Genotyping of Multidrug-Resistant Strains of *Mycobacterium tuberculosis* in the Limpopo Province

by

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DISSERTATION
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DECLARATION

I declare that the dissertation Genotyping of multidrug resistant strains of *Mycobacterium tuberculosis* in Limpopo Province hereby submitted to the University of Limpopo, for the degree of Master of Science in Medical Microbiology has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

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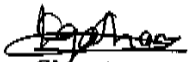

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17/05/2013 — **Date**

DEDICATION

I would like to dedicate this work to my Family, The Royal House, my Son, and my Husband. To you it was a journey to be taken all the time before getting to the top, thanks I made it.

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ABSTRACT

Genotyping of TB is essential to investigate and confirm transmission of the m*ulti-drug resistant tuberculosis* and of great value in optimizing strategies for the determination of strains causing the increased mortality rates of TB outbreaks. Sputum samples (207) were collected from National Health Laboratory Services in Polokwane laboratory for determining mutations and genotypes of the *Mycobacterium tuberculosis* strains using GenoType®MTBDRplus (Hain LifeScience, Germany) and Real-Time PCR (Roche, South Africa) techniques. Of the 207 samples, 28 (13.5%) exhibited drug resistance. Thirteen of the 28 (46%) MDR-TB strains belonged to the *non-Beijing* family, with mutations at codons *rpoB* 516 and *rpoB* 526 for RIF and *katG* 315 and *inhA* 15 for INH resistance. The *Non-Beijing* strains 75% (21/28) were monoresistant to RIF 14% (3/21) at codons 516, 526, 531 of *rpoB* gene and INH 19% (4/21) at codon 315 of *katG* and codon 15 of inhA 5% (1/21). Of the eight *Beijing* strains, 3(8%) were INH- resistant at codon 315 for *katG* and codon 15 for *inhA* and 3(8%) were RIF-resistant with mutations at codons 516 and 526. Two samples were typed as MDR for the Beijing strains with codon 315 for INH and codons 526 and 531 for RIF. The sample with a co-infection for *Beijing* and *non-Beijing* was an MDR-TB strain with mutations in *rpoB* codons 526, 531, *katG* 315 and *inhA* 8, 15 and16. The study showed a high rate of drug resistance with the non-Beijing compared to Beijing strains and mutations in specific codons for RIF and INH are variable for the TB families.

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ABBREVIATIONS

1. AFB^{+}	Acid fast bacillus
2. BCG	Bacillus- Calmette- Guérin
3. bp	Base pair
4. *CAS*	*Central Asian strains*
5. CRISPR	Clustered regularly interspaced short palindrome
6. ^{o}C	Degrees Celsius
7. DGM	Dr. George Mukhari Hospital
8. DNA	Deoxyribose nucleic acid
9. DOT	Direct observed therapy strategy
10. DOTS –plus	Direct observed therapy strategy-plus
11. DRs	Direct repeats
12. DST	Drug sensitivity testing
13. *EAI*	*East African-Indian*
14. *E.coli*	*Escherichia coli*
15. EMB	Ethambutol
16. ETH	Ethionamide
17. FRET	Fluorescence Resonance Energy Transfer
18. GFP	Green Fluorescent Protein
19. HIV	Human immunodeficiency virus
20. INH	Isoniazid
21. KZN	KwaZulu Natal
22. KatG	Catalase-peroxidase gene
23. *LAM*	*Latin American and Mediterranean*
24. M.avium	*Mycobacterium avium*
25. M.smegmatis	*Mycobacterium smegmatis*

26. M.tuberculosis	*Mycobacterium tuberculosis*
27. *MDR-TB*	*Multidrug-resistance tuberculosis*
28. $MgCL_2$	Magnesium chloride
29. MGIT	Mycobacterial growth indicator tube
30. MIC	Minimal inhibitory concentration
31. MIF	Migration inhibitory factor
32. Min	Minute
33. ml	Milliliter
34. MRC	Medical Research Council
35. MUT	Mutation
36. NAD+	Nicotinamide adenine dinucleotide
37. NADH	Nicotinamide adenine dinucleotide hydride
38. NaOH	Sodium hydroxide
39. NHLS	National Health Laboratory Services
40. PCR	Polymerase chain reaction
41. POA	Pyrazinoic acid
42. PZA	Pyrazinamide
43. RFLP	Restriction fragment length polymorphism
44. RIF	Rifampicin
45. RNA	Ribonucleic acid
46. rpoB	RNA polymerase B-subunit gene
47. R-T-PCR	Real-Time Polymerase chain reaction
48. SA–MRC	South African Medical Research Council
49. STR	Streptomycin
50. T	TB strains
51. TB	Tuberculosis
52. TUB	Tuberculosis Band

53. UV	Ultra Violet
54. WHO	World Health Organization
55. WT	Wild type
56. *XDR-TB*	Extensively drug resistance tuberculosis
57. µl	Microliter

DEFINITION OF TERMS

1. **Monoresistance:** refers to resistant to at least one of the first line antituberculosis drugs **[60, 62, 66]**.

2. "**Primary" resistance:** refers to drug resistance in a patient who has never received antituberculosis therapy **[82].**

3. **Primer**: is a short piece of DNA complementary to a given DNA sequence that acts as a point at which replication can proceed, as in a polymerase chain reaction **[30].**

4. **Probe**: a substance such as DNA that is radioactively labeled or otherwise marked and used to detect or identify another substance in a sample **[30].**

5. **Surrogate marker:** is a measure of the biological activity inside the body that indicates the effect of treatment on the state of disease **[84].**

6. **"Secondary" resistance**: refers to the development of drug resistance during or following chemotherapy for previously drug-susceptible tuberculosis **[82].**

7. **Heteroresistance:** simultaneous presence of both drug resistant and sensitive TB bacilli in clinical samples **[86]**

1. INTRODUCTION

1.1. GENERAL BACKROUND

Tuberculosis (TB) is a common and deadly infectious disease of humans caused by the mycobacterium complex, mainly *Mycobacterium tuberculosis (M. tuberculosis)*. Other mycobacterium such as bovis, africanum, avium and microtti can also cause tuberculosis, especially in immunocompromised patients **[1].**

The emergence of drug-resistance TB is often attributed to failure to implement proper TB control programmes to correctly manage TB cases **[1, 2].** Non-adherence to therapy and inappropriate prescribing of anti-tuberculosis drugs has been associated with the development of multidrug-resistant tuberculosis *(MDR-TB). MDR-TB* is resistance to isoniazid (INH) and rifampicin (RIF) and poses a serious threat to the success of TB control programmes **[1-3].** An *MDR-TB* surveillance system observed by World Health Organization (WHO) for global monitoring of the burden of the diseases is followed in South Africa. With the guidelines from WHO, a surveillance study was conducted by the Medical Research Council of South Africa (MRC-SA) reporting the prevalence of *MDR-TB* for all provinces of South Africa **[4].**

Understanding the etiologic organism, antimicrobial resistance mechanisms and the transmission of *MDR-TB* are of great value in optimizing strategies to control and prevent its development **[5, 6].** Rapid detection, adequate treatment and contact tracing to prevent further transmission are the key factors needed in the control of this infectious disease **[5, 6].** Selective TB genotyping has been used for outbreak investigations and to identify isolates resistant to isoniazid and rifampicin. Tuberculosis genotyping was essential to investigate and confirm transmission in a number of settings and to confirm or exclude laboratory contamination **[7].** When the first study on detection of mutations was published, hopes were raised that early detection of resistance in *M. tuberculosis* would soon be a routine clinical practice **[8]** but to date mutation detection analysis is still not yet in place. With frequency of mutations associated with drug resistance

varying by geographical locations, it is important for country and province to report the data **[8].**

The development of new molecular genotyping techniques
has revealed the presence of mixed *Mycobacterium tuberculosis* infections, which may accelerate the emergence
of drug-resistant strains. There are some studies describing the local distribution of circulating strains in
South Africa, but to date, reports describing the frequency and distribution of *M. tuberculosis* genotypes, and
specifically MDR genotypes, across the different provinces are limited.

Several studies have associated the *Beijing* genotype of *M. tuberculosis* with drug resistance **[7-12].** The *Beijing* genotype is a distinct genetic lineage of *M .tuberculosis*, which is distributed worldwide **[9].** Strains of the *Beijing* genotype family of *M. tuberculosis* have caused large outbreaks of tuberculosis, sometimes involving multidrug resistance and this conserved family of *M .tuberculosis* strains predominates in some geographical areas. The *W-Beijing* genotype has been strongly associated with drug resistance in several settings and in regions with high incidence of *MDR-TB* **[9].** It has been proposed that certain lineages of *M. tuberculosis*, such as the Beijing lineage, may have specific adaptive advantages **[10].** Genotypes such as non-Beijing strains were ignored for the drug resistance association **[10]** but the study conducted from KwaZulu-Natal Province in 2007 has shown that the non-Beijing strains are spreading and associated with drug resistance [**11].** This strain was named F15/LAM4/KZN which was associated with drug resistance and forming part of the non-Beijing family called Latino-American and Mediterranean (LAM**) [11].** In 2009, a study conducted by the Medical Research Council of South Africa, Limpopo Province was shown to be dominated by a non-Beijing strain called X-lineage **[11].**

1.2 AIM

To detect mutations and families of *Mycobacterium tuberculosis* associated with multi-drug resistant tuberculosis in the Limpopo Province.

1.3 OBJECTIVES

- Detection and characterization of types of mutations for rifampicin and isoniazid using GenoType®MTBDRplus assay (Hain LifeScience, Germany).

- Identification of the most common genetic mutations (*rpoB and KatG*) associated with drug resistance and associated resistance codons in the Limpopo Province.

- Detection of *Beijing* and *non-Beijing* families of *Mycobacterium tuberculosis* using real-time PCR assay with probes designed from Roche (South Africa).

2 LITERATURE REVIEW

2.1 General background of tuberculosis

Tuberculosis commonly attacks the lungs (as pulmonary TB) but can also affect the other sites of the body (central nervous system, genitourinary system, lymphatic system, bones, joints and the Skin). Other *mycobacteria* such as *africanum*, *avium*, *bovis* and *microtti* can also cause tuberculosis, but these species do not usually infect healthy adults. Not everyone infected with *M. tuberculosis* develops fully blown disease. Asymptomatic, latent infection is common and one in ten latent infections will progress to active TB disease, which if not treated, kills more than half of its victims **[1-3].**

2.2 Management and control of M. tuberculosis

The identification of mycobacterium species is of utmost important in the establishment of appropriate treatment. It has become evident that, many non-tuberculous mycobacteria (NTMs) are resistant to TB drugs **[11].** The first line treatment of active tuberculosis is by administering isoniazid, rifampicin, pyrazinamide, and ethambutol for two months followed by isoniazid and rifampicin for a further four months. The patient is considered cured at six months although there is still a relapse rate of 2 to 3% **[12]**. For latent tuberculosis, the standard treatment is six to nine months of isoniazid alone **[13].**

Interruption during treatment using these first-line drugs leads to *MDR-TB,* which is a specific form of drug resistant tuberculosis that develops as a result of response failure to the drugs that are used to treat TB **[14].** The resistance is conferred on the basis of response failure to rifampicin and isoniazid **[14].** The resistance could be either acquired on the basis of failure of patients to comply with four drugs given for treatment or primary based on the resistant *M.tuberculosis* acquired from other patients. The resistance is attributable to the mutations in the drug target genes **[15].** The factors that attribute to the outbreaks of MDR include delayed diagnosis, inadequate treatment regimens, non-compliance and significant rates of transmission **[16].** Second line drugs which have toxic side effects, scarce in developing countries and very expensive

are used to treat MDR strains. The second line drugs which include capreomycin, kanamycin, ethionamide, cycloserine, ciprofloxacin, amikacin and clofazimine are prescribed specifically based on the case of the patient. Tuberculosis strains resistant to second line drugs are called extensively drug resistance tuberculosis (*XDR-TB*). The XDR-TB is resistance to the first line drugs, more especially, rifampicin and isoniazid as well as a single fluroquinolone and any of the three injectable drugs such as amikacin, kanamycin or capreomycin **[17].**

2.3 *Epidemiology of* MDR-TB *and* XDR-TB

Globally, TB is a threat, causing three million deaths annually, with 8 million new active cases reported per year **[1]**. World Health Organization (WHO) estimated that an alarming 2,4 million people were infected with TB in 2001; making TB the most infectious agent in the world **[2].** According to the WHO of global tuberculosis control report 2006, there were 8.9 million new TB cases (140/100 000) and approximately 1.7 million (27/100 000) TB deaths in 2004 **[1]**. In South Africa the incidence of all cases in 2004 was estimated at an alarming 718/100 000 and the mortality rate was 1345/ 100 000 **[18-20]**. The 22 high-burden countries collectively account for 80% of the global tuberculosis burden. In 2007, the countries with the highest prevalence were India (with 2.0 million cases), China (1.3 million), Indonesia (530,000), Nigeria (460,000), and South Africa (460,000). Of the estimated 1.37 million cases in HIV-positive subjects, 79% were in Africa and 11% in Southeast Asia **[18]**. South Africa was rated 4th among the 22 high-burden TB countries by WHO report in 2008 [**18].**

The increase in the burden of disease is due to inefficient TB control programmes with low cure rates, resulting from inadequate and interrupted treatment **[1].** The success of TB control program is also threatened by the emergence of drug resistant strains of *M. tuberculosis*, especially multidrug-resistance strains and the rising HIV epidemic **[3].** Non-adherence to therapy and inappropriate prescribing of anti-tuberculosis drugs has been associated with the development of *MDR-TB* **[3].** Early detection of MDR-TB is a primary importance for both patient management and control of infection **[3].** In 1995, World Health

