CHAPTER ONE

INTRODUCTION

Tuberculosis (TB) is a disease caused by a bacterium called *Mycobacterium tuberculosis* (*M. tuberculosis*). The bacteria can cause infection not only in lungs; it can extend to other parts of the body such as kidney, spine and brain. Worldwide, TB is one of the leading causes of death among people living with Human Immunodeficiency Virus (HIV) (CDC, 2013). Currently, the World Health Organization (WHO) estimates that over 13 million people have TB and about 1.5 million die each year from the disease (WHO, 2013)

TB can be successfully treated with appropriate antibiotics for at least six months. First-line anti-TB drugs include isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA) (Palomino, Leão & Ritacco, 2007). These drugs are mostly effective against actively replicating populations therefore a lengthy treatment is needed in order to eradicate the dormant bacilli too (Ehlers, 2009). In some patients failing first-line therapy, or diagnosed with multi-drug resistant (MDR)-TB or extensively-drug resistant (XDR)-TB; second line drugs such as Aminoglycosides, Polypeptides, Fluoroquinolones, Thioamides, Cycloserine and Teriziodone are used and considered as reserved therapy (WHO, 2010). There are other drugs such as Rifabutin, Clarirthromycin and Linezolid that can be considered as the third line TB drugs. However, these drugs are not endorsed by the WHO.

Among all the drugs available for use in the treatment of TB, PZA is unique as it is used in first, second and novel drug regimens. It is an oral drug used for TB short course chemotherapy and is metabolized by the liver and excreted by the kidneys. It is one of the few anti-TB drugs that have a unique effective role against persistent tubercle bacilli and it is usually used in combination with other drugs such as INH and RIF. In the host, it is only active against *M. tuberculosis* and *M. africanum*, however *M. bovis* is inherently resistant to it (Bhuju *et al*., 2013). In *M. tuberculosis*, PZA is converted into its active form pyrazinoic acid (POA) by the enzyme pyrazinamidase (PZase) which is coded by the *pnc*A gene (Blanchard,

1996). Susceptible *M. tuberculosis* strains possess functional PZase enzyme. However, possible mutations in the *pnc*A gene may lead to the reduction or the complete loss of PZase activity therefore making the strain resistant to PZA (Butler and Kilburn, 1983).

Several in vitro biochemical tests have been developed to detect PZA resistance in *M. tuberculosis*. These include PZase test (Wayne's assay), Resazurine Microtitre Plate assay (REMA), and Nitrate Reductase assay (NRA) (Siddiqi, Hawkins & Laszlo, 1985; Espinal, 2003; Kontos *et al.*, 2004). Additionally, conventional phenotypic methods such as proportion method, Bactec Radiometric 460 method and Bactec mycobacteria growth indicator tube (MGIT) 960 method have also been used for the detection of PZA resistance. In general, performance of the aforementioned methods is not easy in the routine settings due to the difficulty of growing *M. tuberculosis* in low pH (5.5) which is an important required standard for PZA testing (Trivedi, Desai, 1987; Mphahlele *et al.*, 2008). These challenges highlight the need for new simpler and rapid tests for the detection of PZA resistance (Ando *et al*., 2010).

High Resolution Melting (HRM) curve analysis is a novel powerful real-time Polymerase Chain Reaction (PCR) molecular technique which is useful in the detection of mutations, polymorphisms and epigenetic differences in double-stranded deoxyriboneuclic acid (DNA) samples. Advances such as faster thermo cycling times, higher throughput, flexibility, expanded optical systems, increased multiplexing and more user-friendly software have expanded its use in research and diagnostic fields (Walker, 2002).The general principle of the test is that after real-time PCR, fluorescent melting analysis of PCR products are analysed according to the difference in melting temperature (Tm) between products (Ririe, Rasmussen & Wittwer, 1997). Unique transitions are shown by different genotypes that are revealed by HRM, shape comparison as well as different plots of the melting curve (Wittwer *et al*., 2003; Graham *et al*., 2005)

There has been an increasing interest in the use of HRM for the diagnosis of PZA resistance in *M. tuberculosis* because PZA is an important foundation drug used in the treatment of TB.

HRM is currently considered to be one of the best molecular techniques because of its rapid results, high sensitivity and specificity, relatively low cost, and its compatibility with several methods of DNA preparation (Hoek *et al*., 2008). This study aims to develop and evaluate HRM-curve analysis as a novel technique for rapid and efficient detection of mutations in the *pnc*A gene. Results obtained will provide information on PZA resistance and possibly impact on current TB treatment regimens.

Aim

The aim of this study was to develop and evaluate high resolution melting (HRM) curve analysis for PZA resistance determination in *M. tuberculosis* isolates.

Objectives

1. To collect 120 *M. tuberculosis* isolates stratified with an equal proportion between MDR and non-MDR from the National Health Laboratory Service TB laboratory- Tshwane Academic Division, within the Department of Medical Microbiology, University of Pretoria (NHLS/UP).

2. To determine the percentage of PZA resistance in *M. tuberculosis* as detected by Bactec MGIT 960 (Becton Dickinson, Sparks, MD, USA) in a limited amount of clinical isolates collected from (NHLS/UP).
3. To perform DNA extraction from Bactec MGIT 960 (Becton Dickinson, Sparks, MD, USA) culture tubes.
4. To optimize HRM-curve analysis for detection of mutations in *pnc*A gene
5. To analyse the sensitivity and specificity of the HRM-curve analysis as a rapid detection method for PZA resistance through the identification of mutations in the *pnc*A gene.
6. To perform DNA sequencing for all possible false resistant and sensitive isolates of the *pnc*A gene mutation.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Background

Tuberculosis (TB) is a serious and deadly infectious disease which has a long history. Consumption, king's evil, lupus vulgaris and phthisis are some of the names that have been used to describe TB in the last several centuries (Daniel, 2006). TB affects almost every part of the human body, but pulmonary TB is the most common form and has the highest mortality rate amongst all infectious diseases in humans (Ducati *et al.*, 2006). The causative agent, *M. tuberculosis*, also known as tubercle bacilli was discovered in 1882 by Robert Koch and may have killed more persons than any other pathogen (Daniel, 2006).

Despite the implementation of TB control programmes globally, the management of TB remains a challenge due to the prevalence of HIV infection in many countries. In most individuals infected with TB, host defences are able to inhibit growth of the pathogen, leading to latent infection with persistent and dormant bacteria. About one third of the world's population are latent carriers of the organism but only 10% develop clinical TB (Bhatt, Salgame, 2007; WHO, 2008; WHO, 2009). The situation is further complicated by a worldwide increase in multi-drug resistant-TB (MDR-TB) and extensively drug resistant-TB (XDR-TB) strains (Pablos-Mendez *et al*., 1998; WHO, 2009). With the current treatment requiring multi-drug regimens and a high degree of patient compliance to reduce the risk of drug resistance which has not been consistently achieved, the number of MDR-TB isolates is increasing in many areas of the world. Moreover, *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG), the only vaccine against TB, has proven to be ineffective against the adult form of TB (lung infection), and its efficacy varies in different geographical settings (WHO, 2008). Thus, more effective prophylactic and therapeutic measures are urgently needed to respond to the global TB emergency, requiring more effective vaccines and drug strategies.

Morphologically, *M. tuberculosis* bacilli appear as straight or slightly curved rods, measuring 1-10 μm in length and 0.2-0.6 μm in width, however, size and shape may differ from

coccobacilli to long bacilli (Figure 2.1). *M. tuberculosis* bacilli are non-motile, non-spore forming and strictly aerobic organisms, the bacilli grow more successfully in tissues with high oxygen content, such as the lungs. *M. tuberculosis* is a facultative intracellular pathogen that usually infects mononuclear phagocytes (e.g. macrophages); is a slow growing organisms (2-6 weeks) with generation time 12-18 hrs and requires 5-10% CO_2 and pH 6.5 to 6.8 for optimal growth. The microscopic appearance only does not allow full differentiation of TB (van Soolingen *et al.*, 1998).

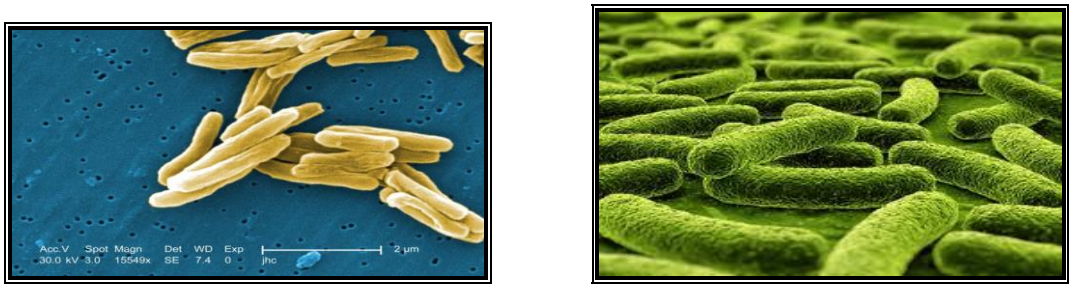

Figure 2.1 Electron micrograph of *M. tuberculosis* cells showing the characteristic rod-shaped bacilli (Todar, 2007; Taggart, 2010).

The cytoplasmic membrane of *M. tuberculosis* is composed of lipopolysaccharides which provide osmotic protection and regulate the movement of specific solutes between the cytoplasm and the environment. It also contains proteins with different functions, i.e. sensors that measure the concentration of certain molecules in the surrounding environment, proteins that transfer signals to the cytoplasm, and carriers that mediate nutrients and ions selective passage. Like other bacteria, the membrane of *M. tuberculosis* is surrounded by a cell wall. This wall protects the cell contents and provides mechanical support to maintain the shape of the bacterium. It constitutes a layer of peptidoglycan, which is suggested to play a role in maintaining the bacterial structure integrity. It resists staining with common dyes and takes up dye either with increased staining time or with application of heat; however, it resists decolourization with up to 3% HCL. This property is known as acid fastness. It can be stained with special agents e.g. Ziehl-Neelsen, Kinyoun and fluorescence (Mahon, 1999) (Figure 2.2).

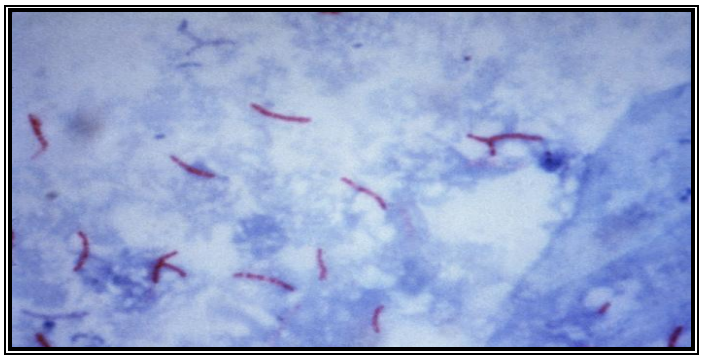

Figure 2.2 Ziehl-Neelsen stain of *M. tuberculosis* (Bryan, 2011)

2.2 Epidemiology

TB is a leading cause of death from an infectious disease worldwide. In young women, TB is responsible for killing more women than any other causes of maternal mortality combined (Borgdorff *et al*., 2000). However, more TB cases are reported among men than women, which may be due to behavioural and cultural practices related to health care seeking behaviour or occupational related risk such as the mining sector with increased risk for TB and is male dominated to the fact that men have more access to health facilities in most settings (Borgdorff *et al*., 2000).

Globally, it is difficult to obtain accurate information regarding the incidence of drug-resistant TB. This mainly due to the fact that culture and drug susceptibility testing (DST) are not routinely done in most developing countries where the disease is more common. However, many surveillance studies have indicated the wide spread distribution of drug-resistant TB (Zignol *et al*., 2006).

In 2012, 8.6 million incident cases of TB were reported. In the Southern African region, more than 50% of TB cases were HIV co-infected. In areas of sub-Saharan Africa and Southeast Asia where there is a high HIV infection and inadequate access of health care, the highest TB incidence rates are found (Wilson, 2013; WHO, 2013). A systematic review on MDR-TB in 2013, reported that there were 450 000 new MDR-TB cases worldwide in 2012 including primary and acquired MDR-TB (WHO, 2013). South Africa was one of the five countries with the highest number of incident cases (0.4 million-0.6 million) and accounted for 10% of the global XDR-TB cases and showed an increase from 767 cases in 2009 to 1596 cases in 2012 (WHO, 2013). In the same year, an estimated 12 million prevalent cases (range, 11

million–13 million) of TB have been reported worldwide, this rate had fallen by 37% between the year 2009 and 2012. In all six WHO regions, TB prevalence rates are declining however the African and Eastern Mediterranean regions are unlikely to reach the target of declining TB prevalence by the year 2015 (WHO, 2013).

