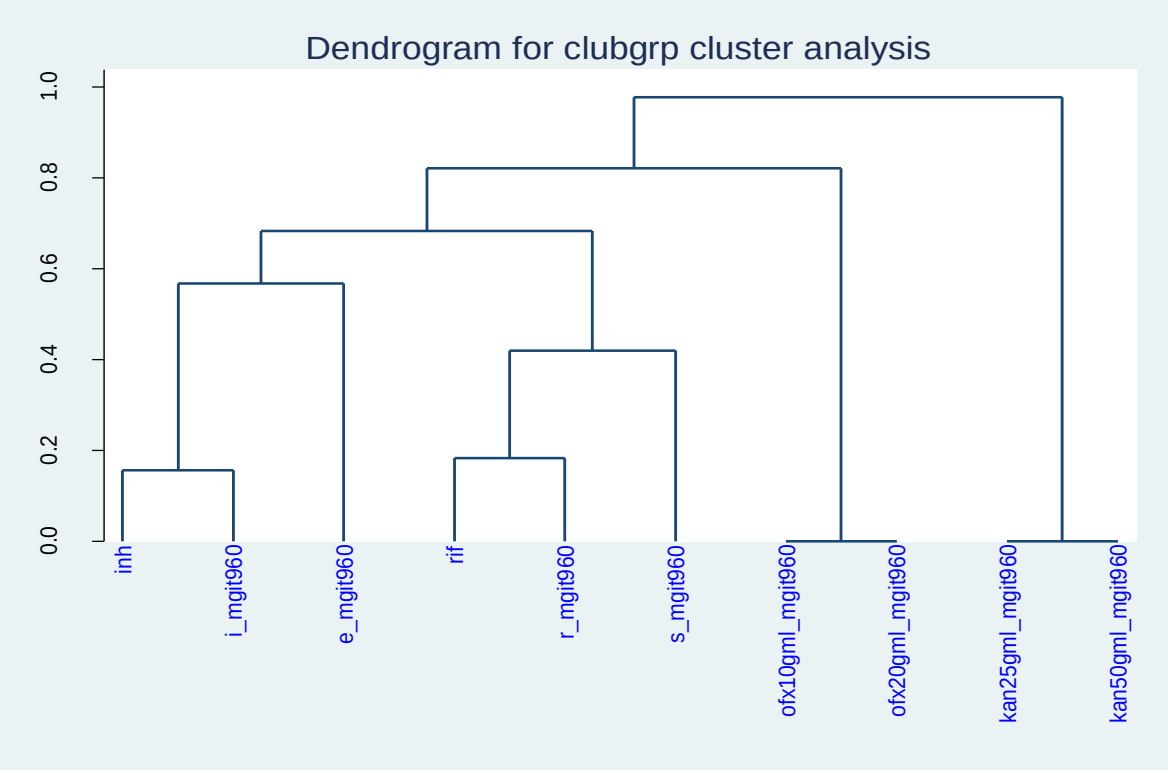


Figure 4.6 **A dendrogram of the cluster analysis showing similarity of Susceptibility and Resistance Characteristics of INH, RIF, OFX and KAN**

CHAPTER 5

DISCUSSION

In the recent years, a major emphasis has been given to the rapid diagnosis of MDR-TB, which poses a great threat to the TB control programs worldwide (Sharma and Mohan, 2006). Effective treatment of MDR-TB is very expensive particularly in low income countries. Therefore, a sensitive and specific diagnostic tool is required to initiate appropriate therapy and reduce the spread of multidrug-resistant *M.* tuberculosis strains (N'guessan, Assi, Ouassa, Ahui-Brou, Tehe, Keita Sow, Guei, Kouakou, Dosso, 2014). This study describes the performance of line probe assay (LPA) compared to BACTEC MGIT 960 system for testing of MDR-TB isolates against INH, RIF, EMB, OFX and KAN in a routine diagnostic laboratory. According to WHO, an effective treatment regimen is dependant on optimal susceptibility testing of *Mycobacterium tuberculosis* to first-line drugs (WHO, 2000). The accuracy of susceptibility testing results varies with the drug tested as well as with the method of drug susceptibility testing used (Said, Kock, Ismail, Baba, Omar, Osman, Hoosen and Ehlers, 2012). In this study, the performance of Genotype$^{(R)}$MTBDR assay (HAIN Lifescience GmbH Genotype$^{(R)}$MTBDR*plus* and Genotype$^{(R)}$MTBDR*sl*) was compared against BACTEC MGIT 960 system for the detection of resistance to INH, RIF, EMB, OFX, KAN as well as MDR-TB and XDR-TB.

Substantial reduction in the time to diagnose drug-resistant TB, the earlier commencement of appropriate therapy and the potential to prevent transmission of drug-resistant strains are major advantages of these newer molecular methods. In comparison to the BACTEC MGIT 960 system, LPA is an accurate and time-efficient method for the detection of resistance. In this study, susceptibility testing with LPA to INH, RIF, KAN and OFX had a rapid turn-around time of 48 hours (Figure 4.5) which is similar to a study in Antewerp, Belgium by Singhal *et al*., (2012) and Turkey by Kiraz *et al*., (2014). However, proper laboratory design, standard biosafety procedures and quality control to avoid cross-contamination are required for LPA which is similar to other nucleic acid amplification assays. The BACTEC MGIT 960 system is cheaper than the LPA but it is not easy to perform, requiring high standards of biosafety. The use of radioactive materials, with the need for disposal of radioactive waste,

represented the major disadvantage of the BACTEC MGIT 960 system (Cruciani, 2004). Furtheremore, there are still no commercial kits for second-line drugs with BACTEC MGIT 960 system. Working solutions of the drugs have to be prepared by the users. Hence, strict quality control measures are even more necessary for second-line drug testing.

Overall concordance of RIF, INH, OFX and KAN results comparing LPA and MGIT 960 system was found to be 89, 96, 92 and 96 per cent respectively (Table 4.5 and Figure 4.6). Other studies have reported good concordance of 84.4 – 98.1 per cent for RIF and 87.3 – 90.14 percent for INH respectively between Genotype(R)MTBDR*plus* and conventional drug susceptibility tests (Makinen, Marttila, Marjamaki, Viljanenand Soini, 2006; Brossier, Veziris, Pernot, Jarlier, Sougakoff, 2006; Balabanova Drobniewski, Nikolayevskyy, Kruuner, Malomanova and Simak, 2009). The sensitivity, specificity, positive and negative predictive values for detection of RIF resistance by Genotype(R)MTBDR*plus* was found to be 100, 58, 87 and 100 per cent respectively (Table 4.5). High sensitivity for RIF has also been found in other studies; 98.1 per cent, (95 % CI 95.9 – 99.1) in the meta–analysis for comparison of Genotype(R)MTBDR*plus* assay with conventional susceptibility testing (Ling *et al*., 2008), the sensitivity was 96.2 per cent in studies from South Africa, Germany and Italy (Hillemann *et al*., 2007; Miotto *et al*., 2008; Barnard *et al*., 2008) and 100 per cent in studies from Uganda and France (Albert *et al*., 2010; Brossier *et al*., 2006). The lower specificity of RIF (58 %) detected in this study is similar to a study carried out in Cote d'Ivoire with a specificity of 73.2 % by N'guessan *et* al., 2014. This suggests that conventional DST should be carried out before making conclusive test result even though Genotype(R)MTBDR*plus* assay has advantage of rapid turnaround time. The sensitivity and specificity for INH resistance for Genotype(R)MTBDR*plus* was found to be 100 and 93 per cent respectively (Table 4.5) which is in accordance to some other studies that have found good sensitivity and specificity to INH (Balabanova *et al*., 2009 91.1 and 92.5 per cent; Huyen *et al*., 2010 92.6 and 100 per cent; Anek-Vorapong *et al*., 2010 95.3 and 100 per cent).

The Genotype(R)MTBDR*plus* assay detects resistance based on the reverse hybridization method (Kiraz *et al*., 2014). In this study, Genotype(R)MTBDR*plus* assay rapidly detected not only MTB, but also the most common mutations associated with RIF and INH resistance in

