CHAPTER TWO

1. REVIEW OF LITERATURE

2.1 GENERAL CHARACTERISTICS OF Mycobacterium tuberculosis

2.1.1 DESCRIPTION

The tubercle bacillus belongs to the genus *Mycobacterium*, of the family Mycobacteriaceae \(^{[25]}\). The *M. tuberculosis* complex includes five species of mycobacteria which can cause tuberculosis (TB): *M. africanum*, *M. bovis*, *M. canetti* and *M. microti* and *M. tuberculosis*. The first two rarely cause disease in humans; the second two do not cause human disease while the last is the major cause of the disease TB in humans. Although for many years TB complex organisms were known to grow aerobically \(^{[25]}\), it has been also shown to persist in conditions of hypoxia \(^{[26]}\). TB is predominantly the disease of the lungs but it can also affect bones and joints, vascular system, central nervous system, genitourinary systems and the lymphatic system \(^{[27]}\).

2.1.2 GROWTH AND PHYSICAL CHARACTERISTICS
*M. tuberculosis* is a slow growing microbe with generation times ranging from 12-24 hours \[^{28}\]. This is extremely slow compared to other bacteria, which tend to have division times measured in minutes. It is primarily a facultative, intracellular pathogen which resides within the phagolysosomes of alveolar macrophages \[^{25}\]. *M. tuberculosis* is typically slightly curved or straight rod shaped. Its typical size when cultured *in vitro* is 1 to 4 mm in length and 0.3 to 0.6 mm in diameter, making it smaller than most bacterial pathogens \[^{28}\].

### 2.1.3 MICROSCOPIC CHARACTERISTICS

*M. tuberculosis* is an acid-fast bacillus (AFB). AFB is gram positive bacilli that stain poorly and are seldom seen in a gram stain. This is due to the lipid content of their cell wall. The primary cell wall structure of *M. tuberculosis* consists of a plasma membrane, which is supported by a peptidoglycan backbone against the osmotic pressure of the interior \[^{25}\]. Attached to the peptidoglycan is an arabinogalactan layer and to which are esterified mycolic acids. The high lipid content of *M. tuberculosis* cell wall makes it highly hydrophobic, which results in the clumping of cells, a characteristic that prevents staining with the usual chemicals. *M. tuberculosis* can be identified microscopically by its staining characteristics. It resists decolorisation with acid after being stained. In the most common staining technique, the Ziehl-Neelsen stain, AFB are stained a bright red which stands out clearly against a blue background (Figure 2.1). AFB can also be visualized by fluorescent microscopy using an auramine-rhodamine stain. The mycobacterial cell wall is impermeable towards many drugs used in the chemotherapy of bacterial diseases \[^{29}\].
2.1.4 TRANSMISSION OF TB

Transmission of TB begins after contact with a human source, almost always an infectious person with cavitary pulmonary TB. The infection is transmitted almost exclusively by the airborne route via a small bacillus-containing particle called a droplet nucleus \[^{28}\]. When a droplet nucleus containing one or two viable bacilli is inhaled, it is deposited on the alveolar surface where the bacilli begin to multiply. Initially, the infecting organism meets only limited resistance from the host, as phagocytosis by alveolar macrophages has little effect on the bacilli. The earliest evidence of host tissue recognition is dilation of the capillaries, followed by a migration of polymorphonuclear leukocytes and macrophages into the infected area. After several weeks of infection, the number of leukocytes in the area decreases and the mononuclear cells predominate. These crowd together and contain pale, foamy cytoplasmic material, which is rich in lipids. The resulting unit is called a tubercle, the fundamental lesion of TB \[^{25}\]. When an
individual is infected with the tubercle bacilli, the organism is taken up by the alveolar macrophages and carried to the lymph nodes. In the lymph nodes it can spread further to other organs. It takes about 2 to eight weeks after infection for the immune system to elicit a response. During this time, cell mediated immunity and hypersensitivity develops which leads to the characteristic reaction of the tuberculin test \[^{30}\]. In immunocompetent individuals containment of infection then follows. About 10% of these infected individuals will develop active disease in their lifetime. The other 90% do not become ill and cannot transmit the organism due to the ability of the normal immune system to contain the infecting organism or even in some instances, to eradicate them. Individuals who are taking immunosuppressive agents or those that are infected with Human Immunodeficiency Virus (HIV) are more likely to develop the disease because of their compromised immunity. Therefore, TB is reported to be rampant in populations that have dual infections with HIV \[^{31}\].

2.2 EPIDEMIOLOGY OF TB

2.2.1 TB INCIDENCE AND PREVALENCE

The global burden of TB remains enormous. In 2012, there were an estimated 8.6 million incident cases of TB and 1.3 million people died from the disease (940,000 deaths among people who were HIV-negative and 320,000 among people who were HIV positive. Among these deaths there were an estimated 170,000 from MDR-TB, a relatively high total compared with 450,000 incident cases of MDR-TB. India and China had the largest number of cases (26% and 12% of the global total, respectively). South Africa and Swaziland had the highest incidence rate per capita (about 1 new case for every 100 people each year) \[^{32}\].
2.2.2 MDR AND XDR TB

A total of 94 000 TB patients eligible for MDR-TB treatment were detected in 2012: 84 000 people with confirmed MDR-TB), plus 10 000 with rifampicin resistance detected using
Xpert MTB/RIF. This was a 42% increase in detected cases eligible for treatment compared with 2011. The largest increases between 2011 and 2012 were in India, South Africa and Ukraine. On average, an estimated 9.6% of MDR-TB cases have XDR-TB \(^{[32]}\).

