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

By

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Submitted in partial fulfilment of the requirements for the degree

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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

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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 Observation 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 CO₂ 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/ (Hain Lifescience, Nehren, Germany) was developed to detect resistance to ethambutol (EMB), fluoroquinolones
and injectable aminoglycosides/cyclic peptides by focusing on the most prevalent *gyrA*, *rrs*, and *embB* 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 assess the ability of the GenoType\(^\text{R}\)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\(^\text{R}\)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>
<thead>
<tr>
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<th>Global rank (by estimated cases)</th>
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<td>Afghanistan</td>
<td>333</td>
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<td>Bangladesh</td>
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<td>Brazil</td>
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<td>Cambodia</td>
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<td>China</td>
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<td>Democratic Republic of Congo</td>
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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 °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 \textit{M. tuberculosis}

The virulence of \textit{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 \textit{et al}, 2002). Some of the general properties of \textit{Mycobacterium tuberculosis} that renders it virulent are:

\textbf{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 \textit{et al}, 2002).

\textbf{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 \textit{et al}, 2002).

\textbf{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 \textit{et al}, 2002).

\textbf{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 \textit{et al}, 2002).

\textbf{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 \textit{M. leprae} causes leprosy, \textit{Treponema pallidum} causes syphilis, and \textit{Borrelia burgdorferi} causes Lyme disease (Beaucher \textit{et al}, 2002).

\textbf{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 recognized 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\textsubscript{H}1 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-\textgamma]), which attract T cells and other inflammatory cells to the site (Tufariello et al., 2003). The recruited CD4 T cells initiate the T\textsubscript{H}1-type immune response over the following 3 to 9 weeks in which IFN-\textgamma 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\textsubscript{H}1 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\textsubscript{H}1 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 14 days; 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 patient 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)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of action</th>
<th>Genes affected by resistant mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First line drugs (Oral)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoniazid (INH)</td>
<td>Inhibit mycolic acid synthesis</td>
<td>KatG, inhA, oxyR</td>
</tr>
<tr>
<td>Rifampicin (RIF)</td>
<td>Binds to RNA polymerase inhibiting RNA synthesis</td>
<td>rpoB</td>
</tr>
<tr>
<td>Pyrazinamide (PZA)</td>
<td>Activated to pyrazinoic acid, which is bactericidal</td>
<td>pncA</td>
</tr>
<tr>
<td><strong>Second line drugs</strong></td>
<td></td>
<td></td>
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<tr>
<td>a. Injectable drugs</td>
<td></td>
<td></td>
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<tr>
<td>Streptomycin (SM)</td>
<td>Binds to ribosomal proteins and inhibits protein synthesis</td>
<td>rrs, rpsl</td>
</tr>
<tr>
<td>Amikacin (AMI)</td>
<td>Disrupts ribosomal function and inhibits protein synthesis</td>
<td>rrs, rpsl</td>
</tr>
<tr>
<td>Kanamycin (KAN)</td>
<td>Binds to 30S ribosomal subunit, inhibits protein synthesis</td>
<td>rrs, rpsl, eis</td>
</tr>
<tr>
<td>Capreomycin (CAP)</td>
<td>Similar to aminoglycosides</td>
<td>rrs, rpsl</td>
</tr>
<tr>
<td>b. Fluoroquinolones</td>
<td></td>
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<tr>
<td>Ciprofloxacin (CFX)</td>
<td>Disrupts the DNA-DNA gyrase complex blocking DNA synthesis</td>
<td>gyrA, gyrB</td>
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<tr>
<td>Ofloxacin (OFX)</td>
<td></td>
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<tr>
<td>Moxifloxacin (MFX)</td>
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<td>Gatifloxacin (GFX)</td>
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<tr>
<td>Levofloxacin (LFX)</td>
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<tr>
<td>c. Oral bacteriostatic anti-TB agents (second-line)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycloserine (CS)</td>
<td>Inhibits cell wall synthesis</td>
<td>-</td>
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<tr>
<td>Ethionamide (ETH)</td>
<td>Inhibits oxygen dependent mycolic acid synthesis</td>
<td>-</td>
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<tr>
<td>P-aminosalicylic acid (PAS)</td>
<td>Disrupts folic acid metabolism</td>
<td>-</td>
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<tr>
<td>Rifabutin (RBU)</td>
<td>Binds to RNA polymerase, Inhibits RNA synthesis</td>
<td>rpoB</td>
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<tr>
<td>Thioacetazone (TAC)</td>
<td></td>
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<tr>
<td>d. Anti-TB agents with unclear efficacy</td>
<td></td>
<td></td>
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<tr>
<td>Clofazimine (CFZ)</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Amoxicillin/Clavulanate (AMX-CLV)</td>
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<tr>
<td>Clarithromycin (CLR)</td>
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<tr>
<td>Linezolid (LZD)</td>
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</table>
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 \textit{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 (\textit{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 \textit{et al.}, 1992). High-level resistance to INH is mainly due to mutations within the \textit{KatG} gene, while mutations in the \textit{inhA} regulatory region confer low-level resistance to INH in \textit{M. tuberculosis} isolates (Almeida Da Silva and Palomino, 2011).