these isolates. In the case of RIF resistant isolates, missense mutations at codons 531, 516 and 526 of *rpoB* gene were observed. The S531L missense mutation that led amino acid substitutions of serine to threonine was most common in *rpoB* gene accounting for 55.4 % (46/83) of all RIF resistant isolates (Table 4.4). The mutation pattern obtained was similar to other studies in this regard (Albert *et al*., 2010; Hazbon *et al*., 2006). Other reports from India have found the S531L mutation to be common in RIF resistant isolates (Huyen *et al*., 2010; Suresh *et al*., 2006; Mani *et al*., 2001; Siddiqi *et al*., 2002) which also correlates with this study. RIF resistance is often considered as a marker for MDR-TB isolates. INH is an anti-TB drug that is used for both prophylaxis and the treatment of MTBC infections (Kiraz *et al*., 2014). Hence, the determination of the susceptibilities against these two drugs is critical (Miotto *et al*., 2006). Studies about DNA sequencing have proved that approximately 95% of RIF-resistant strains have a mutation within the 81-bp hotspot region of the *rpo*B gene. When gene mutations causing RIF resistance occur in a limited region, better results can be obtained by molecular methods developed for the determination of RIF resistance (Bicmen *et al*., 2008; Al-Mutairi *et al*., 2011; Cavusoglu *et al*., 2002; Cho, 2007). This study suggested a similar result where Genotype$^{(R)}$MTBDR*plus* detected 75.4 % RIF mono-resistance. Similarly, a study done by Kiraz *et al*., 2014 reported ten isolates that were susceptible to RIF by BACTEC MGIT 960 system which was also confirmed by the Microplate agar proportion method, whereas Genotype$^{(R)}$MTBDR*plus* assay identified them as resistant to RIF. It was indicated in previous studies that the correlation between phenotypic resistance and genotypic mutations is not absolute (Al-Mutairi *et al*., 2011). Moreso, resistance mutation may be associated with the variable phenotypic expression of drug resistance (low, moderate or high-level). Silent mutations may occur at the genetic level with no change in drug susceptibility pattern (Chan *et al*., 2007). Genotype$^{(R)}$MTBDR*plus* assay could incorrectly identify silent or neutral mutations that are phenotypically susceptible. Nevertheless such mutations are considered as an insignificant problem (Morgan *et al*., 2005; Cavusoglu *et al*., 2006). According to the gold standard (agar proportion) the RIF resistance is a false resistance which can also be interpreted that there is no phenotypic resistance in the 10 discordant isolates or there is low-level phenotypic resistance than the level of BACTEC MGIT 960 DST which could be detected.

Mutations causing INH resistance are not limited to a specific gene region. It is reported that mutations on the position codon 315 of *kat*G gene are responsible for INH resistance in 60 % of cases worldwide. Furthermore, INH resistance is caused by mutations in the *inh*A regulatory and coding region and the *ahp*C-*oxy*R, *ndh* and *kas*A genes (Kiepiela *et al*., 2000). The most frequent mutation causing INH resistance is observed to be in the S315T1 region (Ling *et al*., 2008). This study also detected a similar result as 59.3 % (32/54) of resistant strains have S315T1 mutations in the *kat*G region that led to amino acid serine substitution to threonine. This study showed 3 discordant INH resistance results by Genotype(R)MTBDR*plus* assay when compared to the BACTEC MGIT 960 system which was detected as false-resistance with the agar proportion method. Brossier *et al*., reported that the Genotype(R)MTBDR*plus* assay has increased the detection of low level INH resistance, however it could not detect some isolates with high level INH resistance. Moreover, WHO recommends the use of both BACTEC MGIT 960 system and line-probe assay in case of first-line anti-TB drugs and confirmation of MDR-TB by conventional solid-based DST is regarded as the gold standard for first-line anti-TB drugs (WHO, 2008).

Genotype(R)MTBDR*sl* assay is a specific test for the detection of resistance to Fluoroquinolones, ethambutol and kanamycin in *Mycobacterium tuberculosis*. The sensitivity of this assay is variable for different drugs; therefore, this test should not be used to "rule out" resistance to second-line drugs, but it is an accurate rapid screening test for the identification of second-line drug resistance, especially in suspected cases of XDR-TB (Kiet *et al*., 2010). The sensitivity for the detection of EMB resistance (63%) in this study is similar to previously reported studies by Kiet *et al* for isolates from Vietnam, Hilleman *et al* for isolates from Germany and Brossier *et al* from France at 64%, 69.2% and 57% respectively. Similarly for Kanamycin resistance, the sensitivity was 100% in this study and 100% in a study by Kiet *et al*. Detection of FQ resistance was (100%) in this study because all the FQ-resistant isolates with mutations were in the *gyr*A region. The low detection rate of ethambutol resistance is consistent with reports from other settings, with *emb*B mutations accounting for between 30 and 70% of ethambutol resistant isolates (Alcaide *et al*., 1997; Zhang and Yew, 2009). This underlines the need to identify other mutations for ethambutol resistance in order to improve the sensitivity of molecular tests, including line probe assays for ethambutol resistance detection (Kiet *et al*., 2010). According to Table 4.3 Genotype(R)MTBDR*sl* 5.5 % (5/90)

XDR-TB which is lower than data from Western Cape and Eastern Cape; 14 % and 26 % respectively(Strauss *et al.*, 2008). This study shows good concordance between Genotype(R)MTBDR*sl* when compared to MGIT 960 system and Microplate agar proportion method in the detection of XDR-TB.

In this study, Line probe assay has shown excellent agreement for INH susceptibility testing when compared to MGIT 960 system while there was good agreement between both methods for RIF, OFX, KAN and EMB was moderate (Table 4.5). Hence, Line probe assay can be effectively used for rapid screening of drug resistant TB.

CHAPTER 6

CONCLUSION

Conclusively, the data obtained from this study has demonstrated that the Line probe assay is a rapid and efficient tool for the diagnosis of MDR-TB and early detection of possible XDR-TB. Genotype(R)MTBDR*plus* showed a good and excellent agreement to RIF and INH respectively when compared to BACTEC MGIT 960 system with a rapid turnaround time. However, initial evaluations of MTBDR*sl* test suggest that it may achieve performance characteristics similar to those of the line probe assays currently available for the detection of MDR-TB and could be implemented for the timely identification of XDR-TB cases in settings already performing the MDR-TB assays.

Furthermore, in this study there was no discordance between Genotype(R)MTBDR*sl* V1.0 and Genotype(R)MTBDR*sl* V2.0 when used to detect resistance to second-line Fluoroquinolones drug and second-line injectable drugs. It is however advisable to always use Genotype(R)MTBDR*sl* V2.0 because it contains more probes. The rapid identification of XDR-TB cases will allow early initiation of appropriate therapy and infection control and prevent increase of drug resistance in the community. The excellent and good agreements found between the LPA and BACTEC MGIT 960 system suggests the reliability of Line probe assay for the drugs tested. In spite of the advances in molecular methods, confirmation of results is still required with conventional methods in laboratories that use molecular tests for the detection of RIF and INH resistance.

Having shown good concordance between the two methods (Genotype(R)MTBDR*sl* and MGIT 960 system) in the detection of extensively drug resistance TB however, Genotype(R)MTBDR*sl* is the only rapid test that detects resistance to second-line Fluoroquinolones and second-line injectable drugs as well as detecting XDR-TB (Theron *et al.*, 2014).

REFERENCES

Abdel Aziz M (2003). Guidelines for surveillance of drug resistance in tuberculosis. Geneva, World Health Organization Document, (WHO/TB/2003.320–WHO/CDS/CSR/RMD/ 2003.3).

Abdel Aziz M, Wright A, De Muynck A and Laszlo A (2003). Anti-tuberculosis drug resistance in the world: Third global report. *World Health Organization.* Available: *http://whqlibdoc.who.int/publications/2004/9241562854.pdf.* Accessed 23 May 2006.

Affolabi D, Odoun M and Sanoussi N (2008). Rapid and inexpensive detection of multidrug-resistant *Mycobacterium tuberculosis* with the nitrate reductase assay using liquid medium and direct application to sputum samples. *J. Clin. Microbiol.* 46(10), 3243–3245.

Ahmad S, Jaber A-A and Mokaddas E (2007). Frequency of embB codon 306 mutations in ethambutol-susceptible and -resistant clinical *Mycobacterium tuberculosis* isolates in Kuwait. *Tuberculosis (Edinburgh).*87:123-9.

Ahmad S and Mokaddas E (2012). New approaches in the diagnosis and treatment of susceptible, multidrug-resistant and extensively drug-resistant tuberculosis. *Kuwait Medical Journal.*44:3-19.

Ahmad S and Mokaddas E (2009). Recent advances in the diagnosis and treatment of multidrug-resistant tuberculosis. *Respir Med.* 103.

Ahmad S and Mokaddas E (2004). Contribution of AGC to ACC and other mutations at codon 315 of the *Kat*G gene in isoniazid-resistant *Mycobacterium tuberculosis* isolates from the Middle East. *International Journal of Antimicrobial Agents*. 23:473-479.

Ahmed N and Hasnain SE (2004). Genomics of *Mycobacterium tuberculosis*: old threats and new trends. *Ind. J. Med. Res.* 120:207-212.

Ahuja SD, Ashkin D, Avendano M, Banerjee R, Bauer M and Bayona JN (2012). Multidrug-resistant pulmonary tuberculosistreatment regimens and patient outcomes: an individual patient data meta-analysis of 9,153 patients. *PLoS Medicine*. 9:e1001300.

Akira S, Uematsu S and Takeuchi O (2006). Pathogen recognition and innate immunity. *PubMed*. 124:783-801.

Akpaka PE, Baboolal S, Clarke D, Francis L and Rastogi N. (2008). Evaluation of methods for rapid detection of resistance to isoniazid and rifampin in *Mycobacterium tuberculosis* isolates collected in the Caribbean. *J. Clin. Microbiol*. 46:3426-3428.

Albert H, Bwanga F, Mukkada S, Nyesiga B, Ademun JP and Lukyamuzi G (2010). Rapid screening of MDR-TB using molecular line probe assay is feasible in Uganda. *BMC Infect Dis.* 10: 41(1-7).

Alcaide F, Pfyffer GE and Telenti A (1997). Role of embB in natural and acquired resistance to ethambutol in mycobacteria. *Antimicrob. Agents Chemother*. 41:2270–2273.