### 2.3 HISTORICAL OVERVIEW OF TB TREATMENT

The treatment of TB goes as far back as the late eighteenth century. However, it was the discovery of streptomycin from *Streptomyces griseus* by Selman Waksman and his colleagues in 1943 that saw the birth of a new era of chemotherapeutic approach to TB therapy or treatment \(^{[33]}\). Although streptomycin proved to inhibit the growth of *M. tuberculosis*, it soon became apparent that resistant mutants thrived in the application of streptomycin monotherapy, resulting in treatment failure. The quest for new drugs to treat TB subsequently lead to the discovery of paminosalicylic acid (PAS) in 1949, isoniazid (INH) in 1952, pyrazinamide (PZA) in 1954, cycloserine in 1955, ethambutol (EMB) in 1962, and rifampicin (RIF) in 1963 \(^{[34]}\). In years to follow, more drugs were discovered, such as aminoglycosides which include capreomycin (CAP), viomycin (VIO), kanamycin (KM), amikacin (AK) and quinolones such as ofloxacin (OFX) and ciprofloxacin (CIP) \(^{[34]}\). Currently, the use of INH, EMB, STR, PZA and RIF constitute the first line drugs for the treatment of TB \(^{[34]}\). The proper use of these drugs should produce a rapid clinical improvement and a significant decrease in bacterial count \(^{[3]}\). The TB treatment regimen comprises of two phases: the intensive and the continuation phase.

The intensive phase takes two months and involves multiple antibiotics such as RIF, INH, PZA and EMB or STR, to ensure that mutants resistant to a single drug do not emerge \(^{[3]}\). The following 4 months is the continuation phase and only RIF and INH are administered to
eliminate any uncleared tubercle bacilli. INH and RIF are the two most potent anti-TB drugs and kill more than 99% of tubercle bacilli within 2 months of initiation of therapy \[35, 36\]. Therefore, resistance to these two drugs was regarded as MDR in \textit{M. tuberculosis} \[3\]. The use of these drugs in conjunction with each other reduces antitubercular therapy from 18 months to 6 months. When TB results from infection with drug susceptible strains of \textit{M. tuberculosis}, the success rate of this treatment is close to 100%, provided that the patient strictly adheres to the treatment regimen \[3\].

2.3.1 RIFAMPICIN

RIF, derived from \textit{Streptomyces mediterranei} was first introduced for use as antitubercular therapy in the early 1970’s and is still a very important component of current regimens \[37\]. It is the most potent sterilizing antibiotic used for the treatment of TB \[38\]. The mode of action of RIF is based on the inhibition of the elongation of transcripts by covalently binding to the beta subunit of RNA polymerase in \textit{M. tuberculosis} thus leading to bacterial death \[39\]. The RNA polymerase beta-subunit is encoded by the \textit{rpoB} gene \[7\].

2.3.2 ISONIAZID

Isoniazid, also called isonicotinic acid hydrazide (INH) was first synthesised in the early part of the 19th century but only introduced as anti-TB drug in the 1950’s \[18\] and its powerful anti-TB activity was discovered in 1951. INH used in treatment of TB is a prodrug and necessitates catalytic activation by KatG to be converted into the active form. KatG is an enzyme
with dual activities of catalase and peroxidise \[40\]. Catalase-peroxidase is a heme containing enzyme encoded by the katG gene of \textit{M. tuberculosis} \[20\], which converts INH to a toxic derivative. INH activation leads to inhibition of mycolic synthesis, a long chain fatty acid-containing component of the mycobacterial cell wall \[41, 42\]. Two enzymes involved in the elongation cycle of the fatty acid biosynthesis, namely an enoyl-acyl carrier protein reductase \[21\] and \(\beta\)-ketoacyl-acyl carrier protein synthase \[43\] are believed to be the targets of activated inhibitor(s).

### 2.3.3 ETHAMBUTOL

EMB is a first line \textit{M. tuberculosis} drug with a broad spectrum of activity. The cell wall, and more specifically mycolic acid synthesis process, is the primary target of EMB \[44\]. They observed effects such as the inhibition of the transfer of precursor molecules in mycolic acid synthesis from the cytoplasm to the cell wall \[45\], the accumulation of the trehalose-, mon- and dimycolates in the cell and the inhibition of the synthesis of arabinogalactan from D-arabinose \[46\]. Other investigators have suggested that the inhibition of glucose metabolism may be involved \[47\]. A resistant mutant of \textit{M. tuberculosis} contained less phospholipids and unsaturated fatty acids and more arabinose, galactose, hexosamine and mycolic acids than the EMB susceptible strain \[48\]. The action and the target of EMB remain less understood.

### 2.3.4 STREPTOMYCIN

STR is a broad spectrum antibiotic of the aminoglycoside family that is bactericidal in action and was the first drug used in treatment of TB. It is an aminocyclitol glycoside that
interferes with prokaryotic protein synthesis. Its main effects are induction of misreading of the genetic code and inhibition of initiation of translation \([49]\). The site of action of streptomycin is the small 30s, subunit of the ribosome, especially the ribosomal protein S12 and the 16S rRNA \([50]\). STR acts at several stages in protein synthesis and its main effects appear to be the inhibition of initiation of mRNA translation, misreading of genetic code and aberrant proofreading by bacterial ribosome.

### 2.3.5 Pyrazinamide

Currently, PZA is one of the essential drugs in short course chemotherapy of TB. PZA is a synthetic derivative of nicotinamide that has been used in short course anti-TB treatment regimens \([37]\). This drug is the least studied of all the drugs of treatment because its activity depends upon an acid pH environment which leads to discrepancies between the in vivo and in vitro investigations. It acts in the acidic extracellular micro-environment found during acute inflammation and kills at least 95% of bacilli during the first two weeks of treatment \([35]\). The target of action of PZA in *M. tuberculosis* is thought to be *fasI* \([51]\). It is known that *M. tuberculosis* converts PZA to its active form, pyrazinoic acid, by using enzyme pyrazinamidase (PZAs) \([52]\).