![Figure 2.1 Chemical structure of Isoniazid (Bwanga \textit{et al.}, 2009)](image)

KatG mutations occur frequently (~50-95%), particularly at codon 315 (\textit{katG}315) in INH-resistant \textit{M. tuberculosis} strains (Ahmad and Mokaddas, 2004). The frequency of mutations in the \textit{inhA} regulatory region also varies in INH-resistant strains (Al-Mutairi, \textit{et al.}, 2011). Mutation in other genes (\textit{acpM}, \textit{ahpC} and \textit{KasA}) 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).

![Chemical structure of Rifampicin](bwanga_et_al_2009)

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

\section*{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 \textit{gyrA} and \textit{gyrB} genes respectively) and cause cell death due to inhibition of DNA replication and repair (Rieder, 2009). Mutations in small regions of the \textit{gyrA} (mostly codon 90 or 94) and \textit{gyrB} genes or changes in drug efflux pumps confer resistance to FQs in \textit{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).

\section*{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 \textit{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 \textit{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).

![Figure 2.1 MDR-TB Prevalence](image)

*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, Bosnia and Herzegovina, Cabo Verde, Comoros, Djibouti, Gabon, Indonesia, Iraq, Libya, Mauritania, Mozambique, Namibia, Niger, Nigeria, Saudi Arabia, Sudan, Syria, Tanzania, and Yemen 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 4,000 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 antituberculosis 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 nontuberculous 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, Odonu 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 *rpoB* 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 *rpoB*. 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 *katG* gene, resulting in failure to activate the prodrug (Zhang and Telenti, 2000). In addition to mutations in *katG*, mutations in the enoylreductase InhA-coding gene, *inhA* may contribute to INH resistance due to overexpression or alteration of the drug target (Piatek, Telenti and Murray, 2000). Mutations within the *oxyR–ahpC* 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 *ndl* 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 *embB* 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, Rüscher-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 pyrazinonic acid that is converted to its active form by the mycobacterial nicotinamidase/pyrazinamidase (PZase) (Zhang and Telenti, 2000). Defective PZase activity due to *pncA* 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 *gyrA* and *gyrB* (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 \textit{rpoB}, \textit{gyrA}, \textit{gyrB} and \textit{pncA} 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 \textit{M. tuberculosis} complex bacteria with simultaneous detection of RMP resistance from specimens (Xpert\textregistered 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 \textit{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 MTBDRplus** detects RIF and INH resistance (MDR-TB strains) in culture isolates and sputum samples targeting *rpoB*, *katG*, and *inhA* 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 MTBDRsl** detects resistance to FQs, injectable drugs (KAN/AMI/CAP) and EMB in culture isolates and clinical specimens (Drobniewski et al., 2012). Pooled sensivities of genotype MTBDRsl 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 MTBDRsl and genotype MTBDRplus 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 *gyrB* (Iwamoto, Sonobe, Hayashi, 2003), *rrs*
(Pandey, Poudel and Yoda, 2008) and rimM (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 extrapulmonary 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,
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\textsuperscript{(R)}MTBDR\textit{plus} V2.0 and Genotype\textsuperscript{(R)}MTBDR\textit{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\textsuperscript{(R)}MTBDR\textit{plus} and Genotype\textsuperscript{(R)}MTBDR\textit{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\textsuperscript{(R)}MTBDR\textit{plus} \textit{and} Genotype\textsuperscript{(R)}MTBDR\textit{sl} assay respectively. Genotype\textsuperscript{(R)}MTBDR\textit{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\textsuperscript{(R)}MTBDR\textit{sl} assay detected 32/90 (35.5\%), 16/90 (17.7\%) and 5/90 (5.5\%) resistance to Ethambutol, Fluoroquinolones and Aminoglycosides respectively. Genotype\textsuperscript{(R)}MTBDR\textit{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\(\text{\textsuperscript{(R)}}\text{MTBDR\text{\textsuperscript{plus}}}\) and Genotype\(\text{\textsuperscript{(R)}}\text{MTBDR\text{\textsuperscript{sl}}})\) 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.