Almeida Da Silva PE and Palomino JC (2011). Molecular basis and mechanisms of drug resistance in *Mycobacterium tuberculosis*: classical and new drugs. *Journal of Antimicrobial chemotherapy* .66:1417-1430.

Al-Mutairi N, Ahmad S and Mokaddas E (2011). Performance comparison of four methods for rapid detection of multidrug-resistant *Mycobacterium tuberculosis* strains. *International Journal of Tuberculosis and Lung Disease*. 15: 110-115.

Altman DG (1999). *Practical Statistics for Medical Research*. London. Chapman & Hall/CRC. 403-409.

Amaral L and Viveiros M (2012). Why thioridazine in combination with antibiotics cures extensively drug-resistant *Mycobacterium tuberculosis* infections. *International Journal of Antimicrobial Agents*. 39:376-380.

American Thoracic Society, CDC (2003). Infectious Diseases Society. Treatment of tuberculosis. *Am J Respir Crit Care Med* 167:603.

Anek-Vorapong R, Sinthuwattanawibool C, Podewils LJ, McCarthy K, Ngamlert K and Promsarin B (2010). Validation of the GenoType MTBDRplus assay for detection of MDR-TB in a public health laboratory in Thailand. *BMC Infect Dis*. 20:123.

Ängeby KAK, Werngren J, Toro JC, Hedström G, Petrini B, Hoffner SE (2003). Evaluation of the BacT/ALERT 3D system for the recovery and drug susceptibility testing of *Mycobacterium tuberculosis*. *Clin. Microbiol. Infect.*9(11):1148–1152.

Aronson J (1999). That's show business. *BMJ*. 319(7215):972.

Balabanova Y, Drobniewski F, Nikolayevskyy V, Kruuner A, Malomanova N and Simak T (2009). An integrated approach to rapid diagnosis of tuberculosis and multi-drug resistance using liquid culture and rapid methods in Russia. *PLoS One*. 4(9):e7129.

Banerjee A, Dubnau E and Quemard A (1994). *InhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. *Science* 263(5144):227–230.

Banu S, Honore N, Saint-Joanis B, Philpott D, Prevost MC and Cole ST (2002). Are the PE-PGRS proteins of *Mycobacterium tuberculosis* variable surface antigens? *Mol. Microbiol.* 44:9-19.

Barnard M, Albert H, Coetzee G, O'Brien R and Bosman ME (2008). Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa. *Am J Respir Crit Care Med*. 177(7):787-792.

Barnard M, Warren R, Van Pittius NG, van Helden P, Bosman M and Streicher E (2012). GenoType MTBDRsl line probe assay shortens time to diagnosis of XDR-TB in a high-

throughput diagnostic laboratory. *American Journal of Respiratory and Critical Care Medicine*. 186:1298-1305.

Bardarov S, Bardarov S Jr, Pavelka MS Jr, Sambandamurthy V, Larsen M, Tufariello J, Chan J, Hatfull G and Jacobs WR Jr (2002). Specialized transduction; an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis, M. bovis* BCG and *M. smegmatis. Microbiology*. 148:3007-3017.

Barnes DS (2000). Historical perspectives on the etiology of tuberculosis. *Microbes Infect.* 2:431-440.

Beaucher J, Rodrigue S, Jacques PE, Smith I, Brzezinski R and Gaudreau L (2002). Novel *Mycobacterium tuberculosis* anti-sigma factor antagonists control sigma F activity by distinct mechanisms. *Mol. Microbiol.* 45:1527-1540.

Becerra MC, Freeman J and Bayona J (2000). Using treatment failure under effective directly observed short-course chemotherapy programs to identify patients with multidrug-resistant tuberculosis. *Int. J. Tuberc. Lung Dis.* 4:108-114.

Bemer P, Palicova F, Rusch-Gerdes S, Drugeon HB and Pfyffer GE (2002). Multicenter evaluation of fully automated BACTEC Mycobacteria Growth Indicator Tube 960 system for susceptibility testing of *Mycobacterium tuberculosis. J Clin Microbiol.* 40:150-154.

Bergmann JS and Woods GL (1998). Evaluation of the ESP culture system II for testing susceptibilities of *Mycobacterium tuberculosis* isolates to four primary antituberculous drugs. *J. Clin. Microbiol.*36:2940–2943.

Bicmen C, Gunduz AT, Coskun M, Senol G, Ozkutuk N and Cirak AK (2008). Molecular identification and characterization of rifampicin-resistant *Mycobacterium tuberculosis* isolates by line probe assay: An approach for rapid diagnosis of multidrug-resistant tuberculosis. *Lett Appl Microbiol*. 47:214-20.

Blumberg HM, Burnam WJ, Chaisson RE, Daley CL, Etkind SC and Friedman LN (2003). Treatment of tuberculosis. *American Journal of Respiratory and Critical Care Medicine*. 167:603:662.

Boehme CC, Nabeta P, Hillemann D, Nicol MP, SHenai S and Krapp F (2010). Rapid molecular detection of tuberculosis and rifampin resistance. *New England Journal of Medicine*. 363:1005-1015.

Botha E, den Boon S and Lawrence KA (2008). From suspect to patient: tuberculosis diagnosis and treatment initiation in health facilities in South Africa. *Int. J. Tuberc. Lung Dis.* 12(8):936-941.

Brossier F, Veziris N, Aubry A, Jarlier V and Wougakoff W (2010). Detection by GenoType MTBDRsI test of complex mechanisms of resistance to second-line drugs and ethambutol in

multidrug-resistant *Mycobacterium tuberculosis* complex isolates. *Journal of Clinical Microbiology*. 28: 1683-1689.

Brossier F, Veziris N, Jarlier V and Sougakoff W (2009). Performance of MTBDR plus for detecting high/low levels of *Mycobacterium tuberculosis* resistance to isoniazid. *Int J Tuberc Lung Dis*. 13:260-5.

Brossier F, Veziris N, Pernot CT, Jarlier V, Sougakoff W (2006). Performance of the GenoType MTBDR line probe assay for detection of resistance to rifampicin and isoniazid in strains of Mycobacterium tuberculosis with low and high level resistance. *J Clin Microbiol* 44 : 3569-4.

Brown-Elliot B and Wallace RJ (2007). *Mycobacterium*: Clinical and laboratory characteristics of rapidly growing mycobacteria. *Manual of Clinical Microbiology*. 9:589-600.

Bwanga F, Hoffner S, Haile M and Joloba ML (2009). Direct susceptibility testing for multi-drug resistant tuberculosis: A meta-analysis. *BMC Infect. Dis*. 9(67):1–15.

Caminero JA, Sotgiu G, Zumla A and Migliori GB (2010). Best drug treatment for multidrug-resistant and extensively drug-resistant tuberculosis. *Lancet Infectious Diseases*. 10:621-629.

Campbell E, Korzheva N, Mustaev A, Murakami K, Satish N and Goldfarb A (2001). Structural Mechanism for Rifampicin Inhibition of Bacterial RNA Polymerase Cell. 104:901-12.

Canetti G, Wallace F, Khomenko A, Mahler HT, Menon NK, Mitchison DA, Rist N and Smelev NA (1969). Advances in techniques of testing mycobacterial drug sensitivity, and the use of sensitivity tests in tuberculosis control programmes. *Bulletin of the World Health Organization*, 41:21–43.

Causse M, Ruiz P, Gutierrez JB, Zerolo J and Casal M (2008). Evaluation of new GenoType MTBDR*plus* for detection of resistance in cultures and direct specimens of *Mycobacterium tuberculosis*. *Int. J. Tuberc. Lung Dis*. 12:1456-1460.

Cavusoglu C, Hilmioglu S, Guneri S and Bilgic A (2002). Characterization of *rpoB* mutations in rifampin-resistant clinical isolates of *Mycobacterium tuberculosis* from Turkey by DNA sequencing and line probe assay. *J Clin Microbiol*.40:4435-8.

Cavusoglu C, Turhan A, Akinci P and Soyler I (2006). Evaluation of the Genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* isolates. *J Clin Microbiol*.44:2338-42.

Cegielski JP and McMurray DN (2004). The relationship between malnutrition and tuberculosis: evidence from studies in humans and experimental animals. *Int. J. Tuberc Lung Dis*. 8: 286 – 298.

Center for Disease Control (2006). Notice to readers: revised definition of extensively drug-resistant tuberculosis. MMWR 55(43):1176.

Chaisson RE and Churchyard GJ (2010). Recurrent tuberculosis; relapse, reinfection and HIV. *J Infect Dis* 201: 653 – 655.

Chan ED and Iseman MD (2002). Current medical treatment for tuberculosis. *Br. Med. Journal*. 325:1282-1286.

Chan RC, Hui M, Chan EW, Au TK, Chin ML and Yip CK (2007). Genetic and phenotypic characterization of drug-resistant *Mycobacterium tuberculosis* isolates in Hong Kong. J Antimicrob Chemother. 59:866-73.

Chang K, Lu W, Wang J, Zhang K, Jia S and Li F (2012). Rapid and effective diagnosis of tuberculosis and rifampicin resistance with Xpert MTB/RIF assay: a meta-analysis. *Journal of Infection.* 64:580-588.