### 2.4 Genetic Mechanisms of Resistance

Generally, resistance arises by the process of mutation and adaptation. In *M. tuberculosis* drug resistance occurs spontaneously i.e. sites for resistance are chromosomally located and not
linked \cite{28}. It differs from most other bacteria in its cell wall composition, and as a result, exchange of genes across this cell wall is difficult. Acquisition of drug resistance in *M. tuberculosis* does not involve plasmids or transposable elements. However, drug resistance is an outcome of changes in protein structure involved in drug uptake or in activation of prodrugs \cite{53, 7, 45, 46}. These changes are due to mutations in genes coding for such proteins. The mutations that occur randomly at chromosomal loci include nucleotide changes such as point mutations, small deletions or insertions which confer resistance to single drugs. *M. tuberculosis* accumulates these mutations in a stepwise manner and this leads to drug resistant TB. Drug resistant strains also emerge when treatment is discontinued or otherwise insufficient, emphasizing the importance of early detection of drug resistance. Resistant organisms (or mutants) evolve in the absence of drug exposure but they are diluted within the vast majority of the drug-susceptible bacilli. During bacterial multiplication, resistance develops through spontaneous mutations at a defined rate e.g. mutations resulting in resistance to RIF occurs at a rate of 10\(^{-8}\) in *M. tuberculosis* in vitro and is thought to be a one step mutational event \cite{42, 36, 37}. For INH and EMB, resistance rate occurs at a rate 10\(^{-6}\) and for STR 10\(^{-5}\) \cite{37}. During antibiotic treatment, the susceptible sub-population of bacilli was killed and allowed the resistant mutants to be selected.
Table 2.1

Genetic loci conferring drug-resistance in *Mycobacterium tuberculosis*

<table>
<thead>
<tr>
<th>Drug</th>
<th>Gene</th>
<th>Product</th>
<th>Frequency of mutations in resistant strains (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td>rpoB</td>
<td>B-subunit of RNA polymerase</td>
<td>&gt; 90</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>katG</td>
<td>Catalaseperoxidase</td>
<td>60-70</td>
</tr>
<tr>
<td></td>
<td>inhA</td>
<td>Enoyl-ACP Reductase</td>
<td>&lt; 10</td>
</tr>
<tr>
<td></td>
<td>ahpC-oxyR</td>
<td>Alky hydroreductase</td>
<td>~ 20</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>rpsL</td>
<td>Ribosomal protein S12</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Rrs</td>
<td>16s rRNA</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>pncA</td>
<td>Amidase</td>
<td>70-100</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>embB</td>
<td>EmbCAB</td>
<td>69</td>
</tr>
</tbody>
</table>

Table from Rattan *et al.*, 1998
2.4.1 RESISTANCE TO FIRST LINE DRUGS

2.4.1.1 RIF RESISTANCE

Resistance to RIF has been shown to be due to the alteration of the beta subunit of the RNA polymerase encoded by the rpoB gene \(^{[7]}\). Several authors have reported specific mutations, insertions, and deletions in this gene \(^{[7, 10, 54]}\). About 96% of RIF resistant isolates of *M. tuberculosis* have point mutations in an 81-bp region of this gene. These mutations are not present in susceptible isolates and are thus an ideal target for development of genetic susceptibility testing methods \(^{[7]}\). Mutations in the rpoB gene lead to an altered structure of RNA polymerase and hence to a largely impaired affinity to RIF for this enzyme \(^{[37]}\).

2.4.1.2 INH RESISTANCE

Complex metabolic changes have been described for INH resistant organisms. Resistance to this drug is associated with a range of mutations affecting one or more genes such as those encoding catalase-peroxidase (*katG*) \(^{[20, 55]}\), the two gene operon (*inhA* locus) encoding the enoyl-acyl carrier protein reductase involved in mycolic biosynthesis \(^{[21]}\) the alkyl hydroperoxidase...
reductase (\textit{ahpC}), which is involved in the cellular response to oxidative stress\textsuperscript{[56, 57]}, the β-ketocyl acyl carrier protein synthase (\textit{kasA}) which is important in fatty acid elongation\textsuperscript{[43]} and the \textit{ndh} gene which encodes an NADH dehydrogenase, causing defects in the enzyme activity that results in an increased NADH/NAD\textsuperscript{+} ratio and co-resistance to INH and ethionamide\textsuperscript{[58]}. However, most studies have demonstrated that INH is most frequently associated with mutations in \textit{katG} gene.

2.4.1.3 EMB RESISTANCE

Resistance to EMB is a result of mutations in the \textit{embB} gene, coding for arabinosyl transferase, which is involved in mycolic acids metabolism\textsuperscript{[45]}. The substitutions on codon 306 in the \textit{M. tuberculosis} gene \textit{embB} have been shown to be the most frequent and most predictive mutations for EMB resistance\textsuperscript{[59]}.

2.4.1.4 STR RESISTANCE

Resistance to STR has been shown to result from three different mechanisms. Firstly, the S12 protein is encoded by the \textit{rpsL} gene and missense mutations in this gene have been shown to confer STR resistance. Presence of mutations results in changes in the conformational structure of the ribosomal subunits. This affects the binding of STR to the ribosome and as a result, the effects of the drug when bound are diminished\textsuperscript{[60]}. Secondly, resistance occurs due to changes in the 16S rRNA\textsuperscript{[61]}. This protein is encoded by the \textit{rrs} gene and mutations in this gene results in changes in the ribosomal structure. Isolates that do not possess mutations in these two genes, have an alternate unknown mechanism of resistance\textsuperscript{[19, 62]}. 
2.4.1.5 PZA RESISTANCE

Numerous investigations have reported that PZA-resistant organisms have lost the pyrazinamidase activity possessed by PZA susceptible strains [63]. Resistance to pyrazinamide is usually caused by mutations in the gene pncA encoding the enzyme pyrazinamidase (PZase) [64, 18]. This enzyme metabolises PZA to pyrazinoic acid and PZA resistant organisms have lost PZase activity [65, 64]. Therefore, it is suggested that PZA resistance may be a result of molecular mechanisms such as loss of PZase structural gene or missense mutation resulting in an altered allele [18]. However, some highly PZA resistant strains do not always lack PZA activity and thus an alternate resistance mechanism may exist [64].

2.5 SECOND-LINE DRUGS FOR TREATMENT OF RESISTANT TB

2.5.1 FLUOROQUINOLONES (FQs)

FQ therapy is associated with an improved outcome in MDR-TB. OFX and CIP have shown to be the most active of the quinolone drugs against M. tuberculosis. OFX has more frequently used compared to CIP because of its increased absorption and half-life [66]. The primary target of FQs in many bacterial species is DNA gyrase which is involved in DNA replication [67].

