The prevalence of isoniazid and rifampicin resistance of *Mycobacterium tuberculosis*

by

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of the requirements for the degree

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I, the undersigned, declare that the dissertation hereby submitted to the University of Pretoria for the degree MSc (Medical Microbiology) and the work contained therein is my own original work and has not previously, in its entirety or in part, been submitted to any university for a degree.

Signed: ________________________this______________day of___________________2009

The most beautiful thing we can experience is the mystical. It is the source of all true art and science – Albert Einstein

Man cannot discover new oceans unless he has the courage to lose sight of the shore – Andrè Gide

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List of Abbreviations

A	Adenine
AFB	Acid-fast bacilli
AIDS	Acquired immunodeficiency syndrome
AMI	Antibody mediated immunity
ARV	Anti-retroviral
BCG	Bacille Calmette-Guérin
bp	Base pairs
C	Cytosine
CD4	Cluster of differentiation 4+
CFA	Component of Freund's
CFP-10	Culture filtrate protein
CMI	Cell mediated immunity
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
DOTS	Directly Observed Treatment Short-Course
DST	Drug Susceptibility Testing
ESAT-6	Early secreted antigenic target 6 kDa protein
ETH	Ethambutol
FDA	Food and Drug Administration
FIND	Foundation for Innovative New Diagnostics
G	Guanine
h	hour
Hain	Hain Lifescience
HIV	Human immunodeficiency virus
hsp65	Heat shock protein 65
IFN-γ	Interferon gamma
INH	Isoniazid
IRIS	Immune Reconstitution Inflammatory Syndrome
IS	Insertion sequences
IU	International units
LAMP	Light Advanced Microscope Project
LTBI	Latent tuberculosis infection
M	million
M. africanum	*Mycobacterium africanum*
M. avium	*Mycobacterium avium*
M. avium-intracellulare	*Mycobacterium avium-intracellulare*
M. bovis	*Mycobacterium bovis*
M. canettii	*Mycobacterium canettii*
M. caprae	*Mycobacterium caprae*
M. conspicuum	*Mycobacterium conspicuum*
M. gastri	*Mycobacterium gastri*
M. intracellulare	*Mycobacterium intracellulare*
M. kansasii	*Mycobacterium kansasii*
M. lepraemurium	*Mycobacterium lepraemurium*

M. marinum	*Mycobacterium marinum*
M. microti	*Mycobacterium microti*
M. pinnipedii	*Mycobacterium pinnipedii*
M. szulgai	*Mycobacterium szulgai*
M. tuberculosis	*Mycobacterium tuberculosis*
M. ulcerans	*Mycobacterium ulcerans*
MAC	*Mycobacterium avium* complex
MDR-TB	Multi-drug resistant tuberculosis
MGIT	Mycobacteria growth indicator
MHC II	Major histocompatibility complex class II
min	minute
ml	milliliter
MOTT	Mycobacteria other than tuberculosis
MPB64	Anti-*Mycobacterium tuberculosis* protein 64
mRNA	Messenger ribonucleic acid
MTBC	*Mycobacterium tuberculosis* complex
MTBDR	*Mycobacterium tuberculosis* drug resistance
NADH	Nicotinamide adenine dinucleotide
NHLS	National Health Laboratory Services
NTCP	National Tuberculosis Control Programme
NTM	Non-tuberculous mycobacteria
PCR	Polymerase chain reaction
PGL	Phenolic glycolipid
PPD	Purified protein derivatives
PZA	Pyrazinamide
QFT	QuantiFERON-TB
RD1	Region of difference 1
RIF	Rifampicin
RNA	Ribonucleic acid
ROC	Receiver Operating Curves
rRNA	Ribosomal ribonucleic acid
SM	Streptomycin
sod	Superoxide dismutase
spp	species
T	Thiamine
TB	Tuberculosis
T-cells	Thymus cells
TDH	Tshwane District Hospital
Th	Thymus helper
TST	Tuberculin skin test
UP	University of Pretoria
WHO	World Health Organization
XDR-TB	Extensively drug resistant tuberculosis

List of publications and conference contributions

Publications:

1. **Veldsman C, Kock Marleen M, Rossouw Theresa, Nieuwoudt Martin, Maeurer Mark M, Hoosen Anwar A and Ehlers Marthie M.** QuantiFERON-TB GOLD ELISA assay for the detection of *Mycobacterium tuberculosis* specific antigens in blood specimens of HIV positive patients in a high burden country (FEMS Immunology and Medical Microbiology Journal (2009) **57**: 269-273)
2. **Veldsman C, Kock MM, Rossouw TM and Ehlers MM.** The use of real-time PCR assays for the detection, identification and drug susceptibility patterns of *Mycobacterium* in sputum and blood specimens of HIV positive patients (the editorial style of FEMS Immunology and Medical Journal was followed in this chapter)

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1. **Veldsman C, Kock Marleen M, Rossouw Theresa, Nieuwoudt Martin, Maeurer Mark M, Hoosen Anwar A and Ehlers Marthie M.** QuantiFERON-TB GOLD ELISA assay for the detection of *Mycobacterium tuberculosis* specific antigens in blood specimens of HIV positive patients in a high burden country. Poster presentation for Faculty day (15-08-2009), University of Pretoria
2. **Veldsman C, Kock Marleen M, Rossouw Theresa, Nieuwoudt Martin, Maeurer Mark M, Hoosen Anwar A and Ehlers Marthie M.** QuantiFERON-TB GOLD ELISA assay for the detection of *Mycobacterium tuberculosis* specific antigens in blood specimens of HIV positive patients in a high burden country. Oral presentation for Faculty day (15-08-2009), University of Pretoria
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4. **Veldsman C, Kock Marleen M, Rossouw Theresa, Nieuwoudt Martin, Maeurer Mark M, Hoosen Anwar A and Ehlers Marthie M**. QuantiFERON-TB GOLD ELISA

assay for the detection of *Mycobacterium tuberculosis* specific antigens in blood specimens of HIV positive patients in a high burden country. Poster presentation FIDSSA at Sun City (20-22 Aug 2009), University of Pretoria

The prevalence of isoniazid and rifampicin resistance of *Mycobacterium tuberculosis*

by

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DEPARTMENT: Medical Microbiology

DEGREE: MSc Medical Microbiology

SUMMARY

The World Health Organization (WHO) estimated that eight million new cases of tuberculosis (TB) occur every year and that one-third of the world's population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*). With the increase in HIV/AIDS in the 1980's, an increase in transmission of TB led to an increase in TB incidence. A study showed that South African adults (ages 15 to 49) will suffer 278 154 deaths between 2008 and 2017 if current control measures are continued.

A *M. tuberculosis* strain that is resistant to isoniazid (INH) and rifampicin (RIF) used in the treatment of TB is known as a multi-drug resistant (MDR-TB) strain. In extensively drug-resistant tuberculosis (XDR-TB) the *M. tuberculosis* strains are not only resistant to INH, RIF and any one of the fluoroquinolones but to at least one of the three injectable second-line drugs such as amikacin or kanamycin. Unfortunately, many people with XDR-TB will die because it is virtually impossible to formulate an effective treatment before the resistance pattern of the *M. tuberculosis* strain has been identified.

Bacteriological culture is considered the diagnostic gold standard and can identify mycobacteria in over 80% of TB cases, with a specificity of over 98%. However, culturing the mycobacteria takes 4 to 6 weeks and makes diagnosis and treatment a prolonged process.

In this study 60 patients suspected of TB disease, from the Anti-retroviral (ARV) clinic at the Tshwane District Hospital (TDH) were collected from October 2008 to April 2009. This study evaluated the use of the QuantiFERON-TB GOLD ELISA assay in a high burden setting. Tshwane District Hospital, South Africa. The sensitivity and specificity of the QFT assay in the clinic were 30% (9/30) and 63% (19/30) respectively when compared to the gold standard culture results. Analysis suggested that the sensitivity of the QuantiFERON assay is determined by a limiting patient CD4 value of between 150 and 200.

Real-time PCR assays were used for rapid identification of *Mycobacterium* spp and to determine the presence of isoniazid and rifampicin resistant genes of *M. tuberculosis* strains. The real-time PCR assay identified 28% (17/60) *M. tuberculosis*, 2% (1/60) *M. kansasii* and 70% (42/60) of the isolates *Mycobacterium* spp negative. No *M. avium* were detected. The 17 *M. tuberculosis* positive specimens were further analysed for the presence of INH and RIF resistance genes. All 17 specimens had either no mutation or one or more mutations at the specific gene targets (*rpo*1, *rpo*2, *kat*G and *inh*A).

This study showed several possibilities for the use of both an immunological assay as well as molecular methods for the diagnosis of TB. This study suggested that in terms of routine diagnosis of TB in high HIV prevalence settings the QFT test should be used with caution. Real-time PCR for both detection and identification showed useful results and can be used together with culture results to improve turnaround times for TB diagnosis.

CHAPTER 1

1.1 Introduction

Tuberculosis (TB) is one of the oldest diseases known to mankind and spread from person to person via aerosols (Valadas & Antunes, 2005). In the 1940's, the discovery of antibiotics against TB led to the belief that the occurrence of TB would rapidly decrease (Herzog, 1998). Studies done in the 1960's on drug-resistance showed an increase in resistance against antibiotics such as isoniazid (INH) and by the end of the 1950's rifampicin (RIF) was introduced with the use of combination therapy (Johnson *et al*., 2006). The decline in the number of TB cases led to a loss of interest in TB control programmes (Johnson *et al*., 2006). During the next 20 years no real monitoring of drug-resistance was carried out (Johnson *et al*., 2006). With the increase in human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) in the 1980's, an increase in transmission of TB led to an increase in TB incidence (Johnson *et al.,* 2006). South Africa has the world's largest TB-HIV co-infected population and a recent experience with XDR-TB outbreaks (Dye *et al*., 1999; Valadas & Antunes, 2005; Dowdy *et al*., 2008). Current control measures are still not enough to prevent the high mortality and will continue to increase if no changes follow.

Multi-drug-resistant TB (MDR-TB) is caused by a *M. tuberculosis* strain that is resistant to isoniazid (INH) and rifampicin (RIF) used in the treatment of TB (Girard *et al*., 2005). The lack of monitoring of resistance development has contributed to the increasing incidence of drug-resistant *M. tuberculosis* (Dowdy *et al*., 2008). Irrational antibiotic use occurs in cases where TB patients are non-compliant and do not complete their treatment regimens leading to the development of drug-resistant mutations in *M. tuberculosis* (Espinal, 2001; Gupta *et al*., 2001). These resistant *M. tuberculosis* bacteria are not killed during drug treatment and are allowed to replicate in the infected individual (Espinal, 2001). Individuals who develop the active disease either fail to contain the primary infection or develop reactivation as a result of relative or absolute immune-suppression at a point from primary infection (Espinal, 2001). A person with MDR-TB will need to change to a regimen containing newer and often less widely available second-line drugs, such as ethanionamide (Girard *et al*., 2005). Treatment with these drugs are

for approximately 24 months and these drugs are more costly, toxic and less effective than first-line drugs used for routine treatment of TB (Shah *et al*., 2007).

In extensively drug-resistant tuberculosis (XDR-TB), the *M. tuberculosis* strains are not only resistant to INH, RIF and any one of the fluoroquinolones but to at least one of the three injectable second-line drugs, such as amikacin, kanamycin or capreomycin (Jassal & Bishai, 2009). Many people with XDR-TB will die because of the difficulty to formulate an effective treatment before a resistant *M. tuberculosis* strain has been identified (Evans *et al*., 2009). A HIV positive status does not in itself increase the chance of drug-resistance, but both MDR-TB and XDR-TB infection are a more severe problem for HIV positive people (Dowdy *et al*., 2008). A weakened immune system means that patients are unlikely to fight off TB naturally and this is often the only hope for those infected with a resistant *M. tuberculosis* strain (Noble, 2006).

The World Health Organization ranked South Africa fourth, based on the estimated number of incident cases in 2006; however, information regarding the number of patients tested positive for MDR-TB is still not available (WHO report, 2008). The emergence of MDR-TB and XDR-TB *M. tuberculosis* bacteria specifically in patients, co-infected with HIV, were observed (Dye *et al*., 2005; Guillerm *et al*., 2006). In 2006, a cluster of XDR-TB cases was identified in KwaZulu-Natal (Casenghi, 2006). Fifty-three patients out of 221 were infected with XDR-TB strains and 52 of those patients died before TB was properly diagnosed (Casenghi, 2006). All of these patients were HIV positive and those who died included patients on antiretroviral treatment (Guillerm *et al*., 2006). Studies have indicated that the fatality rate in people with TB and HIV is nearly 80% (Salyers & Whitt, 2002). However, according to the outbreak in KwaZulu-Natal the fatality rate was closer to 98% (Guillerm *et al*., 2006).

Control of TB depends mainly on the rapid and accurate diagnosis and management of TB (Aragón *et al*., 2006). Over the years, nucleic acid amplification (NAA) technologies have gradually improved and real-time-based platforms currently seem to offer numerous advantages based on specificity and sensitivity when compared to culture which still remains the gold standard (Drouillon *et al*., 2007). Real-time PCR assays can be applied for the rapid detection and identification of mutations confined in specific DNA regions of the bacterial genome

(Aragón *et al*., 2006). The main advantages with the use of real-time PCR are the ability not only to identify the specific *Mycobacterium* spp but to detect INH and RIF resistance of *M. tuberculosis* strains within 48 to 72 h after the specimens have been collected (Espasa *et al*., 2005; Parashar *et al*., 2006; Lim *et al*., 2008). Bacterial culture however, can take up to 4 weeks for identification with an additional three weeks for susceptibility testing (Farnia *et al*., 2002).

It is clear that urgent research concerning the rapid and accurate identification of *M. tuberculosis* strains and the determination of multi-drug resistant species are crucial. The purpose of this study was to evaluate real-time PCR assays for the rapid identification of *Mycobacterium* spp and to determine the presence of INH and RIF resistant genes of *M. tuberculosis* strains directly from clinical specimens. These specimens were obtained from the Anti-retroviral (ARV) clinic at the Tshwane District Hospital (TDH). Valuable new information was obtained with regards to the *Mycobacterium* spp present in HIV positive patients and the antibiotic resistance patterns of *M. tuberculosis* strains identified in these patients.

The aims of this study were:

1. To collect sputum and blood specimens (until 120 *M. tuberculosis* positive specimens have been obtained) from HIV positive patients presenting with the clinical signs and symptoms of tuberculosis obtained from the ARV clinic at TDH
2. To manually extract DNA from sputum specimens (using FDA approved protocol: Protocol 1: Specimen preparation; Prototype LightCycler® TB Kit)
3. To extract DNA from blood specimens using an automated system (MagNaPure LC Compact: MagNA Pure Compact Nucleic Acid Isolation Kit I – Large Volume)
4. To use a real-time PCR assay for the identification of *Mycobacterium* spp from the DNA obtained from both sputum and blood specimens
5. To determine the presence of mutations in the *inh*A and *kat*G genes for INH resistance of *M. tuberculosis* positive specimens
6. To determine the presence of the mutation in the *rpo*B gene for RIF resistance of *M. tuberculosis* positive specimens

7. To analyse blood specimens using QuantiFeron® TB Gold for the identification of *M. tuberculosis* positive specimens
8. Data analysis

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Tuberculosis (TB) is one of the oldest diseases known to mankind and globally accounts for 2 million deaths annually (Seagar *et al*., 2008). In the 1940's, the discovery of antibiotics against TB led to the belief that the occurrence of TB would rapidly decrease (Herzog, 1998). However, TB remains a major cause of morbidity and mortality and it is increasing (Hermann *et al*., 2009). The World Health Organization (WHO) reported 9.2 million new cases of tuberculosis in 2006 (Hermann *et al*., 2009) and that one-third of the world's population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*) (Lawn *et al*., 2006). Control of TB depends mainly on rapid and accurate diagnosis and management of the disease (Aragón *et al*., 2006).

Multi-drug resistant TB (MDR-TB) is caused by a *M. tuberculosis* strain that is resistant to two or more first-line antibiotics, such as isoniazid (INH) and rifampicin (RIF) drugs used in the treatment of TB (Girard *et al.*, 2005). Irrational antibiotic use, poor-quality anti-TB drugs and the human immunodeficiency virus (HIV) pandemic have contributed to the increasing incidence of drug resistant TB (Gupta *et al*., 2001). Irrational antibiotic use occur in cases where TB patients are non-compliant and do not complete their treatment regimens leading to the development of drug-resistant mutations in the TB bacteria (Espinal, 2001). A person with MDR-TB will need to change to a regimen containing newer and often less widely-available second-line drugs (Girard *et al*., 2005). Treatment with these drugs requires use of drugs for up to 24 months and these drugs are more costly, toxic and less effective than first-line drugs used for routine treatment of TB (Shah *et al*., 2007).

A person infected with an extensively drug resistant TB (XDR-TB) strain is not only resistant to INH and RIF and any fluoroquinolone but also to at least one of the three

injectable second-line drugs capreomycin, kanamycin and amikacin (Barnard *et al*., 2008; Jassal & Bishai, 2009). Extensively resistant TB has been detected in all regions of the world (Shah *et al*., 2007). A HIV positive status does not in itself increase the chance of drug resistance however; these patients more frequently have resistant mycobacterial strains (Valadas & Antunes, 2005).

Microscopy is a cheap and rapid method for identification but culturing the mycobacteria is necessary, which takes 4 to 6 weeks and make diagnosis and treatment a prolonged process (Farnia *et al*., 2002). Microscopy of *M. tuberculosis* only detects less than half of all patients with active TB in Africa (Farnia *et al*., 2002; Kaufmann & Parida, 2008). Bacteriological culture is still considered the diagnostic gold standard (Jafari & Lange, 2008). Molecular technologies have improved and seem to offer numerous advantages, such as specificity and sensitivity comparable to the gold standard (Kim, 2005).

Molecular assays can be applied for the detection of mutations confined in short base pair (bp) deoxyribonucleic acid (DNA) regions (Aragón *et al*., 2006). The main advantage with the use of real-time PCR is the additional application for the detection of INH and RIF resistance within 48 to 72 h after sample collection (Cho, 2007). Despite the availability of the effective short-course chemotherapy, a vaccine and knowledge about the resistance mechanisms, the tubercle bacillus continues to claim more lives than any other single infectious agent (Johnson *et al*., 2006).