Chapin KC, Lauderdale T, Murray PR, Baron EJ, Jorgensen JH, Pfaller MA and Yolken RH (2003). Reagents, stains, and media: bacteriology, *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C. p 358.

Cho SN (2007). Current issues on molecular and immunological diagnosis of tuberculosis. *Yonsei Med J*. 48:347-59.

Choi JH, Lee KW, Kang HR, Hwang YI, Jang S and Kim DG (2010). Clinical efficacy of direct DNA sequencing analysis on sputum specimens for early detection of drug-resistant *Mycobacterium tuberculosis* in a clinical setting. *Chest*. 137:393-400.

Comas I, Borrell S, Roetzer A, Rose G, Malla B and Kato-Maeda M(2011). Whole-genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains identifies compensatory mutations in RNA polymerase genes. *Nature Genetics*.44:106-10.

Cox H and Ford N (2012). Linezolid for the treatment of complicated drug-resistant tuberculosis: a systematic review and meta-analysis. *International Journal of Tuberculosis and Lung Diseae.*16:447-454.

Cruciani M, Scarparo C, Malena M, Bosco O, Serpelloni G and Mengoli C (2004). Meta-analysis of BACTEC MGIT 960 and BACTEC 460 TB, with or without solid media, for detection of mycobacteria. *J Clin Microbiol*. 42:2321-2325.

Dalton T, Cegielski P, Akksilp S, Asencios L, Caoili JC and Cho SN (2012). Prevalence of and risk factors for resistance to second-line drugs in people with multidrug-resistant tuberculosis in eight countries: a prospective cohort study. *Lancet*. 380:1406-1417.

Devasia RA, Blackman A and May C (2009). Fluoroquinolone resistance in *Mycobacterium tuberculosis*: an assessment of MGIT 960, MODS and nitrate reductase assay and fluoroquinolone cross-resistance. *J. Antimicrob. Chemother.*63 (6):1173–1178.

Diel R, Loddenkemper R and Nienhaus A (2010). Evidence-based comparison of commercial interferon-gamma release assays for detecting active TB: a meta-analysis. *Chest*. 137(4):952-968.

Dinnes J, Deeks J and Kunst H (2007). A systematic review of rapid diagnostic tests for the detection of tuberculosis infection. *Health Technol. Assess.* 11(3):1-196.

Di Perri G and Bonora S (2004). Which agents should we use for the treatment of multidrug-resistant *Mycobacterium tuberculosis*? *J. Antimicrob. Chemother*. 54:593-602.

Dooley KE, Obuku EA, Durakovic N, Belitsky V, Mitnick C and Nuermberger EL (2013). World Health Organization group 5 drugs for the treatment of drug-resistant tuberculosis: unclear efficacy or untapped potential? *Journal of Infectious Diseases*. 207:1352-1358.

Dorman SE and Chaisson RE (2007). From magic bullets back to the magic mountain: the rise of extensively drug-resistant tuberculosis. *Nat Med* 13: 295 – 298.

Dou HY, Tseng FC, Lin CW (2008). Molecular epidemiology and evolutionary genetics of *M. tuberculosis* in Taipei. *BMC Inf. Dis.* 8:170.

Draper P and Daffe M (2005). The cell envelope of *Mycobacterium tuberculosis* with special reference to the capsule and outer permeability barrier. *Manual of Clinical Microbiology*. 261-273.

Drobniewski FA and Balabanova YM (2002). The diagnosis and management of multiple-drug resistant tuberculosis at the beginning of the new millennium. *Int. Journal of Infectious Disease*.6:S21-S31.

Drobniewski F, Eltringham I, Graham C, Magee JG, Smith E and Watt B (2002). A national study of clinical and laboratory factors affecting the survival of patients with multiple drug resistant tuberculosis in the UK. *Thorax*. 57:810-816.

Drobniewski F, Nikolayevskyy V, Balabanova Y, Bang D and Papaventsis D (2012). Diagnosis of tuberculosis and drug resistance: what can new tools bring us? *International Journal of Tuberculosis and Lung Disease*. 16:860-870.

Dye C, Scheele S, Dolin P, Pathania V and Raviglione MC (1999). Consensus statement. Global burden of tuberculosis. Estimated incidence, prevalence, and mortality by country. *WHO Global Surveillance and Monitoring Project*. *JAMA* 282: 677-686.

Dye C, Williams BG (2009) Slow elimination of multidrug-resistant tuberculosis. *Sci Transl Med* ; 1:3ra8

El-Etr SH and Cirillo JD (2001). Entry mechanisms of mycobacteria. *Front Biosci*. 6:737-747.

Espasa M, Salvado M, Vincente E, Tudo G, Alcaide F and Coll P (2012). Evaluation of the VersaTREK system compared to the Bactec MGIT 960 system for first-line drug susceptibility testing of *Mycobacterium tuberculosis*. *J Clin Microbiol*. 50:488-491.

Espinal MA, Kim SJ, Suarez PG, Kam KM and Khomenko AG (2000). Standard short-course chemotherapy for drug-resistant tuberculosis: Treatment outcomes in 6 countries. *JAMA* 283: 2537-2545.

Farmer P and Kim JY (1998). Community based approaches to the control of multidrug resistant tuberculosis: Introducing DOTS-plus. *BMJ* 317: 671-674.

Freixo MI, Caldas PC and Said A (2004). Antimicrobial susceptibility determined by the E test, Löwenstein-Jensen proportion, and DNA sequencing methods among *Mycobacterium tuberculosis* isolates discrepancies, preliminary results. *Mem. Inst. Oswaldo Cruz.* 99(1):107-110 (2004).

Furin J (2007). The clinical management of drug-resistant tuberculosis. *Current Opinion in Pulmonary Medicine*. 13:212-217.

Gandhi NR, Andrews JR, Brust JCM, Montreuil R, Weissman D and Heo M (2012). Risk factors for mortality among MDR and XDR-TB patients in a high HIV prevalence setting. *International Journal of Tuberculosis and Lung Disease*. 16:90-97.

Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T and Lalloo U (2006). Extensively drug-resistant tuberculosis as a cause of death in patients co- infected with tuberculosis and HIV in a rural area of South Africa. *Lancet*. 368(9547):1575-80.

Gegia M, Kalandadze I, Kempker RR, Magee MJ and Blumberg HM (2012). Adjunctive surgery improves treatment outcomesamong patients with multidrug-resistant and extensivelydrug-resistant tuberculosis. *International Journal of Infectious Diseases*. 16:e391-e396.

Georghiou SB, Magana M, Garfein RS, Catanzaro DG, Catanzaro A and Rodwell TC (2012). Evaluation of genetic mutations associated with *Mycobacterium tuberculosis* resistance to amikacin, kanamycin and capreomycin: a systematic review. *PLoS ONE*. 7:e33275.

Goble M, Iseman MD, Madsen LA, Waite D, Ackerson L and Horsburgh CR, Jr (1993). Treatment of 171 patients with pulmonary tuberculosis resistant to isoniazid and rifampin. *N Engl J Med.* 328(8):527-32.

Gupta R, Raviglone MC and Espinal MA (2001). Should tuberculosis programmes invest in second-line treatments for multidrug-resistant tuberculosis (MDR TB). *Int J Tuberc Lung Dis* 5: 1078-1079.

Harada N, Higuchi K, Yoshiyama T, Kawabe Y, Fujita A, Sasaki Y, Horiba M, Mitarai S, Yonemaru M, Ogata H, Ariga H, Kurashima A, Wada A, Takamori M, Yamagishi F, Suzuki K, Mori T and Ishikawa N (2008). Comparison of the sensitivity and specificity of two whole blood interferon-gamma assays for M. tuberculosis infection. *Journal of infection*. (56)5:348-353.

Hain Lifescience GmbH GenoType MTBDRplus V2.0 products insert. Hain Lifescience GmbH, Nehren, Germany. http://www.hain-lifescience.com

Harding CV, Boom WH (2010). Regulation of antigen presentation by *Mycobacterium tuberculosis*: a role for Toll-like receptors. *Nature Reviews Microbiology*. 8(4):296-307.

Hausdorfer J, Sompek E, Allerberger F, Dierich MP and Rüsch-Gerdes S (1998). E-test for susceptibility testing of *Mycobacterium tuberculosis*. *Int. J. Tuberc. Lung Dis.* 2(9):751–755.

Hazbón M17. H, Brimacombe M, Valle MB, Cavatore M, Guerrero MI and Varma-Basil M (2006). Population genetics study of isoniazid resistance mutations and evolution of multidrug- resistant Mycobacterium tuberculosis. *Antimicrob Agents Chemother*. 50 : 2640-9.

Heifets LB (1991). *Drug Susceptibility in the Chemotherapy of Mycobacterial Infections*. CRC Press, Boca Raton, FL, USA.

Helb D, Jones M and Story E (2009). Rapid detection of *Mycobacterium tuberculosis* and rifampin-resistance using on-demand, near patient technology. *J. Clin. Microbiol.* 48(1):229-237.

Hernandez-Pando R, Jeyanathan M and Mengistu G (2000). Persistence of DNA from *Mycobacterium tuberculosis* superficially normal lung tissue during latent infection. *The Lancet*. 356:2133-213.