2.5.2 CAPREOMYCIN AND VIOMYCIN
CAP and VIO are mostly used together for treatment of drug resistance *M. tuberculosis* strains \[68\]. They are polypeptide antimicrobial agents and are structurally similar, such that cross-resistance between the two drugs in *M. tuberculosis* has been shown \[69\]. CAP is a macrocyclic peptide antibiotic produced by *Saccharothrix mutabilis* subspecies *capreolus* \[70, 71\]. This appears to hinder the process of translation in mycobacteria. It was shown to inhibit phenylalanine synthesis in an in vitro translation assay using mycobacterial ribosome’s \[72\]. CAP is very expensive but very useful in cases with tubercle bacilli resistant to STR, KM and AK. VIO affects the dissociation of the 70S ribosome of *M. smegmatis* subunit by binding to both the 30S and 50S ribosome subunits \[73\]. Furthermore, it inhibits ribosomal translocation by stopping peptidyl-tRNA in the ribosomal acceptor site \[74\].

### 2.5.3 KANAMYCIN AND AMIKACIN

KM is an aminoglycoside that is used in treatment of organisms that are resistant to first line drugs. KM is the least expensive, but largely used for indications other than TB in some countries. Amikacin (AK) is as active as KM and better tolerated but much more expensive. KM and the closely related AK are commonly used for treatment of MDR-TB \[28\]. Although strains that acquire resistance to CAP also generally remain susceptible to other anti-TB medication, cross resistance with KM and VIO can occur.

### 2.5.4 ETHIONAMIDE
ETH, one of the most frequently used second-line drugs in the treatment of MDR-TB and is a structural analog of INH \cite{75}. Both compounds are known to target the enoyl-acyl carrier protein reductase enzyme involved in biosynthesis of mycolic acid and is encoded by the \textit{inhA} gene \cite{42}. The structural similarity and existence of cross-resistant phenotypes suggests that ETH and INH share a common molecular target \cite{21}.

2.5.5 D-CYCLOSERINE

DCS is an effective antimycobacterial agent but is rarely prescribed and is seldomly used due to its adverse effects. Few studies have been conducted on the mode of action and mechanisms of DCS resistance in mycobacteria. It is believed that DCS inhibits the formation of peptidoglycan \cite{76}. It is also an inhibitor of D-alanine: D alanine branch \cite{77}.

2.6 DRUG SUSCEPTIBILITY TESTING IN \textit{M. tuberculosis}

Diagnostic mycobacteriology plays a significant role in the control of the spread of TB especially MDR-TB. Rapid methods of diagnosis and determination of drug susceptibility are particularly important. Conventional methods using solid media, either agar or egg based, require long incubation periods before the results are available. A significant reduction of turnaround times for susceptibility results (from 3-6 weeks to 3-15 days) as a result of introducing manual and automated methods for susceptibility testing in liquid media was also reported \cite{78}.

2.6.1 SUSCEPTIBILITY TESTING USING SOLID MEDIA
Three conventional methods that utilise solid media to determine whether an *M. tuberculosis* isolate is susceptible or resistant have been established, namely: the absolute concentration method, the resistant ratio (RR) method and the proportion method \(^{79}\).

### 2.6.1.1 ABSOLUTE CONCENTRATION METHOD

Drug free media and media containing graded concentrations of the drug to be tested are inoculated with a standardised inoculum. The drug is included into solid agar or Lowenstein-Jensen medium or in broth as two-fold dilutions. Resistance is defined as the lowest concentration of the drug that inhibits growth. The major limitation in this method is the variability in inoculum size \(^{80, 81}\).

### 2.6.1.2 RESISTANCE RATIO (RR) METHOD

This method is the refinement of the absolute concentration method that controls for variations in the Minimum Inhibitory Concentration (MIC) of a given isolate when tested on different batches of media containing drugs. It is defined as the MIC of the test isolate divided by the MIC of a standard susceptible strain such as H37Rv. Testing is conducted at three concentrations of the drug and is greatly affected by the inoculum size as well as the viability of the strains. In addition, any variation in the susceptibility of the control strain also affects the RR of the test strain \(^{80}\).

### 2.6.1.3 PROPORTION METHOD
The proportion method based on culture is the ‘gold standard’ for the detection of resistance in *M. tuberculosis* isolates \cite{82}. In this method, the isolate is classified as susceptible below a critical proportion of resistant bacteria and as resistant above it. The proportion of drug resistant mutants in a population is calculated from a ratio of the number of colonies growing in drug containing medium and on drug free medium \cite{80}. It is an inexpensive and relatively simple technique, which provides results in 3 weeks from a cultured isolate or from AFB smear positive sputum specimen.

### 2.6.2 SUSCEPTIBILITY TESTING USING LIQUID MEDIA

It is widely known that drug susceptibility testing of *M. tuberculosis* is more rapid in liquid compared to solid media. Liquid media systems use an increase in biomass as a growth marker of TB and hence predicting resistance to the drug by the test organism. Radiometric methods were first introduced in mycobacteriology by Cummings *et al*, 1975\cite{83}. A major advancement was made in 1977, when Middlebrook introduced a liquid 7H12 medium containing 14C-labelled palmitic acid for radiometric detection of mycobacterial growth \cite{84}.

#### 2.6.2.1 BACTEC 460 TB METHOD

The BACTEC 460 method is a radiometric variant of the proportion method and was first described by Siddiqi *et al*, in 1981\cite{85}. The method is based on detection of growth radiometrically. During growth, bacteria utilize a radiolabelled source of carbon (14C) and
produces radiolabeled CO2. The instrument records the level of radioactivity as growth index. This method has shortened the duration of susceptibility testing to 5 to 7 days. The BACTEC system provides results in only 5 days but it is costly and requires disposal of radioactive material. In 1983, Roberts et al reported 98% agreement for BACTEC 460TB in comparison with conventional susceptibility testing of *M. tuberculosis* with a turnaround time of approximately 5 days [86]. These results were concordant with those of Siddiqi et al (1981) [85].