2.3 Laboratory diagnosis of TB

Rapid identification of new TB cases is crucial in reducing the high rates of incidence and prevalence of TB (Wysocki *et al*., 2013). TB diagnosis is mainly based on the combination of clinical, radiological, and microbiological testing (Palomino, 2005). Globally, especially areas with inadequate health care access, many hospitals and clinics rely mainly on poor diagnostic tools such as sputum smears, chest radiography and tuberculin skin testing to make the diagnosis of TB, without the ability to perform cultures and DST (Perkins, Cunningham, 2007; Pai, O'Brien, 2008; Wilson, 2013). Laboratory diagnosis of TB can be divided into phenotypic methods, molecular methods and serological methods.

2.3.1. Phenotypic methods

2.3.1.1 Ziel-Neelson/Kinyoun and Auramine O stain

Ziel-Neelson (ZN) is a stain used for the identification of acid-fast bacilli (AFB). The method includes a heating step in order to facilitate the accessibility of the dye through the mycolic acid present in the mycobacterial cell wall. In this method, carbol fuchsin is used as a primary stain, acid alcohol as a decolourizer and methylene blue as a counter stain. In microscopic examination, AFB will appear as red and non-AFB as blue (Bartelt, 1999). Kinyoun stain is similar to the ZN method however it doesn’t involve the application of heat.

The principle of Auramine O stain is mainly dependent on the high affinity of the mycolic acid to the fluorochromes enabling the dye to bind to the cell wall of mycobacteria. When mycobacteria are examined by the fluorescence microscope they appear as bright yellow rods. Auramine O is a fast and highly sensitive method as compared to the ZN method, therefore it is generally recommended for the microscopic detection of mycobacteria (Isenberg, 1998; Salfinger, Heifets, 1988; Ebersole, 1992).

2.3.1.2 Light-emitting diode (LED) microscopy

Light-emitting diode (LED) microscopy is based on the conversion of electric energy into light through a reversible injection electroluminescence process which is facilitated by semi-conducting doped materials (Schubert, 2006). In a comparison study, LED detected more scanty smears than ZN on conventional light microscopy therefore LED has been recommended by WHO to be a good alternative to conventional microscopy (WHO, 2011; Xia *et al*., 2013). The increased sensitivity, rapid examination time, low cost and good user acceptance make LED a preferable technique in peripheral laboratories and poor settings (Xia *et al*. 2013). However, when LED was used to recheck AFB smears without re-staining within a month, false negative results were observed, this is probably due to the AFB colour fading (Radhakrishnan *et al*., 2013).

2.3.1.3 Culture methods

The low specificity of the staining techniques of *M. tuberculosis* has made the confirmation of TB diagnosis using culture methods very important. Specimens collected from sterile body sites can be cultured directly onto the culture medium, while contaminated specimens from non-sterile sites of the body have to be decontaminated and concentrated before culture to eliminate the microbial flora that may interfere with mycobacterial growth. The most common decontamination solutions used are two or four percent sodium hydroxide, N-acetyl-cystein sodium chloride, and oxalic acid (Palomino, Leão & Ricatto, 2007).

Solid culture media used for *M. tuberculosis* isolation are mainly egg-based media with high concentration of malachite green, this reagent is added to overcome bacterial contamination during growth e.g. Lowenstein-Jensen (LJ) medium and Ogawa medium. Other solid culture media such as Middlebrook 7H10 and 7H11 are agar-based (Palomino, Leão & Ricatto, 2007; Piersimoni *et al.*, 2013), while Middlebrook 7H9 is broth-base culture media that also support the growth of *M. tuberculosis*, it is supplemented with some nutrients such as glycerol, oleic acid, albumin, and dextrose. In addition to the importance of Middlebrook 7H9 in the growth of *M. tuberculosis*, this medium is also very useful in the antimicrobial assay, biochemical tests and maintenance of stock strains (MacFaddin, 1985; Metchock, 1999). Sauton media is another broth-based media that contains L-asparagine and Tween 80,

it is used for identification of *M. tuberculosis* based on the utilization of carbon of asparagine for growth and protein synthesis in *M. tuberculosis* (Lyon, Hall & Costas-Martinez, 1969).

Generally, conventional culture methods for the diagnosis of *M. tuberculosis* are usually very slow, taking up to eight weeks for growth to appear and required viable microorganisms. The introduction of the broth systems, such as Bactec TB-460 and Bactec MGIT 960 (Becton Dickinson, Sparks, MD, USA), facilitates an earlier diagnosis of TB.

2.3.1.4 Microscopic observation drug susceptibility assay (MODS)

Microscopic observation drug susceptibility assay (MODS) is a liquid culture method based on microscopic detection of characteristic *M. tuberculosis* morphology-like tangles or cords. With the aid of an inverted light microscope, mycobacterial growth can be detected long before it would be visible to the naked eye (Park *et al.*, 2002; Moore *et al*., 2004). Automated systems are expensive and not widely used in resource-poor settings therefore MODS appears to have the potential to become a tool which may improve TB management in such settings and can be a promising alternative to these methods (Makamure *et al*., 2013; Trollip *et al.*, 2014)

Many studies have been done to evaluate MODS as a rapid, inexpensive, sensitive, and specific method for *M. tuberculosis* detection as well as susceptibility testing. It has been found to be appropriate for use in developing countries with high TB burden (Huang *et al*., 2013).

2.3.1.5 Colorimetric assays

The colorimetric assay is based on the changing of the yellow colour of the dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide or MTT) into a purple colour by the enzymatic activity of the growing *M. tuberculosis* bacilli which can be detected visually or by use of spectrophotometer (Abate, Mshana & Miorner, 1998). This assay is very simple to use, rapid, showed low contamination levels and can be used as an alternative to MGIT culture in limited resource settings (Boum *et al*., 2013). There are many examples of other colorimetric methods which are useful in the diagnosis of TB and they are based on the inhibition of *M.*

tuberculosis growth in the presence of certain antibiotics such as: Wayne's assay, (Resazurine Microtitre Plate assay) REMA, and (Nitrate Reductase assay NRA) (Palomino, 2005).

2.3.1.6 Phage-amplification assays

Phage-amplification assay is based on the infection of *M. tuberculosis* cells present in clinical specimens by specific mycobacteriophages. Only phages that have infected TB cells will continue their replication, while the free ones will be inactivated by the action of virucidal solution (virusol) (Albert *et al*., 2002). The infected cells are further tested on an agar plate containing fast growing sensor cells (*M. smegmatis*) to detect progeny mycobacteriophages. Phages then infect and lyse sensor cells making holes or zones in the plate. The number of zones is directly proportional to the number of viable *M. tuberculosis* in the clinical specimen (Veena Sharma *et al*., 2013).

Phage-amplification assay has become more popular in developing countries, because it is the only developed method to detect viable *M. tuberculosis* cells in clinical specimens within 48 hrs in addition to being cost-effective (Albert *et al*., 2002).

2.3.2 Molecular methods

Molecular techniques primarily target the detection of genetic determinants of organism or related resistance. Generally, molecular or genetic methods rely on performing two main steps: amplification of nucleic acid of *M. tuberculosis* genome by polymerase chain reaction (PCR), and secondly testing the PCR amplified products for the detection of any mutation causing the resistance (Palomino, 2005).

2.3.2.1 AccuProbe *M. tuberculosis* Assay

AccuProbe M. tuberculosis complex culture identification test (GenProbe, San Diego, CA) is the method that depends on direct detection and identification of *M. tuberculosis* from positive broth cultures, this shortens the overall time needed for the diagnosis of TB (Cloud *et*

al., 2005; Said *et al*., 2011). It is a rapid DNA probe test that relies on the principle of hybridization and the ability of complementary nucleic acid strands to specifically align to the ribosomal ribonucleic acid (rRNA) of the target organism. The test identifies TB from positive broth cultures in less than one hour (Kohne, Stegetwalt & Brenner., 1984; Said *et al*., 2011). Although the test is easy, and rapid with high sensitivity and specificity, it is also laborious, expensive, and technically demanding, requiring relatively large amounts of specimen (two to three ml).

2.3.2.2 Roche LightCycler Mycobacterium detection kit

Roche LightCycler Mycobacterium detection kit is a real-time PCR based assay which is a very useful tool in the detection of *M. tuberculosis*. Real-time PCR is considered as a highly sensitive, specific, and rapid molecular method in addition to its property of species identification due to its ability to detect melting temperatures of double stranded DNA during heat dissociation of DNA. Roche LightCycler® system has been widely used as a platform of real-time PCR in high-burden settings due to its high-throughput. Roche LightCycler® technology can detect rRNA gene of *M. tuberculosis* in clinical specimens as well as positive cultures. This kit has shown to be rapid (90 min.), robust, sensitive, specific and accurate for rapid diagnosis of TB (Omar *et al*., 2011).

2.3.2.3 Line probe assays

Line probe assays are the techniques which are based on using nitro-cellulose strips that have specific probes attached to them allowing simultaneous molecular identification of TB and also for the detection of single nucleotide polymorphisms (SNPs) associated with resistance in *M. tuberculosis* complex (O'Grady *et al*., 2011). The most common example of line probe assays is the Genotype MTBDR*plus* assay (Hain Lifesciences GmbH, Nehren, Germany), it is used for the detection of high level of resistance to RIF and INH through mutation detection in *rpo*B gene and *kat*G gene, respectively (Lacoma *et al*., 2008)

The test is based on hybridization of labelled PCR products of the amplified *M. tuberculosis* genome with specific oligonucleotide probes immobilized on a strip. If a mutation is present,

the amplicon cannot hybridize with the relevant probe. Drug resistance is detected by the development of coloured bands on the strip only at the sites of probe binding, and can be observed by the naked eye. However, though the MTBDR assay showed a high sensitivity and specificity in detection of *M. tuberculosis* complex and drug resistance (WHO, 2008), it identifies only the most frequent mutations, is labour intensive and requires highly trained personnel and dedicated laboratory space and equipment (Makinen *et al*., 2006; Nicol, 2010).

2.3.2.4 Loop-mediated isothermal amplification

Loop-mediated isothermal amplification is a novel molecular technique which is used for the amplification of a single copy of *M. tuberculosis* gene under isothermal conditions using six sets of primers. The amplification can be detected easily just by observing fluorescence or turbidity without the need of using labelled probes or gel (Boehme *et al*., 2007; Sethi *et al*., 2013). Loop-mediated isothermal amplification assay is a very rapid, specific and effective method in the diagnosis of positive smears as well as confirmation of negative smears. Since it is a closed-tube system, it minimizes the risk of contamination and can be used in less sophisticated settings (Sethi *et al*., 2013).

2.3.2.5 Oligoneuclotide microarray

The principle of the oligonucleotide microarray is based on the digestion of the *M. tuberculosis* genome by restriction enzyme and ligating these fragments from their sticky sites with adapters and amplifying them by PCR. After PCR, the restriction fragments which are representing the entire genome will be labelled with different fluorophores and co-hybridized to a microarray genome (Sarmiento *et al*., 2003; Lima *et al*., 2008). Then, the data will be analysed using specific software. Classically, the software used three colours together with control to differentiate between different gene fragments. A green colour represents the up-regulated gene, red represents the down-regulated gene, and yellow represents the genes of equal abundance in both test and control (Bartelt, 1999). Oligonucleotide microarray allows the detection of mutations in genes (substitutions, deletions and insertions) related to drug resistance i.e. MDR and XDR-TB (Volokhov *et al*., 2009). Detection of full resistance profile can be achieved in a single day (Sarmiento *et al*., 2003; Lima *et al*., 2008).

2.3.2.6 Automated nucleic acid amplification tests

Fully automated nucleic acid amplification test (NAAT) is based on hemi-nested real-time PCR amplification of a target gene in *M. tuberculosis* and the forerunner in this technology is the Xpert MTB/RIF assay (Cepheid, Suunyvale, USA) which has become one of the common tests used in the diagnosis of TB and endorsed by the WHO as a replacement test for smear microscopy. This assay was designed to overcome the problems of the manual NAAT and simplifies molecular testing by integrating and automating all essential processes for real-time PCR examination (sample preparation, amplification, and detection). It has a short turnaround time and can detect RIF-resistant *M. tuberculosis* in less than three hours. Its rapid diagnosis allows the start of effective treatment in TB patients much sooner than waiting for the results of other DST methods (CDC, 2009; Zeka, Tasbakan & Cavusoglu, 2011). Considering the increase in MDR-TB rates globally, Xpert MTB/RIF assay has become a good replacement to microscopy (Ebersole, 1992; Isenberg, 1998; Salfinger, Pfyffer, 1994; WHO, 2013). The test is easy to perform and requires minimum training. There are some shortcomings of Xpert MTB/RIF assay such as the need of a stable electricity supply, the need of annual calibration of the instrument and high cost of the test (Trebucq *et al*., 2011).