Organization estimated that 50 million people were infected with drug-resistant strains of *M. tuberculosis* and it is estimated that 300,000 new cases of *MDR-TB* occur each year worldwide **[9, 10].** With the DOTS-plus strategy in place since 1999, South African health services implemented the strategy in year 2000 at a gold mining company of North West Province **[1, 4].** More than 450 000 multidrug resistant cases are estimated to occur globally each year **[19]** while 1-2% occurs in South Africa **[1, 4, 19].**

Disturbingly, there were an estimated 500,000 cases of MDR-TB in 2007 (including 289,000 new cases). Of these 131,000 were in India, 112,000 in China, 43,000 in Russia, 16,000 in South Africa, and 15,000 in Bangladesh. Fifty five countries had reported cases of *extensively drug resistant tuberculosis* (*XDR-TB*) by the end of 2008 **[18]**. For example, 28% of all people newly diagnosed with TB in one region of north western Russia had the multidrug-resistant form of the disease in 2008, the highest level ever reported to WHO as compared to the previous highest level of 22% at Baku City, Azerbaijan in 2007 **[20].**

A *MDR-TB* surveillance system observed by WHO for global monitoring of the burden of the diseases is followed in South Africa. With the guidelines from WHO, a surveillance study was conducted by the Medical Research Council of South African (MRC-SA) reporting the prevalence of MDR-TB for all provinces of South Africa **[4, 8].** The surveillance system for *MDR-TB* covered the nine provinces of South Africa. The study reported an increase from 1.5% of *MDR-TB* in new patients of Mpumalanga during 1997 to 2.6% in 2001. Prevalence of patients retreated for *MDR-TB* increased from 8.1% to 13.9%. The prevalence of *MDR-TB* in Limpopo Province was 2.4% among new patients and 6.8% in re-treatment patients as reported in a drug resistance survey conducted by MRC during 2001-2002 **[4, 8].** A 2001 study showed that eight of the nine South African Provinces (no data available for the 9^{th} province) had an estimated incidence rate of greater than three per 100,000 of the population for multidrug-

resistant tuberculosis. In the Western Cape Province, the estimated incidence rate for *MDR-TB* was reported as 8.39 per 100.000 **[4, 8].**

In the new WHO's *Multidrug and Extensively Drug-Resistant Tuberculosis:* 2010 Global Report on Surveillance and Response, it is estimated that 440, 000 people had MDR-TB worldwide in 2008 and that a third of them died. Almost 50% of MDR-TB cases worldwide are estimated to occur in China and India **[20].** In Africa, estimates show that 69, 000 cases emerged, the vast majority of which went undiagnosed **[20].** The low percentages of *MDR-TB* reports of Africa are due to limited laboratory capacity to conduct drug resistance surveys. Previous reports from WHO found high levels of mortality rates among people living with HIV and infected with *MDR-TB* and *XDR-TB* **[13].**

In 2006, in Tugela Ferry KwaZulu Natal (KZN), South Africa, an outbreak of XDR-TB has killed 52 out of 53 people within three weeks representing 98% mortality rates most of whom were HIV positive **[13, 20].** Drug sensitivity testing of TB bacterial isolates in KZN has revealed that 2476 patients had *MDR-TB* compared with just 124 cases in the United States for the year 2006 **[20]**. In several instances, *MDR-TB* outbreaks were reported to be the results of the spread and transmission of a particular strain of *M.tuberculosis* **[19].** Genotyping studies conducted on isolates from 46 patients with *XDR-TB*, 74%-95% of patients with XDR-TB were infected with a genetically similar strain, unique to KwaZulu-Natal **[13].** The strain involved was referred to as "the KwaZulu-Natal (KZN)".

The strain was already present in the KZN Province during *M. tuberculosis* finger printing activities conducted in 1994. It was found that although the IS6110 fingerprint of the strain was unique, the spoligotype has been reported before and was listed in the Spoligotyping data base as ST60. This spoligotypes have been found in 12 countries on the European, North and South American continents. After the South African classification of *M. tuberculosis* strain, the organism was found to belong to the F15 family which forms part of the LAM

family in correspondence with the LAM4 subgroup. Therefore, it was renamed F15/LAM4/KZN as called to date **[11, 13].**

2.4 MOLECULAR MECHANISMS OF DRUG RESISTANCE

In order to control the drug resistance epidemic it is necessary to gain insight into how *M. tuberculosis* develops drug resistance. This knowledge help us to understand how to prevent the occurrence of drug resistance as well as identifying genes associated with drug resistance strains. *M.tuberculosis* and other members of the *M. tuberculosis* complex use several strategies to resist the action of antimicrobial agents. However, genetic studies have shown that resistance of *M. tuberculosis* to antimicrobial drugs is a consequence of mutations in genes that encodes either the target of the drug or enzymes that are involved in drug activation **[6**]. Mutations in the genome of *M. tuberculosis* that can confer resistance to anti-tuberculosis drugs occur spontaneously with an estimated frequency of 3.5×10^{-6} for INH and 3.1×10^{-8} for RIF **[6].** With the chromosomal loci responsible for various drug resistances, the risk of a double spontaneous mutation is extremely low: 9×10^{-14} for both INH and RIF **[6, 21].**

Any drug used in the anti-TB regiment is supposed to have an effective sterilizing activity that is capable of shortening the duration of treatment. Resistance to first line anti-TB drugs has been linked to mutations in 9 genes. They are *KatG, inhA, ahpC, KasA* and *ndh* for INH resistance; *rpoB* for RIF resistance; *embB* for EMB resistance; *pncA* for PZA resistance and *rpsL* and *rrs* for STR resistance **[22].**

2.4.1 Rifampicin:

Rifampicin was first introduced in 1972 as an anti-TB drug and has excellent sterilizing activity **[5, 23].** RIF interferes with transcription by the DNA-dependent RNA polymerase. RNA polymerase is composed of four different subunits (α, β, β' and σ) encoded by *rpoA, rpoB, rpoC* and *rpoD* genes, respectively. Characterization of the *rpoB* gene in *Escherichia coli (E.coli*) demonstrated that rifampicin specifically interacted with the β-subunit of RNA transcription which is required for chain initiation and extension. The step is achieved by RIF binding to the β-subunit hindering transcription and thereby killing the organism.

Ribonucleic acid (RNA) polymerase plays a critical role in the information transfer within bacteria by transcribing the messages of all the genes into RNA. It represents an ideal target for chemotherapy with its inactivation causing an ultimate total inhibition of cellular functions **[24].** Rifampicin interacts with the RNA polymerase holoenzyme resulting in abortive ignition of transcription. Resistance to high levels of rifampicin occurs in a “single-step” in *M. tuberculosis* **[24].**

Mutations in the *rpoB* locus from *M. tuberculosis* were characterized and mutations conferring the resistant trait were identified **[12].** Most mutations were determined to be restricted to an 81-bp core region and are dominated by single nucleotide changes, resulting in a single amino acid substitution, although in frame deletions and insertions also occur at lower frequencies. From extensive studies on *rpoB* gene in RIF resistance of *M. tuberculosis* a variety of mutations and short deletions in the gene are identified. A total of 69 single nucleotide changes, 3 insertions, 16 deletion and 38 multiple nucleotide changes have been reported **[25].** With more than 95% of the missense mutations of RIF occurring at the *rpoB* gene between codon 507-533, most common changes occur in codon Ser531leu, His526Tyr and Asp516Val **[5,23].** Most mutations occur spontaneously in strains that were not previously exposed to the antibiotic at a frequency of 10^{-8} which results from missense mutations near the centre of the *rpoB* gene **[24].** Furthermore, the minimal inhibitory concentration (MIC) showed that high level of RIF resistance is associated with mutations in codon 526 and 531, whereas alterations in codon 511,516,518 and 522 result in low level RIF resistance **[24].**

2.4.2 Isoniazid:

Previous studies have identified resistance-associated mutations in *katG*, *inhA*, *kasA*, *ndh*, and the *oxyR-ahpC* intergenic region of INH. Isoniazid induces several genes in *M. tuberculosis* that encode proteins physiologically relevant to the drug’s mode of action **[25].** It is proposed that INH enters *M. tuberculosis* as a prodrug by passive diffusion and is activated by catalase-peroxidase, encoded

by *katG*, to generate free radicals, which then attack multiple targets in the cells **[26].** Recent studies have shown that an NADH-dependent enoyl acyl carrier protein (ACP) reductase, encoded by *inhA*, and a -ketoacyl ACP synthase, encoded by *kasA*, are two potential intracellular enzymatic targets for activated INH **[26-30].** Both enzymes are involved in the biosynthesis of mycolic acids **[30].**

Resistance-associated amino acid substitutions have been identified in the *katG*, *inhA*, and *kasA* genes of INH-resistant clinical isolates of *M. tuberculosis* **[27, 28].** Additional genetic and biochemical studies have shown that certain promoter mutations of alkylhydroperoxide reductase, encoded by *ahpC*, in INH-resistant isolates result in overexpression of *ahpC* as a compensatory mechanism for the loss of catalase activity due to *katG* mutations **[25].**

Resistance to INH in *M. tuberculosis* is attributed to mutations in several genes. The *katG* gene which encodes catalase-peroxidase is the most commonly altered, with the majority of mutations occurring at codon 315. Mutations in the promoter regions of *inhA* and *oxyR-ahpC* genes have been identified in INH-resistant strains but not INH-susceptible strains **[5].**

INH resistance genes:

a. *KatG* gene.

Isoniazid enters the cell as a prodrug that is activated by a catalase peroxidase encoded by *KatG* gene. The peroxidase activity of the enzyme is necessary to activate INH to a toxic substance in the bacterial cell **[29]**. This toxic substance subsequently affects intracellular targets such as mycolic acid biosynthesis which are important components of the cell. A lack of mycolic acid synthesis eventually results in loss of cellular integrity and the bacteria die **[25].** A loss of catalase activity can result in INH resistance **[30]**. Subsequent genetic studies demonstrated that *KatG* deletions and transformation of INH-resistant *M. smegmatis* and *M. tuberculosis* strains with functional *KatG* gene restored

susceptibility give rise to INH resistance **[29]**. However, mutations in this gene are more frequent than deletions and these can lower the activity of the enzyme. Most mutations are found between codons 138 and 328 with the most commonly observed gene alterations being at codon 315 of the *KatG* gene **[29].** With an estimation of 30-60% of INH resistance, a common substitution occurs in the Ser315Thr **[5]**. The *KatG* 463 (CGC-CTG) (Arg-Leu) amino acid substitutions is the most common polymorphism found in the *KatG* gene and is not associated with IHN resistance **[31].**

b. *ahpC* gene.

It has been observed that a loss of *KatG* activity due to the S315T amino acid substitution if often accompanied by an increase in expression of an alkyl hydroperoxide reductase (*ahpC*) protein that is capable of detoxifying damaging organic peroxides **[32].** Five different nucleotide alterations have been identified in the promoter region of the *ahpC* gene, which lead to over expression of *ahpC* and INH resistance **[5].** Alkyl hydroperoxide reductase overexpression exerts a detoxifying effect on organic peroxides within the cell and protects the bacteria against oxidation damage but does not provide protection against INH. Catalase-peroxidase can also be up regulated under conditions of oxidative stress. The correlation between polymorphic sites in the *ahpC* regulatory region with INH resistance in *M. tuberculosis* requires further examination **[5].**

c. InhA gene.

One of the targets for activated INH is a protein encoded by the *inhA* locus. Enoyl-acyl carrier protein (ACP) reductase (inhA) is proposed to be the primary target for resistance to INH and ethionamide (ETH**) [26].** Activated INH binds to the *InhA*-NADH complex to form a ternary complex that result in inhibition of mycolic acid biosynthesis. Six point mutations associated with INH resistance within the structural *inhA* gene have been discovered (IIe16Thr, IIe21Thr, IIe21Val, IIe47Thr, Val78AIa and IIE95Pro) **[5]**. A Ser94AIa substitution result in a decrease binding affinity of *inhA* for NADH, resulting in mycolic acid synthesis

inhibition. Although these mutations in the structural *inhA* gene are associated with INH resistance, it is not frequently reported in clinical isolates. *InhA* promoter mutations are more frequently seen and are present at position -24(G-T), -16(A-G), or -8(T-G/A) and -15(C-T). These promoter mutations result in over expression of *inhA* leading to low level INH resistance. To date approximately 70-80% of INH resistance in clinical samples of *M.tuberculosis* can be attributed to mutations in the *KatG* and *inhA* genes **[5].**

d. *KasA* gene

Considerable disputes within the literature are present as to the role of *KasA* as a possible target for INH resistance **[32].** This gene encodes a β-ketoacyl-ACP synthase involved in the synthesis of mycolic acids. Mutations have been described in this gene that confers low levels of INH resistance. Genotypic analysis of the *KasA* gene reveals four different amino acid substitutions involving codon 66 (GAT-AAT), codon 269 (GGT-AGT), codon 312 (GGC-AGC) and codon 413 (TTC-TTA) **[5, 33].** Nevertheless, the possibility of *KasA* constituting an additional resistance mechanism should not be excluded completely.

e. *ndh* gene

The *ndh* gene encodes NADH dehydrogenase that is bound to the activated INH. Structural studies have shown that a reactive form of INH-NAD (H) co-factor generates a covalent INH-NADH adduct. Mutations in the *ndh* gene, encoding NADH dehydrogenase cause defects in the enzymatic activity. Thus, defects in the oxidation of NADH to NAD^+ result in NADH accumulation and NAD^+ depletion [**32].** These high level of NADH can then inhibit the binding of the INH-NAD adduct to the active site of *inhA* enzyme **[34,35].**