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 \textit{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 \textit{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\textit{plus} (Hain Lifescience, Nehren, Germany) for rapid detection of high-risk MDR-TB cases (Akpaka \textit{et al}., 2008). GenoType MTBDR\textit{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 \textit{et al}., 2007). The assay detects mutations in the \textit{rpoB} gene for RIF resistance, in the \textit{katG} gene for high-level INH resistance and in the \textit{inhA} regulatory region gene for low-level INH resistance (Causse \textit{et al}., 2008).

GenoType®MTBDR\textit{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\textit{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 \textit{et al}., 2014). Drug susceptibility testing by the agar proportion method as described by Canetti, Wallace, Khomenko, Mahler, Menon, Mitchison, Rist and Smelv, (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)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration of drug after reconstitution *</th>
<th>Volume added to MGIT tube</th>
<th>Final concentration in MGIT tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>STR</td>
<td>83 μg/ml</td>
<td>100 ml</td>
<td>1.0 μl/ml</td>
</tr>
<tr>
<td>INH</td>
<td>8.3 μg/ml</td>
<td>100 ml</td>
<td>0.1 μl/ml</td>
</tr>
<tr>
<td>RIF</td>
<td>83 μg/ml</td>
<td>100 ml</td>
<td>1.0 μl/ml</td>
</tr>
<tr>
<td>EMB</td>
<td>415 μg/ml</td>
<td>100 ml</td>
<td>5.0 μl/ml</td>
</tr>
</tbody>
</table>

*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®MTBDRplus and GenoType®MTBDRsl assays were performed respectively according to the instructions provided by the manufacturer (Hain Lifescience, Nehren, Germany). Firstly, Genolyse\textsuperscript{(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 \degree 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\degree C, followed by 10 cycles comprising 30 seconds at 95\degree C and 120 seconds at 65\degree C; an additional 20 cycles comprising 25 seconds at 95\degree C, 40 seconds at 50\degree C and 40 seconds at 70\degree C and a final extension at 70\degree 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®MTBDRplus assay strips contain 27 reaction zones; 21 of them 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 \textit{rpoB} amplification control, a \textit{KatG} amplification control and an \textit{inhA} amplification control. For the detection of RIF resistance, the probes cover the \textit{rpoB} gene while the INH resistance specific probes cover positions in \textit{KatG} and \textit{inhA} genes, as shown in Fig. 4.1. Furthermore, GenoType®MTBDRsl V1.0 and GenoType®MTBDRsl V2.0 assay strips were used in this study for second-line drugs. The GenoType®MTBDRsl
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 gyrA and gyrB 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®MTBDRsl 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 gyrA amplification control for detection of resistance to FQ, *rrs* amplification control for the detection of aminoglycosides resistance and *embB* 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°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.
### 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 summarized 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; *katG* and *inhA*. There were mutations in 54 of 97 (55.6%) resistant isolates. Specific mutations in codon S315T1 of *katG* gene was found in 32 isolates. Mutations in *inhA* 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