2.3.3 Immunological tests

Immunological tests are based on the detection of humoral or cell mediated immune response to *M. tuberculosis* antigens in patient's using blood samples or skin tests (Palomino, Leão & Ricatto, 2007). Advantages of immunological tests include its rapid diagnosis, simplicity of their formats, and modest training requirements in comparison to microscopy (Makamure *et al*., 2013).

2.3.3.1 Tuberculin skin test (Mantoux test)

In 1882, Robert Koch discovered the treatment of *M. tuberculosis*. He obtained heat-deactivated filtrate of TB cultures, injected it experimentally into guinea pigs and observed cure from TB. This product was later named as Koch's old tuberculin, it triggers the delayed

type hypersensitivity reaction (DTH) of the human skin within 24-72 hrs., the resultant reaction is then measured and used for TB diagnosis (Palomino, Leão & Ricatto, 2007).

In 1934, Siebert made a simple protein precipitate of old tuberculin and named it purified protein derivative (PPD) which is nowadays used as an injectable antigen for detecting the latent and active TB infection (Palomino, Leão & Ricatto, 2007). There are many challenges facing the use of PPD based tests such as false-positive results, which are caused by other environmental mycobacteria that share common antigens with *M. tuberculosis*, false-negative results in immunocompromised patients, inaccurate and difficult interpretation among health care workers and difficulty in application and lack of reproducible results. Due to all the above mentioned shortcomings, several new assays have been developed to assess the response of TB specific antigens and evaluate them as diagnostic tools (Tat, Polenakovik & Herchline, 2005).

2.3.3.2 Interferon-gamma (INF-γ) release assays (IGRAs)

Interferon-gamma (IFN-γ) release assays (IGRAs) are in vitro immune tests which are introduced as an alternative to the tuberculin skin test (TST) for the diagnosis of the latent tuberculosis infection (LTBI) (Moon, Hur, 2013). They are blood tests based on the production of IFN-γ by T-lymphocytes from sensitized individuals when exposed to *M. tuberculosis* antigens i.e. detect the presence of persistent cellular immune responses towards the *M. tuberculosis* (Halevy, Cohen & Grossman, 2005; Trollip *et al*., 2014). There are many IFN-γ assays commercially available; these include the enzyme-linked immunospot (ELISPOT) and Tspot-TB (Oxford Immunotec, UK) (Palomino, Leão & Ricatto, 2007). These assays have some limitations as they do not distinguish between active and latent TB infections, they also need sufficient amounts of blood to be drawn from patients and processed within limited time frame, and this is more difficult in children.

2.3.3.3 Enzyme linked immunosorbent assays (ELISA)

ELISA is a serological test used for the detection of *M. tuberculosis* antibody using specific antigen attached to the specific surface. Briefly, *M .tuberculosis* antigen possibly present in the clinical specimen e.g. (serum, plasma, or cerebrospinal fluid (CSF)) binds to the

corresponding antibody, then the antigen-antibody complex is detected by a colour change that occur due to the presence of a substrate attached to the antibody. There are some commercially available ELISA tests which are used in developing countries and used for the detection of specific antigens such as the 38 kDa protein. Nowadays, ELISA has become a very useful tool for the detection of immunological response to *M. tuberculosis* antigens, this is mainly because of its speedy results and good sensitivity and specificity (Palomino, Leão & Ricatto, 2007; Huang *et al*., 2013).

2.3.3.4. Immunochromatographic (ICT) assays

Immunochromatographic (ICT) assays, also known as lateral-flow tests or simply rapid strip tests, are based on the detection of Immunoglobulin G (IgG) antibodies directed against highly purified antigens secreted by actively growing *M. tuberculosis* (Bartoloni *et al*., 2003). Simply, serum or plasma of a TB patient is applied into test area in the card followed by the addition of two drops of specific diluents and reading the result within 20 min. Rapid strip tests are very useful due to its rapid results, low cost, high sensitivity (99.1%) and specificity (100%), however these tests may be not available in low-resource countries and cannot differentiate active from latent disease nor susceptible from drug resistant cases (Schubert,. 2006; Palomino, Leão & Ricatto, 2007; Maurya *et al*., 2012).

2.4 Drug resistance in *M. tuberculosis*

M. tuberculosis is inherently resistant to various antibiotics and chemotherapeutic agents. The alteration of drug targets, putative drug efflux systems, the ability to produce hydrolytic and drug-modifying enzymes such as aminoglycoside acetyltransferases as well as the presence of a highly hydrophobic cell wall, make treatment of TB more difficult (Ducati *et al*., 2006). The development of clinical drug resistance in TB is classified as primary resistance and acquired resistance. Primary resistance is when a patient is infected with a resistant strain (Cantwell *et al*., 1994), while acquired resistance is when drug resistant mutants are selected as a result of ineffective treatment (Cantwell *et al*., 1994), irregular drug supply, inappropriate prescriptions, it occurs mainly when the patient was put on monotherapy (Vareldzis *et al*. 1994). The genetic resistance is usually due to spontaneous chromosomal mutation in the process of mycobacterium replication (Zhang, Yew, 2009). The classification

of drug resistance into primary and acquired is very clear, but is preferable to use the term "initial drug resistant" for the primary resistance especially when the previous treatment cannot be readily determined (Akolo *et al*., 2010).

Resistance to first line anti-TB drugs has been linked to mutations in at least 10 genes; *kat*G, *inh*A, *ahp*C, *kas*A and *ndh* for INH resistance; *rpo*B for RIF resistance, *emb*B for EMB resistance, *pnc*A and *rps*A for PZA resistance and *rps*L and *rrs* for STR resistance. The phenotypic methods as well as molecular mechanisms to detect resistance of second line drugs are less well established and the molecular mechanisms of resistance are also less clearly defined (Johnson *et al*., 2006).

2.4.1 Multi-drug resistance TB (MDR-TB)

MDR-TB is TB which is resistant to both INH and RIF (Minion *et al*., 2013). Globally, an estimated 3.6% of new cases and 20.2% of previously treated cases have MDR-TB (WHO, 2013). In 2012, a total of 94 000 TB patients were eligible for MDR-TB treatment, 84 000 of them with confirmed MDR-TB and the remaining 10 000 with RIF resistance alone. Between 2011 and 2012, the largest increases were in India, South Africa, and Ukraine (WHO, 2013).

Incomplete and inadequate treatment is the most important factor leading to the development of drug resistance, suggesting that it is often a man made tragedy. Efficiently run TB control programmes based on a policy of directly observed treatment, short course (DOTS) are essential for preventing the emergence of MDR-TB (WHO, 2012).

2.4.2 Extensively drug resistance TB (XDR-TB)

XDR-TB is TB which is resistant to INH, RIF, fluoroquinolones and one or more injectable drugs (amikacin, kanamycin, or capreomycin) (CDC, 2007; Minion *et al.*, 2013). In 2012, an average of 9.0% of MDR-TB cases were identified as XDR-TB in 84 countries. Treatment success rate of XDR-TB was only 22% in 26 of these countries, which excludes South Africa which had 44% mortality (WHO, 2013). Effective treatment of XDR-TB is challenging as it requires proper selection of one of the second-line drugs by an expert clinician (Zhang *et al*., 2003), however, in a recent study, the combination therapy of linezolid, moxifloxacin, and

thioridazine was used with a high success rate, though it was tested in HIV negative TB patients (Abbate *et al*., 2012) .

2.5 Pyrazinamide (PZA)

2.5.1 Introduction

PZA is one of the most important first-line anti-TB drugs as it has bactericidal activity (Palomino, Leão & Ricatto, 2007). PZA, together with INH and RIF, represent the cornerstone of TB therapy (Zhang *et al*., 2003). PZA was first introduced in 1980 for further reduction of the anti-TB treatment from 12 to six months, (Zhang *et al*., 2003; Palomino, Leão & Ricatto, 2007). This is mainly because of its unique role in killing the semi-dormant bacilli that are not killed by other drugs (Konno, Feldmann & McDermott, 1967; Heifets & Lindholm-Levy, 1992; Palomino, Leão & Ricatto, 2007). PZA is more active against old *M. tuberculosis* bacilli than young and newly replicating mycobacteria, especially in acidic pH which is produced due to inflammation (Tarshis, Weed, 1953; Zhang, Permar & Sun, 2002).

PZA and INH combination is considered to be rapidly bactericidal for *M. tuberculosis*, thus it is a critical component to short-course treatments such as DOTS (Konno, Feldmann & McDermott, 1967; Palomino, Leão & Ricatto, 2007). PZA has no activity against other mycobacteria (Heifets, 1996; Scorpio, Zhang, 1996). Regardless of the importance of PZA in the treatment of TB, its mechanism of action is still not fully understood and its resistance mechanism is largely unknown (Konno, Feldmann & McDermott, 1967; Palomino, Leão & Ricatto, 2007).

2.5.2 Mechanism of action

The mechanism of action of PZA is the least understood among all anti-TB drugs because of its unique and unusual properties (Zhang *et al*., 2003). PZA, a nicotinamide analogue, is a pro-drug that has to be converted intracellularly to its active form POA, by bacterial enzyme PZase. PZase is encoded by *pnc*A gene found in *M. tuberculosis* genome (Davies *et al*., 2000; Wade *et al*., 2004). This was proven by introducing a wild type plasmid copy of the gene into

resistant strains and both PZase activity and PZA susceptibility were restored (Scorpio, Zhang, 1996). The product of this gene also has nicotinamidase activity, which explains the association between these two enzymes (Singh *et al*., 2006). When the extracellular pH of *M. tuberculosis* cells is acidic, POA accumulates inside the bacilli and lowers the intracellular pH which is thought to be responsible for inactivation of a vital target enzyme such as fatty acid synthase I (fasI) as shown in figure 2.3 (Konno, Feldmann & McDermott, 1967; Zhang *et al.*, 1999; Zhang, Mitchison, 2003). It is suggested that PZA diffuses into *M. tuberculosis* cells in a passive way and accumulates in large amounts inside the mycobacterial cytoplasm due to the insufficient efflux system (Zhang *et al.*, 1999). The POA efflux rate is also affected by many factors such as: the ability of PZA to enter the bacilli, the concentration and activity of the PZase enzyme, the concentration and efficiency of the efflux pumps in the bacterial cell membrane (Zimic *et al*., 2012). *M. smegmatis* possesses highly active efflux mechanism by which it has the ability to dispose POA out of the cell and thus, is naturally resistant to PZA (Zhang *et al.,* 1999).

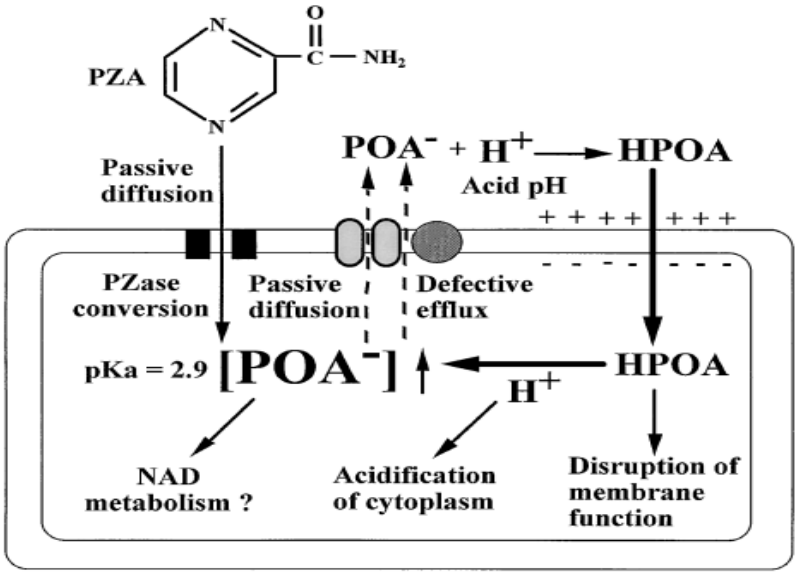

Figure 2.3 Mechanism of PZA action (Zhang, Mitchison, 2004)

In 2003, Zhang *et al*. described another mode of action of PZA, which proved that PZA and POA could inhibit the protein and RNA synthesis which leads to disturbance of the membrane transport function at acidic pH, de-energizing and collapsing the membrane (Zhang *et al.,* 2003). It is also suggested that PZA kills the dormant bacilli through the

accumulation of POA in the acidic environment which leads to its binding to ribosomal protein S1 (*Rps*A) and inhibits the trans-translation process in *M. tuberculosis* (Ahmady *et al*., 2013).