2.4.3 Pyrazinamide:

Pyrazinamide (PZA) is a nicotinamide analog which was discovered in 1952 to have anti-TB activity. Pyrazinamide targets an enzyme involved in fatty acid synthesis and is responsible for killing persistent tubercle bacilli in initial intensive phase of chemotherapy **[36].** However, during the first two days of treatment, PZA has no bactericidal activity against rapidly growing bacilli **[37]**. Pyrazinamide has effective sterilizing activity and shortens the chemotherapeutic regiment from 12 months to 6 months. *M. tuberculosis* has the enzyme pyrazinamidase (PZase) which is only active in acidic conditions. Pyrazinamide is a prodrug which is converted to its active form, pyrazinoic acid (POA) by the pyrazinamidase (PZase) encoded by *pncA* **[38].**

The activity of PZA is highly specific for *M.tuberculosis*, as it has no effect on other mycobacteria. *Mycobacterium bovis* is naturally resistant to PZA due to a unique C-G point mutation in codon 169 of the *pncA* gene. Pyrazinamide is only active against *M. tuberculosis* at acidic pH where POA accumulates in the cytoplasm due to an ineffective efflux pump. Accumulation of POA results in lowering of intracellular pH to a level that inactivates a vital fatty acid synthase and disrupts membrane and interferes with energy production, necessary for survival of *M. tuberculosis* at an acidic site of infection **[38, 39].** Cloning and characterization of *M. tuberculosis pncA* gene studies showed that *pncA* mutations have been identified in more than 70% of *pncA* resistant clinical isolates scattered through the *pncA* gene but thus far no mutational hot spot has been identified **[40].**

A study conducted in Peru showed that 95% of MDR patient also had *M. tuberculosis* resistant to PZA **[41].** In many countries, PZA susceptibility is not done routinely due to technical difficulties. Thus the extent of PZA resistance is globally not known. A study that was conducted by Louw et al showed that PZA resistance is common amongst drug-resistant clinical isolates from South Africa **[40].** PZA resistance was shown to be strongly associated with *MDR-TB* and therefore it was concluded that PZA should not be considered or relied upon in

managing patient with *MDR-TB* in this setting **[40]**. Pyrazinamide resistant isolates had diverse nucleotide changes scattered throughout the *pncA* gene. Mutations in the *pncA* gene correlate well with phenotypic resistance to PZA. However, PZA resistant isolates without *pncA* mutations were also observed suggesting that another mechanism may be involved in conferring PZA resistance in these isolates **[40].** Mutations were identified at codon 63,138,141 and 162 **[38].** A pncA mutation resulting in structural changes in the *pncA* has been identified. It was presumed that structural changes detrimentally change the enzyme function by altering conversion of PZA to its bioactive form **[38].**

2.4.4 Ethambutol:

Ethambutol (EMB) is a bactericidal first line drug, used in combination with other drugs and is specific to the mycobacteria. Ethambutol inhibits an arabinosyl transferase (*embB*) in the cell wall biosynthesis **[38, 42].** Ethambutol has a three gene locus (*embCAB*) that encodes homogenous arabinosyl transferase enzymes involved in EMB resistance. Various studies have identified five mutations in codon 306 [(ATG-GTG), ATG-CTG), (ATG-ATA), (ATG-ATC) and (ATG-ATT)] which results in three different amino acid substitutions (Val, Leu and lle) in EMB resistant isolates **[43, 44]**. These five mutations are reported to have 70-90% associated with EMB resistant isolates **[5].** Missense mutations were also identified in three additional codons: Phe285leu, Phe330Val and Thr630 substitutions than those organisms with Met306lle substitutions.

2.5 Distribution of mutations.

Emergence of *MDR* and *XDR-TB* is a growing problem in many countries **[1].** It is not known whether epidemic drug resistant strains of *M. tuberculosis* have evolved special mechanisms that facilitate drug resistance acquisitions, maintaining fitness, or promoting person to person transmission **[45].** Conventional wisdom has held that *M. tuberculosis* acquires *MDR* and *XDR-TB* through step-wise acquisition of chromosomal mutations that each confers resistance to individual drugs **[5].** Single-step mutations of MDR-TB have not

been identified **[45].** Resistance mutations have been shown to be associated with fitness “cost” in many bacteria, including *M. tuberculosis*, where the cost appears to be dependent on specific mutations and strain types **[45].** When the first study on detections of mutations was published, hopes were raised that early detection of resistance in *M.tuberculosis* would soon be a routine clinical practice but to date mutations detection analysis is still not part of clinical practice **[22].**

Nine genes are known to be linked to resistance to first-line-anti-TB drugs to date. They are *katG, inhA, aphC and kasA* for INH resistance; rpoB for RIF resistance; rpsL and *rrs* for streptomycin (STR) resistance; *embB* for ethambutol (EMB) resistance; and pncA for pyrazinamide (PZA) resistance **[5]**. Molecular patterns of mutations conferring resistance to rifampicin (RIF) in 81-bp hot spot region of the *rpoB gene* and isoniazid (INH), mainly in the *katG*, *oxyR-ahpC* of *M. tuberculosis* strains isolated worldwide in 1998 have been explored in previous investigations **[5,47].** For the other drugs mutations are in a random not excluding South Africa. Since that time, a shift in the population structure of *MDR-TB* strains is observed and documented by its rising proportions **[48].**

Studies worldwide have demonstrated that >95% of RIF resistant *M. tuberculosis* strains have a mutation within the 81-bp hot-spot region (codons 507-533) of the RNA polymerase β subunit (*rpoB*) gene **[12].** Unlike RIF resistance, INH resistance is apparently controlled by a more complex genetic system that involves several genes **[12].** However, extensive studies have demonstrated that INH resistance is more frequently associated with a specific mutation in katG (codon 315), a gene that encodes the catalase-peroxidase enzyme in *M. tuberculosis* **[12].** The most frequently observed alteration in this gene is a serine-to-threonine substitution at codon 315 (S315T) located within the active site of the catalase moiety. Also, *inhA* (enoyl-ACP-reductase), an enzyme involved in mycolic acid biosynthesis, was identified as the main target of INH. Mutations in the regulatory region of the inhA gene have been linked to INH resistance **[12].**

Studies in Poland have shown that mutations in *rpoB* and *katG* genes were comparable to those reported as common mutations from other parts of the world, thus reflecting a global pattern **[12].** The *rpoB* codons 531, 526 and 516 are the most frequently mutated codons worldwide, although variations in the relative frequencies of mutations in these codons have been described for *M.tuberculosis* from different geographical locations. The most common mutations of RIF are at 531TTG worldwide, while 526GAC and 516 appear to be prevalent in only few countries, including Italy, Greece and Mozambique **[1]**.

A 2008 study in South Africa reported the same mutations as encountered throughout the world but with an addition of mutations found at codon 314 of *katG* gene. This mutation was reported for the first time in Africa and codon 530 of *rpoB* gene for isolates collected from Dr George Mukhari Hospital (DGM) **[49]**.With most of the studies done in Western Cape, South Africa, MDR-TB was first recorded in 1985, but no genotype data are available from these early isolates **[50].** In agreement with other studies worldwide, the most common mutations found in Cape Town for RIF are at codon 531,526, and 516 with a higher proportion of RIF resistance due to S531L mutations **[49, 51, 52].** The prevalence of mutations in the *inhA* and *katG* genes was shown to be widely varying in different geographical locations. For katG, mutations were found in 97% (77/79) and inhA, mutations were in 24% (19/79) of INH-resistant isolates from KwaZulu-Natal **[53]**,whereas van Rie and colleagues reported *katG* mutations in 72% of INH resistant isolates (41/57) and mutations in the inhA gene in only 2% (1/57) isolates in the Western Cape Province **[52].**

2.6 Detection of *Beijing* and Non-*Beijing* genotypes

Since early 1990s, selective TB genotyping has been used in New York City for outbreak investigations, to identify isolates resistant to isoniazid and rifampicin **[7, 48].** Tuberculosis genotyping was essential to investigate and confirm transmission in a number of settings and to confirm or exclude laboratory contamination. A number of programmes demonstrated the utility of universal genotyping, which influenced the development of this service in New York **[48]**.

In 2001, the New York City bureau of TB control began genotyping isolates for every new TB case with Spoligotyping and IS600-based restriction fragment length polymorphism (RFLP) to improve the efficiency of TB control **[7].**

The control of TB has been more complicated by the recent emergence of *XDR-TB* which had devastating effects. *Extensively drug resistance tuberculosis* is an indicator of more increased drug resistance to known TB treatment drugs which include second line drugs **[13].** A Genotyping study conducted in 2006 on isolates from 46 patients with *XDR-TB*, 74%–95% of patients were infected with a genetically similar strain, unique to KwaZulu Natal **[13].** However, the *MDR-TB* outbreak that occurred in New York in 2001 among 267 patients, majority of patients were coinfected with human deficiency virus (HIV) and the MDR-TB strain was unique to the city **[7].** The strain was named W-like Beijing strain which only constitute a small branch on the phylogenetic tree of genotypic strain **[24].** Identification of the diversity of the strains provides a means to identify instances of recent transmission of TB as well as chains of transmission that occur among persons with TB and also helps to elucidate the patterns and dynamics of TB transmission **[51, 52].**

Two families of the *M. tuberculosis* complex are identified throughout the world which is Beijing and non-Beijing. A total of eight potential non-Beijing super families or clades of *M.tuberculosis* complex are identified (*Mycobacterium africanum*, *Mycobacterium bovis*, *East African-Indian (EAI), Central Asian strains (CAS), TB strains (T), Haarlem I, X,* as well as *Latin American* and *Mediterranean (LAM).* The *Beijing* family accounted for the most reported *MDR-TB* outbreaks **[53-55]** with W-strain and W-like strain**.** *Beijing* strains are however still uncommon in Africa, except for South Africa **[56].** Studies in Cape Town, (South Africa) between the year 2000-2003 have shown that the *Beijing* clade is a cause of disease in children and has increased from 13% to 33% suggesting a selective advantage in transmissibility and disease production **[57, 58].** Other studies in Cape Town have shown that the *Beijing* and the *Haarlem* families are dominating in this part of South Africa, with no reports for the other provinces, the

prediction of dominance of families remains unclear **[57-59].** It has been globally reported that the population structure of *M. tuberculosis* is defined by six phylogeographical lineages, associated with specific populations which are *Beijing, Haarlem I, CAS, LAM, T* and *EAI* **[50, 53, 58]**.

The *Beijing* or *W*-like family of *M. tuberculosis* continues to be the focus of extensive investigations because strains are widely spread throughout the world including South Africa and Russia. In these countries it constitutes the major family of *MDR-TB* isolates **[57].** It is therefore possible that the drug-resistant *Beijing* strain or other MDR outbreak associated strains are widespread but has not been recognized and reported as such **[58].** Among the genotypes that prevail in the modern spectrum of *M.tuberculosis* strains, the *Beijing* genotype is the one that causes major concern, as it is geographically widespread and it is considered hypervirulent **[58].** *Mycobacterium tuberculosis* strains of the *Beijing* genotype were first identified in China and neighboring countries and have attracted special attention due to their global emergence and association with drug-resistance **[58].** A recent systematic review of the published literature demonstrated the worldwide ubiquity of the *Beijing* strains and their association with outbreaks and drug resistance **[59].**

It has been suggested that the *Beijing* isolates are more frequently resistant due to their ability to mutate more rapidly than other strains **[57].** The mechanisms underlying this epidemiological finding have not been clearly elucidated and knowledge may have an important role in the control of *MDR-TB* **[57]***.* At a molecular level, Beijing strains have specific properties in terms of proteins and lipid structures and their interaction with the host immune system **[60].** This genetically highly conserved family of *M.tuberculosis* strains predominates in some geographic areas **[9].**The *Beijing* genotype has been linked to polymorphisms in a human immune gene, suggesting the possibility of human-mycobacterial co-evolution **[61].** The *Beijing* genotype could have an enhanced ability to acquire drug resistance due to the polymorphism in genes coding for DNA repair enzymes. However, *Beijing* strains have shown similar rates of

mutations-conferring resistance to RIF compared to non-*Beijing* strains suggesting that they do not mutate more frequently than non-*Beijing* strains **[61, 62].**

As Beijing strains from different geographical areas show a remarkable degree of genetic conservation in comparison to other *M. tuberculosis* strains a hypothesis was made that this genetic lineage has selective advantages over other genotypes of *M.tuberculosis*, and have started spreading significantly **[47]**. Molecular epidemiological studies have shown that there is predominance and successful transmission of specific mutations, such as Ser315Thr in *KatG* and Ser531Leu in *rpoB*, associated with *MDR-TB* in several populations **[47].** However, in a study conducted in Brazil, the comparison of fitness between in vitro *rpoB* mutants showed that *Beijing* and non-*Beijing* strains had similar biological cost for the same rpoB mutations **[63].** It is known that the *Beijing* family and non-*Beijing* family strains represent the same genotype of *M. tuberculosis*, which was concurrently identified in the American and Asian continents **[63]**. The widely distributed (but not universal) associated drug resistance and the *Beijing* genotype suggest that these strains may have a particular propensity for acquiring drug resistance **[64].** Recently, it was reported that *Beijing* strains carry mutations in putative genes and this may explain a higher adaptability of these bacteria to stress conditions such as exposure to anti-tuberculosis drugs and the hostile intracellular environment **[65].**

Many researchers have examined possible associations between the *Beijing* genotype and the distribution of mutations in the genes underlying resistance to anti-tuberculosis drugs. Genotypic studies focusing at *katG*315, the most important mutation encoding for resistance to INH, were indeed valuable, reporting the higher rates of mutation among *Beijing* strains **[66].** This was shown to be less clear for mutations in the *rpoB* gene, which account for >90% of drug resistance to rifampicin. Some researchers found that the *Beijing* strains did not show significant differences in frequency of the most commonly encountered mutations in the *rpoB* gene compared to non-*Beijing* genotypes strains **[67-69].** A

significantly higher proportion of the *rpoB* S531L mutation in *Beijing* genotype strains was found in Germany and Russia, while in Korea the opposite was found **[69].** It was hypothesized that certain specific characteristics of the cell wall structure of the *Beijing* strains lead to suboptimal intracellular concentrations of anti-tuberculosis drugs and acquisition of drug resistance **[70].**

A conceptual frame work on the success of the *Beijing* stains was generated on the underlying factors of its success. A Bacillus- Calmette- Guérin (BCG) vaccination is thought to be less protective against typical *Beijing* strains than against other strains **[71].** This has lead to more latent infection and more progression from latent infection to active TB **[71].** It was suggested that anti-TB treatment may similarly be less effective in eradicating *Beijing* strains than other strains, however, it has been suggested that the spread of *Beijing* strains already started long before the introduction of vaccination and antibiotic treatment. This implies that *Beijing* strains have an intrinsic advantage over other *M. tuberculosis* genotypes in terms of transmission, progression from latent to active tuberculosis, acquisition of drug resistance and disease chronicity **[72]. Figure1** shows the conceptual framework of the factors underlying the success of *Beijing* genotype.