It is clear that urgent research concerning the identification of the different *M. tuberculosis* strains and the characteristics of multi-drug resistant species in HIV positive patients are crucial. The purpose of this study was to rapidly determine the prevalence of *M. tuberculosis* strains showing INH and RIF resistance genes obtained from specimens from HIV positive patients in the Pretoria region.

2.2 History of tuberculosis

In the 19th century a new method of treatment was introduced namely the sanatoria (an immune place) (Herzog, 1998). Tuberculosis patients would spend many months to years in the sanatoria (Figure 2.1) which was a cross between a hotel and a hospital (Davies, 1999). Sunlight, diet and gentle exercise was prescribed to insure survival of TB patients (Herzog, 1998). However, with the removal of infectious patients and thus reducing transmission, an improved survival rate was reached (Herzog, 1998). Surgery became the most important method of treatment in the 1930s by attempting to obliterate the cavities which formed in the lung (Davies, 1999). Drug therapy was introduced in the 1950s and reduced the need for sanatoria beds (Herzog, 1998). Hospitalisation for all but the most ill tuberculosis patients were eliminated when it was realised that treatment could be given at home (Davies, 1999). As a result, great expenses of hospitalisation, in resource poor countries, were avoided (Herzog, 1998).

Figure 2.1 The sanatoria where patients would spend months to years for the treatment of tuberculosis during the 19th century (Herzog, 1998)

Anti-tuberculosis drugs, such as para-aminosalicylic acid, streptomycin (SM), INH, pyrazinamide (PZA), cycloserine and RIF were introduced as combination therapy in the

1950s and by the 1980s, there was a 98% chance of cure (Herzog, 1998). By the late 1980's, TB reappeared as a serious threat to public health because of factors that are related to a high TB burden, such as the HIV pandemic, homelessness and poverty (Valadas *et al*., 2005). Unfortunately, the very success of the drug treatment of TB has been the medium for the emergence of a new wave of drug resistance (Davies, 1999). Patients were allowed to take their medication home completely unsupervised, drugs were singularly administered and patients didn't use all the prescribed drugs (Davies, 1999). Thus, a combination of poor compliance and poor medical supervision resulted in multi-drug resistance (Davies, 1999). Treatment had to be continued with good quality drugs (INH and RIF) for long periods of time to guarantee cure, but with the difficulties of ensuring that this was followed through, an increased incidence of TB resistance to the most effective drugs (INH and RIF) occurred during the 1990s because of non-compliance (Valadas *et al*., 2005). This resulted in the emergence of the multi-drug resistant TB strains (Girard *et al.*, 2005). The WHO declared TB a global emergency in 1994 and therefore, further research and new developments into rapid diagnostic methods were encouraged (Valadas *et al*, 2005).

2.3 Classification of *Mycobacterium* species

The genus *Mycobacterium* contains species that can cause serious diseases in humans and animals (Devulder *et al*., 2005). Human infections are caused mainly by slow-growing strains and there have been an increase in infections caused by mycobacteria other than *M. tuberculosis* (MOTT) (Devulder *et al*., 2005). The *M. tuberculosis* complex (MTBC) comprises of *M. tuberculosis*: the causative agent in the vast majority of human tuberculosis cases, *M. bovis*: which infects a wide variety of mammalian species such as badgers, cattle, deer, elephants, lions and humans, *M. bovis* Bacille Calmette-Guérin (BCG): an attenuated variant of *M. bovis*, which is still used today as a vaccine against tuberculosis, *M. africanum* an agent of human TB in sub-Saharan Africa and *M. microti* the agent of TB in voles (Cole, 2002).

Mycobacteria are abundant in soil and water and it was generally believed that TB was acquired from cattle (Cole, 2002). Colonies of *M. bovis* and *M. tuberculosis* (Figure 2.2) grow as rough, raised, thick off-white to yellow with wrinkled surfaces on solid media, such as Löwenstein-Jensen medium (Agarwal *et al*., 2005). *Mycobacterium bovis* BCG tends to be raised, more compact white colonies with irregular edges and a granular surface when cultured on solid media (Cole, 2002). *Mycobacterium microti* forms tiny yellow colonies, whereas *M. canettii* produce smooth yellow colonies due to the overproduction of phenolic glycolipid (PGL) (Cole, 2002).

Figure 2.2 Typical morphology of *M. tuberculosis* cultured on Löwenstein-Jensen medium (Agarwal *et al*., 2005)

There are four species of the *Mycobacterium* genus, belonging to the *Mycobacteriaceae* family that cause atypical mycobacterial infections: *M. avium-intracellulare*; *M. kansasii*; *M. marinum* and *M. ulcerans* (Ngan, 2006). There are currently, 143 valid species of mycobacteria that have been described using nucleic acid sequencing, excluding the six members of the MTBC (Euzéby, 2009).

The MTBCs are obligate pathogens growing most successfully, at a temperature of 37ºC, in tissues having the highest partial pressure of oxygen, such as the lung apices (Clark,

2005). *Mycobacterium avium* complex (MAC) is slow-growing and is referred to as non-photochromogenic as these bacteria do not produce pigmented colonies either during light or dark incubation (Acidfast/Branched_Rods, 06/03/2007). *Mycobacterium marinum* are non-pigmented and if exposed to light when the colonies are young, the bacterium becomes yellow (photochromogenic) (Ngan, 2006). *Mycobacterium marinum* is known as the cause of fishtank granulomas when abrasions occur in people exposed to contaminated fresh or saltwater (Ngan, 2006). Fishtank granulomas (Figure 2.3) affect the elbows, knees, feet, knuckles or fingers of immunocompromised patients (Ngan, 2006).

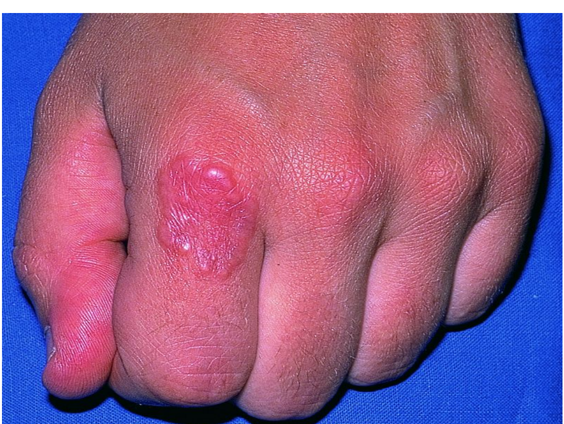

Figure 2.3 *Mycobacterium marinum* is seen frequently in epidemic form as skin lesions resulting from abrasions incurred in swimming pools or fish tanks (Wayne & Kubica, 1986)

Mycobacterium kansasii is non-pigmented and photochromogenic and produces a disease resembling pulmonary tuberculosis (Acidfast/Branched_Rods, 06/03/2007) but it is not considered contagious (Wayne & Kubica, 1986). *Mycobacterium ulcerans* is known as the cause of Buruli ulcers and is common in Central and West Africa, around areas of lush vegetation and swamps (Ngan, 2006). An itchy nodule of 1 to 2 cm develops about 7 to 14 days after infection through broken skin and severe infection may destroy blood vessels, nerves and invade bone (Ngan, 2006). The *M. avium* complex (MAC) group *M. avium*, *M. intracellulare*, *M. avium* subspecies *paratuberculosis, M. avium* subspecies

silvaticum and *Mycobacterium lepraemurium* (*M. lepraemurium*) are specifically important in patients with disseminated diseases, such as AIDS (Turenne *et al*., 2007). Molecular identification and classification methods have led to insightful modifications in the classification of the *Mycobacterium* species (Devulder *et al*., 2005). With the use of sequencing analysis of the 16S ribosomal ribonucleic acid (rRNA) gene, different phylogenies for the *Mycobacterium* genus have been developed (Devulder *et al*., 2005).

2.4 General characteristics of mycobacteria

Characteristic features of the tubercle bacillus include its slow growth, dormancy in the lungs of healthy individuals, complex cell envelope, intracellular pathogenesis and genetic homogeneity (Hett & Rubin, 2008). The generation time of *M. tuberculosis* is typically more or less 24 h when grown in a synthetic solid medium, such as Middle-brook agar (Cole *et al*., 1998). The long generation time of the bacillus contributes to the chronic nature of the disease, which imposes lengthy treatment regimens and represents an obstacle for researchers (Cole *et al*., 1998).

The phenotypic characteristics of *M. tuberculosis* are important for identification in the laboratory because of the differences in colony formation, optimum temperature and optimum pH (Wayne & Kubica, 1986). The optimum temperature of *M. tuberculosis* and *M. kansasii* are 37ºC but some growth does occur between 30 to 34ºC (Wayne & Kubica, 1986). Growth is stimulated by incubation in air with 5 to 10% added carbon dioxide (CO_2) and by inclusion of glycerol to 0.5% in the medium (Wayne & Kubica, 1986). Bacilli grown under highly aerobic conditions die rapidly on abrupt shift to anaerobiosis but when allowed to grow and settle slowly through a self-generated oxygen gradient, they adapt a tolerance to oxygen deprivation (Wayne & Kubica, 1986). *Mycobacterium avium* can also be grown under the same optimum conditions as *M. tuberculosis*, making culturing in the laboratory less labour intense (Wayne & Kubica, 1986).

Mycobacterium species is characterised by slender, straight or slightly curved bacilli (0.2 x 10 µm) that do not form spores and are non-motile (Cole, 2002; Salyers & Whitt, 2002; Hett & Ruben, 2008). The cell-wall structure of *M. tuberculosis* is unique and a

major determinant of virulence for the bacterium (Todar, 2005). More than 60% of the cell-wall contains lipid which consists of three major components: mycolic acids, cord factor and Wax-D (Todar, 2005).

Mycolic acids are a type of lipid found only in the cell-walls of *Mycobacterium* and *Corynebacterium* species (Figure 2.6) (Salyers & Whitt, 2002). These acid fast bacilli contain peptidoglycan (murein) in its cell-wall and do not have the chemical characteristics of either Gram-positive or Gram-negative bacteria (Hett & Ruben, 2008). Mycolic acids are defined as ß-hydroxy fatty acids with a long α-alkyl side chain (Takayama *et al*., 2005) and contribute to 50% of the dry weight of the cell envelope of *Mycobacterium* species (Huang *et al*., 2002). Mycolic acids are strong hydrophobic molecules that form a lipid shell around the bacteria and prevent attack of the mycobacteria by cationic proteins, lysozyme and oxygen radicals in the phagocytic granule (Todar, 2005). The cell envelope of Gram-positive bacteria, such as mycobacteria contains an additional layer beyond the peptidoglycan that is exceptionally rich in unusual lipids, glycolipids and polysaccharides (Hett & Ruben, 2008). Cell-wall components, such as mycolic acids and arabinogalactan can contribute to mycobacterial longevity and trigger inflammatory reactions in hosts (Hett & Ruben, 2008). The virulent strains produce a 'cord factor' causing the bacterium to grow in a rope-like fashion, which is called 'serpentine cords' (Huang *et al*., 2002). The bacterium can linger in the lungs for decades after infection waiting for a decrease in the host's defence mechanism (Noble, 2006). Tuberculosis explodes into full-blown disease once reactivated and causes infection throughout the lungs (Cole, 2002; Timbury *et al*., 2002).

Wax-D in the cell envelope is the major component of Freund's complete adjuvant (CFA) (Todar, 2005). Freund's adjuvant is an antigen solution that is emulsified in mineral oil and is used as a booster of the immune system (Chiu, 2006). The high concentration of lipids in the cell-wall has been associated with resistance to many antibiotics and survival inside the macrophages (Todar, 2005). *Mycobacterium tuberculosis* is naturally resistant to many antibiotics, making treatment difficult (Cole *et al*., 1998; Hett & Rubin, 2008). Many potential resistance determinants are encoded in the genome and these include hydrolytic or drug-modifying enzymes, such as ß-

lactamases and aminoglycoside acetyl transferases (Fleischmann *et al*., 2002). Currently, INH and RIF resistance is the key factor in determining the effectiveness of the currently recommended standard treatment regimen (Abate *et al.,* 2001). Mutations in the *inh*A and *kat*G genes as well as *rpo*B are responsible for the resistance against INH and RIF respectively (Aragón *et al.,* 2006).

2.5 Pathogenesis of mycobacteria

It is important to know the difference between TB infection and TB disease. Tuberculosis infection means that *M. tuberculosis* is in the body but the immune system is keeping the bacteria under control (Todar, 2005). The immune system produces macrophages that form a hard shell around the bacilli keeping the bacilli under control (Todar, 2005). People that are infected with the TB bacilli cannot spread the infection from person to person but people with activated TB are infectious (Todar, 2005). Tuberculosis infected people do not show any symptoms and will have normal chest X-rays (Todar, 2005). Only a small percentage of TB infections will progress to disease and it should be realised that this not only because of a compromised immune system (Todar, 2005).

2.6 Immunological response to tuberculosis infection

Tubercular infection and disease begins in most cases with the inhalation of the tubercle bacilli (Timbury *et al*., 2002; Clark, 2005). The usual inoculum is between 1 to 3 bacterium cells, which are taken up by the alveolar macrophages and are carried to regional lymph nodes (Clark, 2005). Between 7 to 21 days after the initial infection, *M. tuberculosis* preferentially resides in the phagosome of macrophages, where mycobacterial peptides have ready access to the major histocompatibility complex class II (MHC II) molecules that are shuttled to the cell surface (Kaufmann, 2001). The tubercle bacillus binds directly to the receptors on the macrophages or indirectly via complement receptors or Fc receptors (Todar, 2005). The MHC II molecules stimulate the cluster of differentiation 4 (CD4) thymus cells (T-cells) (Kaufmann, 2001). Other macrophages begin to accumulate from peripheral blood but these macrophages are

inactivated and cannot destroy *M. tuberculosis* (Andersen *et al*., 2000; Todar, 2005). Major histocompatibility complex molecules recognise *M. tuberculosis* and infiltrate the T-cells (Todar, 2005). The T-cells and macrophages are activated and can destroy the *M. tuberculosis* as shown in Figure 2.4 (Todar, 2005).

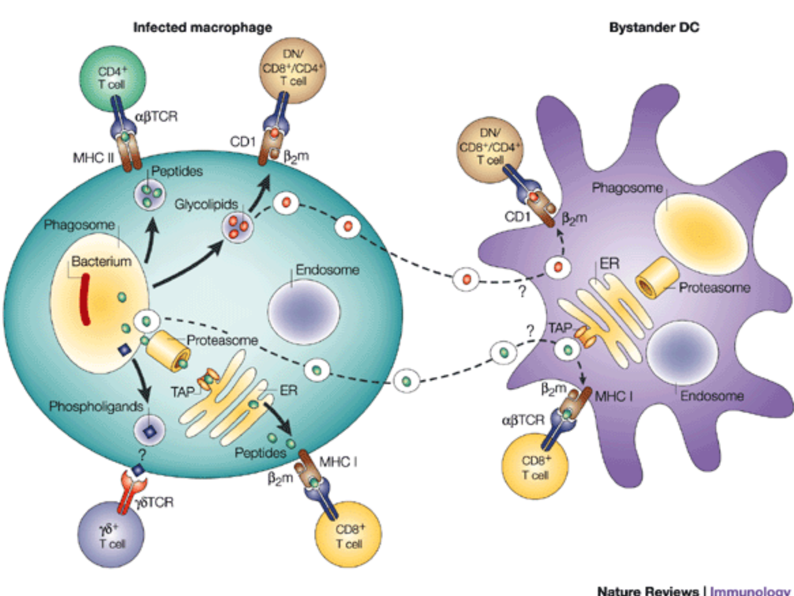

Figure 2.4 The different T-cell-processing pathways that result in activation of distinct T-cell populations in the immune response against *M. tuberculosis* (Kaufmann, 2001)

The cell mediated immune (CMI) response is essential to control the infection but the antibody mediated immunity (AMI) will not aid in the control because *M. tuberculosis* is intracellular (Todar, 2005). The infection occurs extracellular and is resistant to complement killing due to the high lipid concentration in its cell-wall (Todar, 2005). Activated macrophages and T-cells secrete cytokines and it is during this stage that a tubercle forms (Clark, 2005; Todar, 2005).

Most of the damage in the lungs, due to tuberculosis, is associated with the inflammatory effects of the cytokines produced by macrophages that are trying to kill the *M. tuberculosis* infected macrophages (Kaiser, 2006). *Mycobacterium tuberculosis* uses the inactivated macrophages to replicate, resulting in the growth of the tubercle (Todar,

2005). Extrapulmonary tuberculosis or a ‘miliary’ pattern of spread can occur in which there are a myriad of small millet seed (1 to 3 mm) sized granulomas (Todar, 2005). Secondary lesions caused by miliary TB can occur at almost any anatomical location, but usually involve the bones, joints and lymph nodes (Todar, 2005). The individuals who develop the active disease either failed to contain the primary infection or developed reactivation as a result of relative or absolute immune suppression at a point remote from the primary infection (Timbury *et al*., 2002; Clark, 2005). Thus, the host immune system has mechanisms to control the pathogen but fails to accomplish the sterile eradication of *M. tuberculosis* (Kaufmann & Parida, 2008). Infection and reactivation most likely occur in immunocompetent adults or individuals with predisposing factors such as poor nutrition and crowded living conditions (Todar, 2005). Reactivation raises the concerns that drug resistant strains are transmitted and therefore enhanced control strategies are needed and a better understanding of the pathogenesis and virulence of *M. tuberculosis* may aid in the diagnosis and treatment of TB (Kurtz & Braunstein, 2005).