<table>
<thead>
<tr>
<th>No. of specimens which turned positive after days. Total 97 (%)</th>
<th>Average time to detect (No. of days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 – 7 days</td>
<td>14 (14)</td>
</tr>
<tr>
<td>8 – 14 days</td>
<td>68 (70)</td>
</tr>
<tr>
<td>15 – 21 days</td>
<td>15 (16)</td>
</tr>
</tbody>
</table>

Table 4.2: Summary of results of Rifampicin and Isoniazid resistance by Genotype®MTBDRplus compared to the BACTEC MGIT 960 System

<table>
<thead>
<tr>
<th></th>
<th>GenoType®MTBDRplus V2.0 (%)</th>
<th>BACTEC MGIT 960 SYSTEM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR</td>
<td>40/97(41)</td>
<td>53/97(55)</td>
</tr>
<tr>
<td>RIF mono-resistance</td>
<td>43/57(75)</td>
<td>33/44(75)</td>
</tr>
<tr>
<td>INH mono-resistance</td>
<td>14/57(25)</td>
<td>11/44(25)</td>
</tr>
</tbody>
</table>

INH = Isoniazid, RIF = Rifampicin, MDR = Multidrug resistance
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<sup>(R)MTBDR<sub>sl</sub>V2.0</sup> 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<sup>(R)MTDR<sub>sl</sub>V2.0</sup> 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<sup>(R)MTBDRplus</sup> 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 gyrA A90V mutant probe hybridisation positivity (with loss of hybridisation band gyrA WT) while the five aminoglycoside resistant isolate had *rrs* A1401G and C1402T mutant probe hybridisation (Table 4.4). There was no mutant hybridisation for gyrB of FQ and *eis* of aminoglycoside. Hence, there was no discordance between GenoType<sup>(R)MTBDR<sub>sl</sub>V1.0</sup> and
GenoType\textsuperscript{(R)} MTBDR\textsubscript{s/l} 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\textsuperscript{(R)} MTBDR\textsubscript{s/l} compared with BACTEC MGIT 960 System

<table>
<thead>
<tr>
<th></th>
<th>GenoType\textsuperscript{(R)} MTBDR\textsubscript{plus} V2.0(%)</th>
<th>BACTEC MGIT 960 SYSTEM(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OFX-resistance</td>
<td>16/90(18)</td>
<td>9/90(10)</td>
</tr>
<tr>
<td>KAN-resistance</td>
<td>5/90(6)</td>
<td>3/90(3)</td>
</tr>
<tr>
<td>EMB-resistance</td>
<td>32/90(36)</td>
<td>32/90(36)</td>
</tr>
<tr>
<td>XDR</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

KAN = Kanamycin, OFX = Ofloxacin, XDR = Extensively Drug Resistance
Table 4.4: Mutation pattern of FQ (gyrA & gyrB), aminoglycoside (rrs & eis), RIF (rpoB), INH (katG & inhA) obtained from Genotype\textsuperscript{RMTBDRsl V2.0 and Genotype\textsuperscript{RMTBDRplus V2.0 assay}}

<table>
<thead>
<tr>
<th>Drug</th>
<th>Locus</th>
<th>Mutation</th>
<th>Frequency (no. of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gyrA</td>
<td>A90V</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S91P</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D94G</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D94A</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WT</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>gyrB</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>OFX</td>
<td>rrs</td>
<td>A1401G</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C1402T</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KAN</td>
<td>rpoB</td>
<td>S531L</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D516V</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H526Y</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H526D</td>
<td>3</td>
</tr>
<tr>
<td>RIF</td>
<td>katG</td>
<td>S315T1</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T8A</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C15T</td>
<td>14</td>
</tr>
<tr>
<td>INH</td>
<td>inhA</td>
<td>M306V</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M306I</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>embB</td>
<td>M306V</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M306I</td>
<td>9</td>
</tr>
</tbody>
</table>
Table 4.5: Performance of LPA result when compared with MGIT 960 for RIF, INH, OFX, KAN and EMB