2.5.3 PZA resistance in *M. tuberculosis*

2.5.3.1 Epidemiology of PZA resistance

Although PZA resistance is poorly investigated, PZA remains one of the important drugs that are used in the treatment of MDR-TB. Studies conducted in Japan, South Africa and Peru have shown that 53%, 54% and 59% of MDR-TB patients respectively are also resistant to PZA (Saravia *et al*., 2005; Mphahlele *et al*., 2008; Ando *et al*., 2010). In 2008, a study conducted in South Africa showed that a high proportion of South African MDR-TB were resistant to PZA, suggesting re-evaluation of the role of PZA in treating MDR-TB as well as its role in previously treated patients (Mphahlele *et al*., 2008). Another recent study from the Eastern Cape Province in South Africa showed that 40% of MDR TB harboured identical mutations concurrently conferring resistance to PZA (Muller *et al*., 2013). In the United States, among nearly 80 000 TB cases between 1999 and 2009, 2.7% of these were PZA resistant (Kurbatova *et al*., 2013).

2.5.3.2 PZA resistance mechanisms

Resistance to PZA in *M. tuberculosis* can be classified as acquired resistance, which is usually due to mutation in the *pnc*A gene (Scorpio, Zhang, 1996), or primary resistance, which is due to a single point mutation (C→G) in the *pnc*A gene. In *M. tuberculosis*, PZA resistance is associated with the loss of PZase activity (Konno, Feldmann & McDermott, 1967). In 1996, *pnc*A was cloned and sequenced for the first time and 72 to 97% of all PZA-resistant clinical isolates tested showed mutations in the structural gene or in the putative promoter region of the *pnc*A gene that alter gene expression (Scorpio, Zhang, 1996). A recent study from MDR patients in South Korea also found that mutation in the *pnc*A gene is the major mechanism of resistance to PZA (Kim *et al*., 2012).

Mutations in PZA-resistant *M. tuberculosis* have been found unusually located and scattered throughout the *pnc*A gene.(Scorpio, Zhang, 1996) Three clustered mutation areas were

identified in the region of amino acids 3 to 71, 61 to 85 and 132 to 142 (Scorpio *et al*., 1997b). These mutations can be substitutions, insertions or deletions of nucleotides (Hirano *et al*., 1997). Furthermore, these regions are more likely to include catalytic sites for the PZase enzyme (Singh *et al*., 2006). *M. bovis* and *M. bovis* BCG carry a unique point mutation (C to G) in codon 169 of the *pnc*A gene which makes these strains naturally resistant to PZA (Scorpio, Zhang, 1996). This unique mutation can be used to differentiate these two species from *M. tuberculosis* (Scorpio *et al*., 1997a).

The wide diversity of *pnc*A mutations is exclusive to PZA resistance, as this degree of diversity is not associated with other anti-TB drug resistant genes (Singh *et al*., 2006). The reasons for the highly diverse *pnc*A mutations is unclear, it is assumed that *pnc*A may be located in a hot spot for mutation or DNA mismatch repair mechanisms are lacking (Mizrahi, Andersen, 1998). Several studies have confirmed varying frequencies of mutations when sequencing of the *pnc*A gene in PZA resistant isolates was done (Scorpio, Zhang, 1996; Scorpio *et al*., 1997b; Sreevatsan *et al*., 1997). The differences in the mutations rates in these isolates might have been due to incorrect susceptibility testing (a common problem with the current techniques), as well as involvement of undefined gene(s) that may be involved in PZA resistance (Hewlett, Horn & Alfalla, 1995).

While the *pnc*A mutations are considered the major mechanism of PZA resistance (Hirano *et al*., 1997), the existence of some PZA resistant isolates without any mutations in the *pnc*A gene suggested the involvement of some other resistance mechanisms such as PZA uptake; *pnc*A regulation and POA efflux pump (Raynaud *et al*., 1999). Furthermore, variable PZase activities were also observed in those resistant isolates, and that highlighted the need of further investigations on PZA resistant isolates which are PZase positive with no *pnc*A mutations (Mestdagh *et al*., 2000; Singh *et al*., 2006). The basis of the PZA resistant isolates with wild type *pnc*A can be due to unknown mutations that reduce both activity and expression of the PZase or strengthen the activity of weak POA efflux pump in *M. tuberculosis* (Singh *et al*., 2006).

A study conducted in 1999 by Raynaud *et al*. represents the importance of lack of PZA uptake in addition to the lack of PZase activity in PZA resistant *M. tuberculosis.* This study concluded that the naturally resistant strains to PZA such as *M. smegmatis* failed to take up

PZA intracellularly, in contrast to susceptible *M. tuberculosis* which exhibit both PZA uptake and PZase activities (Raynaud *et al.*, 1999). POA efflux rate is also an important factor in resistance to PZA especially in *M. tuberculosis*. When the POA efflux rate of *M. smegmatis* was tested, it was 900 fold higher than that in *M. tuberculosis* strains, which suggests that the accumulation of POA itself is not of importance (Zimic *et al*., 2012). After adjusting other factors, POA efflux rate is a better predictor for PZA resistance in *M. tuberculosis* than *pnc*A mutation and PZase activity (Sheen *et al*., 2013).

PZA also inhibits the trans-translation process in *M. tuberculosis* by binding to the ribosomal protein S1 (*Rps*A). The ribosomal protein (*Rps*A) has an important action in the translation and trans-translation processes by specifically binding to tmRNA (Bycroft *et al*., 1997). POA binds to 3' terminus of the tmRNA preventing its binding to (*Rps*A) which leads to the inhibition of the trans-translation process in the mycobacterium cell (Shi *et al*., 2011).

In 2009, Sheen *et al*. examined the correlation between the kinetic parameters of recombinant mutated PZase cloned from PZA resistant *M. tuberculosis* clinical strains and the microbiological PZA resistance level. The study showed that the efficiency of the mutated enzymes accounts for some strains but not all the inconsistency of resistance level, which suggests the involvement of an alternate mechanism other than the alteration of the PZase function (Sheen *et al.*, 2009).Understanding these mechanisms of *M. tuberculosis* resistance to PZA or other anti-TB drugs helps in developing more rapid diagnostic tests for diagnosis. (Zhang, Yew, 2009).

2.5.3.3 PZA resistance tests

2.5.3.3.1 Phenotypic tests

Generally, all phenotypic methods on solid medium are based on the cultivation of *M. tuberculosis* on egg-based or agar-based solid media in the presence of antibiotics to assess the inhibition of growth in order to distinguish between susceptible and resistant strains (Palomino, Leão & Ricatto, 2007).

• The proportion method

The proportion method is based on the number of colonies obtained in both drug-containing and drug-free (control) media. Hundred-fold serial dilutions of *M. tuberculosis* are inoculated in the above mentioned media, colonies obtained are enumerated and the proportion of resistance is then calculated. When the test is performed using LJ medium, the result will be read after 28 days incubation at 37°C. If the test is performed in a Middlebrook 7H10 or 11 medium, the result will be interpreted after 21 days at 10% CO_2 atmosphere incubation. If the proportion of resistant bacteria is higher than 10% for PZA, the isolate is considered resistant (Palomino, Leão & Ricatto, 2007). A recent study reported that the inoculum size that is used in the proportion method is the main factor leading to errors due to changing of the pH of the medium caused by production of ammonia during the metabolism process of the bacteria which causes inactivation of PZA (Bhuju *et al*., 2013).

• The Bactec radiometric 460 method

The Bactec radiometric 460 method (Becton Dickinson, Sparks, MD) uses enriched Middlebrook 7H9 liquid medium that contains 14C-labeled palmitic acid as a sole source of carbon. Consumption of carbon by growing mycobacteria leads to production of CO_2 which is detected by the instrument and expressed as a growth index. Susceptibility of *M. tuberculosis* to the antimycobacterial agents, e.g. PZA, is measured by the daily increase in the growth index (Palomino, Leão & Ricatto, 2007).

Susceptibility testing in the radiometric Bactec 460 TB system was considered the reference method for susceptibility testing. Recently, the fully automated Bactec MGIT 960 system (Becton Dickinson, Sparks, MD, USA) has been demonstrated to be an excellent alternative for the radiometric system. It is as sensitive and rapid as the radiometric system; however the Bactec MGIT 960 system does not require radio labelled compounds. Therefore, the routine use of the Bactec MGIT 960 system has been recommended (Kontos *et al*., 2003).

- **The Bactec MGIT 960 method**

The Bactec MGIT 960 system is an automated, continuously monitoring system based on the detection of bacterial growth in the drug-containing and drug-free media (control tube) (Lakes, 2002; Said *et al.*, 2012). The Bactec MGIT 960 system is based on measurement of oxygen consumption and has been thoroughly evaluated for susceptibility testing of *M. tuberculosis* to first- and second-line drugs showing a good concordance with the gold standard proportion method (Siddiqi, 2006).

PZA susceptibility testing can be performed based on the same principle. Two MGIT tubes are inoculated with the test culture. A known concentration of PZA (100 µg/ml) is added to one of the tubes, and growth is compared to the MGIT tube without the drug (growth control). If PZA is active against the isolated mycobacteria, it will inhibit the growth and thus there will be suppression of fluorescence, while the growth control will grow uninhibited and will have increasing fluorescence. Growth is monitored by the Bactec MGIT 960 instrument that automatically interprets results as susceptible or resistant (Siddiqi, 2006).

In 2002, Tortoli *et al* evaluated the automated Bactec MGIT 960 system for susceptibility testing of *M. tuberculosis* to four major anti-TB drugs (STR, INH, RIF and EMB) in comparison with the radiometric Bactec 460-TB method and the agar proportion method (as a gold standard method). The overall agreement between Bactec MGIT 960 system and Bactec 460-TB method was 96.7%. The average time to a final result was 6.9 (range, 5 to 16) days for Bactec 460 and 9.4 (range, 5 to 14) days for Bactec MGIT 960.

In 2004, Kontos *et al* evaluated the fully automated Bactec MGIT 960 system for susceptibility testing of *M. tuberculosis* to first-line drugs. The accuracy of the system for susceptibility testing of 177 clinical isolates was compared with the agar proportion method. The sensitivity, the ability to detect resistance, of the Bactec MGIT 960 system was 100%, while the specificity, the ability to detect susceptibility, ranged from 98.6% to 100% for all drugs tested. The median time for obtaining susceptibility results was 7.12 days (range 5–13 days). Bactec MGIT 960 system is regarded as an excellent method that provides rapid and reliable results of susceptibility testing of first- and second- line TB drugs (WHO, 2007; Parrish, Carrol, 2008).

Currently, there are major errors of false resistance results that are reported by Bactec MGIT 960 system when testing the susceptibility of *M. tuberculosis* to PZA. These errors are prevented by using a smaller inoculum size of the isolate than that recommended by the manufacture i.e. from 0.5 ml to 0.25 ml. Results of the reduced inoculum were confirmed by comparing them to the results of *pnc*A mutation analysis and Bactec 460. The sensitivity and specificity of the reduced inoculum assay with above mentioned methods was 92% and 100% respectively (Piersimoni *et al*., 2013).

2.5.3.3.2 Biochemical tests

- **Wayne's assay (PZase test)**

The study of anti-TB activity of PZA *in vitro* is challenging, as it is active only at an acidic pH, which itself suppresses the growth of *M. tuberculosis* (Salfinger, Heifets, 1988). This difficulty makes the detection of PZase activity an alternative to the conventional methods (McClatchy, Tsang & Cernich, 1981). Wayne's assay is a colorimetric assay which detects PZase activity by the hydrolysis of PZA to POA. Briefly, a loopful of a positive culture is inoculated onto the surface of Middlebrook 7H9 agar containing 100µg/ml PZA and two µg/ml sodium pyruvate. The tubes are then incubated at 37°C for seven days. After incubation, one ml of freshly prepared 1% ferrous ammonium sulphate solution is added, followed by incubation at 4°C for four hours. The presence of a pink band in the upper part of the butt of the 7H9 agar indicates positive PZase activity which translates to PZA resistance. The absence of a pink band means there is a lack of PZase activity and thus the isolate is sensitive to PZA (Figure 2.4).