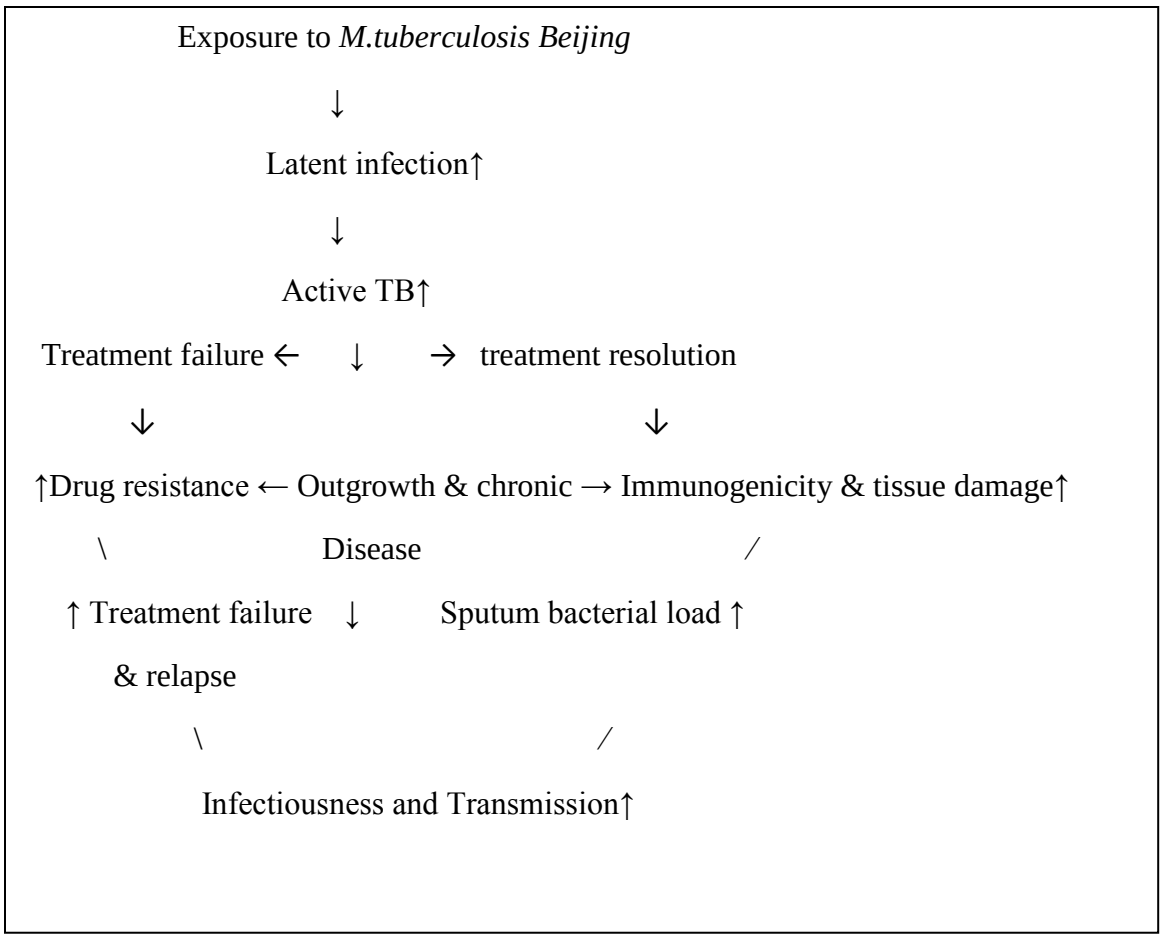

Figure 1: A conceptual framework of factors underlying mechanism of success of *Mycobacterium tuberculosis Beijing* genotype **[72]**. ↑- increased. ↓- decreased.

With the current era of tuberculosis-treatment, drug resistance may drive the spread of a particular *M. tuberculosis* genotype **[73].** Epidemiological studies around the world have examined the association of drug resistance with *Beijing* strain (**Table 1**)

Table 1: Approaches to examine the relation of *M. tuberculosis Beijing* genotype with drug resistance.

No	Approach	Finding	Reference
1	Epidemiological studies of phenotypic drug resistance	Associations with drug resistance in certain parts of the world, but not all	**[63]**
2	Mutations in drug resistance genes	Higher rates of mutations reported for rpoB codon S531L, katG, embB different distributions of rpoB mutations	**[48,67,68]**
3	In vitro exposure to anti-TB drugs	No difference in acquisition of drug resistance	**[75]**
4	Examination of underlying molecular mechanisms	More mutations in 'mutator genes' reported by some but not all	**[62]**

A hypothesis was made that specific characteristics of the cell-wall structure of *Beijing* strains lead to suboptimal intracellular concentrations of anti-tuberculosis drugs and acquisition of resistance **[74-76].**

2.7 Biochemical Characteristics and Immunogenicity of *Beijing* genotype

Mycobacterium tuberculosis Beijing genotype strains may also be more virulent, as a result of intrinsic biochemical properties and their interaction with human host defense **[70].** One of the proteomic studies showed that the *Beijing* strains has an increased expression of a crystallin protein homolog or the 16-kDa protein, a *M.tuberculosis* virulence factor, and decreased expression of Hsp65,

PstS1, and the 47 kDa protein compared to other clinical isolates and control strain $H_{37}Rv$ **[59].** Using human monocytic cell line U-937, a study has shown that two proteins, Mb1363 (probable glycogen phosphorylase GIgP) and MT2656 (Haloalkane dehalogenase LinB), had a higher expression after phagocytosis of *M.tuberculosis* K-strain (a *Beijing* strain) compared to *$H_{37}Rv$*, *$H_{37}Ra$* and *M.bovis* BCG **[77]**. It was hypothesized that highly GIgP protein in K-strains induces macrophage migration inhibitory factor (MIF) activation, which may be advantageous for the bacteria. Although the role of these proteins is not yet clear, they may have a critical function in the pathogenesis of tuberculosis **[77].**

2.8 Prevention and Control

Conquering TB would require more than improved diagnostic tools, better medication, and Direct Observed Therapy (DOT) **[78].** Preventive strategies including prophylaxis for infected patients, case finding and infection control, and vaccination strategies (Bacillus- Calmette- Guérin), are critical to the effect of control and eliminate TB worldwide. Control of tuberculosis may include isolation methods in hospitals, medical and dental offices, nursing homes, and prisons **[78, 79].**

Vaccination using Bacillus- Calmette- Guérin (*BCG*) is the currently available preventive tool for TB. It is an attenuated strain of *M.bovis* which has been used for more than 80 years, but it does not seem to have a major effect in decreasing the epidemic of TB **[14].** It does not elicit a lifelong immunity to protect people from getting active TB. The developments of new vaccines that are under study include live attenuated *M. tuberculosis* vaccine, recombinant *BCG*, DNA vaccines and subunit vaccines in an attempt to have a lifelong immunity will help in the reduction of development of active TB **[70,80,81].**

2.9 Experimental Proposal

The emergence of anti-tuberculosis drug-resistance, especially *Multidrug-resistant tuberculosis (MDR-TB)*, resistance at least to isoniazid (INH) and rifampicin (RIF), poses a serious threat to the success of TB control programs. National Health Laboratory Services (NHLS) in Polokwane receives sputum

samples for microscopy, culture and susceptibility testing and those for microscopy only, referred from all the regional hospitals in the Limpopo province, South Africa. Understanding the etiologic organism, antimicrobial resistance mechanisms, and the transmission of *MDR-TB* are of great value in optimizing strategies to control and prevent its development. Rapid detection, adequate treatment, and contact tracing to arrest further transmission are the key factors needed in the control of this infectious disease. When the first study on detections of mutations was published, hopes were raised that early detection of resistance in *M. tuberculosis* would soon be a routine clinical practice but to date mutations detection analysis is still not part of clinical practice. With frequency of mutations associated with drug resistance varying by geographical locations, it is important for country and province to report the data. Selective TB genotyping has been used for outbreak investigations, to identify isolates resistant to isoniazid and rifampicin (*MDR-TB*). This study will attempt to use genotyping (molecular) assays for the detection of mutations and families of *M. tuberculosis* associated with *MDR-TB* by the use of GenoType®MTBDRplus and Real Time PCR assays, which are recommended as the best in the detection of genotypes.

The aim of this study was to detect mutations and families of *Mycobacterium tuberculosis* associated with multi-drug resistant tuberculosis in the Limpopo province. Characterization of *MDR-TB* strains will provide basic epidemiological information. The information obtained will help on the development of rapid methods for identification of *MDR-TB*. Genotyping will determine whether the families circulating are associated with drug resistant strains of *M. tuberculosis*.

2.10 Expected significance of the study.

Detection and characterization of *MDR-TB* strains will fundamentally help the patients to get the appropriate treatment that will specifically combat the *MDR-TB* strains. Furthermore, this will help to reduce the transmission rate drastically in the community and to persons in close contact with the patients. Detection of mutations in the province using GenoType®MTBDRplus will help in determining the rate of drug resistance. Genotyping using Real-Time Polymerase Chain

Reaction (R-T PCR) allows determination of Families (lineages) which are associated with drug-resistance strains of *M.tuberculosis* circulating in the Limpopo Province. The genotypes are not all associated with drug resistance, so determination of genotypes associated with drug resistance will help in the intervention of epidemiological control management of the genotype implicated in the province. The key feature of Real-Time PCR is that the amplificated DNA molecule is detected as the reaction progresses in real time. Real-Time PCR is used to amplify and simultaneously quantifying a targeted DNA molecule and in one or two specific sequences in a DNA sample, Real-Time PCR enables both detection and quantification. This molecular method (R-T PCR) will help determine the families in terms of *Beijing* and *non-Beijing* genotypes in the province.

3 MATERIALS AND METHODS

3.1 Study Design

The research was a cross sectional study, it involved observation of a representative subset of the population of Limpopo Province, during the time of the study.

3.2 Study Site

The research took place in the Limpopo Province on samples from all regional TB hospitals.

3.3 Study Population

The study targeted all samples from patients diagnosed with pulmonary TB admitted and/or out patients to tuberculosis wards in all the regional hospital of each of the five different regions of Limpopo Province [Capricorn (Polokwane/Mankweng hospital Complex), Mopani (Letaba Hospital), Vhembe (Tshilidzini and Elim hospitals), Waterberg (Mokopane hospital), and Sekhukhune (St.Rita Hospital)].

3.3.1 Sputum Specimen

Sputum specimens were collected from National Health Laboratory Services (NHLS) Polokwane, which serves as a referral laboratory for the five regional TB hospitals of the Limpopo Province.

3.3.2 Sample Size

The sample size estimation using proportion method was determined on the basis of 95% confidence interval at +/- 5% with a power of 80% of sputum TB specimens received monthly in the laboratory; a total number of 207 samples were obtained from January 2009 to August 2010.

3.3.3 Sampling Method

The sputum samples were randomly selected weekly until a total of 207 smear positive or AFB^+ (2^+ to 3^+) samples was obtained.

3.4 Sputum Specimen Processing

3.4.1 Decontamination and Concentration of the sputum samples [83].

Principle of method:

Sodium Hydroxide digest sputum debris, normal flora, and other infectious organisms and the remainder will be highly resistant organism such as Mycobacterium. Mycobacteria have high quantity of mycolic acids which resist entry of Sodium Hydroxide. The remaining cells were washed by saline (distilled water).

Procedure: Refer to appendix 1a

3.4.2 DNA Extraction [84].

Principle of method:

Here, the cells are lysed partially allowing plasmids to escape, whereas the bacterial chromosomal DNA remains trapped in the cell debris. High temperature was then used to denature the chromosomal DNA, after which reannealing allows the plasmids to reassociate. Centrifugation removes the chromosomal DNA along with the cell debris, leaving the plasmid in suspension.

Procedure: Refer to appendix 1b

3.5 Polymerase Chain Reaction [54]

Principle of method:

Temperature-sensitive Taq polymerase splits the double stranded DNA into two single strands and simultaneously adds complementary dideoexyribonucleic acids with respect to base-pairing. New strands of DNA are synthesized and the DNA amplified.

Procedures refer to appendix $1c_1$.