2.7 Genomics of *M. tuberculosis*

The genome of *M. tuberculosis* provides important insight into the biology of the species and the presence of sequence diversity (Fleischmann *et al*., 2002). This knowledge provides a basis for understanding the pathogenesis, immune mechanisms and bacterial evolution of *M. tuberculosis* (Fleischmann *et al*., 2002). The genome of the *M. tuberculosis* laboratory strain H37Rv was completely sequenced and comprises of 4 411 532 bp and has a mean Guanine (G) + Cytosine (C) content of 65.6 mol% (Cole, 2002; Fleischmann *et al*., 2002). The genome contains roughly about 4 000 genes and account for >91% of the potential coding capacity (Cole, 2002). Over 51% of the genes have arisen as a result of gene duplication or domain shuffling events and 3.4% of the genome is composed of insertion sequences (IS) and prophages (phiRv1 and phiRv2) (Cole, 2002). There are 56 copies of IS elements belonging to IS3, IS5, IS21, IS30, IS110, IS256 and IS*L*3 families (Cole, 2002). Genome sequences can be used for the development of diagnostic tools for the rapid and unambiguous identification of members of the MTBC (Cole, 2002). The objective of comparative genomics of *Mycobacterium* spp is to identify genes or loci that are different from virulent or attenuated strains (Cole,

2002). Since the characterisation of these strains would help in defining the molecular mechanisms of pathogenicity, as well as contribute to new information for vaccine development (Cole, 2002). Early secreted antigenic target 6 kDa protein and CFP-10 are contained within the RD1 (region of difference) of the mycobacterial genome (Ravn *et al*., 2005). These proteins are absent from *M. bovis* BCG, *M. avium* and most other nontuberculous mycobacteria making it easier to distinguishing between mycobacteria spp (Ravn *et al*., 2005).

2.8 Clinical manifestation of *Mycobacterium* infections

Tuberculosis can be seen in three forms: primary, latent and post-primary (Timbury *et al*., 2002). The primary form is generally the more invasive stage, with marked lymph node involvement (Timbury *et al*., 2002).

2.8.1 Tuberculosis

The second phase is the latent or asymptomatic dormant phase while the active disease can be initiated after as long as 10 to 80 years (Timbury *et al*., 2002). The post-primary phase includes the development of delayed-type hypersensitivity, which modifies the infection and increase the difficulty of appropriate treatment (Timbury *et al*., 2002).

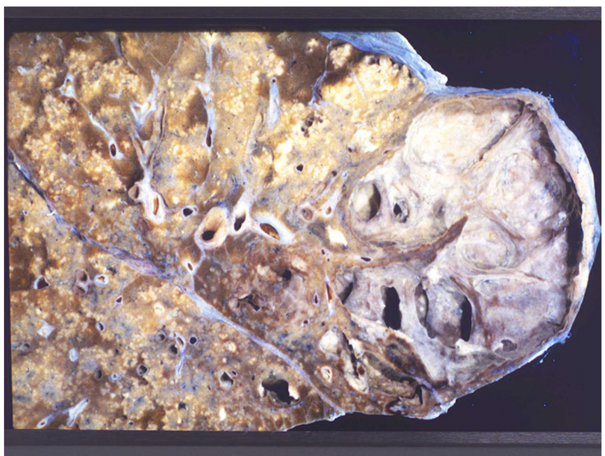

Figure 2.5 Lesion of tuberculosis forming in the lungs (www.fao.org/docrep/003/t0756e/T0756E03.html)

Typical symptoms of TB include: a low-grade fever, night sweats, tiredness, weight loss and a constant cough (often with bloody sputum) (Noble, 2006). However, the main effect of TB is damage to the lungs (Noble, 2006). Lesions in the tip of the lung (Figure 2.6) form and if untreated, chronic disease can develop (Timbury *et al.*, 2002).

2.8.2 Other infections

Other parts of the body can also be affected, for example the lymph nodes, kidneys, liver, brain, bones and joints (Salyers & Whitt, 2002). People infected with *M. kansasii* can experience fever, swollen lymph nodes, wheezing and skin lesions may occur (Ngan, 2006). *Mycobacterium kansasii* infections in middle-aged people can cause lung infections with chronic lung conditions (Turkington, 2002). Buruli ulcers (Figure 2.7), usually occurs on the arms and legs especially in immunocompromised individuals also infected with *M. marinum* (Ngan, 2006; Turkington, 2002).

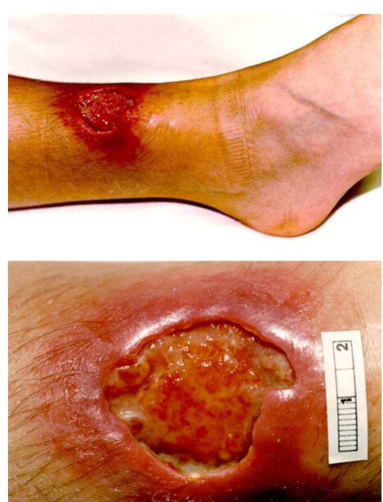

Figure 2.6 Ulcers appear in immunocompromised individuals infected with mycobacteria (Johnson *et al.*, 2005)

Painless and itchy nodules develop about 7 to 14 days after infection by *M. ulcerans* through broken skin and form an ulcer that will spread rapidly over the patient's skin (Ngan, 2006; Portaels *et al.*, 2009). Atypical mycobacterial infections can occur without

causing any symptoms, making detection of the infection difficult (Turkington, 2002; Portaels *et al*., 2009).

Symptoms, signs and laboratory abnormalities can be common in disseminated mycobacterial infections but are unfortunately not specific indicators of the disease and thus needs molecular confirmation (Yoon *et al*., 2009). In patients with disseminated *M. avium* complex, the diagnosis is made on an average of 9 to 12 months of the development of an index AIDS diagnosis (Kerilikowske *et al*., 1992; Friedland, 2009). Localised infections (pneumonia, skin nodules and diarrhoeal syndromes) with *M. avium* complex tend to occur in HIV-infected patients with better immune function (Kasperbauer & Daley, 2008). Patients infected with HIV who are not severely immunocompromised usually present clinically with typical pulmonary tuberculosis with apical infiltrates or cavitations on chest X-rays (Kasperbauer & Daley, 2008). Although infection with *M. tuberculosis* is indicated by a positive tuberculin reaction, isolating *M. tuberculosis* from culture is required to confirm the disease (Kasperbauer & Daley, 2008).

Tubercle bacilli are readily phagocytosed but can multiply within mononuclear cells and resist digestion (Timbury *et al*., 2002). This intracellular parasitism is associated with the development of delayed hypersensitivity and of activated macrophages, which modifies the host response to a second and third challenge (Timbury *et al*., 2002).

2.9 Tuberculosis and HIV infection

The increase in HIV infection has contributed significantly to the rise in the worldwide incidence of TB (Friedland, 2009). Human immunodeficiency virus is the single most important risk factor for TB and the collision of TB and has resulted in an estimated 12 to 14 million people co-infected with TB and HIV (Friedland, 2009). Co-infection with HIV significantly increases the risk of developing TB by over 100-fold, as well as the possibility of developing resistant mycobacterial strains (Davies & Pai, 2008).

Worldwide, a further increase in TB rates can be expected in areas with a high prevalence of HIV infection, considering that: i) the reactivation rate is much higher in HIV-infected individuals (10% a year) when compared to an individual not infected with HIV (10% during life) and an HIV-infected individual progresses faster from infection with *M. tuberculosis* to full-blown TB (Valadas & Antunes, 2005). In addition, HIV patients with TB have a shorter survival and develop AIDS faster than those without TB and this emphasise the importance for an early diagnosis and effective treatment of TB (Valadas & Antunes, 2005).

Clinicians must maintain a high index of suspicion for TB when evaluating HIV-infected patients with respiratory complaints (Finch & Beaty, 2007). Human immunodeficiency virus infected patients require hospitalisation and these patients must be placed in respiratory isolation until the public health risk they pose can be assessed (Finch & Beaty, 2007). Immune reconstitution inflammatory syndrome (IRIS) is emerging in developing countries, as an important complication of the combination of anti-retroviral (ARV) and anti-TB drugs (Meintjes *et al*., 2009). Immune reconstitution inflammatory syndrome occurs in 8 to 43% of patients who initiate ARV's while receiving TB treatment (Meintjes *et al*., 2009). Currently it is believed that the mechanism underlying TB IRIS is related to the ARV-induced suppression of viral replication and immune recovery, which results in the reactivation of TB-specific immune responses (Friedland, 2009). Predictors of IRIS include low CD4 cell count, early initiation of ARV and extrapulmonary TB (Friedland, 2009). The clinical presentations vary widely with regard to the manifestations and severity but include some of the following: recurrent TB-related symptoms; new/recurrent TB meningitis, worsening granulomatous hepatitis and/or worsening tuberculous lesions (Meintjes *et al*., 2009). To make matters worse, clinicians may have difficulty distinguishing IRIS from other HIV-related opportunistic diseases and studies are urgently needed to manage IRIS (Meintjes *et al*., 2009). A study conducted by Baba and colleagues, (2008) determined the correlation of a very low CD4 count (median of 59 cells/μl) and diagnosis of patients with TB and HIV co-infection. The results of the study indicated that the adenosine deaminase activity can be used as a sensitive marker for patients with a low count (Baba *et al*., 2008). This is an important

finding for countries with a high burden of TB and HIV co-infection (Baba *et al*., 2008). As HIV-infection progresses and the CD4 count declines, extra-pulmonary disease occurs with increasing frequency (Davies & Pai, 2008). *Mycobacterium tuberculosis* disease is a unique infection in patients with AIDS because it is communicable and virulent and yet preventable and curable (Friedland, 2009).

2.10 Treatment of tuberculosis

Tuberculosis is usually treated with four different antimicrobial agents and the therapy lasts from 6 to 9 months (Jassal & Bishai, 2009). The most commonly used drugs (known as the first-line medication) are INH, RIF, PZA and EMB or SM (Todar, 2005; Jassal & Bishai, 2009). Effective treatment regimens need second-line medications, such as the following: aminoglycosides (amikacin or kanamycin); polypeptides (capreomycin) and or fluoroquinolones, such as moxifloxacin, levofloxacin or gatifoxacin (Jassal & Bishai, 2009). Typical MDR-TB regimens consist of up to five drugs and the WHO recommends their use for a minimum of 18 months of treatment after culture conversion to a negative result (Jassal & Bishai, 2009).

2.10.1 First-line medication used for TB

Isoniazid is the most commonly used anti-tuberculosis drug and is highly effective against actively dividing bacilli (Hershfield, 1999). The administration of INH can lead to asymptomatic elevations of serum transaminases or even fatal hepatitis and all patients taking INH should be monitored for adverse reactions (Hershfield, 1999). Pape and colleagues, (1993) found that INH delayed progression of symptomless HIV infection to disease and death. A possible explanation for the delay in the progression of HIV infection could have been the effect of INH on the development of clinical TB (Pape *et al*., 1993).

One of the most important drugs in the treatment of tuberculosis, RIF was discovered in the late 1960s (Davies, 1999). Rifampicin is able to kill the very slow dividing mycobacteria in a way that other drugs cannot do (Davies, 1999). Rifampicin is a potent

agent against actively dividing intracellular and extracellular TB bacteria and has activity against semi-dormant bacilli (Hershfield, 1999). Rifampicin works primarily by inhibiting DNA-dependent RNA polymerase (bacterial RNA polymerase) and blocks RNA transcription and therefore no messenger ribonucleic acid (mRNA) is synthesised (Hershfield, 1999). Rifampicin is called the 'persister' as the drug is able to kill the very slow dividing bacteria while other drugs, such as INH cannot (Davies, 1999). By combining INH with RIF, the length of treatment can be reduced to as little as six months (Davies, 1999). An oral dose is given daily and this may cause a harmless discolouration of urine and other body fluids (Hershfield, 1999).

Pyrazinamide are used to shorten the therapy from previously 9 to 12 months to 6 months (Zhang *et al.,* 2003). This commonly used anti-TB drug kills a population of semi-dormant tubercle bacilli in acidic pH environments that are not killed by other TB drugs (Zhang *et al.,* 2003). This front-line drug is only active against *M. tuberculosis* during active inflammation when the pH of the environment is acidic (Zhang *et al*., 2003).

Ethambutol inhibits the selection of resistant mutants, is active against both intracellular and extracellular bacteria and prevents the transmission of the infection (Blaivas, 2004). One tablet is taken daily in the morning and EMB is usually administered in cases in which INH resistance is a possibility (Hershfield, 1999).

Streptomycin is an antibiotic drug, the first of a class of drugs called aminoglycosides to be discovered in the 1940's and was the first antibiotic remedy for tuberculosis (Hershfield, 1999). An adverse effect of this medicine is its ototoxicity and it impairs hearing or balance and may lead to permanent hearing loss (Hershfield, 1999).

Ethionamide is an antibacterial agent that works by inhibiting the growth of the TB cells by disruption of the mycolic acids, which results in cell death (Hershfield, 1999). Ethionamide has a bacteriostatic activity against actively growing TB bacilli; it works by obstructing the formation of the cell-wall (Hershfield, 1999).

2.10.2 Second-line medication used for TB

Fluoroquinolones are broad-spectrum antibiotics and are less effective than first-line agents in treating TB but are used mainly for the treatment of drug-resistant tuberculosis (Jassal & Bishai, 2009). Moxifloxacin, levofloxacin and gatifoxacin are three fluoroquinolones that are part of the second-line regimen used in the treatment of TB (Jassal & Bishai, 2009). The National Tuberculosis Control Programme has indicated that specific guidelines need to be followed for the treatment of TB in adults (Table 2.1). Treatment of XDR-TB should include drugs to which the *M. tuberculosis* strains has proven to be susceptible to, any first-line drug to which the isolate has shown to be susceptibile and this regimen should be continued for a minimum of 18 to 24 months (Jassal & Bishai, 2009).

Table 2.1 The South African National Tuberculosis Control Programme (NTCP) guidelines for the treatment of TB in adults (www.ecdoh.gov.za/uploads/files/150906152233.pdf)

Pre-treatment body weight	Initial two months	Four month continuation phase	
	When given 5 times a week	When given 5 times a week	When given 3 times a week
	INH 75 mg, RIF 150 mg, PZA 400 mg, EMB 275 mg	INH 150 mg RIF 300 mg	INH 150 mg RIF 150 mg
30 – 37 kg	2 tabs each	2 tabs each	2 tabs each
38 – 54 kg	3 tabs each	3 tabs each	3 tabs each
55 – 70 kg	4 tabs each	2 tabs each	3 tabs each

However, the development of new TB drugs has increased significantly over the last 40 years (Casenghi, 2006). Clinical trials to register a TB drug represent a lengthy and expensive process and could take a minimum of six years (Casenghi, 2006). Diarylquinoline TMC_{207} is just one of the examples of a promising member of a new class of anti-mycobacterial agents (Casenghi, 2006). The active compound of the diarylquinolines as well as its spectrum is unique in its specificity to mycobacteria (Casenghi,

2006). This implies low probability of cross-resistance with existing TB drugs, which will aid in the fight against MDR and XDR-TB infections (Casenghi, 2006; Jassal & Bishai, 2009).

Tuberculosis drugs should not be administrated as a single drug because this can lead to the development of a bacterial population resistant to the drug (Todar, 2005). The simultaneous use of two or more drugs helps prevent the emergence of resistant tubercle bacilli (Timbury *et al*., 2002). However, it can be difficult to select two agents where the susceptibility of *M. tuberculosis* strains from the patient is not known (Todar, 2005). Improper selection of drugs might result in the development of additional drug-resistant bacteria complicating diagnosis and treatment of TB positive patients (Todar, 2005).

2.11 Drug resistant tuberculosis

Antibiotic resistance is a growing obstacle in the control of infectious diseases worldwide (Agdamag *et al*., 2003). The emergence of MDR-TB strains of *M. tuberculosis* poses a serious problem for the detection and diagnosis of tuberculosis (Agdamag *et al*., 2003). The drug resistance mechanisms of the bacilli causing TB are due to a variety of mechanisms (Wade & Zhang, 2004). These mechanisms are generally divided into five main categories: i) decreased uptake or impermeability; ii) increased efflux; iii) enzymatic inactivation; iv) modification of the antibiotic target and v) reduced pro-drug activating enzyme activity (Wade & Zhang, 2004). An example of the decreased uptake or impermeability is the hydrophobic cell surface, which provides a barrier for some antibiotics (Wade & Zhang, 2004). A study by Wade and Zhang, (2004) concluded that resistance mechanisms to anti-tuberculosis drugs are by means of spontaneous mutations in chromosomal genes rather than by any type of gene transfer.

A person with MDR-TB will need to change to a regimen containing newer and often less widely-available second-line drugs (Girard *et al*., 2005). Multi-drug resistant TB and XDR-TB can be transferred from an infected person to a non-infected person (Barnard *et al*., 2008). Treatment of resistant tuberculosis requires use of drugs for approximately 24 months, which makes it more expensive, toxic and less effective than first-line drugs used

for routine treatment of TB (Jassal & Bishai, 2009). Extensively resistant TB has been detected in all regions of the world (Shah *et al*., 2007). The Tugela Ferry outbreak, 2006, in Kwazulu Natal, served as a serious warning when a cluster of XDR-TB was identified (Casenghi, 2006).

Mutations develop spontaneously due to the natural mutation rate of genomic DNA (Riska *et al*., 2000). *Mycobacterium tuberculosis* drug resistance is mainly due to mutations in genes encoding drug target or drug converting enzymes: *kat*G and *inh*A are the most frequently associated with isoniazid resistance (Espasa *et al*., 2005). Isoniazid resistance develops at a rate of 10^{-5} to 10^{-7}, while resistance to RIF develops less frequently at a rate of approximately 10^{-9} (Riska *et al*., 2000). However, the molecular mechanisms of INH resistance are highly complex because INH resistance involves a variety of additional mutations at the *ahp*C-*oxy*R intergenic region (Hillemann *et al*., 2005; Aragón *et al*., 2006). These mutations will have an effect on one or several genes involved in mycolic acid biosynthesis or over-expression as a response to cellular toxicity of INH (Aragón *et al*., 2006).