**2.6.2.2 MYCOBACTERIA GROWTH INDICATOR TUBE (MGIT) METHOD**

Palaci et al, (1996) reported the Mycobacteria Growth Indicator Tube (MGIT) to be time saving, safe, simple and reliable in the detection of drug resistance [87]. The method uses tubes containing 4.0 ml of Middlebrook 7H9 broth with an oxygen sensitive fluorescence sensor embedded in silicone to indicate microbial growth. The broth is enriched 7H9 (modified Middlebrook) broth base with 0.25% glycerol and 10% CO2 [88]. Actively respiring mycobacteria use the oxygen available within the tube, thereby exciting the fluorescence reaction. In the presence of oxygen the fluorescent indicator is quenched. Fluorescence indicating mycobacterial growth is then detected when the MGIT is viewed with a 365-nm ultraviolet (UV) light from transilluminator. Antimycobacterial susceptibility testing can be performed by comparing the growth in an antibiotic-containing MGIT with that of a growth control MGIT without antibiotics [88].

**2.6.2.3 MB/BacT SYSTEM**
The MB/BacT system has been reported to be a rapid, sensitive method for the growth and detection of mycobacteria from clinical specimens and has shown good performance in comparison with BACTEC 460 TB [89, 90]. In this closed system, mycobacterial growth is indicated by a colorimetric CO2 detection device. Unlike the BACTEC 460 TB, the cumbersome handling of bottles during incubation is avoided. Brunello and Fontana concluded that MB/BacT system was suitable to test the antimicrobial susceptibility of *M. tuberculosis* to first line drugs [91]. The advantage of this method is the absence of radioactivity when compared to BACTEC 460 TB method.

2.6.3 MICROSCOPY BASED DRUG SUSCEPTIBILITY TESTING

2.6.3.1 MICROSCOPIC OBSERVATION DRUG SUSCEPTIBILITY (MODS) ASSAY

The simple microscopic observation drug susceptibility assay was developed by Caviedes *et al*, in 2000 [92]. This method uses the two properties of *M. tuberculosis*: (i) the growth rate of *M. tuberculosis* is rapid in liquid medium compared to the growth rate in solid medium, and (ii) its morphology in liquid culture is recognizable as consisting of tangles or cords of organisms. Middlebrook 7H9 broth inoculated with decontaminated sputum in 24-well plates is examined under an inverted light microscope. Direct susceptibility testing on clinical specimens is performed by incorporating anti-TB drugs at the onset. Park *et al*, 2002 and Moore *et al*, 2004 demonstrated similar results when comparing the MODS assay to gold standard proportion method showing a 100% agreement between the two methods [93, 94].
2.6.4 COLORIMETRIC METHODS

Bacterial species have the ability to reduce an indicator and produce a change of visual colour. This has enabled researchers to exploit this characteristic to develop methodologies for DST such as the Alamar blue assay \([95, 96]\), MTT assay \([97-99]\), the nitrate reductase assay \([100]\), and resazurin assay \([101, 102]\).

2.6.4.1 ALAMAR BLUE ASSAY

Alamar blue is a dye that indicates cellular growth-viability due to the oxidation-reduction process during metabolism of viable organisms. During metabolic activity the blue oxidised form becomes pink upon reduction. This method was first used by Pfaller et al (1994) \([103]\) for drug susceptibility testing of yeast and was first used on mycobacteria by Yajko et al (1995) \([95]\). Collins and Franzblau (1997) modified the assay format to create the microplate alamar blue assay or MABA \([104]\). This assay has been used for DST of \(M. tuberculosis\) to RIF and INH \([105]\).

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

MTT is a yellow tetrazolium salt that is converted into blue formazan by dehydrogenases of live cells \([106]\). The amount of blue or purple formazan formation is proportional to the number of live mycobacteria in a sample \([98]\). Abate et al (1998) and Moore et al (1998) applied this
assay to detect RIF resistance directly from sputum specimens \cite{97, 107}. They found it to shorten the turnaround time for detection of RIF resistance.

### 2.6.4.3 NITRATE REDUCTASE ASSAY

The nitrate reductase assay (NRA) was initially developed at the Central Tuberculosis Research Institute in Moscow Russia \cite{108}. It was then called the Griess method, after J. P. Griess, who discovered the chemistry of the detection method \cite{109}. *M. tuberculosis* has the ability to reduce nitrate to nitrite and this is routinely used for biochemical identification of mycobacterial species. The presence of nitrite can easily be detected with specific reagents, which produce colour change \cite{110}. Angeby *et al* evaluated this method for DST of *M. tuberculosis* and found it to be rapid, inexpensive and easy to perform \cite{100}.

### 2.6.4.4 RESAZURIN ASSAY

The resazurin reduction test was first used to demonstrate bacterial and yeast contamination in milk \cite{111, 112}. Resazurin is a blue dye that does not flouresce but becomes pink and fluoresces as it is reduced to resofurin by oxidoreductases within live cells. This method produced the most reliable and accurate results for DST of INH and RIF \cite{101, 102}. It has been used for detection of both first line \cite{101} and second line antibiotic resistance of *M. tuberculosis* \cite{113}.

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

2.7.1 PHENOTYPIC MOLECULAR TECHNIQUES

Phenotypic molecular methods use markers of viability instead of an increase in biomass as in the case of liquid media based susceptibility testing. Anti-TB drugs also inhibit bacterial metabolic activities in susceptible isolates but not in resistant isolates. Strategies for the detection of resistance that have been used include mycobacteriophages such as the phage assay \cite{114, 115} and the luciferase reporter phages (LRPs) \cite{116}. Drug susceptibility is assessed without reference to the genetic basis of resistance.