3.6 Hybridization

GenoType®MTBDRplus Assay **[8, 85]**

Principle of method:

The GenoType®MTBDRplus assay is based on reverse hybridization between amplicons derived from a multiplex PCR and nitrocellulose-bound probes covering overlapping wild-type (WT) sequences of the hot-spot regions (*rpoB* WT 1-*rpoB* WT 5, katG WT), the four most frequent mutations for *rpoB* *(rpoB* MUT probes), and mutations at codon 315 in katG (*katG* MUT probes). The presence of a mutation is indicated by the lack of hybridization on one or more of WT probes with or without hybridization on the MUT probes. For the step wise procedure refer to appendix $1c_2$

3.7 Real-Time PCR [59]

Principle of the method

Temperature-sensitive Taq polymerase splits the double stranded DNA into two single strands and simultaneously adds complementary dideoexyribonucleic acids with respect to base-pairing. New strands of DNA are synthesized and the DNA is amplified. Amplified DNA is then detected as the reaction progresses in real-time, rather than in a standard PCR where the product of the reaction is detected at its end.

For step wise procedure refer to in appendix 1d.

4 RESULTS

4.1 Detection of mutations

Detection of mutations in the *rpoB* and *katG* genes using GenoType®MTBDRplus Assay (Refer to table 2 at appendix 2 for the whole results (Excel entered)

Readable GenoType®MTBDRplus assay results were obtained for 207 DNA extracts of *M.tuberculosis* strains. Of these 207 DNA Strips, 12.6 % **(26/207)** strips had no TB band (No TUB) indicating the presence of non-TB mycobacteria (When the MGIT, liquid culture, which was used as a gold standard, were unblended, one strain was confirmed to be belonging to the *M.avium* complex while the remaining were contaminated for culture. With 10.1% **(21/207)** repeated contaminated samples with no bands showing either unreadable bands in the rpoB, katG and / or inhA sections appeared, they were recorded as contaminated. Resistant strains accounted for 13.5% **(28/207)** and of these 28 samples, 50.0% **(14/28)** indicated to be MDR strains while monoresistance in the RIF and INH was 25.0% **(7/28).** The remaining strips 64.2% **(132/207)** were sensitive to either RIF or INH or both.

Table 1: GenoType®MTBDRplus Assay results for RIF and INH resistance

	RIF			INH			
Gene affected	Affected codons	No. of mutations	Affected strains %	Gene affected	Affected mutations	No. of codons	Affected strains %
RpoB	Mut 1 D516V	516	**42.85% (9/21)**	*katG*	Mut 1 S315T1	315	**61.9% (13/21)**

	Mut2A&B H526Y & H526D	526	**33% (7/21)**	*inhA*	Mut 1	-15	**19% (4/21)**
	Mut3 S531L	531	**38% (8/21)**		Mut2& Mut3A &Mut3B	-16 & 8	**14% (3/21)**

Mutations and codons affected with percentages. Mutations conferring resistance to RIF and INH were detected in 7 DNA samples extracted from sputum samples respectively, i.e. monoresistance. In fourteen DNA samples resistance to both INH and RIF were noted indicating MDR strains, **Figure 1, 2, 3.** In most of the RIF resistance isolates 42.85% (9/21) of our study, codon 516 was affected (including one strain were a mutation in codon 516 was combined with mutations in other codons). Mutations in other codons of rpoB gene were not less affected, however, 531with 38% (8/21) and 526 19.0% (4/21), combined alone are indicative of being the majority resistance isolates.

Mutations associated with INH resistance were more diverse: thirteen strains 61.9% (13/21) had mutations in katG (codon 315) gene only, 4 strains 19% (4/21) had mutations in inhA gene only, position (-15), accounting for majority) in the mabA-inhA promoter, position (-16) and position 8 and the remaining 4 strains had mutations in the katG and inhA genes, respectively. One strain had all the mutations of katG, inhA and rpoB genes except that for the rpoB gene, only mut1 (516) was missing. According to the manufacturer's recommendations (Hain LifeScience), these results may be indicative of either the presence of heterogeneous strain or mixed populations of mycobacteria in the initial sputum specimen and was interpreted as resistance to the relevant drugs **figure 2: lane 2**.

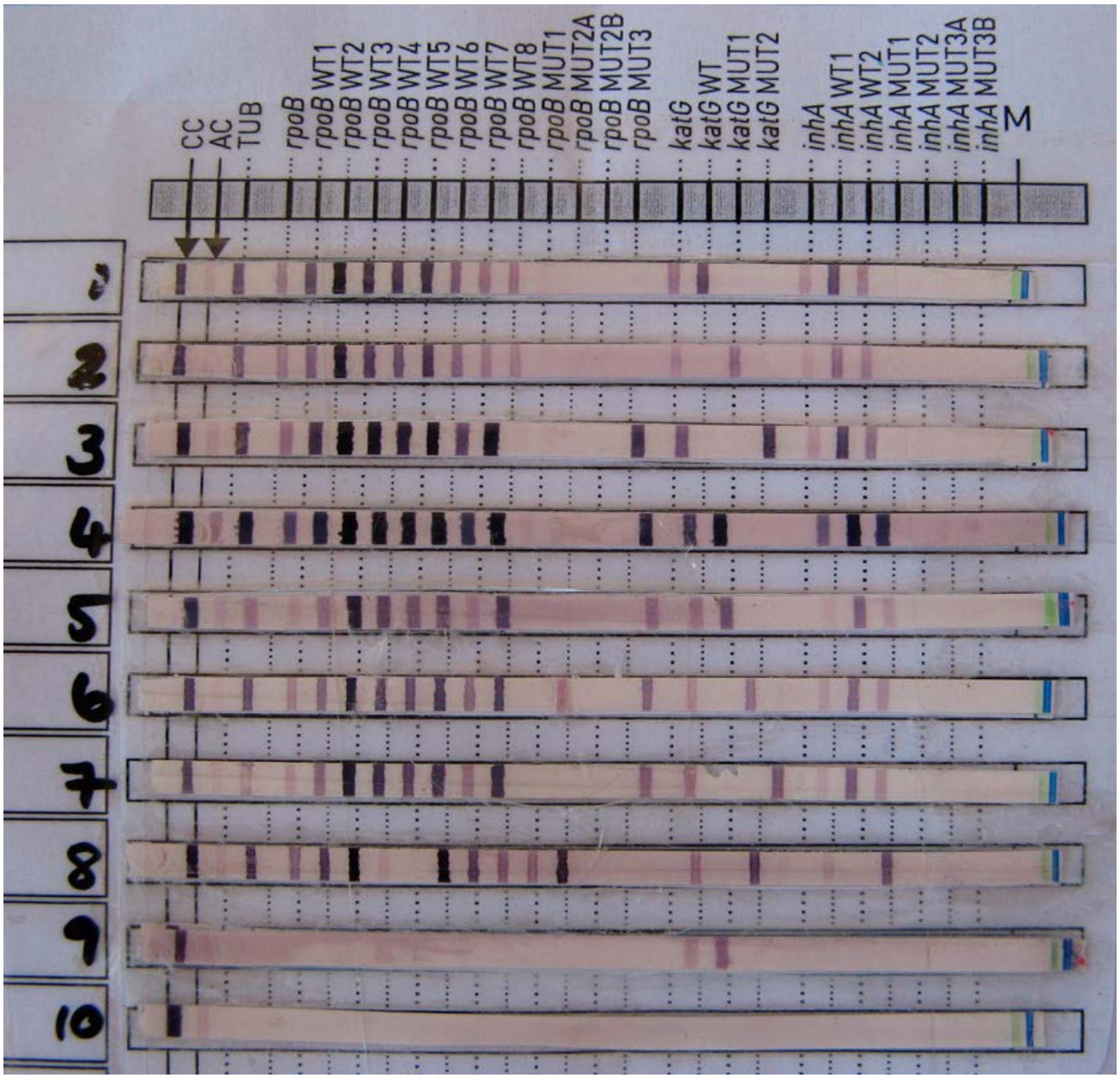

Figure 1: Mutation on the DNA Strips. Examples of GenoType®MTBDRplus strips (Hain LifeScience, Nehren, Germany). **(Lane 1)** *Mycobacterium tuberculosis*, susceptible to isoniazid (INH) and Rifampin (RIF). **(Lane 2)** *M. tuberculosis*, INH monoresistant (katG S315T1 mutation). **(Lane 3)** *Multidrug-resistant tuberculosis (MDR-TB)*, rpoB S531L mutation and katG S315T2 mutation. **(Lane 4)** MDR-TB rpoB S531L mutation and katG S315T1 and inhA C15T mutations. **(Lane 5)** *M. tuberculosis*, RIF monoresistant (mutation in rpoB 530–533 region). **(Lane 6)** MDR TB, rpoB D516V and katG S315T1 mutations. **(Lane 7)** MDR TB, rpoB S531L, and katG S315T2 mutations. **(Lane 8)** MDR TB, rpoB, D516V, katG S315T1 mutation and inhA mutation at 215/216. **(Lane 9)** Uninterpretable result, no *M. tuberculosis* complex (TUB) band. **(Lane 10)** Negative control.

One of the MDR strains identified had two codons of the *rpoB* gene mutated (516, 526) while the other one from the same group of MDRs had three codons of *rpoB* gene mutations (516,526, & 531). Some samples were recorded as non-*M.tuberculosis* resulting from unbound or lack of TUB binding e.g. lane 9 from figure 1.

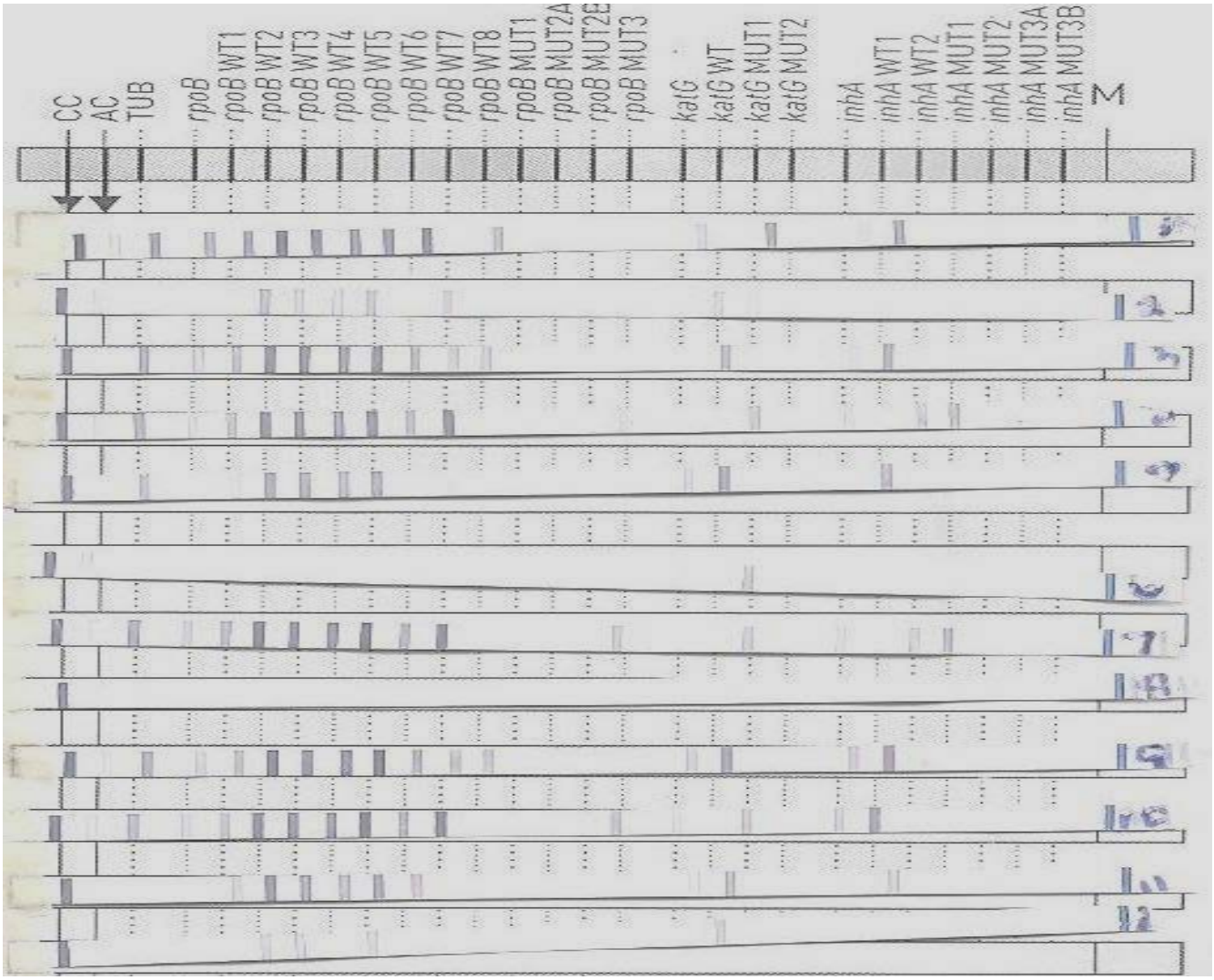

Figure 2: GenoType® MTBDRplus results for mutations with most conferring *MDR-TB* (Hain LifeScience, Nehren, Germany). The DNA strips are numbered from top to bottom with a blue paint as 1-12. Some bands are too light to be seen even after shaded. **(Lane 1)** *M. tuberculosis* is resistant to RIF and INH with mutations at mut2A (H526D) of rpoB gene and mut1 (S315H) of katG gene conferring MDR-TB. **(Lane 2)** *M. tuberculosis* is resistance to RIF and INH with mutations at mut2A (H526Y), 2B (H526D) & 3(S531L) of rpoB gene, mut1 (S315T1) & 2 (S315T2) of katG gene and mut1 (C15T), 2 (A16G), 3A (T8C), & 3B (T8A) of inhA gene conferring heterogeous sample. **(Lanes 3, 5, 7, 9, 11 and 12)** *M. tuberculosis* indicates susceptibility to RIF and INH. **(Lane 4)** *M. tuberculosis* resistance to RIF and INH with mutations at mut3 (S531L) of rpoB gene, mut1 (S315T1) of katG gene and mut1 (C15T), of inhA gene conferring MDR-TB. **(Lanes 6, 8)** No TUB band with mutation at mut1 (S315T1) of katG gene conferring non-*M .tuberculosis* strain are indicated as **Lane 9** of figure 1. **(Lane 10)** *M.*

tuberculosis resistance to RIF and INH with mutations at mut3 (S531L) of rpoB gene and mut1 (S315T1) of katG gene conferring *MDR-TB*.