Although this assay is inexpensive and reliable, the presence of a pink band in Middlebrook 7H9 agar can be subjective due to the fact that some strains can produce bands with faint colours. To make the assay easier to interpret and to identify strains with low PZase activity, it has been recently recommended that for strains that are negative at four and seven days, the incubation should be extended to 10 days (Mirabal *et al*., 2010). In addition, when the sensitivity of PZase was compared with other tests for PZA resistance such as the Bactec MGIT 960, REMA and molecular drug susceptibility test, PZase had a lowest sensitivity among above mentioned test but had a good specificity. The above mentioned study

concluded that detection of PZase is less accurate and requires large quantities of bacteria and the Bactec MGIT 960, REMA, and molecular drug susceptibility tests are superior to the PZase assay (Cui *et al*., 2013) .

Figure 2.4 Pyrazinamidase test: A brownish pink colour indicates formation of POA and positive Pzase reaction (Fukushima *et al*., 2011)

- **Resazurin microtitre plate assay (REMA)**

REMA is a colorimetric method which employs oxy-reduction of a blue dye resazurin that is used as an indicator to detect mycobacterial growth by comparing different minimum inhibitory concentration (MIC) (Ong *et al*., 2010; Campanerut *et al*., 2011; Dixit *et al*., 2012). The colour change of the indicator is directly proportional to the number of viable mycobacterium in the medium and a result can be obtained within eight days (Nour *et al*., 2013). In addition to its short time to give results, REMA is simple, very efficient in detecting PZA resistance with high specificity, low-cost, easy to perform and interpret and doesn't need special equipment or reagents (Campanerut *et al*., 2011). A study conducted in 2006 demonstrated a sensitivity of 100% and specificity of 98% when using REMA for the detection of PZA resistant in *M. tuberculosis* (Martin *et al*., 2006).

- **Nitrate reductase assay (NRA)**

NRA is a simple colorimetric technique that is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite. NRA is an old technique and is also known as the Griess method. Conventionally, it has been used to distinguish between *M. tuberculosis* and other

mycobacterial species (Palomino, Leão & Ricatto, 2007), however it has been also introduced as a rapid tool for susceptibility testing in TB (Angeby, Klintz & Hoffner, 2002). In previous studies, the sensitivity and specificity of NRA was tested against the Bactec 460 system for various *M. tuberculosis* strains with different susceptibility patterns, they were 100% and 100% for RIF, 97% and 96% for INH, respectively. Most of the results were available after 7 days of incubation (Syre *et al*., 2003). Later on, NRA was modified to determine PZA resistance using nicotinamide in media at natural pH. This gave opportunity to avoid acidification of the media which suppress *M. tuberculosis* growth leading to non reliable results (Martin *et al*., 2008). The sensitivity and specificity of NRA using nicotinamide were 91% and 94%, respectively. Results were produced within 10 days (Martin *et al*., 2008).

2.5.3.3.3 Genotypic methods

Genotypic methods have several advantages over phenotypic methods, these include rapid generation of results (few hours instead of days and weeks); the option of direct application on clinical specimens (no need for growth of the organism); reduction of biohazard risks and possibility for automation. Unfortunately, they also have disadvantages such as the presence of inhibitors when applied on clinical specimens (Palomino, 2005).

Several molecular techniques such as PCR, single-strand conformation polymorphism (SSCP) (Scorpio *et al*., 1997b), dideoxy fingerprinting (Felmlee *et al*., 1995), heteroduplex formation (Thomas, Williams & Soper, 2001) and amplification refractory mutation system (ARMS)-PCR (Fan *et al*., 2003) have been developed for rapid screening of mutations in genes associated with anti-TB resistance. However, these techniques do not demonstrate the required sensitivity or high-throughput sample-screening capability. The main disadvantage is that these techniques are only able to detect known mutations in a defined site or region. DNA sequencing remains the most reliable, accurate and commonly used method for detection of mutations (Denkin *et al*., 2005).

- **DNA sequencing**

Sequencing of the DNA of the amplified product has become the most widely used genotypic method for detecting drug resistance in *M. tuberculosis*; it is accurate and reliable and it has

become the reference standard for mutation detection. Several years ago, the process was performed manually; however, currently there has been a move from manual to automatic sequencing (Victor *et al.,* 2001). DNA sequencing has been widely used for characterising mutations in the *rpo*B gene in RIF resistant strains to detect mutations responsible for resistance to other anti-TB drugs (Telenti *et al.*, 1993; Garcia de Viedma, 2003; Jalava, Marttila 2004).

Routine sequencing of the *pnc*A gene that related to the resistance of PZA is considered a rapid and effective tool for verification of PZA susceptibility results. Although the *rps*A gene may also contribute to the resistance to PZA, *rps*A sequencing is not a useful tool yet as it requires the analyses of five overlapping PCR products and no phenotypically informative mutations are identified (Alexander *et al*., 2012). DNA sequencing assay for *pnc*A gene has a very high sensitivity (99.4%) making it a very accurate assay in predicting PZA sensitive or resistant *M. tuberculosis* (Dormandy *et al.,* 2007). DNA sequencing also has some limitations, such as false-positive and false-negative results, which is mainly due to the high sensitivity of DNA, which makes it very liable to contamination, as well as the low sensitivity of intrinsic amplification reactions (Vaneechoutte, Van Eldere, 1997).

- **High resolution melting (HRM) curve analysis**

Real-time PCR is a molecular biological technique which is quite popular (Walker, 2002). It was developed in the mid-1990s for the analysis and quantification of nucleic acids; and continues to play a major role across many disciplines of the biological sciences. Advances such as faster thermal cycling times, higher throughput, flexibility, expanded optical systems, increased multiplexing and more user-friendly software have expanded its use in research and diagnostic fields (Walker, 2002). In 1997, real-time PCR on the LightCycler® (LC) was introduced using fluorescent melting analysis to examine PCR products based on their differences in melting temperatures (*T*m) (Ririe, Rasmussen & Wittwer, 1997). Many reports including genotyping and scanning for small sequence variants with SYBR Green (I) are available, but with questionable reliability of the methods (Marziliano *et al*., 2000; von Ahsen, Oellerich & Schutz, 2001). There has been an increasing interest in the use of melting analysis in molecular biology laboratories. In the last decade, techniques for high-resolution amplicon melting have appeared together with the introduction of a new family of LCGreen®

dyes (Wittwer *et al*., 2003). Unique transitions are shown by different genotypes that are revealed by HRM, shape comparison as well as different plots of the melting curves (Wittwer *et al*., 2003; Graham *et al*., 2005)

A) Principle of HRM

HRM curve analysis is a post-PCR technique for the detection of the variation in DNA sequence by demonstrating fluorescence changes in the melting of the amplified double-stranded DNA amplicons (Taylor, 2009). This novel technique of genotyping does not require the use of specific probes, only the LC green dye during melting curve analysis (Choi *et al*., 2010). As shown in figure 2.5, HRM generates different plot curves (Herrmann *et al*., 2006; Montgomery *et al*., 2007), the difference in the melting curve shapes is then analyzed by software (Choi *et al*., 2010). Temperature plots are generated by converting the wild type melting profile to a horizontal line and normalizing the melting profiles of the examined isolates against the *M. tuberculosis* wild-type profile. *M. tuberculosis* isolates with mutations in the *pnc*A sequence are distinguished from the wild type based on the difference in melting temperature observed. At the end of the analysis, the software either reports the *M. tuberculosis* isolates as a wild type (PZA susceptible) or mutant (PZA resistant).

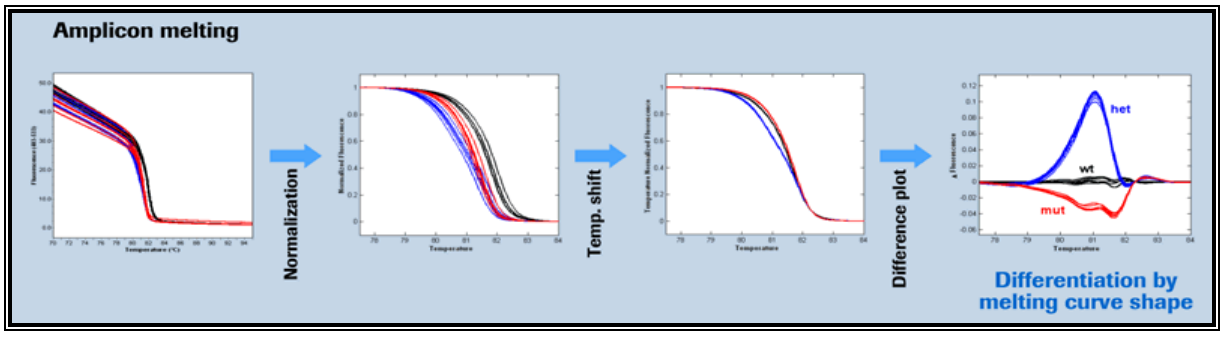

Figure 2.5 Basic principle of High resolution melting (HRM) curve analysis (editor@gene-quantification.info)

B) Current applications

HRM technique enables researchers to discover genetic variations (e.g. SNPs, mutation, methylation) very rapidly and efficiently with outstanding specificity and sensitivity.

Currently, HRM is mostly used in the gene scanning applications, i.e. the identification of heterozygote in order to discover new variations in target-gene-derived PCR amplicons (Roche, 2008).

In 2011, Ong *et al* reported 89%, 98% and 100% sensitivities of HRM for the detection of resistance to RIF, INH and Ofloxacin respectively. HRM curve analysis is also used for SNPs detection which can be easily distinguished based on melting temperature (Tm) differences (Chen *et al*., 2011). In 2012, HRM was also used for detection of mutations in *gyr*A gene of fluoroquinolones and *rps*L and *rrs* genes for STR. HRM detected the mutations within the *gyr*A gene in only 74.1% fluoroquinolone-resistant isolates while having a sensitivity and specificity of 87.5% and 100% respectively for STR-resistance. There was a 100% concordance between the results of HRM and DNA sequencing for all genes that were analysed in this study (Lee *et al.*, 2012). Very recently, HRM curve analysis was used in the detection of mutations in the *pnc*A gene. This study was conducted in China and showed good correlation between HRM curve analysis and Bactec MGIT 960 (the original known procedure without reduction in the inoculum size). The author concluded that HRM curve analysis can be used as an accurate screening test to identify the resistance to PZA with an 85.5% and 98.5% sensitivity and specificity respectively (Hong, Wang & Liu, 2013).

C) Advantages and limitations of HRM

HRM curve analysis is a rapid, inexpensive, cost-effective, sensitive and specific technique (Vossen *et al*., 2009; Ong *et al*., 2010). It takes less than five hours, including DNA preparation, to generate final results (Hoek *et al*., 2008). More importantly, HRM doesn't require additional culture after initial isolation. Additionally, it can be applied to the specimen directly reducing the time required for susceptibility with fewer biohazard risks (Nour *et al*., 2013). HRM curve analysis is a closed-tube process which reduces the risk of contamination (Garritano *et al*., 2009).

There are some challenges in performing HRM in that the isolation method of the DNA should yield very pure extracted DNA which then needs to be optimized to the same concentrations (five to 30 ng/µl) in order to obtain cut off point (Cp) values of less than 30 for all isolates. A high degree of accuracy is required when performing melting curve analysis as minimum errors can lead to incorrect interpretation of results (Roche, 2008).

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CHAPTER THREE

High resolution melting curve analysis for rapid detection of pyrazinamide resistance in *Mycobacterium tuberculosis* clinical isolates

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Abstract

Background: Pyrazinamide (PZA) is one of the most important drugs for tuberculosis (TB) treatment, however its susceptibility is not routinely performed. High-resolution melting (HRM) curve analysis has been widely used for many applications. In this study, HRM assay was developed and evaluated for the detection of PZA resistance in *Mycobacterium tuberculosis* clinical isolates.

Methods: Ninety five *M. tuberculosis* clinical isolates with different susceptibility patterns to anti-TB drugs were used to evaluate this assay. Isolates were phenotypically (Bactec MGIT 960) and genotypically (HRM and *pncA* gene sequencing) analysed for PZA resistance.

Results: Bactec MGIT 960 analysis revealed that 29 of the 95 *M. tuberculosis* isolates were PZA resistant. In comparison to the Bactec MGIT 960, HRM showed a sensitivity of 47.7% and specificity of 74.6%, the overall agreement between the two methods was 68.4%. Based on DNA sequencing, a correlation of 66.7% between phenotypic resistance to PZA and *pnc*A mutations was observed. PZA resistance was strongly associated with multi-drug resistant (MDR)-TB as it was shown in 79.3% of the MDR isolates included in the study.

Conclusion: HRM is simple and useful for screening clinical *M. tuberculosis* isolates for PZA resistance however further modifications to improve its performance are required.