The majority of INH resistant strains have been found to contain AGC to ACC point mutations in codon 315 of the *kat*G gene (Ahmad & Mokaddas, 2004; Ruiz *et al*., 2004). Most mutations occur between codons 138 and 328 with the most common gene alteration at codon 315 of the *kat*G gene (Johnson *et al*., 2006). The *kat*G gene encodes catalase-peroxidase activity and complete deletion of the catalase-peroxidase gene has been shown to cause a high level (60 to 70%) of INH resistance (Evans *et al*., 2009). However, mutations in this gene are more frequent than deletions in clinical isolates and these lower the activity of the enzyme (Johanson *et al*., 2006). The Ser315Thr substitution is estimated to occur in 30 to 60% of INH resistant isolates (Johnson *et al*., 2006). The primary target of activated INH is a nicotinamide adenine dinucleotide (NADH)-dependent enoyl-acyl carrier protein reductase, designated *inh*A (Evans *et al*., 2009). Activated INH binds to the *inh*A-NADH complex and form a ternary complex that results in the inhibition of mycolic acid biosynthesis (Johnson *et al.,* 2006). Frequently reported mutations in clinical isolates, such as the *inh*A promoter mutations, are present at positions -24(G-C), -16(A-G), or -8(T-G/A) and -15(C-T) resulting in the

conversion of a serine to an alanine residue at amino acid 94 (Johnson *et al*., 2006). Strains with mutations in the structural gene *inh*A have been less commonly described but are associated with not only INH but ethambutol (ETH) resistance as well (Morlock *et al*., 2003). Furthermore, there are at least 18 alternative genes that have been implicated in the mechanism of resistance against INH (Aragón *et al*., 2006).

Mutations, in RIF resistant strains are confined to a short 81 bp DNA region and encodes for the β-subunit of the RNA polymerase (Zhang *et al*., 2007). More than 95% of all missense mutations are located in a 51 bp core region of the *rpo*B gene between codons 507 to 533 (Evans *et al*., 2009). The most common changes occur in codons Asp515Val, His526Tyr and Ser531Leu (Johnson *et al*., 2006). These changes occur in more than 70% of RIF resistant isolates with a high level of RIF associated with mutations in codon 526 and 531 (Evans *et al*., 2009). However, alterations in codon 511, 516, 518 and 522 resulted in a low level of RIF resistance (Johnson *et al*., 2006).

The resistance to RIF can be assumed to be a surrogate marker for MDR-TB, since more than 83% of the RIF-resistant isolates are also INH resistant (O'Riordan *et al*., 2008). The introduction of routine *rpo*B gene testing improved the time to diagnose MDR-TB by 6 to 7 weeks compared to relying on culture and sensitivity testing (O'Riordan *et al*., 2008). Rifampicin resistance in the presence of INH sensitivity was found in 17.5% of *rpo*B mutant strains and this justifies the practice of continuing INH therapy until full culture and sensitivity results are available (O'Riordan *et al*., 2008). The introduction of rapid molecular testing for all acid-fast bacilli (AFB) positive cases, for routine diagnostics, has the potential to aid in treatment and prevention in the spread of MDR-TB (O'Riordan *et al*., 2008).

2.12 Detection and diagnosis of tuberculosis

There are a series of diagnostic techniques for the detection of TB in humans for example microscopy, radiological and bacteriological diagnosis, tuberculin test, serological diagnosis and molecular assays (Wanchu, 2005). Molecular methods have enhanced the detection and identification of common mycobacteria but unfortunately, smaller

laboratories do not have the resources to accurately identify these bacteria (Hall & Roberts, 2006).

2.12.1 Clinical detection of TB

A complete medical evaluation for TB must include a medical history, a chest X-ray and a physical examination (Wanchu, 2005). Chest radiography is the cornerstone of diagnosis of pulmonary TB (Wanchu, 2005). Infiltrates and cavities are typically seen in reactivation TB and lower lobe diseases are seen in primary tuberculosis (Wanchu, 2005). The chest radiographs of people infected with HIV may appear normal in 7 to 14% of the cases (Wanchu, 2005). Chest X-rays prove to be useful to physicians to determine whether or not a patient has active TB disease and the changes on the X-ray can help confirm the diagnosis (Taragin, 2007). However, X-rays cannot be used as a single diagnostic tool but must still be used together with the routine laboratory diagnosis (Taragin, 2007).

2.12.2 Microscopy detection of *Mycobacterium* spp

Laboratory diagnosis of TB starts with the fluorochrome acid fast stain of a specimen and culturing (Heymann, 2006). Culture positive specimens are stained to detect typical bacilli by means of the Ziehl-Neelsen stain (Somoskövi *et al*., 2001). The fluorochrome acid-fast stain is used to aid in early diagnosis of mycobacterial infections and assists in the monitoring of patients on anti-mycobacterial therapy (Kumar *et al*., 1998). The acid-fast bacteria fluoresce yellow to orange after staining and this stain is preferred for smears because of the increased sensitivity (Hall & Roberts, 2006). Even though the Ziehl-Neelsen stain cannot distinguish one species from another, it is still an important method to determine whether a bacterium is acid-fast (Hall & Roberts, 2006). The AFB retains the red colour and the non acid-fast bacteria will stain a blue colour (Carson, 1990).

Light microscopy is a cheap and rapid method for identification but culturing the mycobacteria, which takes 4 to 6 weeks, is still necessary for final diagnosis and

treatment (Farnia *et al*., 2002). An advantage of microscopy is that any clinical material smeared on a glass slide can be used for microscopy for example sputum, bronchial washings or aspirates (Heymann, 2006). A disadvantage of microscopy is the low sensitivity (46%) and specificity (90%) (Farnia *et al*., 2002). Microscopy requires a large number of bacilli to be present in order for the result to be positive (5 000 to 10 000 per ml of sputum) (Heymann, 2006). Unfortunately, this limits the sensitivity, ranging between 34 to 80%; reducing the sensitivity even more in patients with HIV co-infection, children and people with disseminated TB (Davies & Pai, 2008). Patients with HIV co-infection has a higher proportion of sputum smear-negative pulmonary and extra-pulmonary TB (Maher *et al*., 2005) because of a weak cough or the inability to produce sputum (Davies & Pai, 2008). The sensitivity of smear microscopy of children and HIV infected patients range between 35 to 70% (Heymann, 2006). The proportion of cases detected by microscopy is often as low as 20 to 30% of all cases and the need for repeat visits (two to three) results in report delays (Heymann, 2006).

2.12.3 Culturing of mycobacteria

Bacteriological culture, with a specificity of over 98%, is considered the diagnostic gold standard and can identify the mycobacteria in over 80% of TB cases (Jafari & Lange, 2008). Specimens submitted for mycobacterial culture are either specimens of pulmonary (sputum) or extra-pulmonary origin (urine) and as few as 10 to 100 viable bacilli per ml are necessary to be detected (Heymann, 2006). Recently, a number of growth indicators have been used, such as automated systems to shorten the detection period to 1 to 3 weeks in most cases (Heymann, 2006). Bacterial culture depends on the viability of the bacterium; however, improper specimen collection can influence results but is still used on account of its simplicity and low cost (Beqaj *et al*., 2007; Morcillo *et al*., 2008).

2.12.3.1 The Bactec System 960 MGIT

The BACTEC MGIT 960 is an automated system supplied for the growth and detection of mycobacteria (Mathur, 2002). Growth detection is based on the acid-fast bacilli's

metabolic oxygen utilisation and the intensification of the oxygen quenched fluorescent dye contained in a tube mycobacteria growth indicator tube (MGIT) (Mathur, 2002). The tubes are incubated in the MGIT system until flagging positive by the instrument up to a period of 8 weeks (Somoskövi *et al*., 2003). Using the BACTEC MGIT 960 lowers the detection time to 1 to 4 weeks in comparison with solid culture media, such as Löwenstein-Jensen (Farnia *et al*., 2002). However, these automated systems are expensive, requires more infrastructure and needs maintenance (Heymann, 2006).

2.12.3.2 Löwenstein-Jensen media

Löwenstein-Jensen culture media is a solid egg or agar-based media, which can be locally prepared at a low cost and can be refrigerated for a long time (Heymann, 2006). However, with this culturing method, you can distinguish between different colonies (Heymann, 2006). Unfortunately, the Löwenstein-Jensen media has a 2 to 6 week detection time and is less sensitive than liquid media (Heymann, 2006).

2.12.4 Assays for the detection of mycobacteria

Substantial progress has been made in the past decade with novel tools for the detection and control of TB (Pai *et al*., 2006). Rapid assays have been investigated and proved to be useful tools as additional tests but the ideal rapid detection test for TB is still not in sight (Pai *et al*., 2006).

2.12.4.1 SD BIOLINE TB Ag MPT64 Rapid assay

Mycobacterium tuberculosis is known to secrete more than 33 different proteins with MPT64 being one of the predominant proteins (Oettinger & Andersen, 1994). The MPT64 can only be found in the culture fluid of *M. tuberculosis* strains (SD BIOLINE TB Ag MPT64 Rapid, package insert, 2008). SD BIOLINE TB Ag MPT64 Rapid is an immunochromatographic identification test to rapidly differentiate between MTBC and MOTTs (Wang *et al*., 2007). The test kit can be used in clinical laboratories in combination with culture systems based on liquid media with the advantage of no technical complexity (Oettinger & Andersen, 1994). The test results are visible in only

15 min after the specimen has been applied onto the test device and interpretation of the test are simple, thus no extensive training are needed (Ismail *et al*., 2009). A definitive clinical diagnosis should not be based on the results of a single test, but should only be made by a physician after all clinical and laboratory findings have been evaluated (Ismail *et al*., 2009).

2.12.4.2 Rapid detection of latent TB infection

Accurate and rapid tests for TB infection based on the detection of T-cells sensitised to *M. tuberculosis* are extremely important (Andersen *et al*., 2000). Latent infection is diagnosed in a non-immunised person by a tuberculin skin test (TST), which yields a delayed hypersensitivity type response to purified protein derivatives (PPD) of *M. tuberculosis* (Wanchu, 2005). Purified protein derivative is a mixture of mycobacterial antigens and *M. bovis* BCG vaccine strains and as a result the TST is not adequate for the diagnosis of LTBI in populations with a high BCG coverage (Eum *et al*., 2008). Advanced TB, malnutrition and other immunosuppressive conditions, such as AIDS may decrease the sensitivity of the TST assay (Liebeschuetz *et al*., 2004; Eum *et al*., 2008). Patients immunised for TB will respond with delayed hypersensitivity parallel to those who are in a state of infection and the test must thus be used with caution (Wanchu, 2005).

2.12.5 Serological assays for the detection of mycobacteria

The detection of antibodies or antigens in blood has so far largely failed to provide sensitive and specific results for the use of serological assays as screening tools in TB (Heymann, 2006). The antibodies in the sample can cross-react with environmental mycobacteria leading to false-positive results (Heymann, 2006). Current available serological tests offer little use overall when compared to the conventional methods; however, a number of assays hold some promise (Heymann, 2006).

2.12.5.1 Whole-blood assay for diagnosing TB

The whole-blood interferon-gamma enzyme-linked immunosorbent assay has been studied and developed mainly for diagnosing active pulmonary TB or latent TB (Song *et al*., 2009). Identification and characterisation of the two *M. tuberculosis*-specific antigens ESAT-6 (early secreted antigenic target 6 kDa protein) and CFP-10 (culture filtrate protein) has led to the development of a specific diagnostic test for patients infected with *M. tuberculosis* (Ravn *et al*., 2005). In high-TB-endemic regions, where there is a high prevalence of both active and latent TB infection (LTBI), showed that 30 to 50% of healthy individuals responded positively to the RD1 antigens (Ravn *et al*., 2005). Another problem is that it cannot be predicted who or when RD1 positive patients will develop TB infection (Ravn *et al*., 2005). However, a positive result may help in identifying candidates for preventative chemotherapy or intensified clinical follow-up (Ravn *et al*., 2005).

2.12.5.2 Detection of *M. tuberculosis* using QuantiFERON®-TB GOLD ELISA

Newer generation test kits have already been developed and reports of their utility are now beginning to emerge (Eum *et al*., 2008). The QuantiFERON-TB GOLD ELISA (Cellestis Limited, Australia) assay was approved by the FDA in 2001 as an aid for detecting the MTBC (Mazurek & Villarino, 2003). This test is an *in vitro* diagnostic assay that measures a component of CMI reactivity to *M. tuberculosis* (Mazurek & Villarino, 2003). Interferon-gamma is released from sensitised lymphocytes in whole blood and incubated overnight with PPD from *M. tuberculosis* and control antigens (Leutkemeyer *et al*., 2007).

This new IFN- γ assay do not discriminate between latent and active TB and a distinction is necessary for better global control of TB (Sauzullo *et al*., 2008). The test is simple to perform and results can be obtained within 24 h, it takes only one patient visit and it does not boost subsequent tests (Ravn *et al*., 2005). A study by Cho, (2007) indicated a higher specificity (97%) and sensitivity (76%) when compared to the TST assay 66% and 71% respectively (Cho, 2007). Sauzullo and colleagues, (2008) proved that about 7% of

patients with active and extrapulmonary TB will have negative IFN- γ results because of the low release of IFN-γ by T-cells (Sauzullo *et al*., 2008). QuantiFERON-TB GOLD ELISA assay testing may be limited by an elevated rate of indeterminate results in patients with CD4 cell counts of less than 100 cells/mm^3 (Leutkemeyer *et al*., 2007). Persons with HIV/AIDS who are on ARV treatment and with certain malignancies may decrease the production of INF-γ in the assay making a negative QuantiFERON result insufficient to exclude *M. tuberculosis* infections (Lie, 2008). Thus, more research on the QuantiFERON-TB GOLD ELISA assay is necessary before the assay can be fully applied to detect LTBI (Cho, 2007).

2.12.6 Molecular based assays for the detection and identification of *M. tuberculosis*

Molecular tests have brought unprecedented opportunities for the rapid diagnosis of TB and can be incorporated into control programmes for TB (Cho, 2007). Research on molecular tests has focused on the following three areas: detection, identification of species and detection of mutations in genes associated with resistance to drugs against TB (Cho, 2007). Direct detection of tubercle bacilli in clinical specimens can be done by a PCR assay (Timbury *et al*., 2002). Conventional and real-time PCR assays allows sequences of mycobacteria DNA present in only a few copies in a specimen to be amplified (Nagesh *et al*., 2001), such that the amount of amplified DNA can be visualised and identified using gel electrophoresis as well as computer software (Mathur, 2002; Kubista *et al*., 2006).

2.12.6.1 Conventional PCR

Conventional PCR assays made it possible to amplify any nucleic acid sequences present in a complex sample in a cyclic process and to generate a large number of identical copies to be analysed (Kubista *et al*., 2006). As an analytical technique the conventional PCR method has limitations, such as post amplification analysis and visualisation by means of an agarose gel, which increase possible contamination as well as time (Kubista *et al*., 2006). Fortunately, this issue was resolved by the development of real-time PCR assays (Kubista *et al*., 2006).

2.12.6.2 Real-time PCR

Real-time PCR, also called quantitative real-time PCR, can be used to simultaneously quantify and amplify a specific part of a given target DNA or RNA (Grassi *et al*., 2006). During the last 8 to 10 years, real-time PCR technology has been used for the following in mycobacterial research: SYBR Green dye that binds non-specifically in the major groove of double-stranded DNA or sequence-specific fluorescent probes can be used for quantification (Grassi *et al*., 2006). The 16S rRNA gene is the target most commonly used for the identification of *Mycobacterium* species (Soini & Musser, 2001). This gene contains both conserved and variable regions, making it an ideal target for taxonomic purposes (Soini & Musser, 2001). The 16S rRNA gene has been sequenced from a large number of mycobacterial species and the sequencing of two hypervariable regions of the 16S rRNA gene allows for identification of the majority of mycobacterial species (Soini & Musser, 2001). The bacteria are identified by comparison of the nucleotide sequence with reference sequences (Genbank) (Soini & Musser, 2001). However, the MTBC cannot be distinguished and additional gene regions, such as the 16S-23S rRNA internal transcribed spacer need to be sequenced to differentiate between the species (Soini & Musser, 2001).

Results of an in-house real-time PCR study by the WHO indicated sensitivity greater than 95% in AFB smear-positive specimens as well as a specificity of 98 to 100% (WHO, 2006). Real-time PCR methods are easier to perform than conventional PCR, involve a minimum of handling steps and require relative small amounts of genomic DNA (Narayanan, 2004). A study by Rebollo and colleagues (2006) investigated the use of blood and urine specimens for the diagnosis of TB, since collection of the sputum is not easy in patients with a non-productive cough or those with disseminated clinical forms. Unfortunately, the study proved that these specimens will still not be used routinely in a diagnostic laboratory (Rebollo *et al*., 2006).

The LightCycler platform (Roche Diagnostics) combines real-time PCR with product detection using fluorogenic hybridisation probes that employ the principle of fluorescence resonance energy transfer to achieve rapid PCR results (Shrestha *et al*.,

2003). An added feature of the LightCycler real-time PCR platform is the post-amplification melting point analysis allowing for differentiation of closely related bacteria (Shrestha *et al*., 2003). Shrestha and colleagues (2003) suggested that the LightCycler assay would be useful for the rapid differentiation of *M. tuberculosis* from MOTTSs by using cultured isolates as well as direct detection in clinical specimens.

However, since the application of these technologies for diagnostic purposes is still in its infancy, these assays should be used with caution in conjunction with the culture 'gold standard' method until sufficient data have been obtained (Parashar *et al*., 2006). The main advantage of real-time PCR is its utility in quantitative analysis, which is not possible by conventional PCR (Parashar *et al*., 2006). Another advantage of real-time PCR is that post PCR detection by electrophoresis or hybridisation can be omitted and users can monitor the amplification of PCR products in real time and decreases the risk of contamination (Parashar *et al*., 2006). The PCR amplification process can be completed in 2 to 4 h after obtaining the processed clinical sample with an additional 2 to 24 h for the detection and identification of mycobacteria resulting in quicker diagnosis in comparison to the 4 to 8 weeks when cultured (Tiwari *et al*., 2007). Its disadvantage is the high cost of system set-up while consumables and reagents became cheaper as more laboratories are using these technologies, exceeding that of a conventional PCR procedure (Grassi *et al*., 2006). False positive PCR results are usually due to laboratory-introduced contamination as well as the lack of the assay to differentiate between viable and dead AFB (Tiwari *et al*., 2007).

2.12.6.3 Cobas Amplicor MTB PCR assay

The Cobas Amplicor *M. tuberculosis* PCR (Roche Diagnostics) is a well-established system for rapid detection of the MTBC (Williams *et al*., 2007). Clinical evaluations of the Roche Amplicor MTB PCR assay have shown high sensitivity (92.8%) and specificity (>99%) when compared to 46% and 98% respectively of the Löwenstein-Jensen culture method (Fernström *et al*., 2003). The necessity for susceptibility testing and epidemiological characterisation of isolates proves that current amplification assays cannot be substituted for culture (Reischl *et al*., 1998).