2.7.1.1 PHAGE ASSAY

The phage assay is fast and easy to perform, yielding results in clinical specimens within 48 hours \cite{114, 117}. Bacteriophages can infect and replicate inside mycobacteria. Once mycobacteria are infected, the number of internalised phages determined indicates the original number of *M. tuberculosis*. This is determined after a number of cycles of infection, replication and release in rapidly growing mycobacteria such as *M. smegmatis*. When drug resistant *M. tuberculosis* is infected with a mycobacteriophage, it remains viable and protected within the bacilli. It then replicates within viable bacilli and eventually lyses its host and lysis is easily seen as clear areas or plaques in a lawn of *M. smegmatis* culture. The number of plaques generated is
directly proportional to the number of protected mycobacteriophages, which is dependent on the number of tubercle bacilli that remain viable after drug treatment. The sensitivity of this assay as well as the presence of inhibitors in sputum poses major problems.

2.7.1.2 LUCIFERASE REPORTER ASSAY (LRP)

Luciferase reporter mycobacteriophages can be used as a simple tool for rapid determination of drug susceptibility profiles of *M. tuberculosis*. Luciferase activity can be monitored with a luminometer or with a photographic film. The luminometer offers higher sensitivity and quantitative results in 54 hours. It is based on the efficient production of photons by viable mycobacteria infected with specific reporter phages expressing the firefly luciferase gene (*lux*). Some reporter phages possess the firefly gene. Riska *et al* reported that these phages can inject their *lux* bearing genes in viable mycobacteria and this would result in production of photons. The injected *lux* gene, when transcribed, using the mycobacterial ATP, catalyses the production of light. This activity can be detected by using the luminometric instrument. Mycobacterial cells killed by anti-TB drugs cannot be infected and thus do not produce light. The shortcoming of this assay is that the mycobacteriophage has a broad host array and will infect many non-tuberculous mycobacteria and therefore may lead to false positive reports of drug resistant *M. tuberculosis*. Although there are techniques for increasing species specificity for this assay, this comes at great cost and complexity. This procedure has shown promising results with clinical isolates and sputum samples. Photographic detection is achieved by using the ‘Bronx box’, which is an expensive light tight box with a Polaroid cassette. This carries a photographic film which is able to detect light emitted by the infected cells carried out in microtiter plates. Qualitative results can be obtained in 94 hours.
2.7.2 GENETIC MOLECULAR TECHNIQUES

The genes coding for the targets of the first-line anti-tuberculosis drugs as well as the mutations associated with the resistant phenotypes have been identified. The detection of these mutations is facilitated since they are localised in limited regions of the genes encoding the drug targets. After PCR amplification, the mutation is identified by sequencing the PCR products or other mutation detecting methods. Molecular studies have indicated that the genome of *M. tuberculosis*, including mutations in the drug resistant genes, is stable in follow-up samples from MDR-TB patients \[^{122}\]. This favours the use of molecular techniques to predict drug resistance by mutation analysis. Generally, resistance arises by a process of mutation and adaptation. Detection of genotypic drug resistance in *M. tuberculosis* isolates provides a rapid and easy alternative to conventional phenotypic susceptibility testing. MDR results from stepwise acquisition of mutations in the genes encoding drug targets or drug converting enzymes \[^{123}\]. This knowledge has paved the way for molecular assays that have potential to provide rapid detection of resistance in *M. tuberculosis* isolates. These techniques are highly sensitive, specific and the fact that they do not rely on mycobacterial growth has shortened the time between detection and the commencement of effective treatment. Genetic loci have been established as sources of resistance by targeting and sequencing these regions believed to be sources of resistance to these drugs. Moreover, the genetic basis of resistance has been exploited to develop rapid tests for drug susceptibility. Molecular assays that have been used to monitor the *rpoB* gene for RIF resistance mutations consist of: DNA sequencing \[^{9}\], heteroduplex analysis \[^{10}\], polymerase chain reaction-single strand conformation polymorphism \[^{11, 124}\], dideoxy
fingerprinting \cite{125}, line probe assay \cite{11, 126, 127, 128}, molecular beacon \cite{13, 129}, mismatch analysis \cite{12}, real-time PCR \cite{130, 131}, high density probes assays \cite{132, 133, 134}, and micro arrays \cite{135}.

2.7.2.1 DNA SEQUENCING

DNA sequencing is the most accurate and reliable method for detection of mutations. It is used as the gold standard for detection of mutations which are believed to be predictive of resistance \cite{9}. The added advantage of this method is that it has been automated, which has simplified the process. Accurate sequence data can be obtained within 48 hours from automated analyzers that use fluorescent chemistry methods. Except for RIF, DNA sequencing is unlikely to be used in the routine detection of drug resistance mutations because it requires several sequencing reactions per isolate which makes it labour-intensive and expensive. However, this method is impractical for use in routine detection of drug resistant mutations because it requires specialised equipment and is labour-intensive and expensive.

2.7.2.2 HETERODUPEX ANALYSIS

Heteroduplex analysis is a method of detecting gene mutations by mixing PCR- amplified mutants followed by denaturation and reannealing \cite{10, 136}. The resultant products are resolved by gel electrophoresis. When two single strand DNA molecules differing in their base matching form a heteroduplex, the resultant conformation is altered, accompanied by a reduced electrophoretic mobility compared to its corresponding homoduplex with no mismatch.

2.7.2.3 DIDEOXYFINGERPRINTING
Dideoxyfingerprinting (ddF) involves dideoxy sequencing followed by non-denaturing electrophoresis and was first described by Sarkar et al. in 1992 [137]. “Fingerprint” or bandshifts where changes in sequences are present is observed in mutated DNA sequences. The theoretical basis for identification of DNA sequence changes is twofold: (i) secondary structural differences of single-strand DNA (full length and strand termination) and (ii) alterations of the termination site of the primer extension product. The time required for detection of RIF-susceptibility using this method was reduced to 2 days [125]. They compared this method to SSCP and found that the increased molecular complexity inherent in ddF increases the efficiency of detecting and discriminating base changes. This same increased complexity contributed to the observation that ddF analysis was less influenced by the location of the mutation, size of the DNA fragment being investigated, temperature and other experimental conditions. These attributes of ddF allowed for greater accuracy in the identification and characterisation of mutations in a reference laboratory setting. This method is not favoured since the use of radioactivity is involved and thus is costly in terms of waste disposal.

