

# CHAPTER 2

## Introduction

**A part of this chapter has been published in the *Journal of Cell and Tissue Research***

OLIVIER I., LOOTS, D.T. 2011. An overview of tuberculosis treatments and diagnostics. What role could metabolomics play? *J. Cell Tissue Res.* 11(1):2655-2671.

## 1. INTRODUCTION

In 2001, the World Health Organization (WHO) declared TB a global health emergency, as one third of the world's population suffered from latent TB infection. Furthermore, despite the availability of effective drugs to treat and cure this disease, an estimated 9.4 million cases of newly diagnosed TB were reported, and 1.8 million people died as a consequence of this in 2008 (WHO, 2010). One of the major contributors to this high incidence is the inability of conventional TB diagnostic and treatment approaches to control TB, especially in HIV-endemic countries (Perkins & Cunningham, 2007).

In the current chapter, we will describe the development and mechanisms of action of the currently used TB vaccines, diagnostic methods and treatment protocols, considering their advantages and drawbacks. We will also discuss a number of newer treatment and diagnostic approaches, and will additionally focus on the potential role that metabolomics could play as a new approach to TB research.

## 2. PATHOPHYSIOLOGY OF TUBERCULOSIS

TB is transmitted via aerosolized droplet nuclei, which are *M. tuberculosis* containing particles with a diameter of 1-5  $\mu\text{m}$ . These droplets are produced and expectorated by patients with pulmonary TB during talking, singing, sneezing, or coughing and may, due to their small particle size, stay airborne for many hours (Frieden *et al.*, 2003). These droplets can also be generated in clinics and hospitals during sputum induction, aerosol treatments, aerosolisation during bronchoscopy, or through tissue or sputum processing (ATC & CDC, 1999).

After inhalation by a previously uninfected individual, the infectious droplet nuclei lodge in the alveoli of the distal airways of the lung. Alveolar macrophages subsequently engulf these nuclei, which sets off a cascade of events leading to either a successful suppression of the infection or the progression to an active TB disease state (Glickman & Jacobs, 2001).

*M. tuberculosis* replicates slowly inside these macrophages and may spread to the hilar lymph nodes via the lymphatic system. In the majority of these infected individuals, a cell-mediated immune reaction develops within 2-8 weeks after infection. Consequently, necrotic, cheese-like granulomas, containing non-viable *M. tuberculosis*, are formed by activated T-lymphocytes and macrophages, which restrict the further replication and spread of the bacteria (Schluger & Rom, 1998). At the cellular level, *M. tuberculosis* infected macrophages interact with T-lymphocytes via a number of host cytokines. These activated T-lymphocytes subsequently release interferon- $\gamma$ , another class of cytokine, which indirectly stimulates phagocytosis of *M.*

*tuberculosis* inside the macrophage (Ellner, 1997). Interferon- $\gamma$  may also stimulate the release of tumor necrosis factor by these macrophages, which in turn, aid in granuloma formation and controls the extent of infection (Flynn *et al.*, 1993). In a host with a strong cell-mediated immunity, a latent infection can be maintained in this form, and active disease may never occur. Even though small amounts of viable *M. tuberculosis* may be present in these granulomas, infected individuals (without active disease) cannot transmit the organism and are, hence, not infectious during this latent phase (ATC & CDC, 1999).

Active disease manifests when the host's immune system can no longer restrain *M. tuberculosis* replication. Although this developing disease most frequently occurs in the parenchyma of the mid and lower lung, it may spread throughout the body and can present in almost any organ system. Several factors may trigger the switch from latent infection to active disease, with HIV co-infection being the single greatest risk factor for this. Other immuno-compromising conditions increasing the risk for developing active disease include: diabetes mellitus; chemotherapy; malnutrition; vitamin D or A deficiency; renal failure, and; extensive corticosteroid therapy (Frieden *et al.*, 2003).

Due to the insidious onset of TB, symptoms might only occur in the later disease stages. These clinical symptoms include: coughing; fever; night sweats; anorexia; weight loss; chest pain; hemoptysis, and; dyspnea (Lobue & Catanzaro, 1997). As inflammation and tissue necrosis develop, sputum, which is widely used as diagnostic material, is produced. Hemoptysis, or the coughing up of blood, is typically a result of preceding disease and does not automatically indicate active TB. Hemoptysis may manifest as a result of: remaining tuberculous bronchiectasis (irreversible dilation of part of the bronchial tree); breakage of a dilated vessel in a cavity wall; bacterial or fungal infections in a residual cavity, and / or; erosion of calcified lesions into the lumen of an airway. TB may eventually result in severe respiratory failure, but dyspnea is atypical, except in extensive disease states (ATC & CDC, 1999; Murray *et al.*, 1979). These symptoms alone are, however, rather non-specific and cannot be used for accurately diagnosing TB, as these correlate with many other lung infections or malignant conditions (Lobue & Catanzaro, 1997).

This strong, cell-mediated immune response, induced by TB infection, forms the basis by which the currently used TB vaccination procedures function.

### **3. TUBERCULOSIS VACCINATION**

The first TB vaccination and successful immunisation was carried out in 1921. This vaccine, the Bacille Calmette-Guerin or BCG, an avirulent *M. bovis* strain, was attenuated for 13 years by

serial passages into glycerol imbibed potato slices (Calmette, 1931). Due to the high demand, the original BCG strain was distributed globally, even before the establishment of an appropriate culture protocol. For this reason, numerous BCG strains, with a variety of antigenic and immunological differences, exist today in various parts of the world. Nevertheless, BCG is currently still the most commonly used TB vaccine worldwide, and BCG immunisation is mandatory in high-incidence TB areas (Delogu & Fadda, 2009). Using 1264 published articles, Colditz *et al.* (1994) performed a meta-analysis on the efficiency of the BCG vaccine. Their study concluded that, when vaccinated with BCG, the risk to develop TB is reduced by only 50%. The newer approaches used towards the development of TB vaccines, like BCG, are also based on the induction of a strong cell-mediated immune response, in order to control bacterial replication and maintain infection in the latent phase. Some of these attempts include: attenuated *M. tuberculosis* mutants; recombinant BCG strains; recombinant proteins, and; DNA vaccines. However, due to the complexity of these studies, it is expected that the results of phase III trials will only be available by 2014-2015 (Delogu & Fadda, 2009).

#### **4. TUBERCULOSIS TREATMENT**

One of the greatest discoveries of the 20<sup>th</sup> century was that of antimicrobial drugs, which have ever since saved millions of lives and are still the major element in the treatment of TB infection and disease. The first effective *M. tuberculosis* antimicrobial agent, streptomycin, was tested as part of the first randomised clinical trial carried out from 1944 until 1948. The publication of the positive outcome of this trial forever changed the international TB treatment policies (Medical Research Council, 1948).

Today many anti-TB drugs are available which can, based on their activity, be classified into 3 groups: 1) those with bactericidal activity; 2) those with sterilising activity; and 3) those preventing drug-resistance. Bactericidal activity is the capacity of a drug to reduce the amount of actively dividing bacilli in the initial therapy stage. Although rifampicin and streptomycin have some bactericidal activity, the most potent bactericidal anti-TB drug is considered to be isoniazid (Hershfield, 1999). Sterilising drugs, such as rifampicin and pyrazinamide, are used to eliminate the putative subpopulation of from which a clinical relapse could potentially occur (Davies, 2010). Subsequently, the most effective treatment regimes consist of two phases including: 1) the initial intensive phase, during which a combination of at least two bactericidal anti-TB drugs (isoniazid and rifampicin) are used to kill actively growing *M. tuberculosis* populations, followed by 2) a continuation phase for the elimination of intermittent dividing and dormant bacteria, using sterilising drugs (Frieden *et al.*, 2003).