The Cobas Amplicor *M. tuberculosis* assay has maintained a reasonable sensitivity and specificity in smear-positive specimens, since its initial approval by the Food and Drug Administration (FDA), but it is still limited by a slow block cycler amplification process and time-consuming procedures (Cheng *et al*., 2005). However, the clear advantages of the pre-fabricated test kits are the standardised procedures and reagents for specimen processing and amplification proving to be well suited for a clinical microbiology laboratory's work flow (Reischl *et al*., 1998).

2.12.6.4 Seeplex® TB Detection 2

Seeplex® TB Detection 2 is designed to detect and identify MTBC in various specimens, such as sputum, bronchial wash and cerebrospinal fluid (Seegene, 2008). A multi-target PCR; *IS*6110 and MPB64 (anti-*Mycobacterium tuberculosis* 64) is used instead of a single-target PCR to prevent false-negative results caused by a lack of the insertion sequence *IS*6110 (Seegene, 2008). This agarose gel-based method can be used as a valuable method for detection and distinguishing of MTBC and MOTTs with the Seeplex® MTB/NTB ACE Detection assay (Seegene, 2008). Seeplex® MTB/BCG ACE detection is made not only to test for TB but also to discriminate BCG simultaneously with a single PCR reaction resulting in a quick test result (Seegene, 2008). These assays would be useful to appropriately diagnose patients, to prevent unnecessary anti-TB drugs and in the process reducing the incidence of drug resistance (Seegene, 2008).

2.12.6.5 Genprobe

The Genprobe (AccuProbe), a acridinium-ester-labeled DNA assay was used in 1994 as the only commercial assay specific for MTBC, MAC, *M. kansasii* and *M. gordonae* (Reisner *et al*., 1994). A study by Reisner and colleagues (1994) provided evidence that the Genprobe used for testing colonies of mycobacteria, are 100% specific and sensitivity varied between species or species complexes (95.2 to 97.2% for MAC and 100% for MTBC). Reisner and colleagues (1994) concluded that the Genprobe is a simple and reliable method for the for identification of most commonly encountered mycobacteria in the clinical laboratory. Since, MAC and *M. kansasii* species were not frequently seen in

the laboratory and the cost of the probes were high, these two probes were excluded from the assay (Reisner *et al*., 1994).

2.12.6.6 artus^{TM} *M. tuberculosis* diff LC PCR assay

The artus^{TM} *M. tuberculosis* PCR Kit CE (QIAGEN, Germany) is an *in vitro* nucleic acid amplification test for the detection of all members of the *M. tuberculosis* complex (Cramer *et al*., 2006; QIAGEN package insert, 2009). This assay allows detection of MTBC directly from clinical samples (such as sputum, bronchial secretion and cerebrospinal fluid) and thus shortens the turn around time (Seagar *et al*., 2008). The real-time PCR assay amplifies the 131 bp region of the mycobacterial nitrate reductase promoter and is detected by fluorogenic probes, which allows differentiation between the members of the *M. tuberculosis* complex (Cramer *et al*., 2006). The artus™ *M. tuberculosis* diff. LC PCR Kit is the first assay, that allows the highly specific identification of the different spp and can give results within 1 h (Cramer *et al*., 2006). Unfortunately the product must be operated by personnel specially trained in the diagnostic procedures (Seagar *et al*., 2008). The assay also had problems with inhibition and decreased sensitivity when compared to an in-house reference method as suggested by a study conducted by Drews and colleagues, in 2008.

2.12.7 Detection of mutations of *M. tuberculosis* resistant strains

The diagnosis of MDR and XDR-TB is based on mycobacterial culture and drug susceptibility testing (DST), with results available in weeks to months (Barnard *et al*., 2008). Culture and DST capacity are severely limited and in response to the growing problem of MDR-TB, the WHO has issued a call for major expansion of culture and DST competence (Barnard *et al*., 2008). Increased understanding of the genetic mechanisms of *M. tuberculosis* drug resistance together with the established diagnostic methods, such as the DST, could allow the development of more rapid molecular assays (Cheng *et al*., 2007).

2.12.7.1 The standard agar proportion method

The reference standard agar proportion method is commonly used for determining drug susceptibility of *M. tuberculosis* isolates in the laboratory (Kim, 2005). The results of this method are reported as the percentage of the total bacterial population resistant to a specific drug, which is defined as the amount of growth-containing medium as compared with growth on a drug-free control medium (Heymann, 2006). The critical concentration is the concentration that inhibits growth in most susceptible cells of *M. tuberculosis* strains and when at least 1% of the bacillary population become resistant to the critical concentration, it is regarded as resistant to that drug (Kim, 2005). The standard agar proportion method is an inexpensive and simple technique that provides results in less than three weeks (Varma *et al*., 2002). Unfortunately, to provide reliable results, the test procedure has to be standardised with maximal simplification to yield results with acceptable reproducibility (Kim, 2005).

2.12.7.2 Detection and identification of mutations of *M. tuberculosis* resistant strains using real-time PCR

Mycobacterium tuberculosis drug resistance is mainly due to mutations in genes encoding drug target or drug converting enzymes (Espasa *et al*., 2005). Mutations, in RIF resistant strains, confined to a short 81 bp DNA region that encodes for the β-subunit of the RNA polymerase and 12 to 75% of INH-resistant strains have been found to contain mutations either in codon 315 of the *kat*G gene or the *inh*A ribosomal binding site (Cherepanov *et al*., 2001; Torres *et al*., 2003). The resistance to RIF can be assumed to be a surrogate marker for MDR-TB, since more than 83% of the RIF-resistant isolates are also INH resistant (Fan *et al*., 2003; O'Riordan *et al*., 2008).

Torres and colleagues (2003) evaluated the use of specific probes to detect RIF resistance between the 510 and 528 regions and INH resistance at the regulatory region of the *inh*A gene (Torres *et al*., 2003). The different melting point temperatures of the previously designed probes allowed distinguishing between susceptible and resistant strains (Torres *et al*., 2002). Probe 1 detected the two most frequent mutations in the *rpo*B gene:

Ser531Leu, a change in codon 531 from coding serine to leucine (TCG-TTG), and His526Asp, a change in codon 526 from coding histidine to aspartic acid (CAC-GAC) (Torres *et al*., 2002). Probe 2 was designed to detect another two mutations in the *rpo*B gene: Gln513Leu, a change in codon 513 from coding glutamine to leucine (CAA-CTA), and Asn518Ser, a change in codon 518 from coding asparagine to serine (AAC-AGC) (Torres *et al*., 2002). Probe 3 was designed to detect the mutation at codon 315 related at the *kat*G gene and probe 4 to detect mutations of the *inh*A gene as result of a nucleotide substitution C209T (Torres *et al*., 2002).

The LightCycler (Version 2.0, Roche Diagnostics) optical device is capable of measuring fluorescence in two separate channels simultaneously (LCRed640 and LCRed 705 fluorophores), thus allowing analysis of different mutations within a single test tube (Torres *et al*., 2002). One limitation of the LightCycler system is the possiblity of a natural variability in the sequence where the sensor probes bind, leading to a change in the probe melting temperature with no association with resistance (Torres *et al*., 2002). However, the study conducted by Torres and colleagues (2002) was the first real-time PCR performed on clinical *M. tuberculosis* resistant isolates and some modifications may be necessary for the use of the assay as a diagnostic tool.

2.12.7.3 GenoType® MTBDR *plus*

The Hain LifeScience GenoType® MTBDR *plus* test is CE approved and can be used both on culture-based isolates and directly on smear positive sputum samples from patients with pulmonary TB (Hillemann *et al*., 2006; Jewel, 2007). The Hain assay is a PCR amplification and reverse hybridisation assay for detection of RIF and INH resistance (Miotto *et al*., 2006; Barnard *et al*., 2008). This test allows an early diagnosis for MDR-TB positive patients (Miotto *et al*., 2006).

Preliminary data from the Foundation for Innovative New Diagnostics (FIND) and Hain LifeScience suggested that the GenoType® MTBDR *plus* test can be used as a new improved molecular test for MDR-TB (Jewel, 2007). The Hain test can detect at least 90% of MDR-TB cases and the decrease in diagnosis time is of great importance

(Barnard *et al.*, 2008). Unfortunately, the performance of the assay is limited to qualified personnel well-trained in the test procedure and familiar with molecular biological methods (GenoType® MTBDR *plus* Manual Ver 1.0). The results of screening patients could allow improved management of MDR-TB positive patients (Miotto *et al.*, 2006).

2.12.7.4 GeneXpert® System

The GeneXpert® System (Cepheid, USA) utilises real-time PCR to amplify and detect the target DNA (Pai & Ling, 2008). This system is fully automated and integrates sample preparation, DNA amplification and detection (Pai & Ling, 2008). An advantage is the potential of this system to amplify even minute amounts of nucleic acid (Pai & Ling, 2008). The system provides PCR test results from a raw sample in more or less 30 min enabling time-critical DNA tests at the point of need (Pai & Ling, 2008). Unfortunately misdiagnosis of extrapulmonary is common, may result in under or over treatment and is expensive (R500 per test) (Pai & Ling, 2008).

2.13 Controlling tuberculosis

Since 1990, the WHO promoted the Directly Observed Treatment Short-Course (DOTS) strategy (Valadas & Antunes, 2005; WHO, 2006). This strategy is to ensure thorough treatment and is based on the recommendation that the patient takes the prescribed antibiotics in the presence of someone who can supervise the therapy (Sterling *et al.*, 2003). The WHO advises that all TB patients should have at least the first two months of their therapy observed and this means an independent observer watching the patient swallow their anti-tuberculous drugs (Snyman, 2006). Directly Observed Treatment Short-Course strategy cures TB in 95% of the cases and the six-month supply of DOTS is not expensive (R4 per day, depending on the patient's weight) (Snyman, 2006).

2.14 Vaccines

Bacille Calmette-Guérin is still the only commercially available TB vaccine (Glatman-Freedman, 2006) and contains live attenuated *Mycobacterium bovis* (Timbury *et al.*, 2002). The BCG vaccine was invented by a French bacteriologist, Albert Calmette and a

French veterinarian, Charles Guérin in the 1930's by noticing that a glycerin-bile-potato mixture grew bacilli that seemed less virulent (Girard *et al*., 2005).

In the developed world, BCG has generally shown very high and consistent efficacy but its effect in developing countries has been far less consistent (Doherty & Andersen, 2005). In South Africa, BCG is administered at infancy (www.health.gov.za, 2007). The vaccine is not 100% effective and has a 60 to 80% effective rate in children (Colditz, 1994; Todar, 2005).

Replacing BCG will not be an easy task and despite its limitations, it is still cheap, safe and well established (Doherty & Andersen, 2005). There are more than 200 new candidate vaccines currently in development (Sohail, 2006). One such vaccine is a recombinant modified vaccinia virus Ankara that expresses the antigen 85A gene from *M. tuberculosis* (MVA85A) (Sohail, 2006). This new vaccine is in phase II of clinical trials in South Africa (Sohail, 2006). A biomarker can be described as a characteristic trait, which is independently measured as an indicator of a normal or pathological process of the response to intervention (Kaufmann & Parida, 2008). These biomarkers could be used as a guideline to determine the efficacy of vaccines prior to the clinical end-point of TB disease outbreak (Kaufmann & Parida, 2008).

The purpose of this study was to evaluate real-time PCR assays for the rapid identification of *Mycobacterium* species and to determine the presence of INH and RIF resistant genes of *M. tuberculosis* strains directly from clinical specimens. These specimens were obtained from the ARV clinic at the Tshwane District Hospital (TDH). Valuable new information has been obtained with regards to the *Mycobacterium* species present in HIV positive patients and the antibiotic resistance patterns of *M. tuberculosis* strains identified in these patients.

2.15 Summary

Mycobacterium tuberculosis is a Gram-positive slender bacillus and a major cause of morbidity and mortality (Lawn *et al*., 2006). South Africa was listed in 2003, as the

country with the eighth highest incidence of TB globally (Lawn *et al*., 2006; Noble, 2006). In the HIV positive community, multi-drug (MDR) and extensively drug-resistant (XDR) TB is on the increase and a weakened immune system means that HIV positive people with MDR or XDR-TB are unlikely to fight off TB naturally and this is often the only hope for those with a resistant strain (Noble, 2006).

Microscopy and bacterial culture is still used as the gold standard but this cheap method for identification takes 4 to 6 weeks prolonging the diagnosing time (Farnia *et al*., 2002). Since microscopy detects less than half of all patients with active TB in Africa, novel technologies are drastically needed to ensure the correct diagnosis is made (Farnia *et al*., 2002; Kaufmann & Parida, 2008).

Interferon gamma release assays, such as the QuantiFERON-TB GOLD ELISA (Cellestis Limited, Australia) assay has also been introduced and studied to aid in the rapid detection of MTBC (Mazurek & Villarino, 2003). This test is an *in vitro* diagnostic aid that measures a component of CMI reactivity to *M. tuberculosis* (Mazurek & Villarino, 2003). Interferon-gamma (IFN- γ) is released from sensitised lymphocytes in whole blood and incubated overnight with PPD from *M. tuberculosis* and control antigens (Leutkemeyer *et al*., 2007). Advantages of the test are the simplicity to perform the test and the results can be obtained within 24 h and takes only one patient visit (Ravn *et al*., 2005). Unfortunately the QuantiFERON-TB GOLD ELISA assay testing may be limited in patients with CD4 cell counts of less than 100 cells/mm^3 and more research on the QuantiFERON-TB GOLD ELISA assay is necessary before the assay can be used as a diagnostic tool (Cho, 2007; Leutkemeyer et al., 2007).

Molecular assays, such as the Cobas Amplicor MTB PCR assay, Seeplex® TB Detection 2 and real-time PCR, seem to offer numerous advantages, such as specificity and sensitivity comparable to the gold standard (Kim, 2005; Heymann, 2006). Molecular assays can be applied for the identification of the different *Mycobacterium* spp and detection of mutations in genes encoding enzymes, such as the *kat*G and *rpo*B (Espasa *et al*., 2005). The reference standard agar proportion method is used for determining drug susceptibility of *M. tuberculosis* isolates in the laboratory (Weyer, 1999). The standard

agar proportion method is an inexpensive and simple technique but only provide results in more or less three weeks (Varma *et al*., 2002).

The Hain "GenoType® MTBDR *plus*" is a new commercial molecular based and easy-to-perform assay and is developed to identify *Mycobacterium* spp and detect RIF and/or INH resistance simultaneously in TB strains using reverse hybridisation (Miotto *et al*., 2006). The simultaneous detection of resistance to both RIF and INH allows an early diagnosis for MDR-TB positive patients (Miotto *et al*., 2006). However, well trained staff is compulsory (Miotto *et al.*, 2006).

Real-time PCR assays can be used to identify the specific *Mycobacterium* spp as well as detecting the resistance patterns of *M. tuberculosis* positive specimens (Grassi *et al*., 2006). These real-time PCR methods involve a minimum of handling steps and identification and resistance profiles results can be available within 48 h (Narayanan, 2004).

In this serological and real-time PCR assays were evaluated specifically in HIV positive patients attending the ARV clinic at Tshwane District Hospital in the Pretoria region to determine the presence of *Mycobacterium* spp in these individuals. Secondly, the presence of INH and RIF resistance patterns of *M. tuberculosis* in sputum and blood specimens were determined.

2.16 References

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CHAPTER 3

QUANTIFERON-TB GOLD ELISA ASSAY FOR THE DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* SPECIFIC ANTIGENS IN BLOOD SPECIMENS OF HIV POSITIVE PATIENTS IN A HIGH BURDEN COUNTRY

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3.1 ABSTRACT

Worldwide, tuberculosis is a life-threatening infection. Despite improvements in therapy it results in 9 M new cases annually and 2 M deaths. This study evaluated the use of the QuantiFERON-TB GOLD ELISA assay in a high HIV/TB burden setting in an ARV clinic at the Tshwane District Hospital, South Africa. The sensitivity and specificity of the QFT assay in the clinic were 30% (9/30) and 63% (19/30) respectively when compared to the gold standard culture results. Analysis also suggested that the sensitivity of the QuantiFERON assay is determined by a limiting patient CD4 value between 150 and 200.

Keywords: Tuberculosis, QuantiFERON-TB GOLD, HIV positive patients

3.2 INTRODUCTION

Until recently, the tuberculin skin test (TST) was the only available screening method for tuberculosis (TB) and is still predominantly used for the diagnosis of latent TB infection (LTBI) (Bakir *et al*., 2009). However, the status concerning a 'gold standard' may be challenged due variability in interpretation and problems with false positive and negative results (Karima *et al*., 2009). The TST is a measure of a delayed-type hypersensitivity response to purified protein derivative (PPD) (Eum *et al*., 2008). Purified protein derivative is a mixture of mycobacterial antigens, some of which are shared between nontuberculous mycobacteria (NTM), *Mycobacterium bovis* Bacille Calmette-Guerin (BCG) vaccine strains and *Mycobacterium tuberculosis* (Eum *et al*., 2008). The BCG vaccination of children at birth, responds immunologically to PPD prepared from *M. tuberculosis* (Kariminia *et al*., 2009). The PPD has been used to support diagnosis of TB in the clinic as well as for screening in national programmes and epidemiological studies (Andersen *et al*., 2000).

The greatest disadvantage of PPD is that most protein components in this substance are shared between mycobacterial species or with unrelated species of bacteria that decreases the specificity of the TST significantly (Kariminia *et al*., 2009). Another reason for the decrease in specificity is due to prior exposure to non-tuberculosis mycobacteria (Kariminia *et al*., 2009). The sensitivity of the test may be low in individuals with a compromised immune function (Eum *et al*., 2008). Sputum microscopy for acid fast bacilli is often negative in an HIV positive population with end-stage disease and the 'gold standard', sputum culture, is time-consuming and can lead to a delay in TB diagnosis (Andersen *et al*., 2000). Limitations such as these emphasise the need for antigen assays specific to *M. tuberculosis* for diagnosis of TB and such alternatives have been recognised and are currently investigated (Andersen *et al*., 2000; Mackensen *et al*., 2008).