Hillemann D, Rusch-Gerdes S and Richter E (2009). Feasibility of the Genotype MTBDRsI assay for fluoroquinolone, amikacin-capreomycin and ethambutol resistance testing of *Mycobacterium tuberculosis* strains and clinical specimens. *Journal of Clinical Microbiology*.47: 1767-1772.

Hillemann D, Rüsch-Gerdes S and Richter E (2007). Evaluation of the GenoType MTBDR*plus* assay for rifampin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* strains and clinical specimens. *J. Clin. Microbiol*. 45:2635-2640.

Hingley-Wilson SM, Sambandamurthy VK and Jacob WR Jr (2003). Survival perspectives from the world's most successful pathogen *"Mycobacterium tuberculosis"*. *Nature Immunology*. 4(10):949-955.

Huang TS, Tu HZ, Lee SS, Huang WK and Liu YC (2002). Antimicrobial susceptibility testing of *Mycobacterium tuberculosis* to first-line drugs: comparisons of the MGIT 960 and BACTEC 460 systems. *Ann Clin Lab Sci*. 32:142-147.

Huyen MNT, Tiemersma EW, Nguyen TNL, Cobelens FGJ, Nguyen HD and Sy DH (2010). Validation of the GenoType MTBDRplus assay for diagnosis of multi-drug resistant tuberculosis in South Vietnam. *BMC Infect Dis*. 10:149.

Ingham CJ, Ayad AB, Nolsen K and Mulder B (2008). Rapid drug susceptibility testing of mycobacteria by culture on a highly porous ceramic support. *Int. J. Tuberc. Lung Dis.* 12(6):645–650.

Iwamoto T, Sonobe T and Hayashi K (2003). Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium* and *M. intracellulare* in sputum samples. *J. Clin. Microbiol.* 41(6):2616-2622.

Jacaban RF (1994). Multiple drug resistant tuberculosis. *Clin. Infect. Disease*. 19:1-10.

Jacobs WR Jr, Barletta RG and Udani R (1993). Rapid assessment of drug susceptibilities of *Mycobacterium tuberculosis* by means of luciferase reporter phages. *Science* 260(5109) 819–822.

Jacobson KR, Tierney DB, Jeon CY, Mitnick CD and Murray MB (2010). Treatment outcomes among patients with extensivelydrug-resistant tuberculosis: systematic review and meta-analysis. *Clinical Infectious Diseases*. 51:6-14.

Jimenez-Corona ME, Cruz-Hervert LP, Garcia-Garcia L, Ferreyra-Reyes L, Delgado-Sanches G and Bobadilla-Del-Valle M (2013). Association of diabetes and tuberculosis: impact on treatment and post-treatment outcomes. *Thorax*. 68:214-220.

Johansen IS, Thomsen VO, Marjamaki M, Sosnovskaja A and Lundgren B (2004). Rapid automated nonradiometric susceptibility testing of *Mycobacterium tuberculosis* complex to four first-line antituberculous drugs used in standard short-course chemotherapy. *Dian Microbiol Infect Dis*. 50:103-107.

Johnson JL, Hadad DJ, Boom WH, Daley CL, Peloquin CA and Eisenach KD (2006). Early and extended early bactericidal activity of levofloxacin, gatifloxacin and moxifloxacin in pulmonary tuberculosis. *International Journal of Tuberculosis and Lung Disease.*10:605-612.

Katoch VM (2004). Infections due to non-tuberculous mycobacteria (NTM). *Indian Journal of Medical Research*. 120(4):290-304.

Kato-Maeda M, Bifani PJ and Krieswirth BN (2001). The nature and consequence of genetic variability in *Mycobacterium tuberculosis*. *Journal Clin. Invest.* 107:533-537.

Kenneth T (2010). *Mycobacterium tuberculosis*: The good, the bad and the deadly. *Todar's online textbook of Bacteriology*.

Kent PT and Kubica GP (1985). Public health mycobacteriology: a guide for the level III laboratory. Atlanta, GA, United States Department of Health and Human Services, *Center for Disease Control.*

Kiepiela P, Bishop KS, Smith AN, Roux L and York DF (2000). Genomic mutations in the *kat*G, *inhA* and *aphC* genes are useful for the prediction of isoniazid resistance

in *Mycobacterium tuberculosis*isolates from KwaZulu-Natal, South Africa. *Tuberc Lung Dis.* 80:47-56.

Kiet VS, Lan NTN, An DD, Dung NH, Ho DV, Chau NV, Chinh NT, Farrar J and Caws M (2010). Evaluation of the MTBDRsl test for detection of second line drug resistance in *Mycobacterium tuberculosis*. *Journal of Clin Microbiol.* 48(8):2934-2939.

Kim SJ, Espinal MA, Abe C (2004). Is second-line anti-tuberculosis drug susceptibility testing reliable? *Int. J. Tuberc. Lung Dis.*9:1157–1158.

Kiraz N, Oz Y, and Saglik I (2014). Evaluation of the Genotype MTBDR assay for detection of rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* complex isolates. *Indian Journal of Medical Microbiology*. 32:318-322.

Klug A (2001). "A Marvellous Machine for Making Messages". Science 292(5523):1844-6.

Kobayashi I, Abe C and Mitarai S (2006). BACTEC MGIT 960 system for drug susceptibility testing of *Mycobacterium tuberculosis*; a study using external quality assessment strains. *Kekkaku*. 81:57-62.

Kontos F, Maniati M, Costopoulos C, Gitti Z, Nicolaou S and Petinaki E (2004). Evaluation of the fully automated Bactec MGIT 960 system for the susceptibility testing of *Mycobacterium tuberculosis* to first-line drugs: a multicenter study. *J Microbiol Methods*. 56:291-294.

Kontos F, Nicolaou S, Costopoulos C, Gitti Z, Petinaki E and Maniati M (2003). Multicenter evaluation of the fully automated Bactec MGIT 960 system for susceptibility testing of *Mycobacterium tuberculosis* to pyrazinamide: comparison with the radiometric Bactec 460TB system. *J Microbiol Methods*. 55:331-333.

Kremer L and Besra GS (2005). A waxy tale by *Mycobacterium tuberculosis. Manual of Clinical Microbiology*. 287-305.

Kruuner A, Yates MD and Drobniewski FA (2006). Evaluation of MGIT 960-based antimicrobial testing and determination of critical concentrations of fi rst- and second-line antimicrobial drugs with drug-resistant clinical strains of M. tuberculosis. *J Clin Microbiol.* 44: 811–818.

Kulaga S, Behr MA and Schwartzmank (1999). Genetic fingerprinting in the study of tuberculosis transmission. *CMAJ*. 161(9):1165-1169.

Lacoma A, Garcia-sierra N, Prat C, Maldonado J, Ruiz-Manzano J, Haba L, Gavin P, Samper S, Ausina V and Dominguez J (2012). GenoType MTBDRsI for molecular detection of second-line and ethambutol resistance in *Mycobacterium tuberculosis* strains and clinical samples. *Journal of Clinical Microbiology.* 50: 30-36.

Lee AS and Ong DC (2012). Molecular diagnostic methods for the detection of *Mycobacterium tuberculosis* resistance: the potential of high-resolution melting analysis. *Expert Reviews on Anti Infective Therapy*. 10:1075-1077.

Lee ASG, Teo ASM and Wong SY (2001). Novel mutations in *ndh* in isoniazid-resistant *Mycobacterium tuberculosis* isolates. *Antimicrob. Agents Chemother.* 45(7):2157–2159.

Lew W, Pai M, Oxlade O, Martin D and Menzies D (2008). Initial drug resistance and tuberculosis treatment outcomes: systematic review and meta-analysis. *Annals of Internal Medicine*. 149:123-134.

Lin SY, Desmond E, Bonato D, Gross W and Siddiqi S (2009). Multicenter evaluation of Bactec MGIT 960 system for second-line drug susceptibility testing of *Mycobacterium tuberculosis* complex. *J Clin Microbiol*. 47:3630-3634.

Ling DI, Flores LL, Riley LW and Pai M (2008). Commercial nucleic-acid amplification tests for diagnosis of pulmonary tuberculosis in respiratory specimens: meta-analysis and meta-regression. *PLoS ONE* 3(2):e1536.

Ling DI, Zwerling AA and Pai M (2008)a. GenoType MTBDR assays for the diagnosis of multidrug-resistant tuberculosis: A meta-analysis. *Eur Respir J*.32:1165-74.

Ling DI, Zwerling AA and Pai M (2008)b. Rapid diagnosis of drug-resistant TB using line probe assays: from evidence to policy. *Expert Rev. Respir. Med.* 2(5), 583–588.

Long JW (1991). *Essential Guide to Prescription Drugs* (1992). New York: HarperCollins Publishers.

Makinen J, Marttila HJ, Marjamaki M, Viljanen MK and Soini H (2006). Comparison of two commercially available DNA line probe assays for detection of multi-drug resistant *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 44:350-352.

Mani C, Selvakumar N, Narayanan S and Narayanan PR.(2001). Mutations in the rpoB gene of multidrug-resistant Mycobacterium tuberculosis clinical isolates from India. *J Clin Microbiol* 39: 2987-90.

Maus CE, Plikaytis BB and Shinnick TM (2005a). Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 49(8):3192–3197.