In an attempt to reduce the global burden of TB, the WHO formulated the Millennium Development Goals (MDGs). The target of the MDGs is to halve the 1990 TB prevalence and death rates by 2015, and furthermore to completely eliminate TB by 2050. In order to achieve this, the WHO developed the DOTS (directly observed treatment, short-course) strategy in the mid-1990s, which was recommended internationally and consequently expanded upon worldwide. DOTS consists of a 6 months therapy regimen, with an initial 2 month treatment phase using four first-line drugs (isoniazid, rifampicin, pyrazinamid and ethambutol), followed by a 4 month continuation phase using only isoniazid and rifampicin. The addition of direct observation therapy (DOT), where patients ingest each daily dose of anti-TB drugs under direct supervision, to the treatment strategy, is strongly recommended. This approach maximises the probability of therapy completion, hence, limiting the emergence of drug-resistance (ATC & CDC, 2003). More or less 90% of all drug-susceptible TB cases are reportedly cured when this regimen is fully adhered to. In the case of MDR-TB, with resistance to at least rifampicin and isoniazid, DOTS alone may not succeed. For these cases, the WHO recommends DOTS-plus, which includes additional second-line drugs to the conventional DOTS program. This regimen is, however, costly, takes up to 24 months, and has a much higher level of toxicity (WHO, 2010). Apart from the recommended treatment regimes, the DOTS program also comprises of a number of other strategies for participating countries, including: dedication from the respective governments for continues TB control; early case detection in symptomatic patients using smear microscopy; the implementation of appropriate drug supply systems; and the development of a standardised system to record and report TB cases (WHO, 2006).

A brief overview of the mechanisms of action and adverse effects of the most commonly used first- and second-line TB drugs will now be discussed.

## **4.1 First-line medications**

### **4.1.1 Streptomycin**

In 1944, Selman Waksman discovered and isolated the first effective anti-TB drug, streptomycin, from *Streptomyces griseus*, and for this, Waksman was awarded the Nobel Prize in 1952 for physiology and medicine (Waksman, 1953).

Streptomycin, an aminoglycoside antibiotic, interferes with protein synthesis of the TB causing bacteria by inhibiting mRNA translation, resulting in the misreading of its genetic code and consequently cell membrane damage (Davies *et al.*, 1965; Zhang, 2005). The exact binding site of streptomycin was found to be in the small 30S subunit of the ribosome, more specifically at ribosomal protein S12 (*rpsL*) and 16S rRNA (*rrs*) (Garvin *et al.*, 1974).

As early as 1946, only two years after its discovery, Klein and Kimmelman already reported the first streptomycin-resistant mutants. These mutants could be classified into two groups depending on whether or not they demonstrated a high or low level of resistance (Klein & Kimmelman, 1946). Ever since then, numerous studies have investigated the underlying molecular mechanism of streptomycin resistance (Honoré & Cole, 1994; Honoré *et al.*, 1995; Ogle & Ramakrishan, 2005), but despite this, the molecular cause of this resistance is, to date, still not yet completely understood. Mutations within both the *rrs* and *rpsL* genes have been linked to a high-level of streptomycin resistance (Honoré & Cole, 1994; Honoré *et al.*, 1995). Most of these resistance causing mutations lead to a hyper-accurate phenotype (increased accuracy of translation), compensating for the effect of the drug without having any effect on the interaction between the ribosome and the drug (Montandon *et al.*, 1986; Okamoto *et al.*, 2007; Pinard *et al.*, 1993). These mutations, nevertheless, have only been identified in just more than one half of all clinical streptomycin-resistant *M. tuberculosis* isolates (Gillespie, 2002; Ramaswamy, *et al.*, 2004). The underlying mechanisms of the other lower-level resistant strains are obscure.

Recently, Okamoto *et al.* (2007) identified a mutation within the gene, *gidB*, conferring low-level resistance in 33% of *M. tuberculosis* isolates. They furthermore confirmed that *gidB* encodes for a conserved 7-methylguanosine (m7G) methyltransferase (GidB) specific for the 16S rRNA. Further studies on the precise function and role of GidB in the pathogenesis of *M. tuberculosis* is, however, still unknown.

Apart from *M. tuberculosis* resistance to streptomycin, other complications, regarding the side effects of streptomycin treatment, should also be noted. Hypersensitivity to streptomycin is not uncommon, and may, depending on the severity, require treatment interruption. Transient dizziness and numbness around the mouth can occur after injection, but does regress and completely disappear when drug administration is stopped. With long-term treatment, chronic toxicity, leading to blackouts, ataxia (lack of coordination of muscle movements), ringing in the ears, and even permanent deafness, have been reported (WHO, 2010).

#### 4.1.2 Isoniazid

Isonicotinic acid hydrazide, or isoniazid, is one of the most efficient anti-TB drugs used to inhibit mycobacterial growth. However, despite the large number of studies dedicated to elucidating its mode of action, the exact mechanism by which it functions still remains largely unknown, mainly due to the fact that it potentially influences a variety of different mycobacterial cellular processes.

Early investigations indicated that when exposed to isoniazid, *M. tuberculosis* loses its acid-fast nature and viability, which led to the suspicion that the drug functions by altering the cell-wall lipids, specifically by inhibiting mycolic acid synthesis (Takayama *et al.*, 1972). Several studies have since confirmed this (Morris *et al.*, 1995; Quemard, *et al.*, 1991) and electronmicroscopy scanning of *M. tuberculosis* exposed to isoniazid determined various altered morphological features (Takayama *et al.*, 1973). Other modes of action of isoniazid include; reactivity with bacterial proteins (tyrosine residues specifically) (Herman & Webber, 1980), and the formation of reactive oxygen radicals during the activation of the drug (Shoeb *et al.*, 1985).

More recently, it has been established that isoniazid is a pro-drug requiring oxidative activation by catalase-peroxidase KatG (Johnsson & Schultz, 1994). An isonicotinoyl radical, the activated form of isoniazid, then reacts with NAD, leading to the covalent adduct INH-NAD, which inhibits the *M. tuberculosis* InhA enzyme (Nguyen *et al.*, 2002). InhA is an enoyl-acyl carrier protein, catalysing the reduction of the trans double bond, conjugated to the carbonyl group of fatty acyl substrates, with NADH acting as the hydrogen donor for these reactions (Dessen *et al.*, 1995). Inhibition of InhA will consequently block fatty acid elongation via the FAS II system, which is essential for mycolic acid synthesis, the main building blocks of the mycobacterial envelope (Scoir *et al.*, 2002).

Resistance to isoniazid entails various mutations (insertions, deletions and point mutations) in a number of genes. The major targets are the *katG* and the coding and regulatory area of *inhA*. Other mutations either have minor roles in isoniazid resistance or are compensatory due to the loss of catalase-peroxidase activity (Ahmad & Mokaddas, 2010).

When recommended isoniazid doses are administered, adverse effects are uncommon, unless the patient has a history of previous kidney or liver failure (ATC & CDC, 2003). Hypersensitivity reactions may, however, occur in the first week of treatment. Peripheral neuropathy is the most common isoniazid associated adverse effect (20% of cases), and is especially prevalent in high-risk patients (pregnant women, alcoholic, malnourished, and diabetic patients), but can be prevented with a complementary low dose of vitamin B6 (WHO, 2010). When isoniazid is used in isolation, hepatitis reportedly manifests in 0.6% of cases, and when used in combination with rifampicin, the incidence increases to 2.7%. This incidence has also been reported to increase with age and in patients with previous liver disease. The fatality rate of isoniazid treated patients due to hepatitis is, however, less than 0.023% (Arbex *et al.*, 2010 (a); ATC & CDC, 2003).