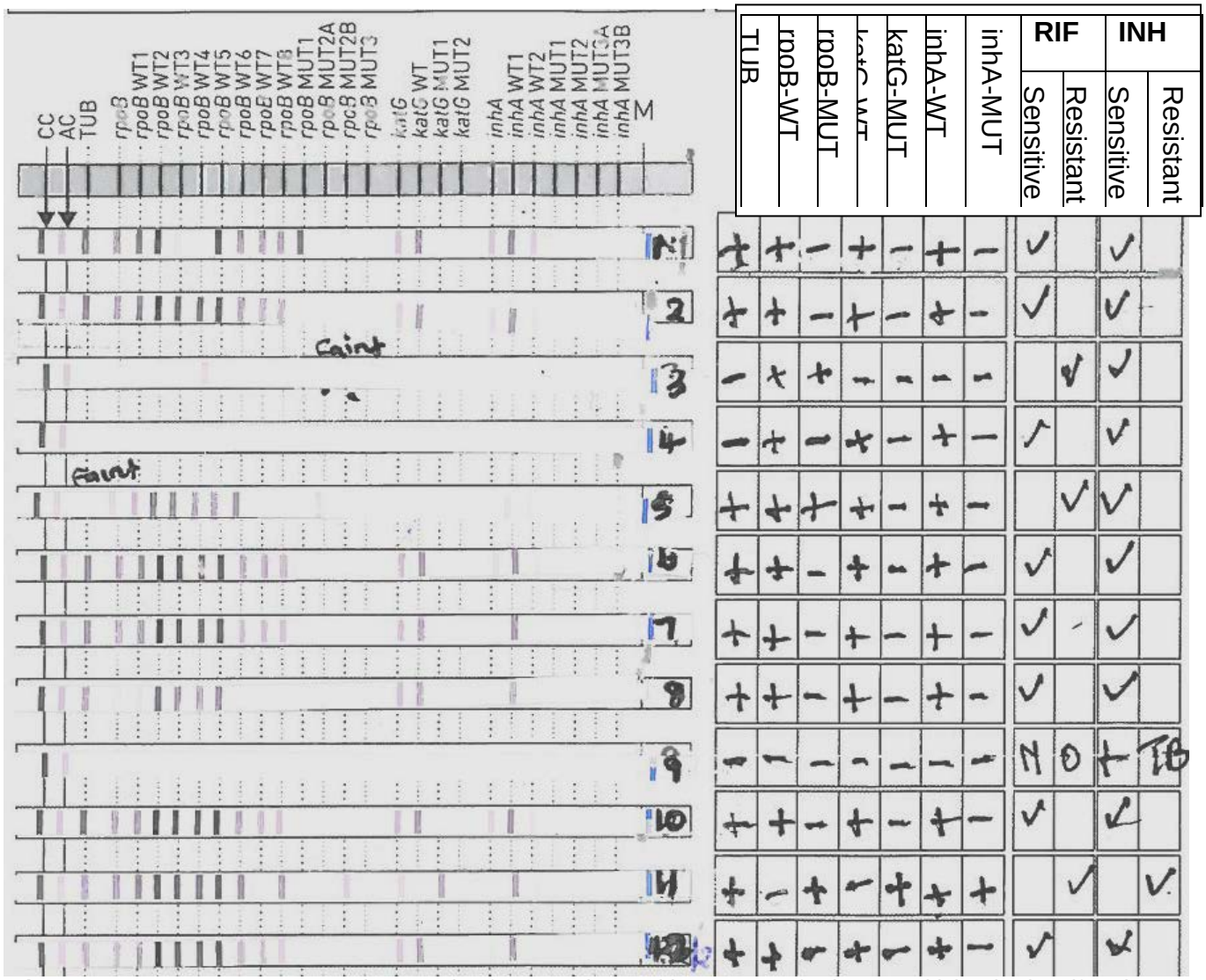

Figure 3: GenoType® MTBDRplus results for mutations with most conferring sensitivity (Hain LifeScience, Nehren, Germany). The DNA strips are numbered from top to bottom as 1-12. Overall this is showing the how the results of the strips are confirmed as multidrug resistance or monoresistance and non-TB strains of TB, i.e. example of monoresistance to RIF in **Lane 3 & 5,** example of *MDR-TB* strain in **Lane 11** and a non-TB strain in **Lane 9. (Lane 1, 2,4,6,7,8,10 and 12)** *M. tuberculosis* indicates to be susceptible to RIF and INH with lane one having a faint wild type 3&4. **(Lane 3)** No TUB band with mutation at mut2A (H526Y) &2B (H526D) of rpoB gene (non-*M. tuberculosis* strain). **(Lane 5)** *M. tuberculosis* resistance to RIF with mutations at mut2A (H526Y) & 2B (H526D) of rpoB gene and susceptible to INH conferring monoresistance. **(Lane 9)** No TUB band conferring non-M.TB. **(Lane 11)** *M. tuberculosis* resistance to RIF and INH with mutation at mut2B (H526D) of rpoB gene and mut1 (315) of katG gene conferring MDR-TB.

4.2 Detection of non-Beijing and Beijing strains using TaqMan probes from Roche

Interpretable results were obtained using the TaqMan probes by Real-Time PCR from Roche. With the selection of the drug resistant strains from the GenoType® MTBDRplus results, 28 DNA extracted specimens which indicated drug resistance either monoresistance or multidrug resistance were subjected to the real-time PCR test.

Mycobacterium $H_{37}R_v$ was used as a positive control for the non-Beijing strains. The unknown samples with an amplification curves as the *M.*$H_{37}R_v$ were an indication of non-Beijing strain: **figure 4**. For the *Beijing* strains, *M.*$H_{37}R_v$ was used as a negative control. Of 29 families, 15 were MDRs, 7 INH monoresistance and 7 RIF monoresistance. Of the 29 strains, 72.4% (21/29) indicated to be *non-Beijing* strains with 86.6% (13/15) of the MDRs and the remaining two MDRs 13.3% (2/15) were recorded as the Beijing strains. Of the 15 MDR strains, 6.6% (1/15) had a combination of the two strains (Beijing and Non-Beijing) indicating heteroresistance. Four of the INH monoresistance were *non-Beijing* strains while only three RIF monoresistance were *non-Beijing* strains.

The remaining 27.5% (8/29) had indicated to be the Beijin*g* strains: **Figure 4 (Examples: Sample no.6, 10, 12, 14 & 17**). This strain was dominated by monoresistance when excluding the one strain with the combination of the two strains tested in this study. Four of the RIF monoresistance were recorded as *Beijing* strains while only three of the INH monoresistance were *Beijing* strains. Of these 27.5% of the *Beijing* strains, the vast majority were monoresistance to INH and RIF with the dominance appearing in codon 315 of *KatG* and 516 of *rpoB,* respectively.

Results

Inc	Pos	Name	Type	CP	Conc	Std	Comb	Calls Target	Control
☑	2	H37Rv	Unknown	11.84			Positive	Positive	Success
☑	3	Sample 3	Unknown	32.33			Positive	Positive	
☑	4	Sample 4	Unknown	23.55			Positive	Positive	
☑	5	Sample 5	Unknown	26.93			Positive	Positive	
☑	6	Sample 6	Unknown				Negative	Negative	
☑	7	Sample 7	Unknown	39.50			Positive	Positive	
☑	8	Sample 8	Unknown	29.08			Positive	Positive	
☑	9	Sample 9	Unknown	30.98			Positive	Positive	
☑	10	Sample 10	Unknown				Negative	Negative	
☑	11	Sample 11	Unknown	31.47			Positive	Positive	
☑	12	Sample 12	Unknown				Negative	Negative	
☑	13	Sample 13	Unknown	26.03			Positive	Positive	
☑	14	Sample 14	Unknown				Negative	Negative	
☑	15	Sample 15	Unknown	39.16			Positive	Positive	
☑	16	Sample 16	Unknown	21.97			Positive	Positive	
☑	17	Sample 17	Unknown				Negative	Negative	

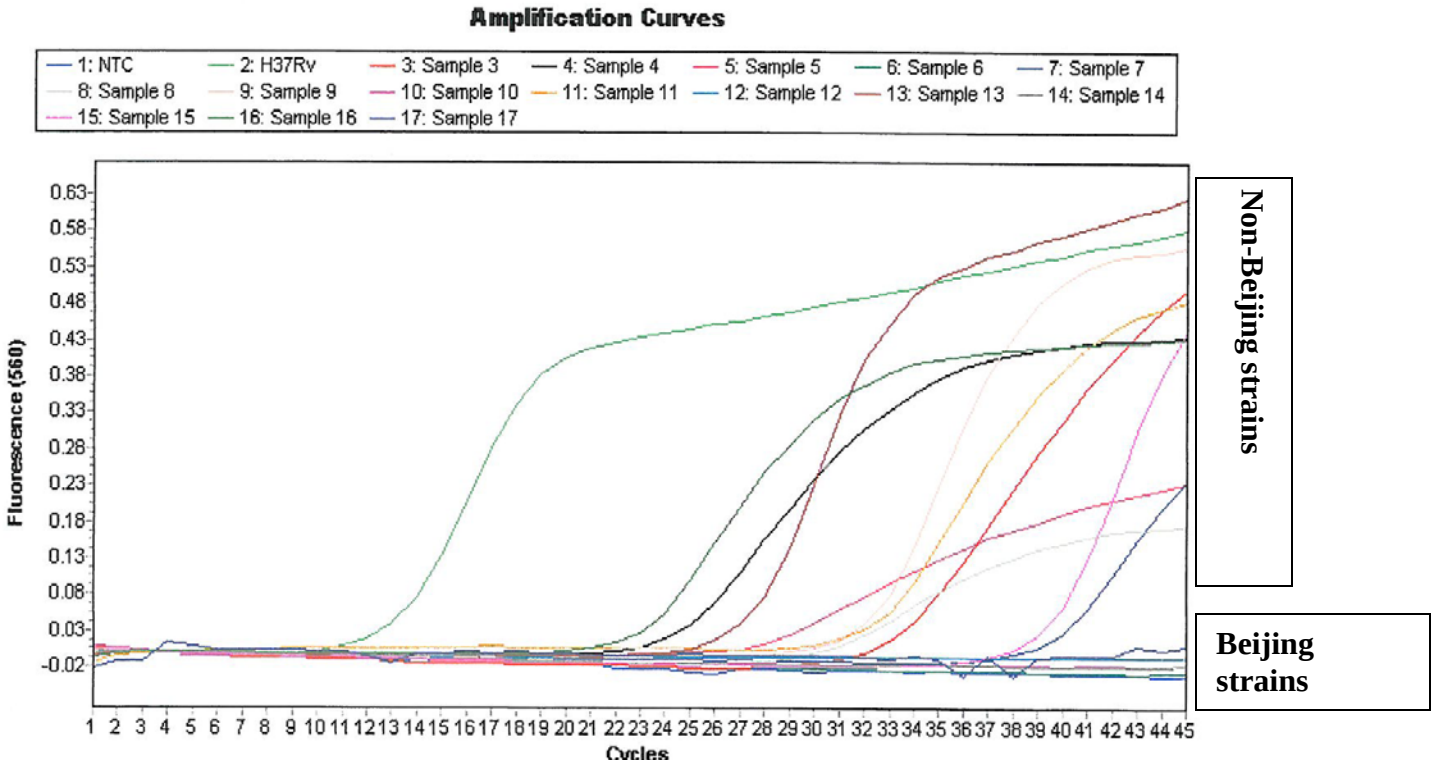

Figure 4: Example of the *Beijing* and *non-Beijing* strains amplification curves (Real-Time PCR Roche /LightCycler® Software Version 4.1). Amplification curves representing the samples which were positive for *non-Beijing* strains and *Beijin*g strains using $H_{37}R_V$ strain as the positive control for the *non-Beijing* strains and negative control for the Beijing strains of *M.tuberculosis* appears in the graphs above. **Pos**; position, **CP**; crossing point or threshold point, **comb**; comparison. Absolute quantification was used in the analysis which gives the exact number of target DNA molecules by comparison with DNA standards. The table shows a count of 17 samples whereby number one is not shown, which was the negative control containing water and is indicated on the graphs as NTC. All the positive samples show its different crossing points or threshold points in different cycle numbers with the negatives showing an empty space.

5 DISCUSSION

The currently study was focused on the determination of Beijing and non-Beijing families of *Mycobacterium tuberculosis* drug strains on sputum samples using the Light Cycler (real-time PCR) collected from NHLS laboratories in Polokwane, Limpopo Province. Moreover, the drug resistance patterns and mutation in the bacterial strains were also identified employing the GenoType®MTBDRplus assay.