Maus CE, Plikaytis BB, Shinnick TM (2005b). Mutation of *tlyA* confers capreomycin resistance in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 49(2):571–577.

Menzies D, Benedetti A, Paydar A, Royce S, Madhukar P and Burman W (2009). Standardized treatment of active tubeculosis in patients with previous treatment and/or with mono-resistance to isoniazid: a systematic review and meta-analysis. *PLoS Medicine*. 6:e1000150.

Mestdagh M, Fonteyne PA and Realini L (1999). Relationship between pyrazinamide resistance, loss of pyrazinamidase activity, and mutations in the *pncA* locus in multidrug-resistant clinical isolates of *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 43(9), 2317–2319.

Meyer H and Mally J (1912). On hydrazine derivatives and pyridine carbonic acids" (in German). *Monatshefte Chemie verwandte Teile anderer Wissenschaften*. 23:393-414.

Miotto P, Piana F, Penati V, Canducci F, Migliori GB and Cirillo DM (2006). Use of genotype MTBDR assay for molecular detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* clinical strains isolated in Italy. *J Clin Microbiol*. 44:2485-91.

Miotto P, Piana F, Cirillo DM and Migliori GB (2008). Genotype MTBDRplus a further step toward rapid identification of drug-resistant *Mycobacterium tuberculosis*. *J Clin. Microbiol.* 46(1):393-394.

Mironova S, Pimkina E, Kontsevaya I, Nikolayevskyy V, Balabanova Y and Skenders G (2012). Performance of the GenoType®MTBDRPlus assay in routine settings: a multicenter study.*European Journal of Clinical Microbiology and Infectious Diseases*. 31:1381-1387.

Mitchison Dand Davies G (2012). The chemotherapy of tuberculosis: past, present and future. *International Journal of Tuberculosis and Lung Disease*. 16:724-732.

Moore DA and Shah NS (2011). Alternative methods of diagnosing drugresistance, what can they do for me. *Journal of Infectious Diseases*. 204 (Suppl 4):S1110-S1119.

Moore DA, Evans CA and Gilman RH (2006). Microscopic-observation drug-susceptibility assay for the diagnosis of TB. *N. Engl. J. Med.* 355(15):1539–1550.

Moore DA, Mendoza D and Gilman RH (2004). Microscopic observation drug susceptibility assay, a rapid, reliable diagnostic test for multidrug-resistant tuberculosis suitable for use in resource-poor settings. *J. Clin. Microbiol.* 42(10):4432–4437.

Morgan M, Kalantri S, Flores L and Pai M (2005). A commercial line probe assay for the rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*: A systematic review and meta-analysis. *BMC Infect Dis*. 5:62.

Morlock GP, Metchock B, Sikes D, Crawford JT and Cooksey RC (2003). *ethA*, *inhA*, and *katG* loci of ethionamide-resistant clinical *Mycobacterium tuberculosis* isolates *Antimicrob. Agents Chemother.* 47(12):3799–3805.

Mukadi YD, Maher D and Harries A (2001). Tuberculosis case fatality rates in high HIV prevalence populations in sub-Saharan Africa. *AIDS* 15: 143 -152.

Musa HR, Ambroggi M, Souto A and Angeby KA (2005). Drug susceptibility testing of *Mycobacterium tuberculosis* by a nitrate reductase assay applied directly on microscopy-positive sputum samples. *J. Clin. Microbiol.* 43(7):3159–3161.

Mwinga A (2001). Drug resistant tuberculosis in Africa. *Ann. N.Y. Acad. Sci.*953:106-112.

Nafees A, James JP and Lawrence WD (2010). Tuberculosis: Treatment. *Sherris Medical microbiology. Fifth edition*. 27:499-500.

Nathanson E, Lambregts-van Weezenbeek, Rich ML, Gupta R, Bayona J, Blondal K, Caminero JA, Cegielski JP, Danilovits M, Espinal MA, Hollo V, Jaramillo E, Leimane V, Mitnick CD, Mukherjee JS, Nunn P, Pasechnikov A, Tupasi T, Wells C and Raviglione MC (2006). Multidrug-resistant tuberculosis management in resource-limited settings. *Emerg Infect Dis.* 12: 1389-1397.

Orenstein EW, Basu S, Shah NS, Andrews JR, Friedland GH and Moll AP (2009). Treatment outcomes among patients with multidrug-resistant tuberculosis: systematic review and meta-analysis. *Lancet Infectious Diseases*. 9:153-161.

Oxford Immunotec (2011) T-SPOT TB Get the facts, 6.8.2011, Available from: http://www.oxfordimmunotec.com/getthefacts/

Pai M, Kalantri S, Pascopella L, Riley LW and Reingold AL (2005). Bacteriophage-based assays for the rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*: a meta-analysis. *J. Infect.* 51(3):175–187.

Palomino JC (2012). Current developments and future perspectives for TB diagnostics. *Future microbiology*. 7:59-71.

Palomino JC, Martin A, Von Groll A, Portaels F (2008). Rapid culture-based methods for drug resistance detection in *Mycobacterium tuberculosis. Journal of Microbiological Methods*. 75:161-166.

Pandey BD, Poudel A and Yoda T (2008). Developement of an in-house loop-mediated isothermal amplification (LAMP) assay for detection of *Mycobacterium tuberculosis* and evaluation in sputum samples of Nepalese patients. *J. Med. Microbiol.* 57(Pt4):439-443.

Park H, Song EJ, Song ES (2006). Comparison of a conventional antimicrobial susceptibility assay to an oligonucleotide chip system for detection of drug resistance in *Mycobacterium tuberculosis* isolates. *J. Clin. Microbiol.* 44(5), 1619–1624.

Parsons LM, Somoskovi A, Urbanczik R and Salfinger M (2004). Laboratory diagnostic aspects of drug resistant tuberculosis. *Front Biosci.* 9: 2086 – 2105.

Pfyffer GE (2007). *Mycobacterium*: General characteristics, laboratory detection and staining procedures. *Manual of Clinical Microbiology.* 9:543-572.

Piatek AS, Telenti A and Murray MR (2000). Genotypic analysis of *Mycobacterium tuberculosis* in two distinct populations using molecular beacons: implications for rapid susceptibility testing. *Antimicrob. Agents Chemother.* 44(1):103-110.

Piersimoni C, Olivieri A, Benacchio L and Scarparo C (2006). Current perspectives on drug susceptibility testing of *Mycobacterium tuberculosis* complex: the automated nonradiometric systems. *J Clin Microbiol*. 44:20-28.

Plinke C, Rüsch-Gerdes S and Niemann S (2006). Significance of mutations in *embB* codon 306 for prediction of ethambutol resistance in clinical *Mycobacterium tuberculosis* isolates. *Antimicrob. Agents Chemother.* 50(5):1900-1902.

Prasad R (2005) MDR-TB: Current status. *Indian J. Tuberc.*52:121-131.

Puustinen K, Marjamaki M and Rastogi N (2003). Characterization of Finnish *Mycobacterium tuberculosis* isolates by spoligotyping. *J. Clin. Microbiol.* 41:1525-1528.

Raja S, Ching J and Xi L (2005). Technology for automated, rapid and quantitative PCR or reverse transcription-PCR clinical testing. *Clin. Chem.* 51(5):882-890.

Raviglione MC, Snider DE, Jr. and Kochi A. (1995). Global epidemiology of tuberculosis. Morbidity and mortality of a worldwide epidemic. *JAMA*. 273(3):220-6.

Raviglione MC, Narain JP and Kochi A. (1992). HIV-associated tuberculosis in developing countries: clinical features, diagnosis, and treatment. *Bull World Health Organ*. 70(4):515-26.

Reid MJ and Shah NS (2009). Approaches to tuberculosis screening and diagnosis in people with HIV in resource-limited settings. *Lancet Infect. Dis.* 9(3):173-184.

Richter E, Rusch-Gerdes S and Hillemann D (2009). Drug-susceptibility testing in TB: current status and future prospects. *Expert Rev. Respir. Med.* 3(5):497-510.

Rieder HL (2009). Fourth-generation fluoroquinolones against tuberculosis. *Lancet*.373:1148-1149.

Rifampicin. [database on the Internet] [cited 22 July 2010]. Available from: http://en.wikipedia.org/wiki/Rifampicin#mw-head.

Robledo J, Mejia GI, Paniagua L, Martin A and Guzmán A (2008). Rapid detection of rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* by the direct thin-layer agar method. *Int. J. Tuberc. Lung Dis.* 12(12):1482-1484.

Rodrigues C, Jani J, Shenai S, Thakkar P, Siddiqi S, and Mehta A (2008). Drug susceptibility testing of *Mycobacterium tuberculosis* against second-line drugs using the Bactec MGIT 960 System. *International Journal of Tuberculosis and Lung Disease*, 12(12):1449–1455.

Ruiz P, Zerolo FJ and Casal MJ (2000). Comparison of susceptibility testing of *Mycobacterium tuberculosis* using the ESP culture system II with that using the BACTEC method. *J. Clin. Microbiol.*38:4663–4664.