### 4.1.3 Ethambutol

Ethambutol is normally included as part of the initial TB treatment regimes, especially when isoniazid resistance is predicted (Hershfield, 1999). Ethambutol is effective against intracellular and extracellular bacteria, however, its exact mode of action is also still unknown. To date, several studies have been done in an attempt to explain this drug's mode of action, however, most of these analyses focused on the actions by which it alters the mycobacterial cell wall structure (Beggs & Andrews; 1973; Takayama, 1989). Takayama and Kilburn (1989) indicated that ethambutol has an inhibitory effect on the transfer of arabinogalactan (arabinosyl transferases) into the *Mycobacterium* cell wall, which in turn leads to the accumulation of trehalose mono- and dimycolates. Silve *et al.* (1993) additionally indicated that ethambutol functions by inhibiting the transfer of (D-14C) glucose into the D-arabinose fraction of arabinogalactan. Consequently, it was suggested that, due to inefficient arabinogalactan transfer into the cell wall, mycolic acids accumulate in these mycobacteria, leading to the previously observed bacterial declumping and morphological alterations following ethambutol treatment (Kilburn *et al.*, 1981). Furthermore, sequence analyses of ethambutol resistant, clinically isolated mycobacteria, indicated that this resistance is primarily linked to a number of missense mutations in the ethambutol resistance determining region of the arabinosyl transferase encoding gene, *embB* (Belanger *et al.*, 1996). Starks *et al.* (2009) determined that these mutations in *embB* codon 306, in particular, are important indicators of ethambutol resistance and may also be useful for confirming isoniazid resistance in 50-70% of all clinical samples. The exact role of the *embB* 306 mutation in the acquisition of isoniazid resistance is, however, still controversial.

A major adverse effect associated with ethambutol treatment, is retrobulbar optic neuritis, with symptoms including: blurred vision, dyschromatopsia (colour blindness), and central scotoma (tunnel vision). These side effects can be prevented by lowering the dosage over a period of 3 months (WHO, 2010).

### 4.1.4 Rifampicin

Rifampicin is a powerful anti-TB drug, reducing actively dividing and semi-dormant organisms. This drug primarily inhibits DNA-dependant RNA-polymerase, an enzyme essential for the transcription of RNA (Wehrli, 1983). Resistance to rifampicin is mainly a result of alterations in the  $\beta$ -subunit of RNA-polymerase due to mutations in the encoding *rpoB* gene. A variety of specific resistance-conferring mutations (accounting for more than 95% of rifampicin-resistant strains) have been described in the 81-bp region of *rpoB* known as the rifampicin-resistance determining region (RRDR) (Anthony *et al.*, 2005). The majority of these are point mutations,

resulting in the replacement of aromatic with non-aromatic amino acids. These replacements lead to drug resistance by interrupting the forces that bind rifampicin to RNA polymerase. These mutations also impair the fitness of these bacteria, which may be restored by secondary mutations (Gagneux *et al.*, 2006). These rifampicin- resistance conferring mutations will be discussed in greater detail in Chapter 4 of this thesis. Additionally, a number of newly proposed mechanisms by which these mutations may influence these bacteria, will be discussed, considering the new metabolite markers identified using the metabolomics research approach developed for this purpose in Chapter 3.

Minor side effects associated with rifampicin treatment include: flu-like symptoms; abdominal pain; fatigue; ataxia; dyspnea; and anorexia, but in most cases, do not warrant the discontinuation of treatment. In combined-treatment approaches however, exanthema (a rash-like reaction) can occur, which requires immediate discontinuation. Furthermore, cholestatic hepatitis reportedly occurs in 2.7% of those patients treated in combination with isoniazid and in up to 1.1% when rifampicin is combined with the other first-line anti-TB drugs (Arbex *et al.*, 2010 (a); ATC & CDC, 2003; WHO, 2010).

#### 4.1.5 Pyrazinamide

Pyrazinamide, together with isoniazid, rifampicin and ethambutol, plays an important role in the initial TB treatment regimes. As it is active at an acidic pH, pyrazinamide is able to kill semi-dormant bacteria not killed by the other previously mentioned anti-TB drugs. The inclusion of pyrazinamide in these multi-drug regimens has been shown to shorten the TB therapy duration from 9 to 6 months (ATC & CDC, 2003). Although pyrazinamide is widely used today, its specific mechanism of action, as is the case with most other TB medication, is still largely unknown. Pyrazinamide, also considered a prodrug, is converted to its active state, pyrazinoic acid (POA), by the bacterial enzyme nicotinamidase / pyrazinamidase (PZase) (Heifets *et al.*, 1989). It has been shown that various mutations in the PZase coding gene, *pncA*, lead to pyrazinamide resistance in *M. tuberculosis* (Hirano *et al.*, 1998). All bacteria are equipped with PZase, however, *M. tuberculosis* is uniquely susceptible to pyrazinamide. This susceptibility is thought to be due to a deficient pyrazinoic acid efflux mechanism in *M. tuberculosis*, in contrast to the natural pyrazinamide resistant *M. smegmatis*, which is capable of rapidly extruding pyrazinoic acid out of the bacterial cell (Zhang *et al.*, 1999). Various studies have suggested that pyrazinoic acid does not necessarily have a specific bacterial target site and most likely kills *M. tuberculosis* due to its weak acid nature (Zhang & Mitchison, 2003). More recent studies, however, suggest that the bacterial membrane is de-energized by pyrazinoic acid and pyrazinamide as a result of a collapsing membrane potential, and that these compounds also influence membrane transport in an acidic environment (Zhang *et al.*, 2003). In 2011, Shi *et al.*

identified RpsA (ribosomal protein S1), as the potential target of POA. The binding of POA to RpsA inhibits trans-translation, a crucial process for freeing rare ribosomes in non-replicating organisms, clarifying the capacity of pyrazinamide as a sterilizing drug for the elimination of dormant bacteria.

Despite its comparatively better capacity for eliminating TB, pyrazinamide is considered to have rather severe side effects. Pyrazinamide treatment may be associated with pruritus, exanthema, or rhabdomyolysis with kidney failure, and myoglobunuria. Pyrazinamide is additionally considered to be the most hepatotoxic of all first-line anti-TB drugs and treatment with this drug should be temporarily discontinued, or replaced with alternative medication, when any of the above side effects are noticed (WHO, 2010; Arbex *et al.*, 2010 (a)).

## 4.2 Second-line medication

### 4.2.1 D-Cycloserine

As is the case with many of the other anti-TB drugs, D-cycloserine (D-4-amino-isoxazolidone), a cyclic structural analogue of D-alanine, is thought to function by inhibiting cell wall synthesis in mycobacteria (Strych *et al.*, 2001). D-amino acids (D-alanine, D-glutamate, and D-aminopimelatein, in particular) are important components of the bacterial cell wall backbone, peptidoglycan. Alanine is typically accessible as the L-stereoisomer and is converted to D-alanine via D-alanine racemase (Alr) in the initial step of alanine branching during peptidoglycan biosynthesis (Walsh, 1989). The dipeptide D-alanyl–D-alanine, is then formed in a D-alanine:D-alanine ligase (Ddl) catalyzed reaction. D-cycloserine blocks mycobacterial cell wall synthesis by inhibiting Alr and Ddl (Strych *et al.*, 2001).

Caceres *et al.* (1997) determined that the over expression of Alr in *M. smegmatis* and *M. bovis* leads to a D-cycloserine resistant phenotype. This resistance was observed to a far higher degree than was seen during Ddl over expression, leading to the suspicion that the primary target of D-cycloserine might be Alr (Feng and Barletta, 2003). Furthermore, an increased sensitivity to D-cycloserine was observed when *alrA* (Chacon *et al.*, 2002) or *ddl* (Belanger *et al.*, 2000) was inactivated. Even though it is presumed that D-cycloserine resistance manifests due to mutations in *alrA* and *ddl*, the exact mechanism of resistance is unclear (Belanger *et al.*, 2000).

Despite the fact that D-cycloserine is a successful anti-mycobacterial drug, it is rarely prescribed and only used in combined therapies due to its severe adverse effects. Neurological side effects including: headaches, vertigo, memory deficiency and mental confusion, only to name a

few, in addition to psychiatric side effects including: depressive and paranoid reactions, and psychotic states, are reported to be rather common (Arbex *et al.*, 2010 (b)). These adverse effects are the result of D-cycloserine binding to neuronal *N*-methyl aspartate receptors in the patient (Thompson *et al.*, 1992), and the inhibition of the enzymes involved in the metabolism and synthesis of the neurotransmitter  $\gamma$ -aminobutyric acid (GABA) (Wood *et al.*, 1978).