A significant proportion of our samples strains 13.5% (28/207) were drug-resistant with Beijing 26% (8/28) and Non-Beijing 75% (21/28). This finding indicates that the Non-Beijing TB family outstrips the Beijing type in our study. A study conducted by researchers in the MRC Pretoria from isolates collected in 2001-2002 in all provinces in South Africa found that out of the 33 clinical isolates collected from Limpopo Province, the majority were of the non-Beijing (X) family **[86].** The current study confirms their findings of the circulating *M. tuberculosis* families in the Limpopo Province. But the candidate's laboratory activities in this case were conducted directly from sputum samples (207) collected from 2009 to 2010 using the Light Cycler (RT-PCR). Pillay *et. al*. **[11]** investigated 41 clinical isolates in Kwa-Zulu Natal Province and found majority of the strains to belong to the non-Beijing family called "F15/LAM4/KZN". And research work conducted by a group in Cape Town found that the Beijing family of tuberculosis clinical isolates dominate and are associated with multidrug resistance **[48, 58].**

Sputum samples collected in the present study exhibited comparable results for isoniazid and rifampicin monoresistance in both Beijing and non-Beijing strains with varying distribution of codons for both mutations. Higher numbers of MDR-resistance was noted for non-Beijing than Beijing strains with varying mutations at the codons. This result assumes that the MDR patterns in both bacterial families, especially non-Beijing may be developing from circulating monoresistant strains. Moreover, increased expression of inhA codon 15 maybe related to non-Beijing multidrug resistance because literature reports that low level expression of inhA is not associated with MDR **[53].** This is not the case in the present study.

The current study differs from observations made by researchers at George Mukhari Hospital relating to differential expression of mutations for isoniazid in which a new codon 314 was reported **[49].** Another study conducted in Cape Town indicates differences in mutation analysis on both the RIF and INH genes as compared to the present study **[58].** This might be due to an observation that the Beijing strains are dominating in Cape Town whereas Limpopo Province is dominating with non-Beijing strains.

The current observations and assumptions are based on comparisons with studies carried out on cultured clinical isolates collected between 2001 and 2002 **[20, 52, 58, 85]** using techniques that are not in current use like RT-PCR and GenoType®MTBDRplus assays. The current study was able to show that using DNA material extracted directly from sputum samples, the candidate characterized the mycobacterial families as well as their drug resistance mutations. Further laboratory work on the extracted nucleic acid material will be used to determine the prevailing non-Beijing clades. The diversity of mutations and genotypes of *M. tuberculosis* strains in the provincial regions are underestimated in South Africa. Because non-Beijing genotype and mutations at codon 516 of rpoB gene showed dominance, further studies are needed to can estimate their epidemiology and clinical impact. The clinical impact of the *non-Beijing* strain will help in the trace of the transmissibility of the strain and its virulent factors as compared to *Beijing* strains. For future studies exploring the biological or clinical implications of the correlation between the genotype and the specific mutation will needed for better understanding of the molecular epidemiology of MDR-TB.

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Appendix -1

Standard operating procedures for methods used

Appendix 1a

Title: Decontamination of sputum samples by Kubica, G. P et al. 1963

Principle

Sodium Hydroxide digest sputum debris, normal flora, and other infectious organisms and the remainder will be highly resistant organism such as Mycobacterium. Mycobacteria have high quantity of mycolic acids which resist entry of Sodium Hydroxide. The remaining cells are washed by saline (distilled water).

On arrival at the University of Limpopo, Turfloop Campus Medical Microbiology laboratory, the sputum specimens were decontaminated using modified Petroff method.

Equipment required:

- Vortex
- Normal Water bath
- Bench top centrifuge

Reagents required:

- N-acetyl L-cysteine (NaLc)
- 2.9% sodium citrate
- 4% NaOH
- Distilled water
- Phosphate buffer (pH6.8)

Procedure:

- Working solution was prepared by dissolving 2g of N-acetyl L-cysteine (NaLc) in 200ml of distilled water, 2.9% sodium citrate and mixed with an equal amount of 4% NaOH.
- A 2X volume of the solution was added to the specimen and mixed by vortexing.
- The mixture was left at room temperature for 15minutes.
- Therefore 5ml of the mixture was transferred into a 15ml centrifuge tube and 10 ml of phosphate buffer (pH6.8) was added to the 15ml tube and centrifuged at 3000xg for 15 minutes.
- The supernatant was discarded into a disinfectant container and +/- 3ml left in the tube for DNA extraction.

Appendix 1b

Title: DNA Extraction as recommended by Eisenach D. et al. 1991.

Principle

Here, the cells are lysed partially allowing plasmids to escape, whereas the bacterial chromosomal DNA remains trapped in the cell debris. High temperature is then used to denature the chromosomal DNA, after which reannealing allows the plasmids to reassociate. Centrifugation removes the chromosomal DNA along with the cell debris, leaving the plasmid in suspension.

Equipment required:

- Vortex
- Normal Water bath

- Bench top centrifuge
- Ultrasonic water bath

Reagents required:

- Distilled water
- Decontaminated sputum samples

Procedure:

- Five hundred microliters of decontaminated samples was pipette into the eppendorf tube.
- Eppendorf tubes with the samples were centrifuged for 15minutes at 10 000 Xg in the desk centrifuge.
- The supernatant was discarded and the pellet was suspended at 100µl of distilled water.
- The tubes were incubated for 20 minutes in 95˚c water bath.
- The tubes were removed and incubated for 15 minutes at an ultrasonic bath.
- The tubes were spanned down at full speed (16 000rpm) for 5 minutes in the desk centrifuge.
- The tubes were removed and the supernatant was transferred in a new tube as DNA.
- Five microliters of the DNA was used for PCR amplification.
- The remaining was stored in -20^{o}C for further processing.

Appendix 1c$_1$

Title: Polymerase Chain Reaction: Telenti A. et al.1997

Principle

Temperature-sensitive Taq polymerase splits the double stranded DNA into two single strands and simultaneously adds complementary dideoexyribonucleic acids with respect to base-pairing. New strands of DNA are synthesized and the DNA is amplified.

Equipment required:

- **Thermocycler**

Reagents required:

- TaqPolymerase **(**HotStar Taq® DNA polymerase) from Qiagen
- Primer Nucleotide Mix (PNMs)
- Distilled / Molecular Biology Graded water
- 10X Polymerase Incubation buffer (containing 15mM $MgCl_2$)
- 25mM $MgCl_2$

Procedure:

Working solutions per tube:

- Thirty five microliters of primer or Nucleotide mix was added into the PCR tube.
- 5μl of 10X polymerase incubation buffer was added to the same PCR tube.
- 2μl of $MgCl^2$ solution was added.
- Zero coma two microliters of thermostable DNA polymerase was added.
- 3μl of water was added to obtain a volume of 45μl.
- 5μl of DNA solution was added leading to final volume of 50μl.
- Polymerase chain reaction tubes with the above solution were incubated in thermocycler for 2hours.

Thermocycler programme: Clinical specimens

- 15 min 95^{o}C 1 cycle

- 30 sec95 ^{o}C 10cycles
  2 min58 ^{o}C

- 25 sec95 ^{o}C
  40 sec53 ^{o}C 30cycles
  40 sec70 ^{o}C

- 8 min70 ^{o}C 1cycle

Amplified products were then subjected to GenoType®MTBDRplus assay

Appendix 1c$_2$

Title: GenoType®MTBDRplus assay: Evans J. et al.2009

Principle

The GenoType®MTBDRplus assay is based on reverse hybridization between amplicons derived from a multiplex PCR and nitrocellulose-bound probes covering overlapping wild-type (WT) sequences of the hot-spot regions (rpoB WT 1-rpoB WT 5, katG WT), the four most frequent mutations for rpoB (rpoB MUT probes), and mutations at codon 315 in katG (katG MUT probes). The presence of a mutation is indicated by the lack of hybridization on one or more of WT probes with or without hybridization on the MUT probes.

Equipment required:

- TwinCubator (Automated water bath from Hain LifeScience)
- Water bath (45^{o}C)

Reagents required:

- Hybridization buffer
- Stringent wash solution
- Rinse Solution
- Distilled water
- Substrate and Conjugate solutions

Working solutions preparation:

- Dilute CON-C (orange) 1:100 with the respective buffer supplied (CON-D)
- Dilute SUB-C (brown) 1:100 with the respective buffer supplied (SUB-D)

Procedure:

- Twenty microliters of the denaturation solution was dispensed at the corner of each well used.
- Twenty microliters of the amplified samples was added to the solution and mixed properly until homogenous mixture was reached and incubated for 5 minutes at room temperature.

-Meanwhile, the strips were taken out of the tube using tweezers and marked with a pencil underneath the colored marker.

- One milliliter of the prewarmed Hybridization buffer was added in each well (Was poured at the top end of the well to avoid cross contamination).

-The tray was gently shaken until the solution reached homogenous state
(Take care not to spill the solution into the neighboring wells.

- The strips were marked and laid into the wells after the 5minutes elapsed.
- The tray (wells with samples) was incubated for 30 minutes in a TwinCubator® bath at 45^{o}C.
- After 30 minutes elapsed, the tray was taken out and solutions aspirated using a new tip for each well.
- One milliliter of stringent wash solution was added to each well with strip and incubated for 15 minutes in TwinCubator® at 45^{o}C.
- After 15 minutes elapsed, the wash solution was poured out.

- One milliliter of Rinse was added and incubated for 1minute in the TwinCubator® 25^{o}C (room temperature).
- Rinse was poured out after 1 minute.
- Preparation of Conjugate and Substrate as referred to the working solutions: 1:100

-Twelve milliliters of ConD was poured in a 15ml centrifuge tube and 120µl of ConC was added. (Due to performing the samples in a group of twelve/twelve)

-Twelve milliliters of SubD was poured in a 15ml centrifuge tube and 120µl of SubC was added and placed in the dark to avoid direct light.

- After 1 minute elapse, the conjugate was added and incubated for 30 minutes in the TwinCubator® at 25^{o}C (room temperature).
- One milliliter of Rinse was added and incubated for 1minute in the TwinCubator® 25^{o}C (room temperature).
- Rinse was poured out after 1 minute.

-Repeat the last two Steps.

- One milliliter of distilled water was added and incubated at 25^{o}C (room temperature) for 1 minute.
- Distilled water was poured out after 1 minute elapse.
- One milliliter of Substrate was added and incubated in the dark for 5 minutes.
- The Substrate was poured out after 5 minutes elapse.
- The strips were washed twice with distilled water.
- The strips were taken out of the wells of the tray and observed.
- Samples which indicated MDR-TB were further genotyped for families with Real-Time PCR.

Appendix 1d

Title: Real-Time-Polymerase Chain Reaction: Hillemann D. et al.2006

Principle

Temperature-sensitive Taq polymerase splits the double stranded DNA into two single strands and simultaneously adds complementary dideoexyribonucleic acids with respect to base-pairing. New strands of DNA are synthesized and the DNA is amplified. Real-Time Polymerase Chain Reaction (R-T PCR) is based on the PCR which is used to amplify and simultaneously quantifying targeted DNA molecule. It enables both detection and quantification (as absolute numbers of copies or relative amount when normalized to DNA input or addition normalizing genes) of one or more specific sequences in a DNA sample. It has the same principle as standard PCR and its key features is that the amplified DNA is detected as the reaction progresses in real-time, rather than in a standard PCR where the product of the reaction is detected at its end.

Equipment required:

- **Light Cycler 2.0**

Reagents required:

- LightCycler®Fast Start Enzyme (**Red Cap,1b)**
- LightCycler® Fast Start Reaction Mix Hydrolysis probe (**Colorless Cap,1a**)

nBjF (5'-AAG CAT TCC CTT GAC AGT CGA A-3')
nBjR (5'-GGC GCA TGA CTC GAA AGA AG-3')
nonBjTMLNA (5'-6FAM-TCCAA+ggT+C+T+T+Tg--BBQ-3')
nonBjTM+ (5'-6FAM-TCA TCA Aag ACC CTC TTg gAA ggC C--BBQ-3')
BjF (5'-CTC GGC AGC TTC CTC GAT-3')

BjR (5'-CGA ACT CGA GGC TGC CTA CTA C-3')
BjTM (5'-HEX-AAC GCC AGA GAC CAG CCG CCG GCT-DABCYL-3')

- PCR graded water provided in the kit (**Colorless Cap,2**)
- Probes and primers:

- **Light cycler program:**

Analysis mode	Cycle Segment		Target Temperature(1)	Hold time	Acquisition mode
Pre-Incubation					
None	1		95°C	10min	None
Amplification					
Quantification	45	Denaturation	95 °C	10s	None
		Annealing	60 °C	30s	None
		Extension	72 °C	1s	Single
Cooling					
None	1		40 °C	30s	None

Working solutions preparations:

- **Enzyme**

-Vial 1a (White cap): Light Cycler Fast Start reaction mix hydrolysis probe

-Vial 1b (Red cap): Light Cycler fast Start enzyme

Add 10µl of Vial 1a into Vial 1b. (Is a 10x concentration)

- **Reconstitution of primers for stock solutions**

-Dissolve 15.6nmol with 156µl of PCR graded water for the Forward primer of the non-Beijing genotype.

-Dissolve 17.3nmol with 173µl of PCR graded water for the Reverse primer of the non-Beijing genotype.

-Dissolve 19.2nmol with 192µl of PCR graded water for the Forward primer of the Beijing genotype.

-Dissolve 17.6nmol with 176µl of PCR graded water for the Reverse primer of the Beijing genotype.

- **Reconstitution of probes for stock solutions**

-For the Beijing probe: 197µl PCR graded H_2O was added to the 3.9nmol of a probe to make a 20nmol concentration

-**For the two non-Beijing probes:**

- nBjTM$^+$: 189µl PCR graded H_2O was added to the 3.8nmol of a probe to make a 20nmol concentration
- nBjTMLNA: 386µl PCR graded H_2O was added to the 7.7nmol of a probe to make a 20nmol concentration

Note: Making a 10X Concentration for the primers and probes.

1:20 [1µl of the primers or probes into 19µl PCR graded water] =10X concentration of each primer and probe.