### 2.7.2.4 Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is a molecular technique that distinguishes mutant amplicons from their wildtype equivalents. This method is more sensitive when compared to DNA sequencing as it can detect point mutations and small insertions and deletions. The principle of this technique is that the melting temperature of DNA changes as its fragments migrates through a gradient of denaturants [138]. A molecular fingerprint is generated based on the characteristic denaturation pattern of different mutational variants within a DNA
fragment. Scarpellini et al, in 1999 successfully applied this technique in the detection of mutations associated with RIF resistance within the *rpoB* gene \[^{[139]}\].

### 2.7.2.5 LINE PROBE ASSAY

By examining the sequence data in sensitive and resistant strains, probes can be designed and immobilised onto a membrane. Detection of susceptibility or resistance can be done by a reverse hybridisation principle \[^{[140]}\]. PCR is performed on the region of DNA in which mutations are associated with drug resistance. The PCR product is then allowed to hybridize with the probes on a membrane. Failure to bind the wild type probe is due to the presence of a mutation and thus is predictive of drug resistance. The commercially available line probe assay for detection of mutations in the *rpoB* core region is based on this principle \[^{[141, 11, 142]}\]. The region is amplified and biotin labelled by PCR. The DNA is detected after hybridisation with a strip in which 5 probes for wild type *rpoB* sequences, 4 for specific *rpoB* mutations, a conjugate control and *M. tuberculosis* control probes are immobilised. The bound DNA is detected with a colour reaction. The advantage of this method is that it can detect mutations directly from clinical specimens. However, its limitations are that it is costly and also detects only four mutations. Oliveira *et al* in 2003 reported an overall accuracy of 97.6% for detection of susceptibility by this assay when compared to conventional resistance testing \[^{[143]}\]. These results were concordant with those of Rossau *et al* (1997) \[^{[144]}\]. The price of this test limits its use in developing countries where the largest pool of resistant isolates exist \[^{[145]}\].

### 2.7.2.6 RNA/RNA MISMATCH ANALYSIS
RNA/RNA mismatch analysis is based on the ability of double stranded RNA to withstand digestion with RNase \[12\]. Target DNA is amplified by using primers which incorporate T7 RNA polymerase and SP6 RNA polymerase promoters in opposite directions, allowing RNA to be transcribed by using the PCR product as a template. A RIF sensitive strain is also amplified by using the same primers but with the SP 6 and T7 promoters incorporated in the strands complimentary to the test strain. The test PCR product and the reference PCR product are combined in a transcription reaction using either T7 or SP6 RNA polymerase. The complimentary transcripts from the test and PCR products are allowed to hybridise and the resulting hybrids are treated with RNase. Any mutations in the test transcript will not pair with the reference transcript and so the hybrid will be cleaved at that point. Undigested transcripts and cleavage products can be detected using agarose gel electrophoresis.

2.7.2.7 REAL TIME PCR

Real time PCR combines both rapid-cycle PCR and real time monitoring of the processing and generation of mutation profiles. Mutations can be monitored using fluorescent probe melting profiles. This method is very expensive and also requires sophisticated equipment. However, real-time based PCR has been shown to be highly specific and sensitive in detection of drug resistant *M. tuberculosis*. This has been applied directly on sputum samples from TB patients, giving results within 3 hours from DNA preparation. In 2000, Torres *et al* described real-time PCR for the detection of resistance associated mutations in *M. tuberculosis* using fluorescence. Methods based on real-time PCR have utilised fluorescence resonance energy (FRET) probes \[14,130\], molecular beacons \[129,13,146\], and Taqman Minor Groove Binding (MGB)
probes. Wada et al in 2004 investigated a real time PCR based system with Taqman MGB probes to detect mutations associated with resistance of *M. tuberculosis* to INH, RIF and EMB. Taqman MGB probes can distinguish single base mismatches. The specificity of MGB probes was shown to be quite high. Drug resistant *M. tuberculosis* can be detected by changes in the cycle threshold values (ΔCt) with Taqman MGB probes in real-time PCR. The shortcoming of this method is its inability to define the exact nucleotide substitution involved in the mutation. However, results can be obtained in 30 min.

### 2.7.2.8 SINGLE-STRAND CONFORMATION POLYMORPHISM

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

### 2.7.2.9 MOLECULAR BEACONS

Molecular beacons are hairpin-shaped probes able to detect the presence of specific nucleic acids. In this assay, the loop portion of the molecule is designed to complement the
target nucleic acid molecule. Complementary arm sequences at the ends of probe sequence can anneal to form a stem. The end of one arm of the molecule has a quenching moiety attached to it whilst the other end has a fluorescent moiety attached to it. Fluorescence of the fluorophore is reduced by transfer of energy which results from close proximity of the two moieties to each other. A longer and more stable hybrid than the stem hybrid is formed when the probe encounters a target molecule. Fluorescence can be detected when the molecular beacon undergoes a spontaneous conformational reorganisation that forces the stem apart. This causes the fluorophore and the quencher to move away from each other restoring fluorescence. This is monitored in real-time, where the fluorescence increases with every cycle in proportion to the amplification of the hybridising target, which is not detected in cases when the target is not complementary to the beacon \[^{151}\]. Beacon assays are performed in sealed wells thus preventing contamination. They are easily implemented, automated and can be used in high throughput analyses. In the case of RIF, a set of 5 beacons has been designed to cover the \textit{rpoB} core region in a single reaction with excellent results \[^{129}\]. The results for RIF resistance were obtained directly from sputum specimens in less than 3 hrs. The assay is sensitive enough to detect 2 bacilli, with results available within 3 hours from sputum collection. Piatek \textit{et al} in 2000 designed a set of beacons to screen for mutations in the regions associated with drug resistance for INH \([\text{katG} \text{ gene (position 315), the promoter region of inhA, the oxyR-ahpC intergenic region and position 66, 269, 312, and 413 of kasA}]. The beacons are highly sensitive and specific: a single mismatch in the target sequence diminishes the beacon-target hybrid stability, allowing the detection of point mutations \[^{146}\].