One such method is the QuantiFERON-TB GOLD In-Tube ELISA assay (QFT) (Cellestis, UK), an assay which measures the release of interferon-gamma (IFN-γ) in whole blood after stimulation by specific TB antigens, for example early secreted antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) (Eum *et al*., 2008; Katiyar *et al*., 2008). Interferon-gamma is

released from sensitized lymphocytes in whole blood and incubated overnight with purified protein derivative from *M. tuberculosis* and control antigens (Kariminia *et al*., 2009). The test is based on DNA strip technology and was approved by the Food and Drug Administration (FDA) in 2001 as an aid for detecting the *M. tuberculosis* complex (MTBC) (Pai *et al*., 2009). This test is attractive since the ESAT-6 and CFP-10 are absent from all BCG vaccine strains and from commonly encountered non-tuberculous mycobacteria (NTM), except *M. kansasii*, *M. szulgai* and *M. marinum* (Kariminia *et al*., 2009). Further advantages of the assay are that the test is simple to perform, results can be obtained within 24 h, it takes only one patient visit and it has no potential for boosting with repeated tests (Miranda *et al*., 2009). A study by Cho (2007) indicated a higher sensitivity (76% versus 71%) and specificity (97% versus 66%) when compared to the TST assay (Cho, 2007).

The overall use of the QFT rapidly expands in a low-burden setting and there is some doubt whether it can distinguish between latent and active TB (Pai & O'Brien, 2008). Furthermore, data on the diagnostic ability of the QFT are lacking in high TB-burden settings (such as South Africa), HIV-infected and immunocompromised populations (Pai & O'Brien, 2008). In India (high-burden and resource-limited setting) a 15 year follow-up study of 280 000 subjects, suggested that the TST remains a useful test and that it continues to serve a useful purpose (Pai, 2005).

QuantiFERON-TB testing may be limited by an elevated rate of indeterminate results in immunocompromised hosts, such as patients with human immunodeficiency virus (HIV), malignancy or renal dysfunction (Leutkemeyer *et al*., 2007; Miranda *et al*., 2009). Indeterminate results do not provide useful information with regard to the likelihood of TB disease (Miranda *et al*., 2009). However, these results reflect either impairment of the immune system and/or technical errors during the testing process (Miranda *et al*., 2009). A study by Miranda and colleagues (2009) showed a decrease in the rate of indeterminate test results by adding another tuberculosis antigen (TB 7.7) and by vortexing the tubes for 10 s. Research conducted by Pai and colleagues (2007), has shown that IFN-γ responses can vary over time within the same individual and that conversions and reversions can occur, making diagnosis even more complicated (Pai *et al*., 2007).

Thus, more research on the IFN-γ release assay is necessary before these assays can be implemented in diagnostic laboratories (Cho, 2007). In this study, the QFT-TB GOLD ELISA assay was used to detect *M. tuberculosis* specific antigens in blood specimens of HIV positive patients in a high burden setting due to the lack of information from a low income, high burden country.

3.3 MATERIALS AND METHODS

3.3.1 Study setting

The study was conducted at an ARV clinic at Tshwane District Hospital in Gauteng (South Africa). This study was approved by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria (45/2008). Human immunodeficiency virus positive patients qualifying for ARV treatment according to the South African National HIV Treatment Guidelines (CD4 counts <200 or WHO Stage 4 disease), older than 18 years of age, attending the ARV clinic, who has given informed consent and clinically presenting with TB symptoms were included in this study.

3.3.2 Clinical assessment

A symptom-screening questionnaire was completed followed by a physical examination for active TB. The presence of any one of the following: coughing more than two weeks, chest pain, recent weight loss, night sweats, fever, swelling of lymph nodes, generalised tiredness and not currently on anti-TB drugs, formed the inclusion criteria. Phlebotomists at the ARV clinic, (Tshwane District Hospital) drew blood (total volume of 3 x 1 ml each) from voluntary patients according to the manufacturer's protocol and the kits were immediately transported to the research division at the Department of Medical Microbiology, University of Pretoria.

3.3.3 Laboratory assays

Blood samples were processed within 4 h of collection. All patients were allocated a unique number and no patient identities were revealed. Blood specimens (total volume of 3 x 1 ml each)

were mixed with antigens and controls for the identification of *M. tuberculosis* and all three tubes were incubated (Shaking incubator, Labcon) for 16 to 24 h at 37˚C. The samples were tested for quantitative IFN-γ (IU/ml) by enzyme-linked immunosorbent assay (BioTek ELx 800, Analytical and Diagnostic Products, South Africa). The ELISA was performed according to the manufacturer's instructions using standard kits (Cellestis). In addition to the QFT assay, samples were sent to the National Health Laboratory Services (NHLS), University of Pretoria, where routine diagnostics, namely sputum microscopy for acid-fast bacilli and TB culture were performed.

The manufacturer's guidelines for the interpretation of results were as follows: the test consists of a negative control (a 'nil' well; whole blood without antigens or mitogen), a positive control (a 'mitogen' well; whole blood stimulated with the mitogen phytoheamaglutinin) and two sample wells (whole blood stimulated with ESAT-6 or CFP-10). QF-TB GOLD values are based on the amount of IFN-gamma released in response to the antigens. The IFN-γ level of the nil well is considered the background value and is subtracted from the mitogen and antigen-stimulated well values. The test result is considered positive and TB infection suspected, if the IFN-γ level in the sample well after stimulation with the specific antigens is > 0.35 IU/ml and > 25% of the nil value. The result is indeterminate if the IFN-γ level in the nil tube is > 8.0 IU/ml and resulting in a value for the sample well that is < = 0.35 IU/ml. A negative test result and no TB infection is suspected, when the sample well value is < = 0.35 and < 25% of nil value.

3.3.4 Statistical methods

Microsoft Excel 2007 was used as the spreadsheet and Statistix 8 (Analytical software, Tallahasee FL, USA) and SPSS 17 (SPSS inc, Chicago, IL, USA) were used as the data analysis packages for all data. To examine the validity of the manufacturer's assignment of all indeterminate values to negatives, Receiver Operating Curves (ROC) were drawn for all QFT results, i.e. all true and false positives and negatives, relative to the sputum cultures. All data was converted to binary values and totals and percentages per category were calculated. Patient CD4 counts were stratified in two different ways to test the QFT results in terms of sensitivity and accuracy/specificity respectively. Firstly, as values < = or > than 100, the same for 150, 200, 250

and 300 and secondly, as values, < = 100, between 100 and 150, 150 - 200, 200 – 250, 250 – 300 and > 300. After cross tabulation, Fischer exact test comparisons of the QFT results with the first set of ranges, or, in the second set of ranges CD4-value stratified Mantel-Haenzel tests comparing the sputum culture with the QFT results. Multivariate and univariate logistical analyses were conducted to examine if age, gender or prior ARV exposure were covariates determining the outcomes of the QFT tests.

3.4 RESULTS AND DISCUSSION

In this study 60 HIV positive volunteers were consecutively recruited to evaluate the use of the QFT-TB GOLD ELISA assay at the ARV clinic, Tshwane District Hospital. The number of patients presenting with positive, negative and indeterminate QFT values relative to the sputum culture results were as presented in Table 3.1. The observed sensitivity and specificity of the QFT-TB GOLD assay in the ARV clinic were 60% (9/24) and 59% (16/27) respectively when compared to the gold standard culture results as indicated in Table 3.1. Twenty-five percent (15/60) culture confirmed TB cases were reported as a QFT negative test result and 18% (11/60) of QFT positive results were negative for culture. However, when all indeterminate QFT results were called negative, the sensitivity decreased significantly to 30% (9/30) with a slight increase noted in the specificity, 63% (19/30). The rates of indeterminate QFT test results were 15% (9/60) and supported the high rate of indeterminate results (11%) revealed by a study by Miranda and colleagues in 2009 (Miranda *et al.*, 2009). ROC curves demonstrated that the manufacturer's assignment of indeterminate values to negatives was justified in each case. Interestingly, a Fischer's exact test comparing sputum culture to the QFT results demonstrated that the relative proportions in the two groups were similar, despite there being 30 positive and negative cultures and 20 positive and 40 negative QFT results. After stratification of all data by CD4 range it was evident that the majority of patients in this study fell into the low CD4 count categories (Table 3.2).

Regarding the sensitivity of the test, Fischer exact test comparisons of the QFT results demonstrated significant differences at < = or > 100 (0.05), < = or > 150 (0.02), but not at < = or > 200 (0.11), < = or > 250 (0.7) or < = or > 300 (1.0). This suggested that the likelihood of a

diagnostic hit (sensitivity), of the QFT assay was determined by a limiting CD4 value lying between 150 and 200. That is, it was more likely that the QFT assay would produce a result of any kind above 200 than below. Unfortunately, a more accurate resolution of this limit was impossible in that only 5 patients had CD4 counts in this range. Since, these diagnostic hits were independent of the results of the sputum culture method. These results say nothing of the accuracy/specificity of the QFT assay.

In terms of the accuracy/specificity, examination of the raw data demonstrated that the majority of diagnostic errors by the QFT assay were found in the lower ranges (Table 3.2 and Figure 3.1). Mantel-Haenzel tests were used to compare the QFT results with the culture sputum results after stratifying the data on CD4 $<=$ 100, 100 – 150, 150 – 200, 200 – 250, 250 – 300 and above 300 ranges. No significant differences were found, suggesting that the QFT test was inaccurate relative to the culture method independently of the CD4 range. In fact, the overall accuracy of the QFT assay was only 46.7 % (Table 3.2). These results would seem to suggest that the QFT assay indicated the presence of the TB antibody rather than active disease. Following binary coding, a comparison of the QFT results individually or in combination of the following independent variables, gender, age ($<=$ or $>$ 40) and prior exposure to ARV, revealed no significant relationships in any circumstance. This was taken as an indication that these variables did not impact the QFT results. Persistent bacteria, even in low numbers, might keep T-cells activated and cause detectable IFN-γ responses (Pai *et al*., 2007). Thus, longer treatment and follow-up may be necessary to completely demonstrate declining T-cell responses (Pai *et al*., 2007).

Additional studies are needed to understand the reproducibility and relative accuracy of the test before its utility in prospective screening programmes can be defined (Perry *et al*., 2008). This study had two limitations. First, the numbers of events were small. A more robust study needs to be conducted within the setting. No HIV negative, TB positive patients were included and thus the real effect of immune suppression could not be determined. In a resource poor country, such as South Africa, the cost-effectiveness of the QFT assay is most likely low and until there is more solid data available about the use of QFT in a high prevalence TB/HIV setting, clinicians should rely on a careful medical history as well as culture results.

3.5 CONCLUSIONS

Despite the limitations, our data offer some interesting insights for the use of the QuantiFERON-TB GOLD ELISA assay. In the high HIV/TB prevalence setting in this study, the QFT test had limited sensitivity and an inability to distinguish between acute and latent infection. This would suggest that in terms of the routine diagnosis of TB by the clinician in a high HIV prevalence setting the test results should be used with caution. It may be advantageous to detect anti-ESAT-6/CFP10 specific T-cells in blood from individuals with TB infection without the need for IFN production but further investigation is necessary to prove the significance.

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Table 3.1 A comparison between the culture and QFT results and an overview of the positive, negative and indeterminate outcomes of the assay

		Sputum culture		
		Positive	**Negative**	**Total**
QuantiFERON	**Positive**	9	11	20
	Negative	15	16	31
	Indeterminate	6	3	9
	Total	30	30	**60**

Table 3.2 Percentage sensitivity, error and specificity/accuracy of the QFT test relative to the sputum culture method

		CD 4 ranges						
		≤ 100	≤ 150	≤ 200	≤ 250	≤ 300	>300	TOTAL
N	patients	51.7(31)	11.7(7)	8.3(5)	11.7(7)	3.3(2)	13.3(8)	100(60)
sensitivity	all negatives	62.5(25)	12.5(5)	5.0(2)	2.5(1)	5.0(2)	12.5(5)	100(40)
	all positives	30(6)	10(2)	15(3)	30(6)	0(0)	15(3)	100(20)
	neg ≤ > CD4: 200			80(32)	20(8)			
	pos ≤ > CD4: 200			55(11)	45(9)			
error	false negatives	52.4(11)	19(4)	9.5(2)	0(0)	9.5(2)	9.5(2)	100(21)
	false positives	36.4(4)	18.2(2)	9.1(1)	18.2(2)	0(0)	18.2(2)	100(11)
	neg ≤ > CD4: 200			81.0(17)	19.0(4)			
	pos ≤ > CD4: 200			63.6(7)	36.4(4)			
	total	46.9(15)	18.8(6)	9.4(3)	6.3(2)	6.3(2)	12.5(4)	100(32)
	total % of N	48.8	85.7	60	28.6	100	50	53.3
specificity/ accuracy	true negatives	73.7(14)	5.3(1)	0(0)	5.3 (1)	0 (0)	15.8(3)	100(19)
	true positives	22.2(2)	0(0)	22.2(2)	44.4(4)	0(0)	11.1(1)	100(9)
	neg ≤ > CD4: 200			78.9(15)	21.1(4)			
	pos ≤ > CD4: 200			44.4(4)	55.6(5)			
	total	57.1(16)	3.6(1)	7.1(2)	17.9(5)	0(0)	17.9(4)	100(28)
	total % of N	51.6	14.3	40	71.4	0	50	46.7

Note: Values given as: % (actual)

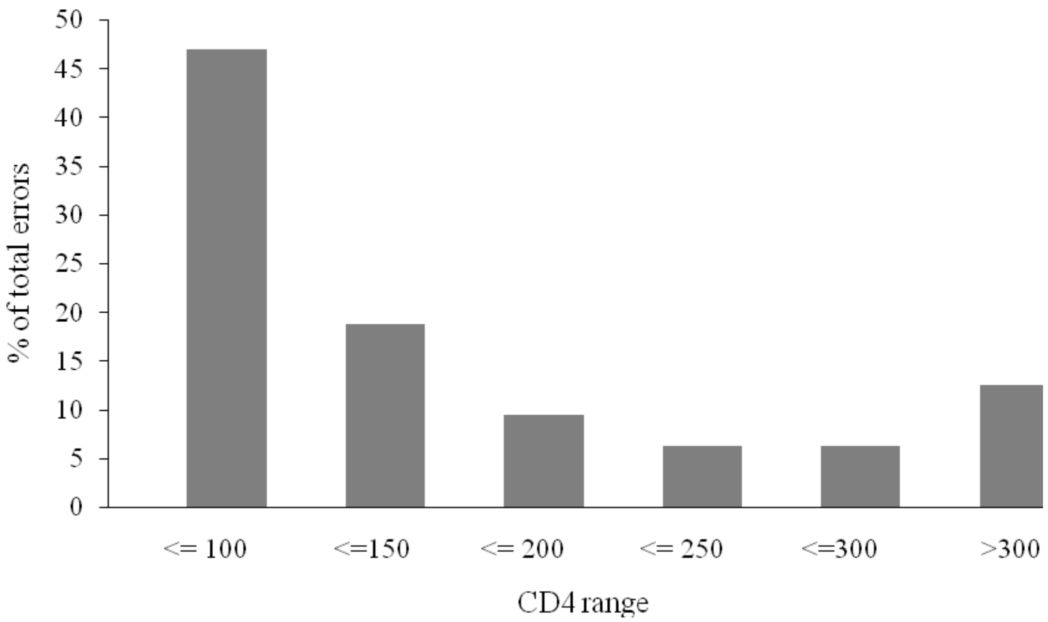

Figure 3.1 Percentage incidences of QFT diagnostic errors relative to the sputum culture method stratified by CD4 value range

CHAPTER 4

THE USE OF REAL-TIME PCR ASSAYS FOR THE DETECTION, IDENTIFICATION AND DRUG SUSCEPTIBILITY PATTERNS OF *MYCOBACTERIUM TUBERCULOSIS* IN SPUTUM AND BLOOD SPECIMENS OF HIV POSITIVE PATIENTS

The editorial style of FEMS Immunology and Medical Journal was followed in this chapter

4.1 Abstract

Rapid detection of *Mycobacterium* spp are essential, since patients are often infected with *Mycobacterium* spp other than *M. tuberculosis,* such as *M. avium* and *M. kansasii*. This study evaluated the use of real-time PCR assay for the detection of *Mycobacterium* spp to identify the *Mycobacterium* spp as well as to determine the prevalence of INH and RIF resistance genes. The real-time PCR assay identified 28% (17/60) *M. tuberculosis*, 2% (1/60) *M. kansasii* and 70% (42/60) of the isolates *Mycobacterium* spp negative. No *M. avium* were detected. The 17 *M. tuberculosis* positive specimens were further used to detect INH and RIF resistance genes. All 17 specimens had either no mutation or one or more mutations at the specific gene targets (*rpo*1, *rpo*2, *kat*G and *inh*A).

Keywords: Tuberculosis, real-time PCR, HIV positive patients

4.2 BACKGROUND

Control of TB depends mainly on rapid and accurate diagnosis and management of the disease (Aragón *et al*., 2006). Rapid detection of the *M. tuberculosis* complex is also essential in patients co-infected with HIV, as they are 50 times more prone to develop TB infection than non-HIV-infected people (Beqaj *et al*., 2007). Immunocompromised patients are often infected with *Mycobacterium* spp other than *M. tuberculosis*, such as *M. avium*, complicating identification or differentiation of the spp (Beqaj *et al*., 2007).

Bacteriological culture, with a specificity of over 98%, is considered the diagnostic gold standard and can identify the mycobacteria in over 80% of TB cases (Jafari & Lange, 2008). As few as 10 to 100 viable bacilli per ml may be detected but unfortunately the sensitivity of culture varies substantially depending on the specimen-processing method (Heymann, 2006). Although culture is routinely used for identification, culture is more expensive and requires more highly trained personnel compared to smear microscopy (Heymann, 2006).

Direct detection of tubercle bacilli in clinical specimens can be done by molecular techniques (Timbury *et al*., 2002). Molecular assays currently seem to offer numerous advantages, such as specificity and sensitivity comparable to culture (Kim, 2005). Real-time PCR assays (Roche Diagnostics, LightCycler® FastStart DNA Master^{PLUS} HybProbe kit) and specifically designed probes as described by Torres and colleagues (2000) have provided major contributions for the rapid and accurate identification of *Mycobacterium* spp (Lim *et al*., 2008). Real-time PCR, also called quantitative real-time PCR, can be used to simultaneously quantify and amplify a specific part of a given target DNA or RNA (Grassi *et al*., 2006).