#### 4.2.2 Ethionamide

Being structurally similar to isoniazid, ethionamide is also considered a prodrug, requiring activation by the infected bacteria. When activated, ethionamide, like isoniazid, disrupts cell wall biosynthesis by inhibiting a mutual cellular target, the enoyl-acyl carrier protein, *InhA* (Banerjee *et al.*, 1994; Vannelli *et al.*, 2002). Gene array studies, indicating similar patterns of gene expression induced by both isoniazid and ethionamide, verifies this shared site of action (Wilson *et al.*, 1999). The fact that isoniazid resistance does not always result in ethionamide resistance, has led to the suspicion that different enzymes may activate these drugs. Little was known about this enzyme, until only recently, when two different research groups almost simultaneously identified *EtaA* (Baulard *et al.*, 2000; Debarber *et al.*, 2000). *EtaA*, a FAD-enclosing monooxygenase enzyme, oxidises ethionamide to its analogous S-oxide, which in turn, is oxidized to 2-ethyl-4-amidopyridine through an unstable oxidised sulfinic acid intermediate (Vannelli *et al.*, 2002). Resistance to ethionamide may occur as a result of various mutations in the gene coding for this activating enzyme, *etaA*, and its target, *inhA* (Morlock *et al.*, 2003).

Ethionamide is classified as a second-line drug due to its severe side effects. Extreme gastrointestinal associated complications, including intense salivation, nausea, loss of appetite, vomiting, and abdominal pain, are reportedly common. These symptoms can be reduced by administering the drug with food or at bedtime. As is the case with isoniazid, ethionamide can cause hepatotoxicity, especially in patients with liver disease and those with a history of alcoholism. Neurological symptoms such as optic neuritis, anxiety, depression, and hallucinations have been reported in only 1-2% of all treated patients. Other adverse effects of ethionamide include: postural hypotension, alopecia, impotence, hypothyroidism, acne, and photosensitivity, only to name a few (Arbex *et al.*, 2010 (b)).

#### 4.2.3 Kanamycin and amikacin

Since the discovery of streptomycin in 1944 (Waksman, 1953), aminoglycosides have played a major role in TB therapy. Kanamycin (isolated from *Streptomyces kanamyceticus*) and amikacin (a semi-synthetic drug derived from kanamycin), are commonly used in MDR-TB

treatment regimens (Arbex *et al.*, 2010 (b); Krüüner *et al.*, 2003). These drugs, like streptomycin and other aminoglycosides, bind to the small 30S ribosomal subunit, in particular the 16S rRNA (*rrs*), consequently inhibiting translation and, hence, protein synthesis (Magnet and Blanchard, 2005). Information regarding the exact mechanism underlying kanamycin resistance in *M. tuberculosis* is scarce. What is known is that similar to streptomycin, mutations in the *rrs* gene, result in a high-level kanamycin resistance, and some mutations may also cause cross-resistance to amakacin and other second-line drugs (Krüüner *et al.*, 2003). However, as much as 80% of the kanamycin resistant clinical isolates exhibit low-level resistance without *rrs* mutations or cross-resistance. Recently, Zaunbrecher *et al.* (2009), determined that in *M. tuberculosis*, an over expression of the survival protein, Eis, due to *eis* promotor mutations, is common in these low-level kanamycin resistant clinical isolates. Eis, an aminoglycoside acetyltransferase, inactivates both kanamycin and amakacin. Kanamycin is reportedly 3-times more efficient than amakacin as a substrate for Eis, explaining the lack of cross-resistance of an *eis* mutation to kanamycin.

Ototoxicity, or damage to the ear, is considered the most severe adverse effect of aminoglycosides. This hearing loss experienced after treatment is as a result of cranial nerve VIII damage, including cochlear and vestibular impairment. Immediate discontinuation of aminoglycoside treatment is advised when ototoxicity occurs. As a result of build-up in the renal tubules, aminoglycosides may additionally cause toxic renal effects including: proteinuria, oliguria (low urine output), and decreased creatinine clearance. In rare occasions, aminoglycoside treatment may bring about hypersensitivity (extreme allergic reaction to the drug) or neuromuscular blockage, resulting in respiratory failure (Arbex *et al.*, 2010 (b)).

#### 4.2.4 Fluoroquinolones

Nalidixic acid, the first quinolone anti-TB medication, was discovered in the early 1960's as an impurity produced during quinine synthesis (Andersson & Macgowan, 2003). Many fluoroquinolone derivatives have since been tested for their antibacterial activity. Levofloxacin, sparfloxacin, ofloxacin, and ciprofloxacin have all shown to be extremely effective against *M. tuberculosis* (Jacobs, 1999), and have therefore been used in TB treatment regimens since the early 1980's (Leysen *et al.*, 1989).

Fluoroquinolones circulate throughout the body and have the extraordinary property of functioning intracellularly, reaching mycobacteria inside the macrophages and, hence, leading to a potent treatment outcome (Ginsburg *et al.*, 2003). Fluoroquinolones function by inhibiting DNA gyrase (Gyr, a type II topoisomerase), an enzyme essential for reducing the tension when double-strand DNA is unwound during DNA replication, recombination and expression. This

inhibition prevents supercoiling of the DNA, leading to uncontrolled mRNA synthesis, exonuclease production, protein synthesis and chromosome degradation, due to free DNA ends (Di Perri & Bonora, 2004; Ginsburg *et al.*, 2003). When used as a monotherapy, mycobacteria quickly develop resistance to fluoroquinolones due to mutations in the DNA gyrase enzyme (*gyr*). Other resistance conferring mechanisms include; 1) the presence of an efflux system, actively excreting the drug from the bacterial cell or 2) an altered cell membrane structure, leading to fluoroquinolone impermeability (Ginsburg *et al.*, 2003). Cross-resistance between fluoroquinolones and other TB-drugs however, does not occur, and even though cross-resistance between various fluoroquinolones has been described (Di Perri & Bonora, 2004; Ginsburg *et al.*, 2003), moxifloxacin and levofloxacin have been used to successfully treat patients resistant to ofloxacin. Furthermore, a study done in India indicated that oxifloxacin, in combination with various first-line drugs, may be effective as a three month, ultra-short course TB treatment regime (Tuberculosis Research Centre, 2002).

Gastrointestinal side effects are most commonly associated with fluoroquinolone treatment. These effects including; vomiting, anorexia, diarrhoea, and abdominal pain, have been reported in 3 - 17% of fluoroquinolone treated patients. A small amount (0.9 - 11%) of patients receiving fluoroquinolone treatment may also develop insomnia, tremors, and headaches. Skin rash, erythema, and pruritus may additionally occur in 0.4 - 2.2% of fluoroquinolone treated patients (Arbex *et al.*, 2010 (b)).

Table 2.1 gives a summary of the modes of action, cellular targets, and resistance conferring genes of the drugs used for TB treatment.