\textbf{2.7.2.10 DNA MICROARRAYS}
DNA microarrays are based on the principle of hybridisation \[^{152}\]. They allow analysis of large numbers of DNA sequences in a single hybridisation step. PCR amplicons labelled with fluorophore moieties are generated from the sample to be hybridised to a large collection of probes bound to a solid surface. The bound amplicons emit fluorescent signals that are scanned with an epifluorescent microscope. Probes are designed to hybridise to fully complementary amplicons. Wildtype and mutant probes are included in the array to determine the presence of specific mutations. Microarrays have been used mainly for species identification and for detection of mutations associated with RIF-resistance, with excellent concordance with sequencing results \[^{118, 132}\]. In 2004, Sougakoff and others detected $rpoB$ mutations associated with RIF-resistance using a high density probe array \[^{133}\]. DNA microarray systems are composed of oligonucleotides synthesized onto a silica slide. Thousands of specific DNA or RNA sequences can be detected simultaneously using this system. Yue $et$ $al$ in 2004 developed an in-house oligonucleotide based microarray for detection of $M.\,$$tuberculosis$ resistant to RIF in clinical isolates \[^{135}\]. They reported this assay to be inexpensive, flexible and easy to perform when compared to high density DNA probe. The widespread application of microarrays is limited to the research setting because they require expertise, sophisticated equipment and are costly.

1.7.2.11 AMPLIFICATION REFRACTORY MUTATION SYSTEM
The amplification refractory mutation system (ARMS) was first introduced for the detection of any point mutations by Newton et al, in 1989 \cite{153}. Fan et al, in 2004 modified this method for the detection of the \textit{rpoB} gene mutations in \textit{M. tuberculosis} \cite{154}. It is simple, rapid and inexpensive. The principle of ARMS is that oligonucleotides which are complimentary to a given DNA sequence except for a mismatch at their 3’-OH residue will not function as primers in PCR under appropriate conditions. This is due to the absence of the 3’- exonuclease proofreading activity associated with Taq DNA polymerase. In this method, the 3’-end of a wild type PCR primer is located at the mutation site. In the case of the wild type template, the DNA polymerase will amplify the DNA efficiently, yielding a clear band during gel electrophoresis. In the case of mutant template, the 3’- end of the primer will not hybridise and the DNA polymerase will not amplify resulting in the absence of the band during gel electrophoresis. The major advantages of this method are that it is convenient, relatively inexpensive and easy to perform since it utilises commonly available reagents and equipment. It can be performed in one day, as it requires only PCR and electrophoresis of PCR products in an agarose gel. Its sensitivity is comparable to the above mentioned molecular assays.

1.7.2.12 GeneXpert MTB/RIF assay (Cepheid)

The GeneXpert system is fully automated and integrates all the steps required for PCR based DNA testing. It is designed to purify, concentrate, detect and identify targeted nucleic acid sequences directly from unprocessed samples. The system consists of an instrument, personal computer, barcode scanner and preloaded software for running tests on collected samples and viewing the results. It requires the use of single disposable GeneXpert cartridges that hold the PCR reagents and host the PCR process. The Xpert MTB/RIF includes reagents for the detection
of TB and RIF resistance as well. A sample processing control ensures adequate processing of the target bacteria and to monitors the presence of inhibitor’s in the PCR reaction. The primers in the Xpert MTB/RIF assay amplify a portion of the \textit{rpoB} gene containing the 81 base pair “core” region. The probes are able to differentiate between the conserved wild-type sequence and mutations in the core region that are associated with RIF resistance. Helb \textit{et al,} in 2010 developed and performed the first analysis of the GeneXpert MTB/RIF assay demonstrating a high sensitivity and specificity to both MTB and RIF resistance detection \cite{155}. In this study they reported a detection sensitivity of 71.7\% and specificity of 84.6\% of smear negative culture positive clinical samples from Vietnam. In a study of retreatment cases in Uganda, the Xpert MTB/RIF detected 98.4\% culture positives and 100\% RIF resistance. Boehme \textit{et al,} in 2010 reported a sensitivity of 98.2\% among smearpositive tuberculosis, 72.5\% of smear negative and a specificity of 99.2\% \cite{156}. For RIF resistance detection, a sensitivity of 97.6\% was reported and a specificity of 98.1\%. In South Africa, the assay was evaluated in a high HIV prevalence region. A sensitivity and specificity of 86\% and 95\% in HIV negative patients while 85\% and 93\% for HIV positive patients. Also 100\% detection in smear positive culture positive and 65\% detection in smear negative culture positive specimens \cite{157}. Although this technique provides quite a number of advantages such as less hand on time on specimen preparation, results are obtained in less than 2 hours and concurrent detection of \textit{M. tuberculosis} and RIF resistance, however it requires sophisticated equipment which is expensive for developing countries. Butkus in his review estimated the cost of this system to be $30 000 and the cost per test to be about $64 \cite{158}.

\textbf{2.7.2.13 REVERSE LINE BLOT HYBRIDISATION}
The reverse line blot hybridisation method is another molecular method that rapidly detects mutations. Morcillo et al, in 2002 applied this method for detection of rpoB mutations hence it was called rifampicin oligonucleotide typing (rifoligotyping in short) \[^{145}\]. A combination of DNA amplification by PCR and reverse line blot hybridisation is involved in this assay. Specific primers are used to amplify the rpoB gene of *M. tuberculosis* by PCR. PCR products are then hybridised to oligonucleotide probes \[^{140}\]. The membrane has oligonucleotide probes encoding consecutive parts of the rpoB gene sequence with the most frequently occurring mutations in RIF resistant strains \[^{145}\]. The rpoB PCR products of the RIF resistant strains will fail to hybridise to one or more of the wild type oligonucleotides and will in most cases show affinity to mutant oligonucleotides \[^{159,145}\]. Resistance is detected within a few hours and 43 samples can be tested at once. Application of these methods directly to clinical specimens will greatly decrease the detection time for rifampicin resistance and will help in predicting multi-drug resistance in *M. tuberculosis*. 