Keywords: Pyrazinamide; Tuberculosis; High resolution melting curve analysis; Drug resistance; *pnc*A gene

Background

Pyrazinamide (PZA) is one of the most important drugs for tuberculosis (TB) treatment. It has a unique sterilizing effect in killing semi-dormant bacilli and is also very effective against multi-drug (MDR)-TB [1-2]. Pyrozinoic acid (POA) is the active form of the drug and is metabolized by pyrazinamidase (PZase) enzyme which is encoded by the *pnc*A gene [3]. Although number of studies suggest that PZA resistance is not caused by a single mechanism, mutations in the *pnc*A gene are considered as the main mechanism of PZA resistance in *M. tuberculosis* [4]. The lack of reliable culture-based methods which require an acidic condition and high cost of the molecular techniques make the testing of PZA resistance generally very difficult to perform [5]. It is very important to develop rapid and accurate drug susceptibility testing (DST) to prevent the spread of multi-drug resistant (MDR)-TB as well as extremely drug resistant (XDR)-TB.

Several molecular techniques have been described for the detection of anti-TB drug resistance-associated mutations, including the line probe assays GenoType MTBDR*plus* (Hain Lifescience GmbH, Nehren, Germany), GenoType MTBDRsl (Hain Lifescience GmbH, Nehren, Germany), INNO-LiPA Rif.TB (Innogenetics, Ghent, Belgium), and Xpert MTB/RIF (Cepheid, Sunnyvale, CA) [6-7] . However, these are probe based assays and only suitable for mutations that are located in hotspot regions of specific genes. Mutations in the *pnc*A are dispersed throughout the gene and its upstream promoter; this makes the development of probe-based methods challenging [8]. Direct sequencing of *pnc*A amplicons remain the best genotypic strategy, however this is costly.

High-resolution melting (HRM) curve analysis is a simple technique and has been widely used for many applications. PCR amplicons are heated and fluorescence loss is monitored in real-time. Variations in sequences are detected by difference in melting point (T*m*) compared to reference DNA; it does not require the use specific probes [9-10]. In the TB field, HRM has been used for detecting rifampin (RIF), isoniazid (INH), streptomycin (STR), and fluoroquinolone resistant *M. tuberculosis* [11]. In this study, we describe an HRM technique

to detect *pnc*A mutations in *M. tuberculosis* clinical isolates and compare its results to the phenotypic PZA susceptibility testing (The Bactec MGIT 960), isolates that had discordant results were sent for sequencing. This method uses the Roche LightCycler 480® and involves simultaneous amplification of three overlapping fragments.

Methods

The study was conducted at the NHLS-TB Laboratory Tshwane Academic Division (Diagnostic division of the Department of Medical Microbiology, University of Pretoria) from July 2013 to October 2013. Ethics approval was obtained (number 57/2012) prior to commencement of the study. The study isolates (95) were a mixture of MDR-TB and non-MDR-TB collected from the National Health Laboratory Service (NHLS) TB Laboratory. All isolates were presumptively identified as *M. tuberculosis* complex using Ziehl-Neelsen (ZN) stain and tested for susceptibility using the GenoType MTBDR*plus* line probe assay (HainLifescience GmbH, Germany). Isolates were then categorized as either MDRs or Non-MDRs (Mono-RIF resistance, Mono-INH resistance or fully sensitive).

Purity of all isolates by ZN staining was confirmed before susceptibility testing was performed. To exclude bacterial and fungal contamination, another amount of a well-mixed suspension was inoculated using sterile glass pipette on chocolate agar (produced in-house, NHLS Laboratory, Tshwane Academic Division) and incubated aerobically at 37ºC. All plates were inspected for bacterial growth every 24 hrs for two days.

Isolates were sub-cultured onto 7H10 agar medium (Becton Dickinson, Sparks, MD, USA) and incubated for three weeks aerobically at 37ºC until typical *M. tuberculosis* colonies appeared (dry, wrinkled, warty, with colourless rough surface). Colonies were picked and sub-cultured into MGIT tubes (Becton Dickinson, Sparks, MD, USA) according to the manufacturer's procedure.

Each isolate was tested for PZA susceptibility when the Bactec MGIT 960 indicated positive culture growth. The day the Bactec MGIT 960 (Becton Dickinson, Sparks, MD, USA) gives a positive signal is considered as day zero. Once positive, tubes were removed from the Bactec MGIT 960 (Becton Dickinson, Sparks, MD, USA) they were transferred into an incubator

37ºC ±1 ºC until susceptibility testing was performed (within five days). Undiluted inoculum was used if the isolate was processed on days one or two. On days three, four or five, the growth culture was diluted 1:5 using normal saline (0.9%) (SABAX Pour Saline, Adcock Ingram, South Africa) prior to susceptibility testing. PZA susceptibility testing was performed according to the manufacturer's procedure. (Becton Dickinson, Sparks, MD, USA). Susceptibility results were available within four to 21 days. Once the test was completed, a report was generated and printed. Each isolate was categorized as 'S' (susceptible) or 'R' (resistant).

DNA extraction of M. tuberculosis isolates

Genomic DNA was extracted from the *M. tuberculosis* isolates using QIAquick PCR Purification Kit (Whitehead Scientific, Brackenfell, South Africa) according to the manufacture's procedure. Extracted DNA was quantified using specific spectrophotometry (NanoDrop; ND-1000; USA), and adjusted to a concentration of five to 30 ng/µl using elution buffer (BioMérieux, France), this range is required by HRM for optimal performance. The extracted DNA was then transferred into a sterile 1.5 ml tube (BioMérieux, France) and used directly for HRM analysis or stored at -80°C.

HRM optimization

The LightCycler® 480 HRM master mix contains all the ingredients required. The two most important contents which needed to be optimized carefully before commencing with the testing process were $MgCl_2$ and primers.

- ***$MgCl_2$***

Because specific amplification is essential for HRM analysis, determination of $MgCl_2$ optimum concentration for each new primer pair is very important. A separate 25 mM $MgCl_2$ stock solution was supplied with the master mix, this is to allow the easy optimization of Mg^{+2} concentration. Optimization was achieved by running a positive sample with serial dilutions of $MgCl_2$ (1.0, 1.5, 2.0, 2.5, 3.0 3,5 mM) and analysis of the PCR products by agarose gel electrophoresis.

- ***Primers***

Primers previously described by Scorpio *et al* were synthesized by Inqaba Biotechnical Industries Pretoria, South Africa to the *M. tuberculosis pnc*A sequence (GenBank accession number U59967) [12]. Prior to the experiment and to ensure specific amplification for HRM analysis, the optimal primer concentration (the lowest concentration that still results in a high rate of amplicon yield with a low cut off point (Cp) and adequate fluorescence dynamics for a given target concentration) was determined. This was achieved by testing different concentrations of each primer (0.1, 0.2 and 0.3µM).

Real-time PCR and HRM conditions

HRM curve analysis including the amplification step was performed using the LightCycler® 480 High Resolution Melting Master Kit and the LightCycler® 480 Instrument II (Roche, Germany). In order to detect all possible mutations in the *pnc*A gene (561 bp) regardless of their locations, and to achieve the recommended size of the gene for HRM analysis (less than 300 bp), the target gene was amplified using three sets of primers to produce three fragments of the target gene.

The reaction mixture was prepared according to the manufacturer's instructions (LightCycler 480® Real-Time PCR manual, 2009). HRM master mix consisted of 2X a concentration hot-start reaction mix that contained FastStartTaq DNA polymerase, reaction buffer, dNTP mix (with dUTP) and HRM Dye. PCR reaction composition is shown in table 1. Inclusion of HRM dye enabled detection of double-stranded DNA by fluorescence, monitoring formation of amplicon during PCR cycling and melting curve analysis.

These components were added into a 1.5 ml reaction tube on ice and mixed carefully by pipetting up and down. The amount in the volume column was multiplied by the number of reactions to be run. Fifteen µl of the PCR mixture was transferred into each well of the LightCycler® 480 Instrument multi-well plate (Roche, Germany) then 5 µl of the DNA template was added, mixed by pipetting up and down. The LightCycler® 480 foil was used to seal the multi-well plate before loading it into the LightCycler® 480 (Roche, Germany) (Table 2).

A non-template control containing sterile distilled water was included in the experiment.

M. tuberculosis wild type strain American Type Culture Collection (ATCC H37) as well as a *M. bovis* strain served as negative and positive controls for the *pnc*A mutation respectively. Collected data were analysed following the final step using Light Cycler 480 software version 1.5. Temperature plots were generated by converting the wild type melting profile to a horizontal line and normalizing the melting profiles of the examined isolates against the *M. tuberculosis* wild-type profile. *M. tuberculosis* isolates with mutations in the *pnc*A sequence were distinguished from the wild type based on the difference in melting temperature observed. At the end of the analysis, the software either reported the isolate as a wild type (PZA susceptible) or mutant (PZA resistant).

DNA sequencing of the pncA gene of discordant M. tuberculosis isolates

The entire *pnc*A gene was sequenced using two primers, P1 and P6, which flank the entire *pnc*A gene and its upstream promoter (700-bp). Purified DNA (10 µl) from each isolate was sent for DNA sequencing which was carried out by a commercial sequencing centre using standard Sanger sequencing (InqabaBiotec, SA). Retrieved sequences were compared with the wild type *pnc*A sequence from *M. tuberculosis* H37Rv for the detection of mutations associated with PZA resistance. Sequence alignment and analysis was performed using special software (CLC Genomics workbench, Denmark). Isolates were either reported as wild type (PZA susceptible) or mutant (PZA resistant). These results were were compared to the HRM results, as well as the phenotypic drug susceptibility results obtained from the Bactec MGIT 960 to assess the HRM against a molecular gold standard as well.

Statistical analysis

Statistical analysis was performed using the Bactec MGIT 960 as a phenotypic gold standard method and DNA sequencing as a molecular gold standard method. Results of HRM and MGIT or sequencing susceptibility testing were entered into 2X2 tables to calculate the sensitivity (ability to detect true resistance) and specificity (ability to detect true susceptibility) of HRM against MGIT as well as the positive predictive values (PPV) and the negative predictive values (NPV).

Results

A total of 120 *M. tuberculosis* isolates were collected from the NHLS-TB Laboratory Tshwane Academic Division (Diagnostic division of the Department of Medical Microbiology, University of Pretoria). Seven isolates were excluded due to duplication. Additional 18 isolates had no HRM results and were excluded from analysis. Susceptibility of the 95 *M. tuberculosis* isolates included in the final analysis to the first-line anti-TB drugs are shown in figure 1. Isolates were categorized as MDR-TB (Resistant to both RIF and INH) and Non-MDR-TB (susceptible to at least RIF or/and INH).

The BACTEC MGIT 960

According to the Bactec MGIT 960, 30.5% (29/95) of the isolates were phenotypically resistant to PZA. Of these 79.3% (23/29) were MDR and 20.7% (6/29) were non-MDR. Pyrazinamide susceptible isolates were detected in 69.4% (66/95) of the isolates, in which 25.8% (17/66) were MDR and 74.2% (49/66) were non-MDR (Table 3). Of the 34 isolates that were phenotypically susceptible to other drugs, 33 were determined to be susceptible to PZA. However, one isolate, which was phenotypically susceptible to other drugs, was PZA-resistant, suggesting PZA mono-resistance (Table 3). The average time of reporting PZA susceptibility results was 8.5 days and 86.3% of isolates were reported in four to 12 days.

HRM analysis

High Resolution Melting curve analysis of the *pnc*A gene was performed in DNA samples isolated from 95 *M. tuberculosis* isolates using three overlapping fragments. Representative normalized melting curves from HRM analysis are shown in figure 2 (A) and 2 (B). In the HRM graphs, each line indicates the melt curve profile for an individual sample. The normalized graph shows melting temperature shifts versus wild-type amplicon indicating lower or higher melting temperature. Based on the differences in the shape of the melting curves in any of the three overlapping fragments, *M. tuberculosis* isolates with mutations could easily be differentiated from the susceptible (wild-type) ones. Wild types are represented by blue lines, whereas mutants are represented by red lines. In each batch, DNA isolated from the susceptible control strain (H37Rv) was included as a reference and shown

to be wild type at all times (blue line). Based on HRM analysis, 77.9% (74/95) of the isolates were susceptible to PZA while 22.1% (21/95) were resistant.

Comparison of PZA susceptibility testing results for The BACTEC MGIT 960 and HRM analysis

Of the 95 isolates included in this study, 55 isolates (57.9%) and 10 isolates (10.5%) were resistant and susceptible by both methods, respectively. Discordant results were observed in 30 isolates (31.6%). When the Bactec MGIT 960 method was considered as the gold standard, HRM showed a sensitivity (ability to detect true resistance) of 47.7% and specificity (ability to detect true susceptibility) of 74.6%. The accuracy, positive and negative predictive value of HRM were 69.5%, 34.5% and 83.3%, respectively. The overall agreement between the two methods was 68.4% (Table 4).