Table 2.1: Mode of action of commonly used anti-TB drugs

| Drug                   | Discovery year | Mechanism of action                                | Cellular target                                                  | Resistance conferring genes |
|------------------------|----------------|----------------------------------------------------|------------------------------------------------------------------|-----------------------------|
| <b>First-line</b>      |                |                                                    |                                                                  |                             |
| Streptomycin           | 1944           | Inhibition of protein synthesis                    | Ribosomal S12 protein and 16S rRNA                               | <i>rpsL</i><br><i>rrs</i>   |
| Isoniazid              | 1952           | Inhibition of cell wall mycolic acid biosynthesis  | Multiple targets including acyl carrier protein reductase (InhA) | <i>inhA</i><br><i>katG</i>  |
| Pyrazinamide           | 1952           | Inhibition of trans-translation                    | Ribosomal protein S1 (RpsA)                                      | <i>pcnA</i>                 |
| Ethambutol             | 1961           | Inhibition of cell wall arabinogalactan synthesis  | Arabinosyl transferase                                           | <i>embB</i>                 |
| Rifampicin             | 1966           | Inhibition of transcription                        | RNA polymerase $\beta$ subunit                                   | <i>rpoB</i>                 |
| <b>Second-line</b>     |                |                                                    |                                                                  |                             |
| D-cycloserine          | 1952           | Inhibition of peptidoglycan synthesis              | D-alanine racemase                                               | <i>alrA</i><br><i>ddl</i>   |
| Ethionamide            | 1956           | Inhibition of cell wall mycolic acids biosynthesis | Acyl carrier protein reductase (inhA)                            | <i>inhA</i><br><i>etaA</i>  |
| Kanamycin and amikacin | 1957           | Inhibition of protein synthesis                    | 16S rRNA<br>Aminoglycoside acetyltransferase                     | <i>rrs</i><br><i>eis</i>    |
| Fluoroquinolones       | 1963           | Inhibition of DNA synthesis                        | DNA gyrase                                                       | <i>gyrA</i><br><i>gyrB</i>  |

Adapted from Zhang, 2005

Although the WHO recommended treatment regimes are highly effective, various studies have demonstrated unwanted interactions between the different anti-TB drugs and between anti-TB drugs and other medications used by TB patients (ATC & CDC, 2003).

Three of the four first-line anti-TB drugs in the DOTS program are hepatotoxic and depending on race, geographic location and socioeconomic status, 1-10% of patients treated with these drugs develop drug-induced hepatitis (Yee *et al.*, 2003). In addition to the severe side effects resulting from these drug interactions, serum drug concentrations are also altered, resulting in a reduced efficiency (Arbex *et al.*, 2010 (a)).

Severe effects due to anti-TB drugs do, however, emerge as a result of variety factors, and the extent of these effects are influenced by, amongst others, the age of the patient, nutritional status, dosage, time of administration, and pre-existing diseases or dysfunctions (such as HIV co-infection, liver or kidney impairment, and alcoholism) (Arbex *et al.*, 2010 (a); ATC & CDC,

2003; WHO, 2010). Changes to the proposed first-line treatment protocols, mainly as a result of drug-resistance, consequently leads to the use of the more toxic and more costly second-line TB drugs, which in turn are usually accompanied by increased hospitalisations and home visits (Yee *et al.*, 2003). The side effects associated with first and second line drugs additionally contribute to the discontinuing of treatment before the recommended 6 month treatment protocol is completed, leading to increased treatment failure and, hence, acquired resistance (Arbex *et al.*, 2010 (a)).

These shortcomings of the current anti-TB drugs and TB treatment regimes are not the only factors driving the TB epidemic. The poor case detection rate of 61% in mid-2010, which fell far short of the global target of 70% (U.S. Global Health Policy, 2010) highlighted the fact that, despite the need for faster acting, less toxic TB treatment procedures, we also urgently require innovative, sensitive and rapid diagnostic approaches in order manage the concurrent HIV epidemic and prevent the rising incidence of MDR-TB cases.

## **5. TUBERCULOSIS DIAGNOSTICS**

The emergence of MDR-TB, and the rising HIV pandemic, has challenged conventional TB diagnostic methods, and the need for new, more accurate, sensitive and quick TB diagnostic approaches are now more crucial than ever. Over the past few years, innovative, high-tech diagnostic procedures such as molecular techniques and rapid culturing methods have entered the market. However, due to their complexity and high costs, these tests are not yet suited for high-burden, low-income settings (WHO, 2006).

The mechanisms by which the currently available and more recently developed TB diagnostic procedures function, will subsequently be discussed, considering each of their advantages and disadvantages. This discussion will familiarise the reader with the currently available methods, in order to understand their limitations in the context of the global TB problem and the need for alternative approaches. The contribution of metabolomics for the development of alternatives approaches, or solutions to these problems, will then be addressed.

### **5.1 Tuberculin skin test**

The tuberculin skin test (TST) is based on the delayed-type hypersensitivity reaction produced by *M. tuberculosis* infected individuals when antigenic compounds (purified protein derivative (PPD), acquired from heat-killed *M. tuberculosis*) are injected intra-cutaneously into the forearm of an infected individual (ATC & CDC, 1999; WHO, 2006). T-cells formed as a result of present or prior infection, then travel to the infected skin area where they release lymphokines. The

induction of local vasodilatation, fibrin deposition, edema and the accumulation of other inflammatory cells, as a result of these lymphokines, cause a thickening of the skin at the injection site, which can be measured (ATC & CDC, 1999). The reported sensitivity and specificity of this technique, however, varies due to a number of reasons. For instance, in a study conducted by Al *et al.* (2009), patients with a TST with a size smaller than the 5 mm threshold, were found to be less likely to have active TB, however, measurements above the threshold, were not necessarily indicative of active disease. Additionally, patients with varying disease states produced almost identical results, considering the size and shape of the TST. Furthermore, false-positive results regularly occur in patients who were previously vaccinated with BCG or are infected with other, non-tuberculous mycobacteria. False-negative reactions, on the other hand, may also occur due to a variety of other technicalities for example, in patients with compromised immune systems due to immunosuppressive drugs (i.e. steroids), AIDS, cancer, age (newborns and adults over 65 years), and supplementary bacterial, fungal or viral infections (Pouchot *et al.*, 1997; WHO, 2006).

## **5.2 Cytokine detection assay**

A newer approach used for the detection of latent TB infection, also making use of the cell-mediated immune response, is the cytokine detection assay. In this test, circulating lymphocytes (extracted from a patient's blood) are exposed to mycobacterial antigens for 6 to 24 hours. *M. tuberculosis* infected lymphocytes recognize these antigens and subsequently produce cytokines, mostly interferon-gamma (INF- $\gamma$ ), which can then be measured (WHO, 2006). The first INF- $\gamma$  release assays (IGRAs) used PPD as the antigen of choice. More recently developed tests however, rely on antigens which are more specific to *M. tuberculosis*, such as early secreted antigen target (ESAT)-6 and culture filtrate protein (CFP)-10 (Pai *et al.*, 2006 (a)). Currently, two IGRAs termed Quanti-FERON<sup>®</sup>-TB Gold and T-SPOT-TB, are commercially available. Several studies indicated that these assays have a high specificity (>95%), but a lower, variable sensitivity (75-97%), and also, results between the two IGRAs vary, limiting their efficacy in routine clinical settings (Ferrara *et al.*, 2006; Pai *et al.*, 2006 (a)).

Considering the fact that both TST and the cytokine detection assay detect markers for *M. tuberculosis* infection only, follow-up diagnostics are required to determine if the patient with an infection does in fact suffer from active TB.

## **5.3 Radiographic methods**

In order to diagnose active pulmonary TB, chest X-rays (CXRs) can be used, and during the interpretation, these may be classified as either: 1) typical of TB, with i) the occurrence of

nodular, alveolar, or interstitial infiltrates predominantly affecting the zones above the clavicles or upper zones of the lung, or ii) the presence of cavitations affecting the upper zones or the apical segment of the lower lobes of the lungs; 2) compatible with TB due to atelectasis, enlarged hilar nodes, pleural exudate, mass lesion, miliary or pneumonic lesion, or; 3) atypical (all other patterns, including normal CXR) (Tattevin *et al.*, 1999). As summarised by the WHO (WHO, 2006), X-rays alone are still widely used as an important tool for TB diagnostics, despite the fact that various studies have proven that TB shows no unique radiographic patterns (ATC & CDC, 1999). Also, numerous other lung diseases have a similar radiographic appearance, which can easily mimic that of TB (Nakamura *et al.*, 1970), leading to a false positive TB diagnosis when used alone. When using culture as the comparative reference, Van Cleeff *et al.* (2005) reported the sensitivity and specificity of CXRs to be 80% and 67% respectively, whereas Arslan *et al.* (2010) reported these values to be 73% and 94% respectively, which is considered rather unsatisfactory. CXR can thus be useful for the identification of abnormalities in the lungs, but to ascertain the tubercular aetiology, further tests, such as bacteriology, are an absolute necessity (WHO, 2006).