Rusch-Gerdes S, Pfyffer GE, Casal M, Chadwick M and Siddiqi SH (2006). Multicenter laboratory validation of the BACTEC MGIT 960 technique for testing susceptibilities of

Mycobacterium tuberculosis to classical second-line drugs and newer antimicrobials. *J. Clin. Microbiol*. 44:688-692.

Said HM, Kock MM, Ismail NA, Baba K, Omar SV, Osman AG, Hoosen AA and Ehlers MM (2012). Comparison between the BACTEC MGIT 960 system and the agar proportion method for susceptibility testing of multidrug resistant tuberculosis strains in a high burden setting of South Africa. *BMC Infectious Diseases.* 12:369.

Sarmiento OL, Weigle KA, Alexander J, Weber DJ and Miller WC (2003). Assessment by meta-analysis of PCR for diagnosis of smear-negative pulmonary tuberculosis. *J. Clin. Microbiol.* 41(7):3233-3240.

Scorpio A and Zhang Y (1996). Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidedase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. *Nature Medicine*. 2:662-7.

Sensi P (1983). History of the development of rifampin. Rev Infect Dis. Suppl 3:S402-6.

Shah NS, Wright A, Bai GH (2007) Worldwide emergence of extensively drug-resistant tuberculosis. *Emerg. Infect. Dis.* 13:380-387.

Sharma SK and Mohan A (2006). Multidrug-resistant tuberculosis: a menace that threatens to destabilize tuberculosis control. *Chest*. 130:261-272.

Sharma SK and Mohan A (2004). Multidrug resistant tuberculosis. *Indian J. Med. Res.*120:354-376.

Sharma SK, Turaga KK and Balamurugan A (2003). Clinical and genetic risk factors for the development of multidrug-resistant tuberculosis in non-HIV infected patients at a tertiary care center in India: a case-control study. *Infect. Genet. Evol.*3:183-188.

Sherman DR, Mdluli K and Hickey MJ (1996) Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. *Science* 272(5268), 1641–1643.

Shi W, Zhang X, Jiang X, Yuan H, Lee JS and Barry CE (2011). Pyrazinamide inhibits trans-translation in *Mycobacteriumtuberculosis*. *Science.*333:1630-2.

Shikama ML, Ferro e Silva R and Villela G (2009). Multicentre study of nitrate reductase assay for rapid detection of rifampicin-resistant *M. tuberculosis*. *Int. J. Tuberc. Lung Dis.* 13(3):377–380.

Siddiqi S, Ahmed A, Asif S, Behera D, Javaid M and Jani J (2012.) Direct drug susceptibility testing of *Mycobacterium tuberculosis* for rapid detection of multidrug resistance using the Bactec MGIT 960 system: a multicenter study. *Journal of Clinical Microbiology*. 50:435-440.

Siddiqi SH and Rusch-Gerdes S (2006). MGIT procedure manual. *FIND.* III:45.

Siddiqi N, Shamim M, Hussain S, Choudhary RK, Ahmed N and Prachee (2002). Molecular characterization of multidrug- resistant isolates of Mycobacterium tuberculosis from patients in North India. *Antimicrob Agents Chemother*. 46: 443-50.

Sirgel FA, Warren RM, Streicher EM, Victor TC, van HeldenPD and Böttger EC (2012). *gyrA* mutations and phenotypic susceptibility levels to ofloxacin and moxifloxacin in clinical isolatesof *Mycobacterium tuberculosis*. *Journal of Antimicrobial Chemotherapy*. 67:1088-93.

Somoskovi A, Parsons LM and Salfinger M (2001). The molecular basis of resistance to isoniazid, rifampin and pyrazinamide in *Mycobacterium tuberculosis. Respir Res* 2:164 – 168.

Spies FS, Ribeiro AW, Ramos DF, Ribeiro MO, Martin A and Palomino JC, (2011). Streptomycin resistance and lineage-specific polymorphisms in *Mycobacterium tuberculosis gid*B gene. *Journal of Clinical Microbiology*. 49:2625-30.

Sreevatsan S, Pan X, Stockbauer KE, Williams DL, Kreisworth BN and Musser JM (1996). Characterization of *rpsL* and *rrs* mutations in streptomycin-resistant *Mycobacterium tuberculosis* isolates from diverse geographic localities. *Antimicrobial Agents and Chemotherapy*. 40:1024-6.

Suresh N, Singh UB, Arora J, Pant H, Seth P and Sola C (2006). RpoB gene sequencing and spoligotyping of multi-drug resistant Mycobacterium tuberculosis isolates from India. *Infect Genet Evol 200.* 6: 474-83.

Takiff H, Salazar L and Guerrero C (1994). Cloning and nucleotide sequence of the *Mycobacterium tuberculosis gyrA* and *gyrB* genes, and detection of quinolone resistance mutations. *Antimicrob. Agents Chemother.* 38(4):773–780.

Tan Y, Hu Z, Zhao Y, Cai X, Luo C and Zou C (2012). The beginning of the *rpoB* gene in addition to the rifampin resistance determination region might be needed for identifyingrifampin/rifabutin cross-resistance in multidrug-resistant*Mycobacterium tuberculosis* isolates from Southern China. *Journal of Clinical Microbiology*. 50:81-5.

Telenti A, Phillipp W, Sreevatsan S, Bernasconi C, Stock-bauer KE and Wieles B (1997). The *emb* operon, a unique genecluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol. *Nature Medicine*. 3:567-70.

Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S and Colston MJ (1993). Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet*.341:647—50.

Tenover FC, Crawford JT, Huebner RE, Geiter LJ, Horsburgh CR Jr and Good RC (1993). The resurgence of tuberculosis: is your laboratory ready? *J. Clin. Microbiol.*31(4):767–770.

Theron, G, Peter J, Richardson M, Barnard M, Donegan, S. Warren R, Steingart KR and Dheda K (2014) The diagnostic accuracy of the GenoType(®)MTBDRsl assay for the detection of resistance to second-line anti-tuberculosis drugs.*Cochrane Database Syst*. :10:CD010705.

Tomita M, Takeno H, Suzuki K, Sakatani M, Kinoshita Y and Kobayashi I (2004). A study on inoculum density and reproducibility of drug susceptibility testing by BACTEC MGIT 960. *Kekkaku*. 79:625-630.

Tortoli E (2003). Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. *Clin. Microbiol. Rev.* 16(2):319-354.

Tufariello JM, Chan J and Flynn JL (2003) Latent tuberculosis: mechanisms of host and bacillus that contribute to persistent infection. *The Lancet Infectious Diseases*. 3(9): 578-590.

Van Deun A, Maug AK, Salim MA, Das PK, Sarker MR, Daru P and Rieder HL (2010). Short, highly effective and inexpensive standardised treatment of multidrug-resistant tuberculosis. *American Journal of Respiratory and Critical Care Medicine*. 182: 684-92.

Van Ingen J, Simons S, de Zwaan R, van der Laan T, Kamst-van Agterveld M, Boeree MJ and van Soolingen D (2010). Comparative study on genotypic and phenotypic second-line drug resistance testing of *Mycobacterium tuberculosis* complex isolates. *Journal of Clinical Microbiology* 48: 2749-2753.

Vilchèze Cand Jacobs WR Jr (2007). The mechanism of isoniazid killing: clarity through the scope of genetics. *Annu. Rev. Microbiol.* 61:35–50.

Vincet V and Gutierrez MC (2007). *Mycobacterium:* Laboratory characteristics of slowly growing mycobacteria. *Manual of Clinical Microbiology.* 9:573-588

Wayne LG (1984) The mycobacteria: a sourcebook part A. *New York Marcel Dekker*. 25-65.

Wehrli W (1983). Rifampicin: Mechanism of Action and Resistance. *Reviews of infectious diseases*. 5.

Wilcke JT, Jensen BN, Ravn P, Andersen AB and Haslov K (1996). Clinical Evaluation of Mpt-64 and Mpt-59; two Proteins Secreted from *Mycobacterium tuberculosis* for skin test reagents, tubercle and lung disease. *Journal of the International Union against Tuberculosis and Lung Disease*, 77(3):250-256.

Wilson SM, al-Suwaidi Z, McNerney R, Porter J and Drobniewski F (1997). Evaluation of a new rapid bacteriophage-based method for the drug susceptibility testing of *Mycobacterium tuberculosis*. *Nat. Med.* 3(4):465–468.

Wood R. Middelkoop K and Myer L (2007). Undiagnosed tuberculosis in a community with high HIV prevalence; implications for tuberculosis control. *Am. J. Respir. Crit. Care Med.* **175(1)**:87-93.

World Health Organization (2013). (WHO/HTM/TB/2013.11). Global tuberculosis report. Geneva.

World Health Organization (2012). Global tuberculosis report. WHO/HTM/TB/2008.393. *Geneva Switzerland.* 1-393.

World Health Organization (2011). Global tuberculosis control. *http://www.who.int/tb/publications/global_report/2011/gtbrII_full.pdf. Geneva, Switzerland.*

World Health Organization (2010). Global tuberculosis control. *http://www.who.int/tb/publications/global_report/2010/en/index.html*

World Health Organization (2010). Multidrug and extensively drug-resistant TB (M/XDR-TB): 2010 global report on surveillance and response. WHO/HTM/TB/2010.3. *Geneva Switzerland.*

World Health Organization (2009) Global tuberculosis control: a short update to the 2009 report. WHO/HTM/TB/2009.426. *Geneva.*

World Health Organization/IUATLD (2008). Global Project on anti-Tuberculosis drug resistance surveillance: Anti-tuberculosis drug resistance in the world, Report No.4, Annex 9.