DNA sequencing of pncA gene

PCR products were obtained from 48 isolates (50.5% of the total sample size) and sequenced. These included all isolates that showed resistance to PZA by MGIT 960 (30), all isolates that showed resistance by HRM (11) and seven isolates that were randomly selected from those which were susceptible by both methods i.e. MGIT 960 and HRM (Table 5).

Different types of mutations in the *pnc*A-encoding region were identified in 27 isolates (56.3%), including nucleotide substitutions, insertions and deletions. No multiple-site mutation was detected (Table 5). Substitution of a single nucleotide was the most common mutation 88.9% (24/27), followed by insertion 7.4% (2/27) and deletion 3.7% (1/27). No mutation was detected in 21 (43.7%) isolates, of these, four (19%) were resistant by both methods, five (23.8%) were resistant by MGIT 960 only, five (23.8%) were resistant by HRM only and seven (33.4%) were susceptible by both methods (Table 5).

As indicated in Table 6, the mutation rate among the phenotypically resistant isolates that were reported by MGIT 960 was 65.5% (19/29), whereas mutations were only detected in 45.5% (5/11) isolates that were initially reported as resistant by HRM. Additionally, mutations were shown in 60% (6/10) of the isolates that were reported resistant by both methods. No mutation was identified in the wild-type (H37Rv) strain. However, two isolates,

despite being susceptible by both MGIT and HRM, showed mutations in the *pnc*A gene. High confidence mutations, i.e. mutations associated with in-vitro documented resistance reported by at least 10 publications were found in 51.8% (14/27) of isolates. The rest, 48.2% (13/27), were new mutations which have not been reported in previous studies.

The most frequently mutated sites were at codon 135, which showed a substitution from thymine to guanine at nucleotide 403, this resulted in a switch at translation from threonine to proline in five isolates (18.5%). Another common site was codon 139 which showed a substitution from adenine to cytosine at nucleotide 416, and resulted in a switch at translation from valine to glycine in three isolates (11.1%). Additional three isolates (11.1%) showed new mutations in codon 14, switching translation from cysteine to tryptophan. These three mutation sites accounted for more than one third of all the mutations identified (Table 5).

Comparison of HRM analysis and The BACTEC MGIT 960 with DNA sequencing

Isolates with discordant results between HRM analysis and the Bactec MGIT PZA susceptibility were compared with DNA sequencing of the *pnc*A gene (Table 6). HRM analysis was concordant with DNA sequencing for 47.9% (23/48) of the isolates. Among the 25 discordant isolates, 16 isolates had mutations in the *pnc*A gene while HRM detected no variation; and 9 had no mutations in the *pnc*A while HRM detected variation. DNA Sequencing of *pnc*A was concordant with the Bactec MGIT 960 PZA susceptibility testing for 66.7% (32/48) of the isolates. Among the 16 discordant isolates, nine were PZA resistant while sequencing detected no mutation, and seven were PZA susceptible while sequencing showed mutations. Overall, there were fewer discrepancies with MGIT than with HRM.

Discussion

Although PZA forms an integral part of both first line and MDR TB management, routine susceptibility testing of this drug is not performed due to various challenges present in the currently available techniques. This study evaluated the rapid technique of HRM for the detection of PZA resistance against the phenotypic gold standard (Bactec MGIT 960)

In this study, the Bactec MGIT 960 results showed a prevalence of PZA resistance of 57.5%

(23/40) among the MDR-TB isolates tested, while the prevalence among non-MDR isolates was 10.9% (6/55). High rates of PZA resistance among MDR-TB have been observed in previous studies, ranging from 49% in Thailand [13], to 50% in Central Africa [14], 52% in South Africa [15], 53% in Japan [16] and 55% in Taiwan [17]. Studies from Pakistan [18], South Korea Kim *et al*., 2012 [19] and India [20] have shown higher rates of resistance, 77%, 85% and 85% respectively. These high rates of PZA resistance in TB endemic countries may be attributed to the widespread use of PZA in re-treatment regimens in these areas, particularly when used without relying on susceptibility testing results. The high occurrence of false resistance by phenotypic susceptibility testing could also cause the overestimation of PZA resistance among MDR-TB [21].

In 2013, Nagai et al developed and evaluated HRM curve analysis for mutation detection in four major anti-TB drugs, namely RIF, INH, EMB, and STR. Their HRM assay was successful in detecting mutations in genes associated with resistance (*rpo*B, *kat*G, *inh*A, *amb*B, *rps*L and *rrs*) and results were completely consistent with those of DNA sequencing. Sensitivity and specificity of HRM were 100% and 100% for RIF, 88.8% and 100% for INH, 100% and 100% for EMB, and 100% and 93.7% for STR, respectively [22].

Comparable results were also achieved by another two recent studies when HRM was used for the detection of PZA resistance. Hong *et al* reported a sensitivity and specificity of 85.5% and 98.5%, respectively, whereas Pholwat *et al* showed concordance of 84% between HRM and phenotypic PZA susceptibility testing [23-24]. The agreement between the phenotypic Bactec MGIT 960 and HRM for PZA susceptibility in this study was only 68.4%. Sensitivity and specificity of HRM were 47.7% and 74.6%, respectively. These values were much lower than previously reported [24-26]. Discrepancies between the two methods detected on 19 PZA-resistant isolates that were reported wild-type by HRM, and on 10 PZA-susceptible isolates that showed mutations by HRM. Among these 19 isolates, sequencing detected mutations in 14 of them suggesting that HRM was not able to detect these mutants which were also phenotypically resistant. The majority variants were SNPs with five of the 19 occurring at codon 135.

Conversely, five of the ten susceptible isolates which were reported as variants by HRM showed no mutation on sequencing. Although this would suggest that these were false on

HRM, it is also possible that the HRM result is true since HRM is PCR based and has a greater likelihood of detecting mixed populations which may not be detected by Sanger sequencing, and these sub-populations may not be large enough to show resistance phenotypically. We did not perform next generation sequencing which may have provided an answer to this uncertainty.

Recent studies have indicated that the new "gold standard" for PZA resistance determination should be sequencing. When considering the discordant isolates only and comparing HRM and MGIT 960 against this "new" gold standard, the accuracy for HRM was 47.9% while that of the Bactec MGIT 960 was slightly better at 66.7% (Table 6).

Phenotypic resistance to PZA has been correlated with mutations in the *pnc*A gene in several previous studies. Some of these studies have reported this correlation to be inconsistent, ranging between 41% and 80% [25-31], whereas other studies showed higher correlation ranging from 91% - 97% [15], [17], [32-34]. In the current study, based on sequencing results, a correlation of 66.7% between phenotypic resistance to PZA and *pnc*A mutation was observed, this is comparable to majority of the previous studies, more especially to the study that was conducted in South Africa by Bishop *et al*, in which a correlation of 67% was also reported [35].

The mutations detected in our study were found to be scattered along the entire *pnc*A gene with no major hot spots identified. However, mutations at codon 135 were found in five isolates, whereas mutations at codons 14 and 139 were shown by three isolates each. These observations of scattered mutations are supported by similar findings in previous studies [36], [27]. The high diversity of *pnc*A mutations limits its inclusion in the current molecular techniques such as GenoType MTBDR*plus* (Hain Lifescience) [37].

Absence of mutations in the *pnc*A gene or its upper promoter in 26.7% of the PZA-resistant isolates we tested correlated with the study that was done by Sreevatsan *et al*, in which they reported no *pnc*A mutations in 28% of PZA-resistant *M. tuberculosis* strains tested [15]. These cases could also be explained by another mechanism of resistance to PZA. Recently, *rps*A gene has been shown to play a role in PZA resistance and has been recommended to be used as an additional target for the molecular detection of PZA susceptibility [38], [9],

however, in another study, none of the PZA-resistant strains harboured mutations in the *rps*A gene [39]. Based on these variations, the association between PZA resistance and *rps*A mutations remains un-established and requires further investigations in the future.

The low sensitivity and specificity of HRM in this study could be due to the use of three sets of primers as compared to five or seven as used in previous studies [40-41]. Unlike other studies, the current HRM was developed and evaluated using the Roche LightCycler® 480. So far, only one study has utilized the Roche LightCycler 480® for PZA susceptibility testing and used the Bactec MGIT 960 as a reference method [42]. They have shown a sensitivity and specificity of 85.5% and 98.5%, respectively and concluded that HRM is a rapid and accurate test for the detection of PZA resistance and can be used as a screening method [26]. Recently, HRM results generated from the Rotor-gene system for the detection of PZA resistance have been found to be easier to interpret than other platforms [25].

Another explanation for the low correlation between PZA resistance and *pnc*A mutations in this study is the possibility of phenotypic false resistance. This observation has been proven elsewhere [25]. PZA phenotypic susceptibility testing by the Bactec MGIT 960 is normally carried out at pH of 5.9, resistance could be falsely reported as a result of the ammonia produced during mycobacterial growth; ammonia elevates the pH of the media thus inactivating the drug [26]. According to the Henderson-Hasselbach equation, *M. tuberculosis* should be tested for PZA susceptibility at a concentration of at least 156 µg/ml [10], rather than the current cut-off used in the Bactec MGIT 960 method (100 µg/ml). PZA resistance could also be caused by the action of efflux pumps which has been shown to play a role in mycobacterial resistance [43]. Detection of *pnc*A mutations in the susceptible isolates suggests that these mutations might not be fully expressed to show phenotypic PZA resistance.

Conclusion

In this study, apart from simplicity and speed, the current HRM genotypic method to determine PZA susceptibility using the gene scanning software of the Roche LightCycler 480 was shown to be moderately specific with low sensitivity. Further modifications to improve its performance are required. Pyrazinamide resistance was common among MDR-TB and the current study results showed low association of the phenotypic PZA resistance with

mutations in the *pnc*A gene. This warrants further studies to determine potential alternate genetic mechanisms of resistance to PZA in our context and re-evaluation of the MGIT 960 for detection of phenotypic resistance with an alternate phenotypic method such as the Wayne's test.

This study provides an important baseline for more extensive evaluation studies to improve and validate the use of HRM in determining PZA susceptibility. In the future, using more than three fragments (six or seven) should be considered as this could increase the sensitivity and specificity of HRM in detecting *pnc*A mutations. However, this might also increase the cost of the test. Additionally, combining such data to results from the biochemical analysis of PZase enzyme (Wayne's test) would add to its value and ease the interpretation of the final results.

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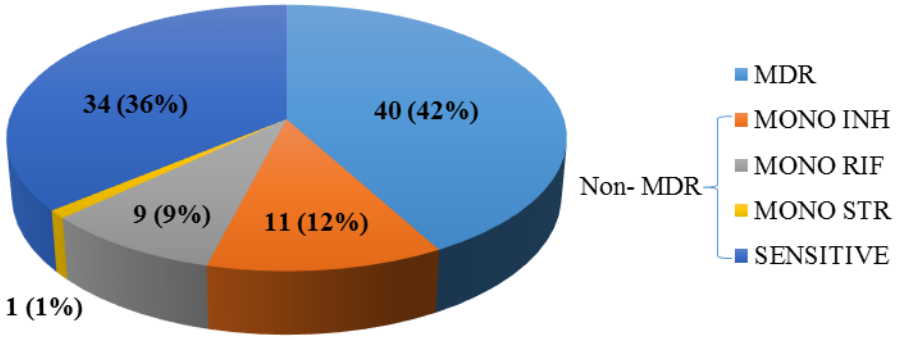
Figure 1: First-line susceptibility profile of 95 *M. tuberculosis* isolates included in the study



MDR = Multi-drug resistant (Resistant to at least RIF and INH); **Mono INH** = Resistant to INH only; **Mono RIF** = Resistant to RIF only; **Mono STR** = Resistant to STR only; **Sensitive** = Susceptible to both RIF and INH

Figure 2: Normalized melting curves from HRM analysis of the *pnc*A gene

Figure 2 (A): Amplicons amplified from susceptible isolate versus wild-type reference strain (H37Rv)

