THE DETECTION OF DRUG RESISTANT MUTATIONS IN *MYCOBACTERIUM TUBERCULOSIS* STRAINS USING ANYPLEX MTB/NTM/MDR-TB PLUS ASSAY IN LIMPOPO PROVINCE

By

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DEDICATION

THIS WORK IS DEDICATED TO MY SUPPORTIVE FAMILY WITHOUT YOUR GUIDANCE AND PERSISTENT HELP THE COMPLETION OF THIS DISSERTATION WOULD NOT HAVE BEEN POSSIBLE.

DECLARATION

I declare that **THE DETECTION OF DRUG RESISTANT MUTATIONS IN *MYCOBACTERIUM TUBERCULOSIS* STRAINS USING ANYPLEX MTB/NTM/MDR-TB PLUS ASSAY IN LIMPOPO PROVINCE** hereby submitted to the University of Limpopo, for the degree of Master of Science in Medical Sciences 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.

Mpanyane D.M **18/09/2015**

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Presentations

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Abstract

Introduction: Multidrug-resistant tuberculosis (MDR-TB) caused by resistance to at least rifampicin (RIF) and isoniazid (INH) drugs is a growing public health concern in South Africa. The detection of MDR-TB still relies on culture despite advancement in molecular diagnostic technology. Currently MTBDR*plus* and GeneXpert are the only available assays used in rapid diagnosis of MDR-TB using chromosomal mutations in drug target regions. Some strains are missed by these assays due to their limitation in mutational detection profile. Novel Seegene Anyplex assays simultaneously detect TB and resistance to RIF and INH using fifteen and six mutational probes, respectively within 3 hours. Limpopo Province has limited information on the circulating strains of TB.

Aim: To determine drug-resistant *Mycobacterium tuberculosis (M. tuberculosis)* mutations using Anyplex™ MTB/NTM/MDR-TB real time assay and characterise the drug-resistant strains.

Methods: We prospectively collected 204 clinical samples at Modimolle MDR-TB unit and retrospectively used 104 culture isolates from MRC laboratory in Pretoria. The MTBDR*plus* assay was used to screen for *M. tuberculosis* and drug resistant mutations to RIF and INH drugs. Anyplex™ MTB/NTM/MDR-TB assay was used for rapid detection of *M. tuberculosis* and drug resistance to RIF and INH within 3 hours. The discordance between phenotypic and genotypic assays was resolved by sequencing and the Anyplex™ resistant profiles were spoligotyped. Diagnostic data was collected from NHLS and MRC databases and analysed using the Microsoft excel and Epi Info version 3.5. Descriptive statistics (percentages and frequencies) were used to explain proportions.

Results: The Anyplex™ MTB/NTM assay detected *M. tuberculosis* in 69/111(62%) and 100/104 (96%) of clinical and culture samples respectively. The sensitivities, specificity, PPV and NPV obtained for both RIF and INH resistance by Anyplex™ MDR-TB assay were 67%, 59%, 67%, 55% and 15%, 100%, 100% and 17%, respectively. Anyplex™ MTB/NTM/MDR-TB resolved 23/45 (51%) of discordant

samples. Sequencing of remaining discordant isolates revealed L511P, L533P and D516Y mutations within *rpoB* gene. A novel R385W mutation within *katG* was also detected. Spoligotyping of Anyplex™ MDR-TB resistant clinical isolates revealed Euro American clade with 20% followed by 15% Manu2, 5% East African Indian, 5% H37Rv, 5% atypical and 50% were orphans.

Conclusion: The novel Anyplex™ MTB/NTM/MDR-TB assay is a rapid and valid technique for detecting *M. tuberculosis* and most common mutations conferring resistance to RIF and INH. However further investigations are required, as the assay has a lower sensitivity as compared to already endorsed techniques.