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

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 MTBDRsl 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.
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)MTBDRplus 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 2 days. 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)MTBDRplus and Genotype(R)MTBDRsl) 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 Antwerp, 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). Furthermore, 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, Viljanen and 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 \textit{rpoB} gene were observed. The S531L missense mutation that led amino acid substitutions of serine to threonine was most common in \textit{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 \textit{rpoB} 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\textsuperscript{(R)}\textsuperscript{MTBDRplus} 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\textsuperscript{(R)}\textsuperscript{MTBDRplus} 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\textsuperscript{(R)}\textsuperscript{MTBDRplus} 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 \textit{katG} gene are responsible for INH resistance in 60\% of cases worldwide. Furthermore, INH resistance is caused by mutations in the \textit{inhA} regulatory and coding region and the \textit{ahpC-axyR}, \textit{ndh} and \textit{kasA} genes (Kiepiela \textit{et al}., 2000). The most frequent mutation causing INH resistance is observed to be in the S315T1 region (Ling \textit{et al}., 2008). This study also detected a similar result as 59.3\% (32/54) of resistant strains have S315T1 mutations in the \textit{katG} region that led to amino acid serine substitution to threonine. This study showed 3 discordant INH resistance results by Genotype\textsuperscript{(R)}MTBDR\textit{plus} assay when compared to the BACTEC MGIT 960 system which was detected as false-resistance with the agar proportion method. Brossier \textit{et al}., reported that the Genotype\textsuperscript{(R)}MTBDR\textit{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\textsuperscript{(R)}MTBDR\textit{sl} assay is a specific test for the detection of resistance to Fluoroquinolones, ethambutol and kanamycin in \textit{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 \textit{et al}., 2010).

The sensitivity for the detection of EMB resistance (63\%) in this study is similar to previously reported studies by Kiet \textit{et al} for isolates from Vietnam, Hilleman \textit{et al} for isolates from Germany and Brossier \textit{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 \textit{et al}.

Detection of FQ resistance was (100\%) in this study because all the FQ-resistant isolates with mutations were in the \textit{gyrA} region. The low detection rate of ethambutol resistance is consistent with reports from other settings, with \textit{embB} mutations accounting for between 30 and 70\% of ethambutol resistant isolates (Alcaide \textit{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 \textit{et al}., 2010). According to Table 4.3 Genotype\textsuperscript{(R)}MTBDR\textit{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)MTBDRsl 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 \( ^{R}\)MTBDRplus 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 MTBDRsl 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 \( ^{R}\)MTBDRsl V1.0 and Genotype \( ^{R}\)MTBDRsl 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 \( ^{R}\)MTBDRsl 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 \( ^{R}\)MTBDRsl and MGIT 960 system) in the detection of extensively drug resistance TB however, Genotype \( ^{R}\)MTBDRsl 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).
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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

<table>
<thead>
<tr>
<th>No of AFB found</th>
<th>Oil immersion fields</th>
<th>Report</th>
</tr>
</thead>
<tbody>
<tr>
<td>No AFB</td>
<td>100</td>
<td>Negative</td>
</tr>
<tr>
<td>1-9 AFB</td>
<td>100</td>
<td>Exact number of AFB</td>
</tr>
<tr>
<td>10-99 AFB</td>
<td>100</td>
<td>1+</td>
</tr>
<tr>
<td>1-10 AFB</td>
<td>50</td>
<td>2+</td>
</tr>
<tr>
<td>&gt;10 AFB</td>
<td>20</td>
<td>3+</td>
</tr>
</tbody>
</table>
APPENDIX II

LÖWENSTEIN–JENSEN MEDIUM (Abdel Aziz, 2003)

Mineral salt base solution

- Potassium dihydrogen phosphate anhydrous (KH$_2$PO$_4$) 2.4g
- Magnesium sulfate (MgSO$_4$7H$_2$O) 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.

Homogenized 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$_2$SO$_4$) 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$_2$SO$_4$) while constantly swirling the flask.
3. Bring the volume to 100ml with 1% H$_2$SO$_4$.
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

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<th>Volume in mL</th>
<th>Standard</th>
<th>1% BaCl₂</th>
<th>1% H₂SO₄</th>
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