Antibiotic resistance is a growing obstacle in the control of infectious diseases worldwide (Agdamag *et al*., 2003). The emergence of MDR-TB strains of *M. tuberculosis* poses a serious problem for the detection and diagnosis of tuberculosis (Agdamag *et al*., 2003). Irrational antibiotic use, poor-quality anti-TB drugs and the HIV pandemic have contributed to the increasing incidence of drug resistant TB (Barnard *et al*., 2008).

The diagnosis of MDR and XDR-TB is based on mycobacterial culture and drug susceptibility testing (DST), with results available in weeks to months (Barnard *et al*., 2008). Culture and DST capacity is severely limited and in response to the growing problem of MDR-TB, the WHO has issued a call for major expansion of culture and DST competence (WHO, 2006). Increased understanding of the genetic mechanisms of *M. tuberculosis* drug resistance together with established diagnostic methods, such as the DST, would allow the development of more rapid molecular methods (Cheng *et al*., 2007). Molecular tests have brought opportunities for the rapid diagnosis of TB and can be incorporated into control programmes for TB (Cho, 2007). The majority of drug resistance is due to mutations in genes encoding drug targets making it possible to develop molecular tests to identify gene mutations associated with the drug resistance (Cho, 2007). Mutations develop spontaneously due to the natural mutation rate of genomic DNA (Riska *et al*., 2000).

Mycobacterium tuberculosis drug resistance is mainly due to mutations in genes encoding drug target or drug converting enzymes: *rpo*B, *kat*G and *inh*A (Espasa *et al*., 2005). The molecular mechanism of INH resistance involves a variety of additional mutations at the *ahp*C-*oxy*R intergenic region complicating the identification of the resistance patterns (Aragón *et al*., 2006). Mutations associated with INH resistant strains contain AGC to ACC point mutations in codon 315 of the *kat*G gene (Ahmad & Mokaddas, 2004; Ruiz *et al*., 2004). The *kat*G gene encodes catalase-peroxidase activity and complete deletion of the catalase-peroxidase gene has been shown to cause a high level (60 to 70%) of INH resistance (Evans *et al*., 2009). The primary target of activated INH is a nicotinamide adenine dinucleotide (NADH)-dependent enoyl-acyl carrier protein reductase, designated *inh*A (Evans *et al*., 2009). Activated INH binds to the *inh*A-NADH complex and forms a ternary complex that results in the inhibition of mycolic acid biosynthesis (Johnson *et al.,* 2006). Frequently reported mutations in clinical isolates, such as the *inh*A promoter regions, are present at positions -8(T-G/A), -15(C-T) or -16(A-G) and -24(G-C) resulting in the conversion of a serine to an alanine residue at amino acid 94 (Johnson *et al*., 2006). Strains with mutations in the structural gene *inh*A have been less commonly described but are associated with not only INH but ethambutol (ETH) resistance as well (Morlock *et al*., 2003). Furthermore, there are at least 18 alternative genes that have been implicated in the mechanism of resistance against INH (Aragón *et al*., 2006).

Rifampicin resistant strains have a mutation confined to a short 81 bp DNA, which encodes for the β-subunit of the RNA polymerase and 95% of all missense mutations are located in a 51 bp core region of the *rpo*B gene between codons 507 to 533 (Zhang *et al*., 2007; Evans *et al*., 2009). These changes occur in more than 70% of RIF resistant isolates with a high level of RIF associated with mutations in codon 526 and 531 (Evans *et al*., 2009). The introduction of rapid molecular testing for all acid-fast bacilli (AFB) positive cases for routine diagnostics has the potential to rapidly treat and in the process prevent the spread of MDR-TB (O'Riordan *et al.*, 2008).

The first aim of this study was to evaluate the use of a real-time PCR assay to rapidly detect and identify *M. tuberculosis* strains from sputum and blood specimens. Secondly real-time PCR assays were used to determine the prevalence of INH and RIF resistance genes obtained from *M. tuberculosis* specimens from HIV positive patients in the Pretoria region.

4.3 METHODS

4.3.1 Sample analysis

The study was conducted at the ARV clinic at Tshwane District Hospital in Gauteng (South Africa). A total of 60 patients HIV positive patients were selected over a six month period (October 2008 to March 2009) and recruited for this study. This study was approved by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria (45/2008). Human immunodeficiency virus positive patients qualifying for ARV treatment according to the South African National HIV Treatment Guidelines (CD4 counts <200 or WHO Stage 4 disease), older than 18 years of age, attending the ARV clinic, who has given informed consent and clinically presenting with TB symptoms were included in this study.

A symptom-screening questionnaire was completed followed by a physical examination for active TB. The presence of any one of the following: coughing more than two weeks, chest pain, recent weight loss, night sweats, fever, swelling of lymph nodes, generalised tiredness and not currently on anti-TB drugs, formed the inclusion criteria. Both blood and sputum specimens were collected and transported immediately to the research division at the Department of

Medical Microbiology, University of Pretoria. All patients were allocated a unique number and were not linked to any patient information.

4.3.2 Manual DNA extraction of sputum specimens

After routine diagnostic analysis by the Diagnostic Division of the National Health Laboratory Service (NHLS), University of Pretoria, the remainder of the sputum specimens was analysed using real-time PCR assays at the Research Division of the Department of Medical Microbiology. The DNA from the undigested sputum specimens was manually extracted according to the FDA approved protocol (Protocol 1: Specimen preparation) supplied by Roche Diagnostics (Roche Applied Science, South Africa). Briefly: 1.5 ml screw-cap tubes (Plastpro Diagnostics, Johannesburg) were labeled and 500 µl of wash solution (RW) (Roche Applied Science, South Africa) were added to each tube. After the RW solution (Roche Applied Science, South Africa) were added, 100 µl of the sputum specimen was added and vortexed (Lasec, Cape Town) for 5 s. The specimens were centrifuged (Allegra X-15 R, Beckman Coulter) at 12 500 *x g* for 10 min and the supernatant was removed. A volume of 100 µl lysis reagent (RL) (Roche Applied Science, South Africa) was added to the pellet and vortexed (Lasec, Cape Town) for 5 s. The specimens and controls were incubated in a 60ºC +/- 2ºC dry heat block (Grant Instruments, Cambridge) for 45 min. Specimens were pulse-centrifuged (Allegra X-15 R, Beckman Coulter) and 100 µl neutralisation reagent (RN) (Roche Applied Science, South Africa) was added. Specimens were vortexed (Lasec, Cape Town) for 5 s at half speed. Extracted DNA was stored at -70ºC until further analysis.

4.3.3 Automated DNA extraction of blood specimens

The DNA_Blood_100_400 protocol (MagNaPure LC Compact) of the MagNA Pure Compact Nucleic Acid isolation Kit 1, Large Volume (Roche Applied Science, South Africa) was used for the automated extraction of DNA. A total of 400 µl of each of the direct blood specimens were used for the automated DNA extraction. The DNA was eluted in a final volume of 100 µl of elution buffer (Roche Applied Science, South Africa). Extracted DNA was stored at -70ºC for further analysis.

The following pre-treatment extraction kits were evaluated to optimise mycobacterial DNA yield from blood. Both the *Mol*ysis Basic sample preparation kit (ProGen, South Africa) and the QIAamp® DNA Mini Blood assay (QIAGEN, Germany) were used on direct blood specimens to remove human DNA before extracting the bacterial DNA using the DNA_Blood_100_400 protocol (MagNaPure LC Compact) of the MagNA Pure Compact Nucleic Acid isolation Kit 1, Large Volume (Roche Applied Science, South Africa). The assays were followed according to the manufacturer's protocol.

4.3.4 Real-time PCR for the detection and identification of *Mycobacterium* spp in sputum and blood specimens

DNA extracted by manual (sputum specimens) and automated (blood specimens) methods were used for the detection and identification of the *Mycobacterium* spp with the use of a real-time PCR assay (LightCycler TB Kit®, Roche Applied Science, South Africa). The oligonucleotide primers and hybridisation probes of this real-time PCR targeted amplification detected a portion of the 16S rRNA gene that included the hypervariable region A. A PCR master mix was prepared as described in Table 4.1.

The protocol is designed for a final reaction volume of 20 µl of which 5 µl of DNA template was added. The real-time PCR assay was performed on a Roche LightCycler version 2.0 (Roche Applied Science, South Africa) according to the protocol indicated in Table 4.2 (LightCycler® 2.0 System protocol). A positive and negative control was included in each run. The resulting T_m indicated the specific *Mycobacterium* spp present in each specimen. The real-time PCR assay was used to identify the following species; *M. tuberculosis* (T_m = 55-57ºC preliminary), *M. kansasii* (T_m = 59-62ºC preliminary) and *M. avium* (T_m = 50-53ºC preliminary).

4.3.5 Real-time PCR for the detection of INH and RIF antibiotic resistance of *M. tuberculosis* strains using the LightCycler® FastStart DNA Master^{PLUS} HybProbe kit (Roche Applied Science, South Africa)

Only DNA of *M. tuberculosis* positive specimens was used in these real-time PCR assays. Specific primers and probes (Roche Applied Science, South Africa) were used, according to a study conducted by Torres and colleagues (2000) for the detection of the mutations in the following genes; the *inh*A and *kat*G gene for INH and the *rpo*B gene for RIF (Table 4.3). Two pair of probes (*rpo*1 and *rpo*2) was simultaneously used to detect the most frequent mutations (Torres *et al*., 2000). The Roche LightCycler version 2.0 (Roche Applied Science, South Africa) was used to perform these assays.

The LightCycler® FastStart DNA Master^{PLUS} HybProbe kit (Roche Applied Science, South Africa) was used for the detection of INH and RIF resistance. The PCR master mix for the detection of INH and RIF resistance of *Mycobacterium* spp was prepared as indicated in Table 4.4. The LightCycler® (Roche Applied Science, South Africa) programme started with a pre-incubation step necessary for the activation of the FastStart DNA Master^{PLUS} HybProbe polymerase (Roche Applied Science, South Africa). Amplification, melting curve analysis and cooling steps followed as indicated in Table 4.5.

4.4 RESULTS AND DISCUSSION

The results of the real-time PCR assays for the detection and identification as well as the resistance patterns of the *M. tuberculosis* positive strains were available on the same day of collection. The DNA extraction procedure required only 1 h, while detection and identification of the *Mycobacterium* spp were completed within 2 to 4 h with an additional 2 to 4 h each for the detection of the INH and RIF resistant strains. The manual extraction of sputum specimens using the Specimen preparation kit (Roche Diagnostics). The internal control results from the real-time PCR showed no inhibition proving the extraction procedures removed any possible inhibitors or that the specimens did not contain any inhibitors.

The LightCycler optical device is capable of measuring fluorescence in two separate channels simultaneously (LCRed640 and LCRed705 fluorophores), which made it possible to detect and identify more than one *Mycobacterium* spp present in one specimen and detect different mutations in one single tube. The resulting T_m (Figure 4.1) indicated the specific *Mycobacterium* spp present in each of the positive isolates from the sputum specimens. The real-time PCR assay identified 28% (17/60) *M. tuberculosis* (T_m = 55-57ºC preliminary), 2% (1/60) *M. kansasii* (T_m = 59 to 62ºC preliminary) and 70% (42/60) of the isolates *Mycobacterium* spp negative.

Amplification and detection of the DNA obtained from blood specimens (using the MagNAPure Compact Nucleic Acid isolation Kit, the *Mol*ysis Basic and QIAamp® DNA Mini Blood assay) indicated no *Mycobacterium* spp positive results as shown in Figure 4.2. No *M. avium*, which was detected in blood of patients suspected of miliary TB were detected. The run was valid since the internal control (LCRed705/Back 530) was positive indicating no inhibitors in the specimen and the positive and negative controls were according to the manufacturer's protocol.

Speculation for negative results were that extra-pulmonary or miliary TB is not that common in HIV positive patients, the extraction method did not work or the levels of DNA were to low to be detected with this specific assay. The TB infection could also have disseminated to specific sites and not be present in the blood. Pre-treatments were performed on the blood specimens to verify that proper procedures were followed and to see if the extraction method could not be improved. However, these pre-treatments did not show any change or improvement in the results. The sputum extractions have been performed directly on the sputum specimen without any decontamination steps as performed routinely in a diagnostic lab and this also could have had led to false negative PCR results.

The sensitivity and specificity of the real-time PCR was determined in comparison with the gold standard culture results from the diagnostic department (NHLS) indicated in Table 4.6. A sensitivity of 76.4% and a specificity of 86.6% were reached using the real-time PCR assay. The DNA of the *M. tuberculosis* positive specimens were further used to detect the resistance genes of INH and RIF. Rifampicin resistance was determined first using the appropriate primers and probes as indicated in Table 4.3 as described by Torres and colleagues (2000). A mutation at a

gene was indicated by a specific increase or decrease in the T_m of the susceptible strain (Torres *et al*., 2000). The T_m for the susceptible strain (wild type) was 64.3˚C and an increase of 2˚C changed the strain from wild type to mutant (TCG to GAC) at codon 531 (Torres *et al*., 2000). A drop in the temperature with more that 6˚C resulted in a mutation at codon 526 (Torres *et al*., 2000). The multiplex PCR assay for the detection of RIF resistance showed no mutation at the *rpo1* gene. However, at the *rpo2* gene all the specimens were positive for a mutation at codon 518 as a result of a drop (in more than 3˚C) in the temperature from the wild type (70.1˚C). Only one specimen showed no mutations at either genes *rpo1* or *rpo2*. The alterations in T_m for a number of the specimens are shown in Figure 4.3.

The TB sensor and TB anchor probes (Torres *et al*., 2000) were used to determine the mutation patterns of the *kat*G gene of INH resistance (Figure 4.4). The T_m for the susceptible strain was 72.8˚C. The change from wild type to mutant (AGC to ACC) at codon 315, resulted in a more than 3˚C decrease. A decrease of 5˚C in the T_m was found in the strains harboring a different mutation at codon 315 (AGC to AAC). An alarming 82% (14/17) of the isolates had a mutation at codon 315 (AGC to AAC) with only 18% (3/17) of the isolates showing no mutation. Another set of primers and probes were used in another PCR assay to determine INH resistance using the *inh* anchor and *inh* sensor (Torres *et al*., 2000). The T_m of the wild type was 62.7˚C and an increase of 5˚C in the T_m indicated a nucleotide substitution at position 209 (C to T) (Torres *et al*., 2000). None of the isolates showed an increase in the T_m value and the results showing the mutations were indicated in Table 4.7.

The emergence of drug-resistant strains of *M. tuberculosis* is an increasing problem for communities and TB control programmes in developing countries, such as South Africa (Torres *et al*., 2000). Control of TB depends mainly on rapid and accurate diagnosis and management of the disease (Aragón *et al*., 2006). Bacteriological culture is still considered the diagnostic gold standard and can identify the mycobacteria in over 80%, with a specificity of over 98% (Jafari & Lange, 2008). Molecular technologies, such as PCR, have improved and real-time-based PCR assay showed numerous advantages such as specificity and sensitivity comparable to the gold standard methods, such as culture and DST for detection, identification and resistance testing (Kim, 2005). Real-time PCR assays can be applied for the detection of mutations confined in

short base pairs (bp) deoxyribonucleic acid (DNA) regions (Aragón *et al*., 2006). The main advantage with the use of real-time PCR is the additional application for the detection of INH and RIF resistance within 48 to 72 h after sample collection (Cho, 2007).

Additional studies are needed to understand the reproducibility and relative accuracy of the direct identification and detection of sputum and blood specimens using real-time PCR assays before its utility in prospective screening programmes can be implemented. Alternative blood extraction methods should be investigated for the removal of the genomic DNA before the detection of *M. avium* from blood. One of the limitations of this study was the small number of events and a larger study needs to be performed in these settings. Secondly, no HIV negative, TB positive patients were included. Since there are currently no solid data available on the use of real-time PCR assays for the detection and identification of *Mycobacterium* spp and the resistance patterns of *M. tuberculosis* positive strains in a resource poor and a high prevalence HIV-TB country, such as South Africa, studies like these are of great importance.

4.5 CONCLUSIONS

The detection, identification and determination of INH and RIF resistance patterns of *M. tuberculosis* using the conventional culture and DST methods are extensive and laborious when compared to real-time PCR. The real-time PCR assays used in this study shorten the analysis period of the detection, identification and determination of INH and RIF resistance genes to same-day results. Novel and rapid techniques are drastically needed to control the TB crisis not only in South Africa but in all high HIV-TB prevalent settings. Despite the limitations of this study, the data is in agreement with the findings of the study conducted by Torres and colleagues (2000) for the diagnostic use of real-time PCR assays to detect, identify and determine resistance patterns of *M. tuberculosis* positive strains. Decreasing the waiting period for patients to commence with the correct TB treatment and preventing the spread of MDR-TB strains in the community are of greatest importance.