#### **5.4 Microscopic examination**

Despite recent advantages in TB diagnostics, smear microscopy, as first demonstrated by Robert Koch in 1882, is still the most commonly used screening method to detect mycobacteria in clinical sputum specimens (Drobniewski *et al.*, 2003). This quick (less than 2 hours), simple and low cost technique, is based on the acid-fast nature of mycobacteria and thus the ability of these organisms to retain dye after treatment with an acid-alcohol solution (ATC & CDC, 1999). The characteristic mycolic acids present in the cell walls of all mycobacteria are responsible for this colour reaction, hence, limiting the ability of the method for *Mycobacterium* species classification. Also, these fatty acids do persist when these bacteria die, and therefore, this technique cannot discriminate a current, active disease state, from a previous *Mycobacterium* infection (Ruiz-Manzano *et al.*, 2008). Furthermore, numerous quantitative studies have shown that high amounts of bacilli (5000 – 10 000 bacteria mL<sup>-1</sup>) are required for the detection of bacteria using smear microscopy tests (Kox, 1995), leading to a reported sensitivity of no more than 35 – 70%. It has, however, been reported that, on occasion, this method detect only 20 – 30% of all TB cases (Urbanczik, 1985), especially in patients with less advanced disease states, TB-HIV co-infection, and in children (WHO, 2006). Nevertheless, although a negative smear does not rule out *Mycobacterium* infection, a positive result almost absolutely verifies a diagnosis, resulting in a technique specificity of more than 95%. This high specificity is limited only by cross-contamination by other environmental *Mycobacterium*, non-tuberculous *Mycobacterium*, and other technical inadequacies (Ruiz-Manzano *et al.*, 2008).

## 5.5 Bacteriological culture

The WHO considers bacteriological culture as the gold standard for TB diagnosis, as it detects over 80% of all TB cases accurately, with a reported specificity of close to 100% (Lee *et al.*, 2003; WHO, 2006). However, it has been reported that negative sputum cultures may occur in 15-20% of adult pulmonary TB cases (Getahun *et al.*, 2007). Additionally, false-positive culture results have been reported to occur in 1-4% of all cases due to a transfer of bacilli from TB-positive to TB-negative samples during laboratory handling (Getahun *et al.*, 2007).

Either solid or liquid media can be used to grow these cultures. Solid media including; egg based media (e.g. Löwenstein–Jensen), or agar based media (e.g. Middlebrook 7H10), are most commonly used due to their low costs. This approach, however, requires a diagnostic turnover time of 4-6 weeks (Hannan *et al.*, 2008). Growth in liquid media is considerably faster (2-4 weeks) and is far more sensitive, but does have the disadvantage of higher contamination rates (8-10%) when compared to solid media (3-5%) (Ruiz-Manzano *et al.*, 2008). The development of automated culture systems, such as BACTEC 460 and mycobacterial growth indicator tube (MGIT) systems, was a major improvement in *Mycobacterium* culture diagnosis (ATC & CDC, 1999). Hanna *et al.* (1999) reported a multicenter evaluation of the BACTEC MGIT 960 and BACTEC 460 systems in comparison to conventional solid culture media for mycobacteria isolation. They found that traditional solid media (Löwenstein–Jensen slopes and Middlebrook 7H11 plates) was capable of a *M. tuberculosis* recovery rate of 79%, in a mean turnover time of 24.1 days, in comparison with BACTEC 460 TB and BACTEC MGIT 960, with recovery rates of 90% and 77%, and mean turnover times of 15.2 and 14 days, respectively. A combination of solid media and BACTEC 460 gave the best *M. tuberculosis* recovery rate (97%). As an added advantage, the above-mentioned methods can differentiate between various clinically important *Mycobacterium* species, including non-tuberculous *Mycobacterium* (Cruciani *et al.*, 2004). These automated systems are, however, expensive and require expensive infrastructure and maintenance, in addition to highly skilled and experienced staff (WHO, 2006).

Bacteriological culture is also the conventionally used method for drug susceptibility testing (DST) in TB. When using solid media, the growth of *M. tuberculosis* in the presence of anti-TB medication can be detected by one of three methods including: 1) proportions; 2) resistance ratios; or 3) absolute concentrations (Ahmad and Mokaddas, 2010). In the proportions method, which is considered the reference standard, the growth of organisms on drug-free media is compared to the growth on media containing an anti-TB drug. The resistance ratio method, on the other hand, determines the minimum inhibitory concentration of a drug-susceptible, reference strain, as compared to that of the patient's strain. Lastly, the absolute concentrations

method utilises media containing various dilutions of the anti-TB drug in order to determine the lowest concentration of the medication necessary to inhibit growth. These results are, however, only available within 2-3 months after sample collection (WHO, 2006).

The gold standard for culture DST, for both first- and second line medication, is the semi-automated radiometric BACTEC 460 TB system (Scarparo *et al.*, 2004; Tortoli *et al.*, 2002), where bacilli are cultivated in liquid media containing various concentrations of anti-TB drugs. This method has been used effectively for over 25 years and has reduced the time required for DST to a mere 4-13 days (Scarparo *et al.*, 2004). Nevertheless, the BACTEC 460 TB system makes use of radioactive materials, leading to many concerns regarding safety during its use and disposal after use. To overcome this problem, the BD BACTEC MGIT 960 SIRE (testing for Streptomycin, Isoniazid, Rifampin, and Ethambutol) assay has been developed and has shown to be an excellent alternative to the radiometric assay (Tortoli *et al.*, 2002). This automated, non-radiometric method uses fluorometric technology to accurately detect the consumption of O<sub>2</sub> in the presence of anti-TB medication, leading to a diagnostic result within 4-12 days (Scarparo *et al.*, 2004). It does, however, suffer the disadvantage of higher contamination rates than the BACTEC 460 TB assay (Scarparo *et al.*, 2004; Tortoli *et al.*, 2002). This contamination may be due to either the richness of the medium used (which is unlikely to change due to the methods growth detection principle) or the use of screw caps instead of rubber septa, which could be resolved in time (Tortoli *et al.*, 2002). A newer liquid-media-based DST method, the microscopic observation drug susceptibility (MODS) assay, is however, more affordable and does not require radioactive isotopes or fluorescent indicators. With MODS, inverted-light microscopy is used to detect early growth of *M. tuberculosis* as strings and tangles of bacterial cells in Middlebrook 7H9 broth medium in the absence or presence of anti-TB drugs, with a diagnostic result achieved in less than two weeks (Caviedes *et al.*, 2000). Six to eight mL of sputum per specimen is, however, required for this analysis, which is difficult to collect even from adults, not to mention children and immuno-suppressed (TB/HIV co-infected) patients. Further disadvantages associated with this method are: the fact that it is an indirect method for the detection of *M. tuberculosis*; the requirement of daily microscopic observations, and; it does not allow for mycobacterial species identification (Ahmad & Mokaddas, 2010).

Colourimetric methods used for DST in TB are based on the reduction of an oxidation–reduction indicator, which is added to liquid culture medium after the exposure of *M. tuberculosis* to various anti-TB medications. A colour change, proportional to the number of viable bacteria, is indicative of drug-resistance (Palomino *et al.*, 2007). These tests are mainly performed on clinical isolates and hence, do not exclude the critical culturing waiting period. Some of these methods have, nevertheless, been tested directly on sputum samples with a sensitivity and specificity ranging between 88% and 100%. These studies did, however, only focus on the

detection of rifampicin- and isoniazid-resistant TB, using highly infectious samples (positive with more than 10 acid-fast bacilli per microscopic field) (Martin *et al.*, 2008). A big concern of these colourimetric tests is the bio-hazardous aerosol generation during the handling of the microtitre plates. Also, phase III and IV diagnostic trails are still required before the clinical implementation of these methods can be considered (Palomino *et al.*, 2007).