WHO/HTM/TB/2008.394 Geneva. World Health Organization (2008) Policy guidance on TB susceptibility testing (DST) of second-line drugs. WHO/HTM/TB/2008.392. *Geneva.*

World Health Organisation (2008). Policy statement. Molecular line probe assays for rapid screening of patients at risk of multidrug resistant tuberculosis (MDR-TB). Geneva, Switzerland.

World Health Organization (2006). Extensively drug-resistant tuberculosis (XDR-TB): recommendations for prevention and control. *Weekly Epidemiol. Rec*. **81**:430-432.

World Health Organization (2006). Guidelines for the programmatic Management of drug-resistant tuberculosis. Document No. WHO/HTM/TB/2006.361.

World Health Organization (2005) Global tuberculosis control: Surveillance, planning, financing *http://www.who.int/tb/publications/global_report/2005/en/index.html*

World Health Organization (2004) The WHO/IUATLD Project on anti-tuberculosis drug resistance surveillance. *WHO/HTM/TB/2004.343. WHO, Geneva, Switzerland*, 1–299.

World Health Organization (2001). Guidelines for drug susceptibility testing for second-line anti-tuberculosis drugs for DOTS-plus. *WHO/CDS/TB/2001.288. WHO, Geneva, Switzerland,* 1–13

World Health Organization (2000) Anti-tuberculosis drug resistance in the world report no 2: prevalence and trends publication. WHO/CDS/TB/2000.278. Geneva.

Wright A, Zignol M, Van Deun A, Falzon D, Gerdes SR and Feldman K (2009). Epidemiology of antituberculosis drug resistance 2002-07: an updated analysis of the Global project on anti-Tuberculosis drug resistance surveillance. *Lancet*.373 (9678):1861-73.

Yee DP, Menzies D and Brassard P (2012). Clinical outcomes of pyrazinamide-monoresistant *Mycobacterium tuberculosis* in Quebec. *International Journal of Tuberculosis and Lung Disease*. 16:604-609.

Zhang Y, Heym B, Allen B, Young D and Cole ST (1992). The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. *Nature*. 358:591-593.

Zhang Y and Telenti A (2000). Genetics of drug resistance in *Mycobacterium tuberculosis*. In: *Molecular Genetics of Mycobacteria.* Harfull GF, Jacobs WR Jr (Eds). ASM Press, Washington, DC, USA, 235–251.

Zhang Y and Yew WW (2009). Mechanisms of drug resistance in *Mycobacterium tuberculosis*. *Int. J. Tuber. Lung Dis*. 13:1320–1330.

Zhu RY, Zhang KX, Zhao MQ (2009). Use of visual loop-mediated isothermal amplification of rim sequence for rapid detection of *Mycobacterium tuberculosis* and *Mycobacterium bovis*.

APPENDIX I

ZIEHL-NEELSEN (ZN) STAINING METHOD

1. Flood the slide with carbol fuchsin
2. Carefully heat the slide from underneath using a torch, until the liquid is steaming (NOT boiling)
3. Stain for 5 minutes
4. Rinse slide gently with tap water
5. Flood the slide with 3% acid-alcohol and decolourise until no more colour drains from the slide (20-30 seconds)
6. Rinse slide gently with tap water
7. Flood the slide with methylene blue and counter stain for 30 seconds
8. Rinse slide gently with tap water
9. Allow to air dry (NOTE: DO NOT BLOT)

INTERPRETATION

1. Examine slide using the 100 x oil immersion objective of the microscope
2. Examine a minimum of 100 fields before reporting the smear as AFB negative.
3. Positive: red/pink bacilli approximately 1- 10 µm long and typically appears as slender, rod-shaped but may appear curved or bent.

   Negative: no red/pink bacilli observed
4. Report clinical smears using the following table

No of AFB found	Oil immersion fields	Report
No AFB	100	Negative
1-9 AFB	100	Exact number of AFB
10-99 AFB	100	1+
1-10 AFB	50	2+
>10 AFB	20	3+

APPENDIX II

LÖWENSTEIN–JENSEN MEDIUM (Abdel Aziz, 2003)

Mineral salt base solution

- Potassium dihydrogen phosphate anhydrous (KH_2PO_4) 2.4g
- Magnesium sulfate ($MgSO_47H_2O$) 0.24g
- Magnesium citrate 0.6g
- Aspargine 3.6g
- Glycerol (reagent grade) 12ml
- Distilled water 600ml

Dissolve the ingredients, in order, in the distilled water by heating. Autoclave at 121º C for 30 minutes to sterilise. Cool to room temperature. This solution keeps indefinitely and may be stored in suitable amounts in the refrigerator

Malachite green solution

- Malachite green dye 2.0g
- Sterile distilled water 100ml

Using aseptic techniques dissolve the dye in sterile distilled water by placing in the incubator for 1–2 hours. This solution will not store indefinitely. If precipitation occurs or the solution becomes less deeply coloured, discard and prepare a fresh solution.

Homogeniszed whole eggs

Fresh hens'eggs (not more than 7 days old), from hens that have not been fed antibiotic containing feed, are cleaned by scrubbing thoroughly with a brush in warm water and a plain alkaline soap. Let the eggs soak for 30 minutes in the soap solution, then rinse them thoroughly in running water and soak them in 70% ethanol for 15 minutes. Before handling

the clean dry eggs, scrub the hands and wash them. Crack the eggs with a sterile knife into a sterile flask and beat them with a sterile egg whisk or in a sterile blender.

Preparation of medium

The following ingredients are aseptically pooled in a large, sterile flask and mixed well:

- Mineral salt solution 600ml
- Malachite green solution 20ml
- Homogenized eggs (20–25 eggs, depending on size) 1000ml

The complete egg medium is distributed in 6-8ml volumes in sterile 14ml or 28ml McCartney bottles or in 10ml volumes in 20 x 150 mm screw-capped test-tubes and the tops are securely fastened. Inspissate the medium within 15 minutes of distribution to prevent sedimentation of the heavier ingredients.

Coagulation of medium

Before loading, preheat the inspissator to 85°C. Place the bottles in a slanted position in the inspissator and coagulate the medium for 45 minutes at 85°C. (Since the medium has been prepared with sterile precautions, this heating is to solidify the medium, not to sterilize it. Inspissation time begins when the inspissation chamber reaches 85°C). The quality of egg media deteriorates when coagulation is carried out at too high a temperature or for too long. Discoloration of the coagulated medium may be due to excessive temperature. The appearance of small holes or bubbles on the surface of the medium also indicates faulty coagulation procedures. Poor quality media should be discarded.

Sterility check

After inspissation, the whole media batch or a representative sample of culture bottles should be incubated at 35-37°C for 24 hours as a sterility check.

Storage

The LJ medium should be dated and stored in the refrigerator and can be kept for several weeks if the caps are tightly closed to prevent drying out of the medium. For optimal isolation from specimens, LJ should not be older than 4 weeks.

APPENDIX III

McFarland Standard No. 1 (Chapin, Lauderdale, Murray, Baron, Jorgensen, Pfaller and Yolken**, 2003)**

Reagents:

1. Sulfuric acid, 1%
2. Barium Chloride, 1.175%

Procedure for the preparation of a 0.5 McFarland Standard:

1. Add approximately 85 ml of 1% sulfuric acid (H_2SO_4) to a 100ml volumetric flask.
2. Using a volumetric pipette, add 1.0ml of 1.175% anhydrous barium chloride ($BaCl_2$) dropwise to the 1% sulfuric acid (H_2SO_4) while constantly swirling the flask.
3. Bring the volume to 100ml with 1% H_2SO_4.
4. Stir or mix for approximately 3 to 5 minutes while examining visually, until the solution appears homogeneous and free of clumps. A magnetic stirrer can be used for this step if available.
5. Check optical density, following the procedure described in the QC section below and record on QC sheet.
6. If QC is acceptable, dispense 2 to 7 ml volumes (depending on volumes routinely used in test) into each glass screw-cap tube.
7. Label the tubes appropriately including the expiration date and the initials of the person preparing the standards. Make sure that the labelling is positioned so that it does not interfere with spectrophotometer readings.
8. Cap the tubes tightly.

9. Draw a line to mark the meniscus on each tube. This mark can be used as a guide to check for evaporation at a later time.
10. Seal the tubes with paraffin or Parafilm.
11. Repeat the procedure to make additional standards using volumes indicated in Appendix A.
12. Store the prepared standards in the dark at room temperature for three months or longer as per QC acceptability.

Table 5.1: Guide for the preparation of McFarland Standards

Volume in mL			
Standard	1% $BaCL_2$	1% H_2SO_4	Number of Bacteria/ mL/(10^8) represented
0.5	0.5	99.5	1.5
1	1.0	99.0	3
2	2.0	98.0	6
3	3.0	97.0	9
4	4.0	96.0	12
5	5.0	95.0	15
6	6.0	94.0	18
7	7.0	93.0	21
8	8.0	92.0	24
9	9.0	91.0	27
10	10.0	90.0	30