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TABLES

Table 4.1 **The PCR master mix prepared for the identification of *Mycobacterium* spp in sputum and blood specimens (LightCycler TB Kit®, Roche Applied Science, South Africa) for the LightCycler® 2.0**

Component	Volume per well
Water, PCR-grade (vial 2, colourless cap)	9 µl
Primer/Probes, 10x conc.	2 µl
Master Mix 5x conc. (vial 1)	4 µl
Final volume	**15 µl**

Table 4.2 **The real-time PCR parameters for the identification of *Mycobacterium* spp in sputum and blood specimens (LightCycler TB Kit®, Roche Applied Science, South Africa) for the LightCycler® 2.0**

	Temperature	Time	Ramp rate	Acquisition Mode
Denaturation	95ºC	60 s	20 ºC/s	None
Amplification	95 ºC	10 s	20ºC/s	None
	50 ºC	10 s	20 ºC/s	Single
	72 ºC	20 s	20 ºC/s	None
Melting Curve	95 ºC	60 s	20 ºC/s	None
	40 ºC	120 s	20 ºC/s	None
	68 ºC	0 s	0.1ºC/s	Continuous
Cooling	40 ºC	30 s	20 ºC/s	None

Table 4.3 **Primers and probes used for the detection of INH and RIF resistance using the LightCycler® FastStart DNA Master^{PLUS} HybProbe kit (LightCycler® 2.0 System protocol, Roche Applied Science, South Africa) (Torres *et al*., 2000)**

Antibiotic	Primer	Sequence	T_m
Isoniazid (*inh*A gene)	TB 86 primer	5'-GAA ACA GCG GCG CTG ATC GT-3'	63ºC
	TB 87 primer	5'-GTT GTC CCA TTT CGT CGG GG-3'	63ºC
	TB anchor probe	5'-CGT ATG GCA CCG GAA CCG GTA AGG ACG C-Fluo-3'	76ºC
	TB sensor probe	5'-LCRed-640TCA CCA GCG GCA TCG AGG TCG T-Pho-3'	68ºC
Isoniazid (*kat*G gene)	TB 92 primer	5'-CCT CGC TGC CCA GAA AGG GA-3'	65ºC
	TB 93 primer	5'-ATC CCC CGG TTT CCT CCG GT-3'	65ºC
	inh anchor probe	5'-CCC CTT CAG TGG CTG TTG GCC AGT C-Fluo-3'	72º
	inh sensor probe	5'-LCRed-640-CCC GAC AAC CTA TCA TCT CGC C-Pho-3'	66ºC
Rifampicin (*rpo*B gene)	TR 8 primer	5'-GTG CAC GTC GCG GAC CTC CA-3'	67.0ºC
	TR 9 primer	5'-TCG CCG CGA TCA AGG AGT-3'	58ºC
	rpo 1 anchor probe	5'-TTC ATG GAC CAG AAC AAC CCG CTG TCG GT-Fluo-3'	73ºC
	rpo 1 sensor probe	5'-LCRed-640-ACC CAC AAG CGC CGA CTG CTG G-Pho-3'	70ºC
	rpo 2 anchor probe	5'-GCT GAG CCA ATT CAT GGA CCA GAA CAA CC-Fluo-3'	72ºC
	rpo 2 sensor probe	5'LCRed-640-CTG TCG GGG TTG ACC CAC AGG CGC-Pho-3'	72ºC

Table 4.4 **The PCR master mix prepared for the detection of INH and RIF of *Mycobacterium* strains (LightCycler® 2.0 System protocol, Roche Applied Science, South Africa)**

Component	Volume per well
Water, PCR-grade (vial 2, colorless cap)	9 µl
Primer/Probes, 10x conc.	2 µl
Master Mix 5x conc. (vial 1)	4 µl
Final volume	**15 µl**

Table 4.5 **The PCR parameters for the detection of INH and RIF resistance of *M. tuberculosis* strains in sputum and blood specimens (LightCycler® 2.0 System protocol, Roche Applied Science, South Africa)**

Analysis Mode	Cycles	Segment	Target Temperature[1]	Hold Time	Acquisition Mode
Pre-incubation					
None	1		95ºC	10 min[4]	None
Amplification					
Quantification	45	Denaturation	95ºC	10 s	None
		Annealing	Primer dependent[2]	5-20 s[5]	Single
		Extension	72ºC[3]	=(amplicons {bp}/25) s	None
Melting curve analysis					
Melting curves	1	Denaturation	95ºC	0 s	None
		Annealing	HybProbe T_m – 5ºC	30 s	None
		Melting	95ºC Slope = 0.1ºC/s	0 s	Continuous
Cooling					
None	1		40ºC	30 s	None

1 Temperature transition rate/slope is 20ºC/s, except where indicated

2 If the primer annealing temperature is low (<55ºC/s), reduce the transition rate/slope to 2-5ºC/s

3 For initial experiments, set the target temperature (i.e., the primer annealing temperature) 5ºC below the calculated primer Tm and calculate the primer TM according to the following formula, based on the nucleotide content of the primer: Tm = 2ºC (A+T) + 4ºC (G+C)

Table 4.6 A comparison between the culture and sputum real-time PCR results and an overview of the positive and negative outcomes of the assay

Culture				
Real-time PCR		+	**-**	**Total**
	+	13	4	**17**
	-	17	26	**43**
	Total	**30**	**30**	**60**

Table 4.7 **The different mutations of each of the 17 *M. tuberculosis* positive specimens using the LightCycler® FastStart DNA Master[PLUS] HybProbe kit (LightCycler® 2.0 System protocol, Roche Applied Science, South Africa) (Torres *et al*., 2000)**

	RIF						**INH**			
	***rpo*1**			***rpo*2**			**TB anchor/sensor (*kat*G)**		**INH anchor/sensor (*inh*A)**	
Unique Number	**WT[1]**	**Codon 531**	**Codon 526**	**WT[1]**	**Codon 513**	**Codon 518**	**WT[1]**	**Codon 315**	**WT[1]**	**Codon 209**
-3						x		AGC-AAC[3]	x	
-6	x[2]					x		AGC-AAC	x	
2	x					x		AGC-AAC	x	
10	x			x				AGC-AAC	x	
13	x					x		AGC-AAC	x	
14	x					x		AGC-AAC	x	
17	x					x		AGC-AAC	x	
27	x					x		AGC-AAC	x	
63	x					x		AGC-AAC	x	
71	x					x	x		x	
73	x					x		AGC-AAC	x	
77	x					x		AGC-AAC	x	
82	x					x	x		x	
84	x					x	x		x	
87	x					x		AGC-AAC	x	
120	x					x		AGC-AAC	x	
123	x					x		AGC-AAC	x	

1 Wild type

2 A positive result for either a wild type or a mutation at the specific codon as indicated

3 Indicating the mutation occurring at codon 315

FIGURES

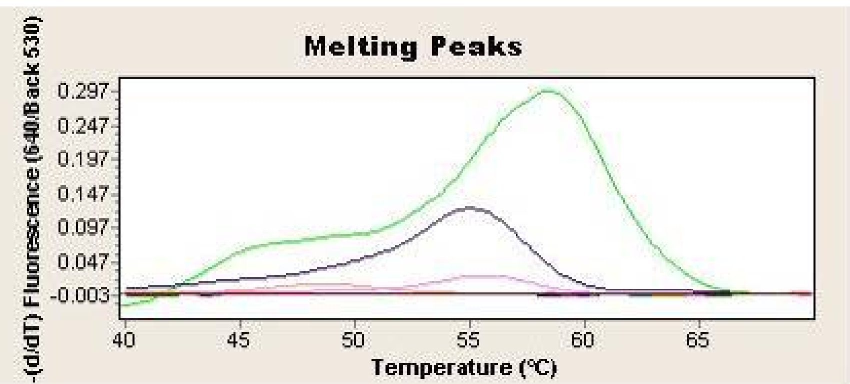

Figure 4.1 **Real-time PCR results for the identification of *Mycobacterium* spp in sputum specimens using the LightCycler TB Kit® (Roche Applied Science, South Africa) with the green line indicating the positive control (T_m = 59-62ºC), the dark purple line a *M. tuberculosis* (T_m = 55-57ºC) positive specimen and the light purple line as the negative control**

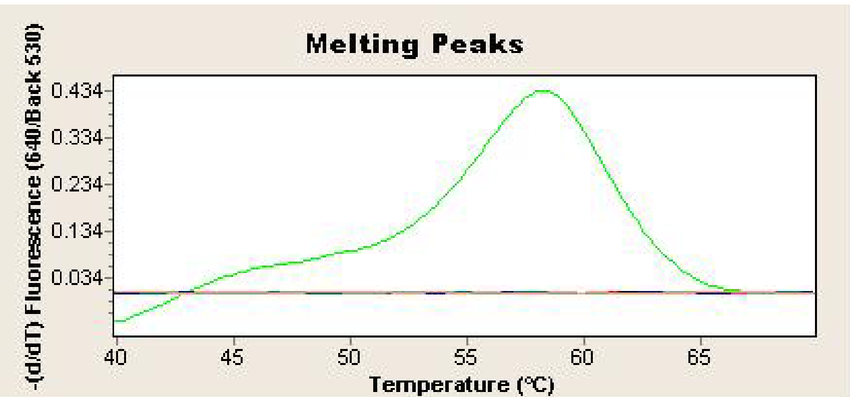

Figure 4.2 **Real-time PCR results for the identification of *Mycobacterium* spp in blood specimens using the LightCycler TB Kit® (Roche Applied Science, South Africa) with the green line indicating the positive control (T_m = 59-62ºC) and the purple line indicating a *M. tuberculosis* negative specimen showing no amplification**

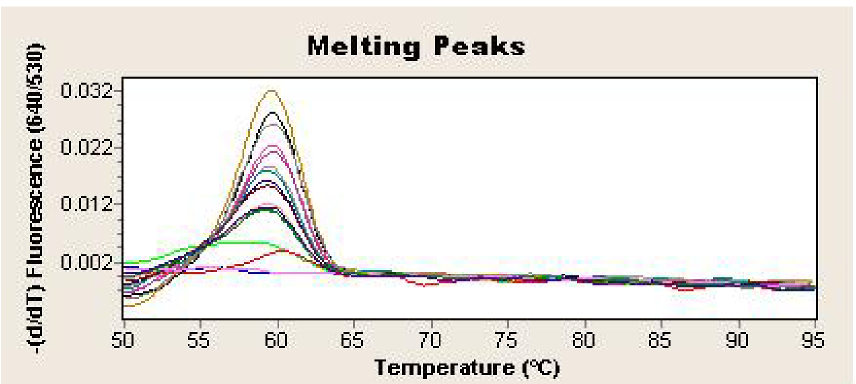

Figure 4.3 **Real-time PCR results for the detection of mutations at the *rpo2* gene using the LightCycler® FastStart DNA Master^{PLUS} HybProbe kit (Roche Applied Science, South Africa). A mutation at codon 518 resulted in a drop of 3˚C or more, in the temperature from the wild type (70.1˚C) indicating thus no mutations for these specimens at codon 518.**

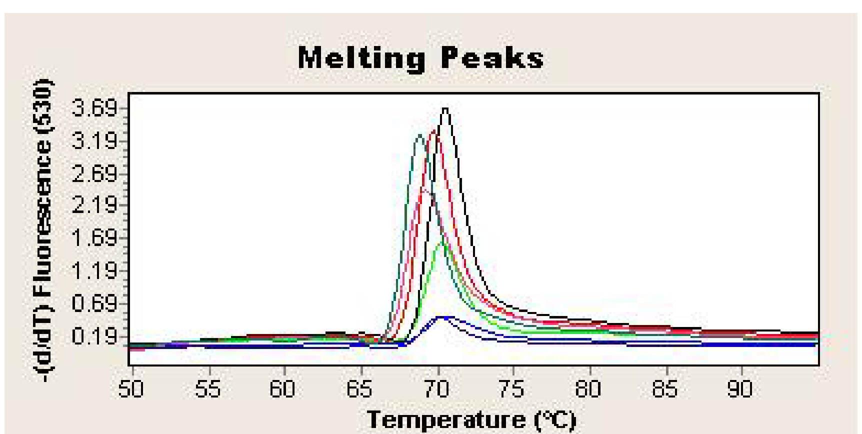

Figure 4.4 **Real-time PCR results using the TB sensor and TB anchor probes to detect the mutations that could lead to INH resistance (LightCycler® FastStart DNA Master^{PLUS} HybProbe kit; Roche Applied Science, South Africa). The T_m for the susceptible strain was 72.8˚C. The change from wild type to mutant (AGC to ACC) at codon 315, resulted in a more than 3˚C decrease. A decrease of 5˚C in the T_m was found in these six strains harboring a different mutation at codon 315 (AGC to AAC)**

CHAPTER 5

CONCLUDING REMARKS

5.1 CONCLUSIONS

More than 4 million people suffer from active tuberculosis (TB) in Africa resulting in an estimated 650 000 deaths per year (Kaufmann & Parida, 2008; Hermann *et al*., 2009). Between 1990 and 2006, TB prevalence in Africa rose from 1.7 million to 4.2 million active cases indicating a three-fold increase (Lawn *et al*., 2006; Kaufmann & Parida, 2008). Poor prognosis can be as a result of the human immunodeficiency virus (HIV) pandemic, immune reconstitution inflammatory syndrome (IRIS) in HIV-TB co-infected patients as well as the increasing incidence of drug resistant TB (Gupta *et al*., 2001).

The term multidrug resistant TB (MDR-TB) are given to patients, which are resistant to INH and RIF and further resistance to any fluoroquinolone and at least one of the three injectable second-line drugs capreomycin, kanamycin and amikacin are termed extensively drug resistant TB (XDR-TB) (Barnard *et al*., 2008; Jassal & Bishai, 2009). Unfortunately, many of these patients will die because of the poor or slow diagnosis and the ineffective treatment and management of the disease (Aragón *et al*., 2006; Noble, 2006).

Diagnosis of TB still depends on microscopy combined with culturing of mycobacteria (Noble, 2006). Although microscopy is cheap and rapid, almost 60% of all TB cases are falsely reported as sputum smear negative (Kaufmann & Parida, 2008). Microscopy has a low sensitivity (46%) and specificity (90%) in immunocompromised patients, such as HIV positive patients because of the non-productive cough (Farnia *et al*., 2002). Bacteriological culture of mycobacteria, which takes 4 to 6 weeks, are also necessary before drug susceptibility can be determined by means of the standard agar proportion method (Kim, 2005). Culture remains the diagnostic gold standard and can identify mycobacteria in over 80% of the cases, with a high specificity of 98% (Jafari & Lange, 2008).

Molecular-based technology has enabled development of new promising diagnostic techniques, such as the use of real-time-based PCR assays for the detection of *M. tuberculosis* (Kim, 2005).

Real-time PCR assays can be applied for identification, characterisation as well as the detection of mutations leading to resistance to TB drugs within 48 to 72 h (Aragón *et al*., 2006; Cho, 2007). Urgent research concerning improved diagnostic assays for TB in HIV positive patients is therefore crucial.

Serological assays have so far failed to provide sensitive and specific results as screening tools in patients with TB infection (Heymann, 2006). Environmental mycobacteria especially, leads to false-positive results making the results unreliable and can thus not be used by a clinician to make a rapid diagnosis and commence with treatment (Heymann, 2006). Unfortunately, the detection and identification of *M. tuberculosis* still relies on conventional methods; however, a number of assays are currently being investigated (Heymann, 2006).

The QuantiFERON-TB GOLD ELISA assay was used to detect TB infection in patients attending the ARV clinic at Tshwane District Hospital. The observed sensitivity and specificity of the QuantiFERON-TB GOLD ELISA assay, when all indeterminates were excluded, were 38% (9/24) and 59% (16/27) respectively compared to the automated culture as gold standard. When all indeterminate QuantiFERON results were called negative, the sensitivity decreased significantly to 30% (9/30) with a slight increase noted in the specificity, 63% (19/30). This is in agreement with results of similar studies confirming the association between different CD4 counts (Heymann, 2006).

The molecular detection and identification of mycobacteria consists of extraction procedures followed by real-time PCR. The manual extraction of *Mycobacterium tuberculosis* from sputum specimens using the Specimen preparation kit (Roche Diagnostics) was an uncomplicated method requiring only 1 h. The DNA obtained from blood specimens (using the MagNAPure Compact Nucleic Acid isolation Kit) indicated no *Mycobacterium* spp positive results. This might be because *Mycobacterium avium*, which was expected to be present in HIV positive patients, was not as prevalent, the extraction was not sensitive enough or the DNA concentration in the specimens was to low. Pre-treatment assays to extract DNA from low numbers of bacteria present (*Mol*ysis Basic and QIAamp® DNA Mini Blood assay) were performed on the blood specimens to optimise the blood extraction method. However, these pre-treatments did not show any change or improvement in the results.

Detection and identification of the *Mycobacterium* spp using the real-time PCR were completed within 2 to 4 h. The real-time PCR assay from direct sputum specimens identified 28% (17/60) *M. tuberculosis*, 2% (1/60) *M. kansasii* and 70% (42/60) of the isolates *Mycobacterium* spp negative. No *M. avium* was detected. A sensitivity of 76.4% and a specificity of 86.6% were obtained using the real-time PCR assay results compared to liquid culture. The prototype (Roche, South Africa) was used for the detection of *M. tuberculosis* directly from sputum specimens and proved useful. However, this prototype was improved and optimised and launched by Germany in May 2009. The PCR assay is being incorporated into laboratories in South Africa with an advantage of low cost of R80 per test compared to R170 for the Hain and R165 for conventional culturing (according to the National Health Laboratory Service).

The DNA of the 17 *M. tuberculosis* positive specimens were further analyzed to detect the resistance genes of RIF and INH. No mutation at the *rpo1* gene was detected in any of the 17 specimens. However, at the *rpo2* gene all 17 specimens were positive for a mutation at codon 518 and only one specimen showed no mutations at either genes *rpo1* or *rpo2*. Determining mutations at the *katG* gene, showed three wild type strains and 14 strains positive for a mutation at codon 315. None of the isolates showed any mutation in the *inhA* gene region.

Further studies regarding the cause of mutations as well as the effect of the mutation on the susceptibility would be useful, since the effect of the mutations found with this assay could not be determined. This study gave interesting new information regarding detection, identification and determining resistance patterns of isolates from patients attending the ARV clinic.

5.2 FUTURE RESEARCH

Future research in TB should be directed towards improvement of surveillance programmes, rapid diagnosis and faster detection of mutations in genes associated with resistance to drugs against TB. This will provide better service to patients and assist clinicians to ensure correct and rapid diagnosis and treatment. Novel assays should have a high sensitivity and high specificity, which would decrease MDR and XDR-TB cases because of better diagnosis. Assays, such as the SD BIOLINE TB Ag MPT64 assay are rapid assays but still require culturing of the specimen.

There is still no assay that could be used in a high TB-HIV prevalent setting, such as South Africa, to differentiate between latent and active TB infection. An assay that could overcome this problem would have an enormous impact on the surveillance programmes. A serological assay, such as the QuantiFERON-TB GOLD ELISA assay holds great promise but its usefulness in a high HIV positive setting is doubtful.

Current molecular techniques, such as the real-time PCR assay used to detect and identify *Mycobacterium* spp, are useful assays, since the assay can be performed directly from the sputum and no culture is necessary. Molecular assays are the future in diagnosing TB since it is more accurate, has a quicker turn-around time and has the advantage of being used to detect, identify and determining resistance patterns. However, there is currently no single ideal assay and future research must focus on combination assays and should still be compared to culture results.

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