## **5.6 High performance liquid chromatography**

For differentiating between various *Mycobacterium* species, high performance liquid chromatography (HPLC) assays can be completed within a few hours, and have a reported sensitivity and specificity of almost 100%. This technique can identify and distinguish between over 50 different *Mycobacterium* species which may be responsible for the infection, and is based on the detection of unique combinations of mycolic acids and  $\beta$ -hydroxy- $\alpha$ -fatty acids present in these different species (Butler & Kilburn, 1988). HPLC however, cannot, distinguish between *M. tuberculosis* and *M. bovis*, and requires at least  $1 \times 10^5$  cultured cells in order to achieve a successful diagnostic outcome (ATC & CDC, 1999).

## **5.7 Nucleic acid amplification**

A more recently developed TB diagnostic approach, nucleic acid amplification tests (NATs), functions by enzymatically amplifying regions of bacterial DNA specific to the *M. tuberculosis* complex. The most widely used NATs are polymerase chain reaction (PCR), transcription mediated amplification (TMA), and strand displacement amplification (SDA) (WHO, 2006). Several commercial NATs tests are available which, in theory, can be used directly on sputum or other clinical samples. In a study conducted by Catanzaro *et al.* (2000), the clinical performance of a NAT, approved by the Food and Drug Administration in 1995, the enhanced *Mycobacterium tuberculosis* Direct (E-MTD) test, was investigated. The sensitivity of the E-MTD test for low, intermediate, and high clinical TB suspicion was 83%, 75%, and 87% respectively, with a corresponding specificity of 97%, 100% and 100%. The positive prediction values of the E-MTD test were 59% (low TB suspicion), 100% (intermediate TB suspicion), and 100% (high TB suspicion) vs. 36%, 30%, and 94% respectively for smear microscopy, hence, confirming NATs to be potentially helpful for the diagnosis of early stage TB. Furthermore, Pounder *et al.* (2010) developed a genomic deletion assay based on multiplex PCR with melting temperature analysis, for the purpose of differentiating between six clinically important *Mycobacterium* species, based on the regions of difference (RDs) in their complete genome sequences. Using a set of 3 primers for each RD, they correctly identified 96% of *Mycobacterium* isolates from cultures. However, because sequence variation may occur at

primer binding sites during the evolution of these organisms, a reselection of the target sequence may be required in future.

Genotyping methods have also been developed for the DST of all first-line and most second-line TB drugs by detecting specific resistance linked mutations in target genes of *M. tuberculosis*. PCR-restriction fragment length polymorphism (PCR-RFLP) analysis is a rapid, low-cost method for the detection of polymorphisms at mutated codons of mostly isoniazid and ethambutol resistant *M. tuberculosis* strains (Ahmad et al., 2004; Cockerill et al., 1995). GenoType® MTBDRplus, a commercially available line-probe assay based on multiplex PCR, reportedly identifies rifampicin-resistance with a sensitivity of 93.1, 92.6% for isoniazid-resistance and 88.9% for a combination of the two, with a concurrent specificity of 100% (Huyen et al., 2010). However, due to the presence of natural PCR inhibitors in sputum, researchers still prefer to use pure cultures as the genomic DNA source for PCR amplification, hence, this diagnostic approach still suffers the time consuming culturing turn around times (Ahmad et al., 2004). Direct DNA sequencing is the most realistic method to use if all resistant strains have mutations in a specific region of a single target gene (as is the case with the RRDR in the *rpoB* gene of rifampicin-resistant strains). DNA sequencing is, however, impractical when analysing large amounts of specimens, particularly in developing countries (Ahmad and Mokaddas, 2010). Espasa et al. (2005) evaluated the potential of real-time PCR for rifampicin and isoniazid susceptibility testing in clinical samples. The sensitivity of the test ranged from 30.4 to 35.3% in smear-negative samples and 95.1 to 99.2% in smear-positive samples, with a 100% reported specificity. The detection limit of real-time PCR for detecting target mutations in clinical samples was found to be  $1.5 \times 10^3$  CFU mL<sup>-1</sup>, compared to 10 CFU mL<sup>-1</sup> in culture. Furthermore, genetic alterations in the target gene sequences were absent in 30% of the isoniazid-resistant isolates, hence, resulting in the low sensitivity of this diagnostic approach. Xpert MTB/RIF, an automated molecular test for *M. tuberculosis* and rifampicin-resistance, based on heminested real-time PCR, amplifies an MTB-specific sequence of the *rpoB* gene, and has been reported to correctly identify 97.6% of sputum samples with rifampin-resistant bacteria and 98.1% of samples with rifampin-sensitive bacteria (Boehme et al., 2010). Commercially available NATs, nevertheless, suffer the further disadvantages of high costs and the need for high-tech infrastructure and well-trained personnel. The high incidence of false-positive results due to laboratory cross-contamination, also limits its performance under field conditions (Catanzaro et al., 2000; WHO, 2006).

## 5.8 Serology

The use of serology (detection of antibodies, antigens and immune complexes) in TB diagnostics has, up to now, failed largely in providing the necessary sensitivity and specificity for

effective clinical use (WHO, 2006). Perkins *et al.* (2003), for example, evaluated the performance of a commercial immuno-chromatographic test kit (ICT Tuberculosis), employing five recombinant *M. tuberculosis* antigens. This kit correctly identified only 64.2% of the smear-positive and of the 46.3% smear-negative, culture confirmed-TB patients. Using a serological approach for TB diagnostics is challenging, as the various stages of the disease e.g. exposure, latent infection, active disease and severe disease, each have their own antibody patterns. Therefore, when developing such a test, one must consider the use of antigens expressed in all stages of the disease by employing, for instance, cocktails of multiple antigens (Pai *et al.*, 2006 (b)). Results may also vary due to the lack of reproducibility in the antigen purifying methods and, additionally, since environmental mycobacteria can cross-react with antibodies in these samples, false positive results frequently occur. Therefore, the use of serological TB diagnostic methods is not yet recommended by the WHO (WHO, 2011).

### **5.9 Phage assay**

Currently, the only commercially available phage assay for the detection of TB, FASTPlaqueTB, makes use of mycobacteriophages (mycobacteria infecting viruses) to signify the incidence of viable *M. tuberculosis* in clinical samples. When using bacteriological culture as the reference test, Muzaffar *et al.* (2002) determined the sensitivity and specificity of this assay to be 87.4% and 88.2% for smear-positive sputum samples, as opposed to 67.1% and 98.4% respectively in smear-negative, culture confirmed cases. Another study, conducted in South Africa, confirmed the poor sensitivity (48.7%) of this approach in smear-negative, culture confirmed specimens (Albert *et al.*, 2002). Although these assay test results can be achieved in 48 hours and is specific for *M. tuberculosis*, it missed other infectious *Mycobacterium* species, and is not able to detect drug resistance (WHO, 2006).

When weighing up the advantages and disadvantages of the above-mentioned TB-diagnostic approaches, it is obvious that no test currently available meets all the specifications of sensitivity, specificity, speed, safety, robustness, training simplicity, and cost. Hence, the world is in need for a new, innovative TB diagnostic method, which will overcome the limitations of the currently used methods, and can easily be implemented in low-income, high-burden countries.