Procedure:

Components		**Volume (one sample) Reaction**
PCR Graded H_2O	9µl	(1)
Primers/probes	2µl	(1)
Master Mix (5X concentration)	4µl	(1)

1. Add 9µl of H_2O PCR graded in an eppendorf tube

Appendix-2: Table1:Comparison results of the study for GenoType®MTBDRplus assay and MGIT			

2. Add 1µl of primers and 1µl probes to make the 2µl needed for one reaction

3. Add 4µl of master mix at a 5X concentration, then mix by vortexing for 1second and centrifuge for 3seconds using a bench top centrifuge.

4. Add 15µl of the mixture above to each capillary tube used and then add 5µl of extracted DNA. **NOTE: DNA addition is done in a dedicated room to avoid cross contamination.**

5. Centrifuge at 800xg for 10seconds, to let the contents to settle down in the capillary tubes.

6. Put the capillary tube in the capillary holder according to your numbering and then let the PCR run ~45-50minutes.

Analysis mode

The Roche LightCycler® Software version 4.1 is used.

The **530 channel** is used at the Absolute Quantification analysis mode and the internal control at the **560 channel**.

NPI no.	GenoType®MTBDRplus	GenoType®MTBDRplus		MGIT	Mutations	Codons Affected
	RIF	INH	RIF	INH		
NPI 2975533	no TUB	no TUB	C	C		
NPI 2952739	S	S	S	S		
NPI 2976459	S	S	S	S		
NPI 2979537	S	S	S	S		
NPI 2955513	S	S	S	S		
NPI 2945148	S	S	S	S		
NPI 2979490	no TUB	no TUB	C	C		
NPI 2988665	S	S	S	S		
NPI 2957526	S	S	S	S		
NPI 2979570	S	S	S	S		
NPI 2976009	S	S	S	S		
NPI 2988361	no TUB	no TUB	C	C		
NPI 2957385	R	R	R	R	***KatG*/mut1*:rpoB*/mut1**	**315: 516**
NPI 2947247	S	S	S	S		

	R	R	R	R	***KatG*/mut1:*rpoB*/mut1**	**315:516**
NPI 2945191						
	R	R	S	S	***KatG*/mut1:*rpoB*/mut3**	**315:531**
NPI 2975542						
NPI 2954468	S	S	S	S		
NPI 2957732	S	S	LVR	LVR		
NPI 2946098	S	S	S	S		
NPI 2948295	S	S	S	S		
NPI 3024298	no TUB	no TUB	not TB	not TB		
NPI 3008373	S	S	S	S		
NPI 2998534	no TUB	no TUB	not TB	notTB		
NPI 2998469	C	C	not TB	not TB		
NPI 3004929	S	R	C	C	***inhA*/mut1**	**-15**
	R	R	R	S	***KatG*/mut1:rpoB/mut2A**	**315:526**
NPI 3004344						
	R	R	R	S	***KatG*/mut1:*rpoB*/mut1**	**315:516**
NPI 2997772						
	R	R	R	S	***KatG*/mut1:*rpoB*/mut3**	**315:531**
NPI 3004592						
NPI 2998476	S	S	C	C		
NPI 3025684	no TUB	no TUB	not TB	notTB		
NPI 3024207	S	S	S	S		

NPI 3022377	no TUB	no TUB	not TB	notTB	
NPI 3020822	C	C	S	S	
NPI 3029305	C	C	S	S	
NPI 3029253	no TUB	no TUB	not TB	notTB	
NPI 3029421	no TUB	no TUB	not TB	notTB	
NPI 3022409	S	S	S	S	
NPI 3022001	no TUB	no TUB	not TB	notTB	
NPI 3021180	S	R	S	S	***inhA*/mut1 -15**
NPI 3020995	C	C	C	C	
NPI 3022351	S	S	S	S	
NPI 3029263	S	S	S	S	
NPI 3023778	C	C	C	C	
NPI 3029254	C	C	C	C	
	S	S	S	S	
NPI 3026990					
NPI 3021181	no TUB	no TUB	not TB	notTB	
NPI 3025869	S	S	S	S	
NPI 3022522	C	C	C	C	
NPI 3023872	no TUB	no TUB	not TB	notTB	
NPI 3000633	no TUB	no TUB	not TB	notTB	

NPI 3036495	S	S	S	S		
NPI 3033598	S	S	S	S		
NPI 3036528	S	R	S	S	***inhA*/mut1**	**-15**
NPI 3036452	C	C	C	C		
NPI 3036514	C	C	C	C		
NPI 3037712	C	C	C	C		
NPI 3036596	S	S	S	S		
NPI 3037488	C	C	C	C		
NPI 3035767	R	S	R	S	***rpoB*/mut2A**	**526**
NPI 3036716	R	R	S	S	***katG*mut1**	**315**
NPI 3035542	R	S	S	S	***rpoB*/mut1**	**516**
NPI 3036590	C	C	C	C		
NPI 3036479	S	S	S	S		
NPI 3030611	S	S	S	S		
NPI 3033597	S	S	S	S		
NPI 2710711	R	S	R	S	***rpoB*/mut2A, B& mut3**	**526: 531**
NPI 2632994	C	C	C	C		
NPI 2634213	R	R	S	S	***katG*/mut1:rpoB/mut3**	**315:531**
NPI 2710911	S	R	C	C	***katG*mut1**	**315**

NPI 2668433	S	S	C	C		
NPI 2710984	R	S	C	C	***rpoB*/mut1**	**516**
NPI 2710885	S	S	S	S		
NPI 2709342	S	S	S	S		
NPI 2710912	no TUB	NO TUB	not TB			
NPI 2663895	S	S	S	S		
NPI 2667664	S	S	S	S		
NPI 2711109	C	C	C	C		
NPI 2665546	no TUB	NO TUB	not TB	not TB		
NPI 2668520	S	S	S	S		
NPI 2665785	S	S	S	S		
NPI 2662731	C	C	C	C		
NPI 2667625	no TUB	NO TUB	not TB	notTB		
NPI 2663895	S	S	S	S		
NPI 2662362	C	C	C	C		
NPI 2709527	C	C	C	C		
NPI 2665543	C	C	C	C		
NPI 2667623	S	R	C	C	***inhA*/mut1**	**-15**
NPI 2638853	C	C	C	C		
NPI 2640764	R	S	S	S	***rpoB*/mut1**	**516**

NPI 2640851	C	C	C	C		
NPI 2637804	S	S	S	S		
NPI 2638869	S	S	S	S		
NPI 2639324	R	R	R	R	***katG*/mut1:rpoB/mut2A**	**315:526**
NPI 2640255	no TUB	no TUB	not TB	notTB		
NPI 2640797	R	R	R	R	***katG*/mut1:rpoB/mut1&2A**	**315:516,526**
NPI 2969768	R	R	R	R	***KatG*/mut1: *rpoB*/mut2A**	**315:526**
NPI 2970932	R	R	R	R	***katG*/mut1&2, inhA/mut1,2&3A3B: rpoB/mut2A, mut2B&mut3**	**315:-15,16,-8:526,531**
NPI 2969823	S	S	S	S		
NPI 2970931	R	R	R	R	***katG*/mut1,inhA/mut1:rpoB/mut3**	**315:-15:531**
NPI 2969923	S	S	S	S		
NPI 2969943	no TUB	NO TUB	C	C	***katG*mut1**	**315**
NPI 2969817	R	R	R	R	***katG*/mut1,inhA/mut1:rpoB/mut3**	**315:-15:531**

NPI 2971049	no TUB	NO TUB		C	C	
NPI 2969835	S	S		S	S	
NPI 2969967	R	R		R	R	***katG*/mut1:rpoBmut3** **315:531**
NPI 2969936	S	S		S	S	
NPI 2969921	S	S		S	S	
NPI 3055752	S	S		C	C	
NPI 3056795	S	S		S	S	
NPI 3047012	S	S		S	S	
NPI 3015439	S	S		S	S	
NPI 3061390	S	*S*	Neg		Neg	
NPI 3063195	S	S		S	S	
NPI 3045924	S	S		S	S	
NPI 3062137	no TUB	no TUB	not TB		not TB	
NPI 3051286	S	S		S	S	
NPI 3063319	S	S		S	S	
NPI 3058315	S	S		S	S	
NPI 3059350	S	S		S	S	
NPI 3052630	S	S		S	S	
NPI 3058450	S	S		S	S	

NPI 3063293	S	S	S	S		
NPI 3055342	no TUB	no TUB	not TB	not TB		
NPI 3064582	S	S	S	S		
NPI 3060725	S	S	S	S		
NPI 3059381	S	S	S	S		
NPI 3056793	S	S	S	S		
NPI 3063297	S	S	S	S		
NPI 3058243	S	S	S	S		
NPI 3046037	S	S	S	S		
NPI3056789	S	S	S	S		
NPI 3052554	no TUB	no TUB	Neg	Neg		
NPI 3059953	R	S	C	C	***rpoB*/mut1**	**516**
NPI 3046270	S	S	S	S		
NPI 3049879	no TUB	no TUB	Neg	Neg		
NPI 3063872	R	S	C	C	***rpoB*/mut1**	**516**
NPI 3054161	S	S	S	S		
NPI 3064595	S	S	S	S		
NPI 3048722	S	S	not done	not done		
NPI 3056790	no TUB	no TUB	*M.avium* complex	*M.avium* complex		

NPI 3055757	S	S	S	S
NPI 2966727	S	S	S	S
NPI 2969966	S	S	S	S
NPI 2969816	S	S	S	S
NPI 2966761	S	S	S	S
NPI 2971167	S	S	S	S
NPI 2967775	S	S	S	S
NPI 2968885	S	S	S	S
NPI 2969841	no TUB	no TUB	negative	Negative
NPI 2967810	no TUB	no TUB	negative	Negative
NPI 2971064	S	S	S	S
NPI 2969838	S	S	S	S
NPI 2970682	C	C	C	C
NPI 2970938	S	S	S	S
NPI 2970443	S	S	LVR	LVR
NPI 2968882	S	S	S	S
NPI 2989635	S	S	S	S
NPI 2990880	S	S	S	S
NPI 3013768	S	S	S	S
NPI 3014608	S	S	S	S

NPI 3015134	S	S	S	S
NPI 3018109	S	S	S	S
NPI 3018155	S	S	S	S
NPI 3016880	S	S	S	S
NPI 3018736	S	S	S	S
NPI 3045924	C	C	S	S
NPI 3047012	S	S	S	S
NPI 3046037	S	S	S	S
NPI 3044571	S	S	S	S
NPI 3055752	S	S	S	S
NPI 3055342	S	S	C	C
NPI 3051286	S	S	S	S
NPI 3051439	S	S	S	S
NPI 3056795	S	S	S	S
NPI 3052630	S	S	S	S
NPI 3058450	S	S	S	S
NPI 3058315	S	S	S	S
NPI 3059350	S	S	S	S
NPI 3063319	S	S	S	S
NPI 3061590	S	S	S	S

NPI 3063193	S	S	S	S
NPI 3062157	S	S	S	S
NPI 3063293	S	S	S	S
NPI29587507	S	S	S	S
NPI2958902	S	S	S	S
NPI2958871	S	S	S	S
NPI2988365	S	S	S	S
NPI2990770	S	S	S	S
NPI2982455	S	S	S	S
NPI2982452	S	S	S	S
NPI2984787	S	S	S	S
NPI2983456	S	S	S	S
NPI2982450	S	S	S	S
NPI2981677	S	S	S	S
NPI2986137	S	S	S	S
NPI2991116	S	S	S	S
NPI2992074	S	S	S	S
NPI2992062	S	S	S	S
NPI2995086	S	S	S	S
NPI2995120	S	S	S	S

NPI2997281	S	S	S	S
NPI2996419	S	S	S	S
NPI2945217	S	S	S	S
NPI2946031	S	S	S	S
NPI2946186	S	S	S	S
NPI2946181	S	S	S	S
NPI2947101	S	S	S	S

Table1: Combined results of the study using MTBDRplus Assay compared to MGIT results. This table gives all the samples tested (n=207). NPI; Identity number of National Health Laboratory Services in Polokwane, S; Susceptible, R; resistant, C; Contamination, No TUB; Absence of TB band, not TB; Absence of TB, Negative; Negative for culture, LVR; Lost Viability recorded, *M.avium* complex; *Mycobacterium avium* complex was detected. Each resistant sample has mutations affected and codons affected for the drugs tested.

NPI no.	Real-Time PCR
NPI 2945385	**Non-Beijing**
NPI 2957191	**Non-Beijing**
NPI2975542	**Beijing**
NPI 3004929	**Non-Beijing**
NPI 3004344	**Non-Beijing**
NPI 2997772	**Non-Beijing**
NPI 3004592	**Non-Beijing**
NPI 3021180	**Non-Beijing**
NPI 3036528	**Non-Beijing**
NPI 3035767	**Non-Beijing**
NPI 3036716	**Non-Beijing**
NPI 3035542	**Non-Beijing**
NPI 2710711	**Non-Beijing**
NPI 2634213	**Non-Beijing**
NPI 2710911	**Beijing**
NPI 2710984	**Beijing**
NPI 2667623	**Non-Beijing**
NPI 2640764	**Beijing**

NPI 2639324	**Non-Beijing**
NPI 2640797	**Non-Beijing**
NPI 2969768	**Non-Beijing**
NPI 2970932	**Non-Beijing and Beijing**
NPI 2970931	**Beijing**
NPI 2969943	**Beijing**
NPI 2969817	**Non-Beijing**
NPI 2969967	**Non-Beijing**
NPI 3059953	**Beijing**
NPI 3063872	**Non-Beijing**
NPI 3004592	**Non-Beijing**

Table 2: All the 28 drug resistance samples found when subjected to GenoType®MTBDRplus assay were subjected to real-time PCR for the genotypes classified as Beijing and non-Beijing strains. 21 strains highlighted in red are non-Beijing strains and 8 highlighted in pink are Beijing strains.