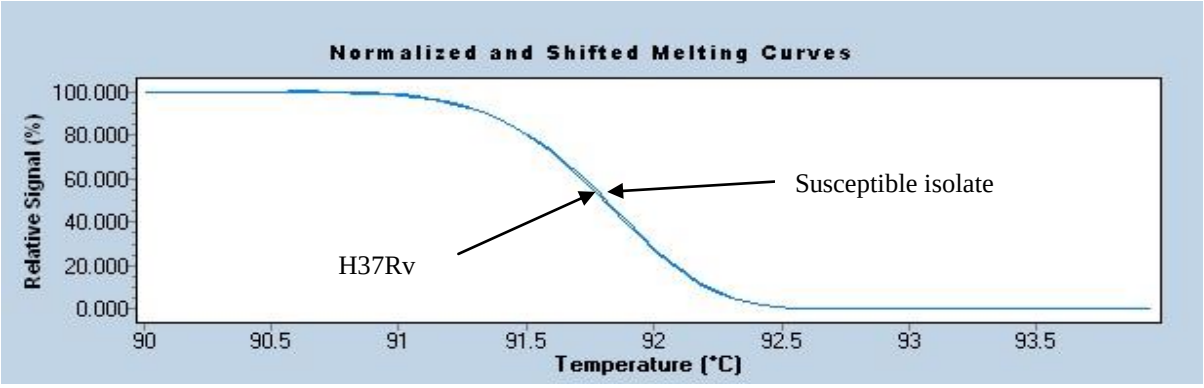


Figure 2 (B): Amplicons amplified from susceptible and resistant isolates versus wild-type reference strain (H37Rv)

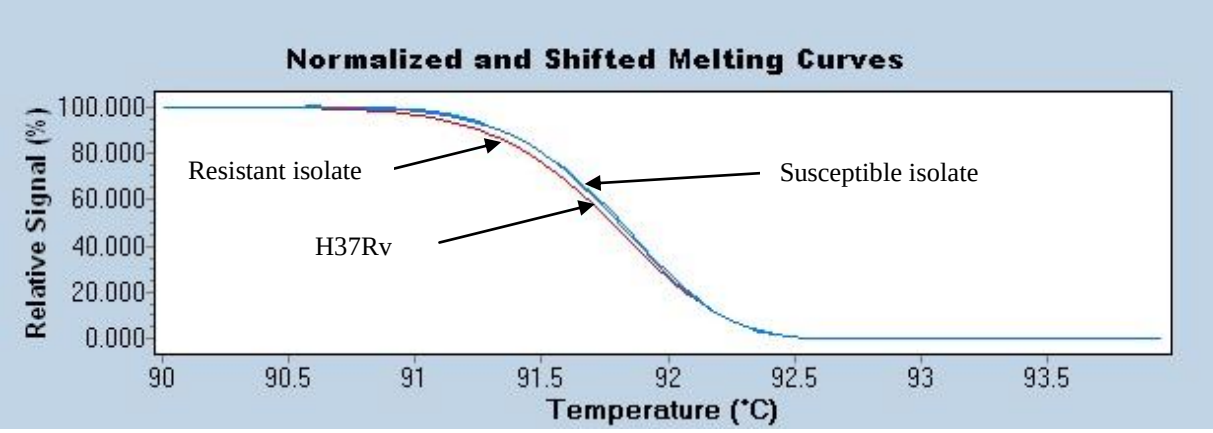


Table 1: Reaction mixture used in HRM analysis for the detection of *pnc*A gene mutation in *M. tuberculosis*

Reagents	Volume (µl)x1	Final Concentration
Master Mix, 2X conc.	10. µl	1X conc.
$Mgcl_2$, 25 mM	1.6µ l	2 mM
Primer mix, 20 X conc. {4µM}	1.0 µl	0.2 µM
Template DNA	5.0 µl	5-30 ng
Water, PCR-grade	2.4 µl	-
Total volume	20 µl	-

Table 2: PCR amplification and HRM conditions

Steps in the PCR cycle	Temperature	Time	No. of cycles
Initial denaturation (FastStartTaq DNA Polymerase)	95ºC	10:00 min	1
Denaturation	95ºC	00:10 sec	45
Annealing	63ºC	15:00 min	
Extension	72ºC	00:16 sec	
For HRM			
Second hold	95ºC	1:00 min	1
	40ºC	00:01 min	
	65ºC	00:01 sec	
Second temperature increase	95ºC	Fluorescent detection (25 acquisition per 1ºC)	
Cooling down	40ºC	00:10 sec	

Table 3: PZA susceptibility results by the BACTEC MGIT 960

***M. tuberculosis* Isolates**	**BACTEC MGIT 960**	
	R	**S**
MDR-TB (*n* = 40)	23	17
Non-MDR-TB (*n* = 55)	6	49
Mono Rif ***(n = 9)***	1	8
Mono INH ***(n = 11)***	3	8
Mono STR ***(n = 1)***	1	0
Sensitive ***(n = 34)***	1	33

MGIT = Mycobacterial Growth Indicator Tube; **MDR–TB** = Multi-drug resistant tuberculosis; **R** = Resistant; **S** = Susceptible; **Mono INH** = Resistant to INH only; **Mono RIF** = Resistant to RIF only; **Mono STR** = Resistant to STR only; **Sensitive** = Susceptible to both RIF and INH

Table 4: Comparison of PZA susceptibility testing results for The BACTEC MGIT 960 and HRM curve analysis

Test	MGIT 960	HRM	Number of isolates with results
Results for indicated test	S	S	**55**
	R	R	**10**
	S	R	**11**
	R	S	**19**

Performance parameters:

Sensitivity = 47.7%

Specificity = 74.6%

Accuracy = 69.5%

PPV = 34.5%

NPV = 83.3%

MGIT =Mycobacterial Growth Indicator Tube; **HRM**: High Resolution Melting; **S** = Susceptible, **R** = Resistant; **PPV**: Positive predictive value; **NPV**: Negative predictive value.

Table 5: Drug susceptibility patterns and DNA sequencing of *pnc*A gene of 48 *M. tuberculosis* isolates

Isolate Number	Resistance Pattern	PZA susceptibility testing		DNA Sequencing of *pnc*A gene				
		MGIT	HRM (F)	Type of change	Codon number ¤	Nucleotide change		Amino acid change
						From	To	
1	RIF, INH,STR, EMB, ETH, KAN, OFX	R	S	Insertion	173	-	C	Glu173Frameshift **
2	INH	S	R (F3)	No change	-	-	-	-
3	RIF, INH	R	S	Substitution	59	A	G	Ser59Pro **
4	RIF, INH, OFX	R	S	Substitution	14	A	G	Cys14Arg *
5	IHN, STR	R	S	Substitution	51	G	C	His51Asp **
6	INH, STR, EMB	R	R (F1)	No change	-	-	-	-
7	RIF, INH	R	R (F1)	Substitution	139	A	C	Va139Gly *
8	INH,STR, ETH	S	S	No change	-	-	-	-
9	RIF, INH	R	S	Substitution	14	G	C	Cys14Trp **
10	RIF, INH	S	R (F1)	No change	-	-	-	-
11	RIF, INH	R	R (F2, F3)	Substitution	14	G	C	Cys14Trp **
12	INH	R	R (F3)	No change	-	-	-	-
13	RIF, INH	S	S	No change	-	-	-	-
14	RIF, INH	R	S	Substitution	134	G	T	Ala134Asp **
15	RIF, INH	S	R (F3)	Substitution	139	A	C	Val139Gly *
16	RIF, INH, ETH	R	R (F3)	Substitution	12	T	C	Asp12Gly *
17	INH	S	R (F3)	Substitution	34	A	C	Tyr34Asp **
18	RIF, INH, EMB, OFX	R	R (F1, F2, F3)	No change	-	-	-	-
19	RIF, INH	R	R (F1, F2, F3)	No change	-	-	-	-

Continued on next page

Isolate Number	Resistance Pattern	PZA susceptibility testing		DNA Sequencing of *pncA* gene				
		MGIT	HRM	Type of change	Codon number ¤	Nucleotide change		Amino acid change
						From	To	
20	RIF, INH	R	R (F1, F2, F3)	Deletion	21	A	-	Val21Frameshift **
21	RIF, INH OFX	S	S	Substitution	10	G	A	Ala10Val *
22	RIF, INH, EMB	R	R (F1)	Substitution	14	A	G	Cys14Arg *
23	RIF, INH	S	S	Substitution	14	G	C	Cys14Trp **
24	STR	R	S	No change	-	-	-	-
25	RIF, INH	R	S	No change	-	-	-	-
26	RIF, INH	S	S	No change	-	-	-	-
27	RIF	R	S	No change	-	-	-	-
28	RIF, INH, STR, EMB	R	S	Substitution	135	T	G	Thr135Pro *
29	RIF, INH	S	R (F3)	Substitution	139	A	C	Val139Gly *
30	RIF	S	R (F1)	Substitution	172	A	G	Leu172Pro *
31	RIF, INH	R	R (F2)	Substitution	59	A	G	Ser59Pro **
32	RIF, INH	R	S	Substitution	135	T	G	Thr135Pro *
33	RIF, INH	R	S	Substitution	135	T	G	Thr135Pro *
34	RIF, INH, EMB	R	S	Substitution	135	T	G	Thr135Pro *
35	RIF, INH, STR	R	S	Substitution	135	T	G	Thr135Pro *
36	RIF, INH	R	S	No change	-	-	-	-
37	RIF, INH	R	S	Insertion	12	-	C	Asp12Frameshift **
38	Susceptible	S	R (F1, F3)	Substitution	122	G	A	Gln122Stop **
39	RIF, INH	R	S	Substitution	12	T	C	Asp12Gly *

Continued on next page

Isolate Number	Resistance Pattern	PZA susceptibility testing		DNA Sequencing of *pnc*A gene				
		MGIT	HRM	Type of change	Codon number ¤	Nucleotide change		Amino acid change
						From	To	
40	RIF, INH	R	S	No change	-	-	-	-
41	Susceptible	S	S	No change	-	-	-	-
42	Susceptible	S	R (F3)	No change	-	-	-	-
43	Susceptible	S	S	No change	-	-	-	-
44	Susceptible	S	S	No change	-	-	-	-
45	Susceptible	S	S	No change	-	-	-	-
46	Susceptible	R	S	Substitution	57	G	C	His57Asp **
47	Susceptible	S	R (F1, F2, F3)	No change	-	-	-	-
48	Susceptible	S	R (F2, F3)	No change	-	-	-	-
H37Rv	Susceptible	S	S	No change	-	-	-	-

PZA = Pyrazinamide; **MGIT** = Mycobacterial Growth Indicator Tube; **HRM**: High Resolution Melting; **(F)**: Fragment showing variation by HRM; **RIF** = Rifampicin; **INH** = Isoniazid; **STR** = Streptomycin; **EMB** = Ethambutol; **ETH** = Ethionamide; **KAN** = Kanamycin; **OFX** = Ofloxacin; **S** = Susceptible; **R** = Resistant; **A** = Adenine; **T** = Thymine; **C** = Cytosine; **G** = Guanine; **H37Rv** = Control strain (Wild-type); **(¤) =** Number of codon position was counted from the start codon (ATG) of the *pnc*A; **(*)** = High confidence mutations (mutations associated with *in-vitro* documented resistance reported by at least 10 publications); **(**)** = New mutations (reported for the first time)

Table 6: Comparison of HRM analysis and The BACTEC MGIT 960 with DNA sequencing

DNA Sequencing	HRM PZA Susceptibility (*n=48*)		DNA Sequencing	MGIT PZA Susceptibility (*n=48*)	
	Susceptible	**Resistant**		**Susceptible**	**Resistant**
Mutation	16	11	Mutation	7	20
No mutation	12	9	No mutation	12	9
Performance parameters:			**Performance parameters:**		
Sensitivity = 40.74%			Sensitivity = 68.97%		
Specificity = 57.14%			Specificity = 63.16%		
Accuracy = 47.9%			Accuracy = 66.7%		
PPV = 55.0%			PPV = 74.07%		
NPV = 42.86%			NPV = 57.14%		

MGIT =Mycobacterial Growth Indicator Tube; **HRM**: High Resolution Melting; **PZA**: Pyrazinamide**; PPV**: Positive predictive value; **NPV**: Negative predictive value.

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Faculty of Health Sciences Research Ethics Committee

15/06/2012

Number : **S57/2012**

Title : High-resolution melting curve analysis for rapid detection of pyrazinamide resistance in *Mycobacterium tuberculosis*

Investigator : Mrs. Fatima Osman; Department of Medical Microbiology; University of Pretoria (SUPERVISORS: Dr. Farzana Ismail / Dr. Nazir Ismail)

Sponsor : NHLS Fund

Study Degree: **MSc. Medical Microbiology**

This Student Protocol was reviewed by the Faculty of Health Sciences, Student Research Ethics Committee, University of Pretoria on 15/06/2012 and found to be acceptable. The approval is valid for a period of 3 years.

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