## **6. THE ROLE OF METABOLOMICS IN TB RESEARCH**

The metabolome is the ultimate downstream result of genome transcription, and may be described as a compilation of all the metabolites (small molecular compounds) present in a specific cell or organism, participating in metabolomic reactions during normal cell function, growth and maintenance. Metabolomics is defined as ‘the non-biased identification and

quantification of *all* these metabolites in a biological sample using highly sensitive analytical procedures (Dunn *et al.*, 2005). During active disease and perhaps even latent infection, TB causing bacilli would potentially disturb the host's biochemical networks and provoke alterations in the quantity and types of metabolites present (Parida & Kaufmann, 2010). Using specialised techniques, the metabolite alterations due to these pathological stimuli can be measured over time. This approach serves as the foundation by which metabolomics functions towards the discovery of new metabolite markers, which in turn may be used for gaining a better understanding of disease mechanisms (including that of drug resistance), elucidating drug mechanisms, monitoring treatment outcomes, and the development of better disease diagnostics

Since TB was discovered in the 1800's, characterisation, diagnosis and treatment relied solely on the identification and traits of the causative organism in clinical samples. Ever since then, technical advances in the sensitivities and detection limits of various analytical techniques have made it possible to diagnose TB from samples containing very low concentrations of bacilli. These analytical techniques do, however, go hand-in-hand with higher levels of complexity and costs (WHO, 2006). Using metabolomics, we are not only able to identify those markers characterising the metabolome of the infected organism present in the diagnostic samples, but we can also measure changes to the host metabolism, due to infection. Over the past few years, metabolomics has been used for identifying markers better characterising a variety of diseases, including, amongst others: coronary heart disease (Brindle *et al.*, 2002); type 2 diabetes (Yang *et al.*, 2004 (a)); epithelial ovarian cancer (Odunsi *et al.*, 2005); Huntington's disease (Underwood *et al.*, 2006); hypertension (Brindle *et al.*, 2003); liver cancer (Yang *et al.*, 2004 (b)); liver failure due to hepatitis B infection (Yu *et al.*, 2007); meningitis and ventriculitis (Coen *et al.*, 2005); Parkinson's disease (Ahmed *et al.*, 2009; Bogdanov *et al.*, 2008); pre-eclampsia (Dunn *et al.*, 2009; Kenny *et al.*, 2008), and schizophrenia (Kaddurah-Daouk, 2006). Recently, two groups have investigated the use of metabolomics for improved TB diagnostics. In 2004, Pavlou *et al.* completed a pilot study investigating the capacity of electronic nose technology (which functions by utilising 14 conducting polymer sensor arrays) for TB diagnosis using both cultured samples and patient collected sputa. By using this approach, they were not only able to discriminate between *M. tuberculosis* and sterile cultures, with a 100% prediction value, but also discriminated between cultured samples of closely related *Mycobacterium* species (*M. avium*, *M. tuberculosis*, and *M. tuberculosis* + *M. scrofulaceum*) and *P. aeruginosa*, with a positive prediction value of 96%. They additionally built a discriminant model, using 36 patient collected sputum samples, 6 of which had culture confirmed *M. tuberculosis*, 8 with *M. avium*, 8 with *P. aeruginosa*, 8 with mixed infection and 6 serving as an infection-negative control group, which correctly identified 90% of the unknown samples used to validate this method. They subsequently investigated the capacity of this approach to correctly diagnose TB

using 330 culture-proven, HIV tested, patient collected sputum samples. This method was able to successfully detect TB with a reported sensitivity of 89% and a specificity of 91%, at a detection limit of  $1 \times 10^4$  bacteria  $\text{mL}^{-1}$ , which is comparable to that of smear microscopy. However, as the nature by which these sensors function is not yet fully understood, the identity and quantities of the differentiating volatiles cannot be determined, which may be seen as a limitation to using this sensor array technology (Fend *et al.*, 2006).

Consequently, Phillips *et al.* (2007) tested their suspicion that a unique pattern of volatile organic compounds (VOCs) may be produced in the lungs and exhaled in the breath of patients suffering from pulmonary TB which, when detected using a GC-MS metabolomics approach, may be used for non-invasive TB diagnostics. This hypothesis was based on the fact that mycobacteria produce unique VOC patterns *in vitro* and the observation that increased oxidative stress in TB patients may also lead to distinct VOC patterns. In their investigation, they compared the VOC patterns in the headspace of *M. tuberculosis* cultures to that of sterile growth media. Headspace VOCs were captured on sorbent traps, and subsequently analysed using automated thermal desorption, gas chromatography and mass spectroscopy (ATD/GC-MS). A set of 130 VOCs, mainly benzene derivatives, naphthalene and alkanes, were constantly detected exclusively in the *M. tuberculosis* cultured samples. Using the same approach, breath VOCs from 42 patients suspected of pulmonary TB, were collected and analysed. Accordingly, their method could distinguish between hospitalised patients (with suspected TB) and healthy controls with a 100% specificity and 100% sensitivity, and additionally differentiated between culture proven TB-positive and TB-negative patients, with a specificity of 78.9% and sensitivity of 95.7%. In a follow-up study, using these characteristic VOCs, Phillips *et al.*, were able to correctly classify 226 patient collected sputum samples, with an achieved reported sensitivity of 84% and specificity of 64.7%, and an additional positive prediction value of 76%, when compared to that of smear microscopy, 68% when compared to that of sputum culture, and 66% when compared to that of chest X-rays (Phillips *et al.*, 2010).

These studies show promise for using metabolomics for identifying unique metabolites characterising the disease, which could potentially be used towards a better understanding of the disease or alternatively for less invasive diagnostics. An added advantage of this approach is that it is relatively quick, taking merely a couple of hours to attain a diagnostic result, in contrast to the current gold standard, bacterial culture, which can take anything from 2-6 weeks (Hannan *et al.*, 2008).

Considering this, the concentrations of these newly identified metabolite markers, may also be used to monitor treatment outcome, or towards the early detection of relapses. This would significantly enhance treatment strategies and may consequently prevent or lower the incidence

of drug-resistance due to non-adherence. Metabolomics may additionally be used to test the performance of newly developed anti-TB drugs using these biomarkers, eliminating the need for the 6 month drug trials currently required. An example of where metabolomics was used towards similar applications was reported by Loots *et al.* (2005), who investigated the effects of combined anti-TB drug therapy (using Rifater, a combination of rifampicin, isoniazid, and pyrazinamide) and the co-administration of an antioxidant melatonin, on the organic acid and free radical profiles of rats. They indicated that Rifater treatment results in increased hydroxyl radicals and abnormal organic acid profiles, characteristic of a multiple acyl-CoA dehydrogenase defect (MADD), which subsequently explained a number of the side effect reported for these anti-TB drugs. Furthermore, they indicated that the co-administering melatonin dramatically reduced these biochemical abnormalities, highlighting melatonin's potential use as part of the standard treatment regimen, thereby increasing drug efficacy and reducing the drug associated side effects. Later, using a similar approach, Huo *et al.* (2009) identified a number of metabolites characteristic of metformin hydrochloride therapy, as potential, treatment-induced biomarkers for monitoring treatment progression and side effects. These studies prove the capacity of metabolomics as a tool to elucidate drug mechanisms and contribute to better treatment protocols.

The fact that the final outcome of alterations to the genome eventually result in an altered metabolite profile, also makes metabolomics an excellent functional genomics tool (Baran *et al.*, 2009). In 2000, Fiehn *et al.* used a metabolomics approach to identify distinct metabolic profiles characterising four different *Arabidopsis* genotypes, including two homozygous ecotypes and a mutant of each. Since then, several other studies have documented alterations in the metabolite profiles of a number of other bacterial species due to a variety of genetic perturbations (Baran *et al.*, 2009). Considering this, metabolomics may additionally be used to identify biomarkers specific to various mutations inducing drug-resistance in TB, contributing to a better understanding of the underlying mechanisms of this occurrence, and potentially the development of better diagnostics and treatment approaches for drug-resistant TB

## **7. CONCLUSION**

Considering the above, it is clear that the still growing TB epidemic is partly fuelled by the inadequate performance of the currently available TB treatment and diagnostic procedures (Perkins & Small, 2006), emphasising the urgent need for new, less toxic, faster acting TB treatment approaches, accompanying more sensitive, specific and rapid TB diagnostic methods. The relatively new research field of metabolomics may possibly be a means to identify new biomarkers, not only leading to innovative approaches for TB diagnostics, but also to a better understanding of the intra-host changes induced by TB infection, active disease, and

the treatment there-of. Considering this, the aim of this study was not only to develop a metabolomics research approach for applications to TB research, but also to test the capacity of such an approach for the purpose of identifying new metabolite markers which would better characterise: 1) various TB and non-TB causing bacteria; 2) the effect of drug-resistance in *M. tuberculosis* (using rifampicin resistance as an example of this); and 3) the underlying mechanism of pulmonary *M. tuberculosis* infection (by analysing patient collected sputa).

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