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ABBREVIATIONS

Ac-	Amplification control
AFB-	Acid Fast Bacilli
BCG-	Bacillus Calmette–Guerin
BP-	Base pair
BSL-	Biosafety level
CC-	Conjugate control
CDC-	Centers for Disease Control
DNA-	Deoxyribonucleic acid
DR-	Direct repeat
DST-	Drug Sensitivity Testing
EDTA-	Ethylene diamine tetra acetic acid
HIV-	Human Immunodeficiency virus
INH-	Isoniazid
LAM-	Latin-American-Mediterranean
LPA-	Line Probe Assay
MDR-	Multi Drug Resistant
MIC-	Minimum inhibitory Concentration
MGIT-	Mycobacterium Growth Indicator Tube
MRC-	Medical Research Council
MREC-	Medunsa Research and Ethics Committee

MTBC-	*Mycobacterium Tuberculosis* Complex
M. tuberculosis-	*Mycobacterium tuberculosis*
NaOH-	Sodium Hydroxide
NaLC-	n-acetyl-L-cysteine
NHLS-	National Health Laboratory Services
NIAID-	National Institute of Allergy and Infectious Disease
NPV-	Negative predictive value
NTM-	non-Tuberculosis Mycobacteria
OADC-	Oleic acid, Albumin, Dextrose and Catalase
PANTA-	Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin
PCR-	Polymerase chain reaction
PZA -	Pyrazinamide
PPV-	Positive predictive value
RIF-	Rifampicin
SDS-	Sodium dodecyl sulphate
SIT -	Shared International Type
SIRE-	Streptomycin, Isoniazid, Rifampicin and Ethambutol
SOP-	Standard operating procedure
SP-	Specificity
SS-	Sensitivity
SSPE-	Saline-Sodium Phosphate EDTA
STR-	Streptomycin
TB-	Tuberculosis
TDR-	Totally Drug Resistance
WHO-	World Health Organization
XDR-	Extensively drug resistant

DEFINITION OF CONCEPTS

1. Mono-resistant- MTB resistance to a single particular drug alone.
2. MDR-TB- is resistance to at least rifampicin and Isoniazid.
3. New cases-patients who had received less or equal to 30 days of TB treatment.
4. Sensitivity- the probability that a TB test will correctly identify all individuals who are infected with TB
5. Specificity- the probability that a TB test will correctly identify all individuals who are not infected with TB.
6. Discordant- the disagreement of results generated from two assays performing the same function.

Nucleotides

Adenine

Cytosine

Guanine

Thymine

TABLE OF AMINO ACIDS

Amino acid	Three letters	One letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoluecine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

CHAPTER 1

1. INTRODUCTION

Tuberculosis (TB) is one of the most infectious diseases with an etiological agent that is widely disseminated (Mathuria *et al.,* 2013). In poorly resourced countries, TB is on the increase with marked socio-economic impact and has received undivided attention from the public and scientific communities (Miranda *et al.,* 2011; Kranzer 2011). There has been 9.0 million new TB cases documented in 2013 and the disease has resulted to 1.5 million deaths (360000 were HIV co-infected) (WHO 2014). In 2013, African region accounted for approximately one quarter of the global TB cases and reported the highest rates of cases and deaths relative to population (more than double the world's average of 126 it had 280 incident cases per 100 000 on average) (WHO 2014).

Globally, TB is a potential challenge and it is further complicated by strains that are resistant to the most effective drugs currently available (Lange 2014). There are TB strains which are resistant to at least rifampicin (RIF) and isoniazid (INH) termed multi drug-resistant (MDR) (WHO 2014). *Mycobacterium tuberculosis* (*M. tuberculosis*) isolates from patients with extensively drug resistance (XDR) are MDR-TB isolates with additional resistance to any fluoroquinolone and one of the injectable drugs (WHO 2014).

The most common form of anti-TB drug resistance is of INH, in isolation or combination with other drugs (WHO 2014). Isoniazid, an anti-bactericidal agent, is used as first line anti-TB drug due to its activity against growing tubercle bacilli through passive diffusion and targets the cell wall mycolic acid of the *M. tuberculosis* (Veluchamy *et al.,* 2013). Resistance to INH is highly associated with mutations found in the gene loci including *katG* (Catalase-peroxidase), the promoter region of *aphC* (alkyl

hydroperoxidase), *inhA* (enoyl-acyl reductase), *kasA* (beta ketoacyl ACP synthase),*mabA* (3-ketoacyl reductase), and *ndh* (NADH dehydrogenase) (Veluchamy *et al.,* 2013).

Rifampicin is a first-line drug for TB treatment that actively antagonizes both growing and stationary phase bacilli with low metabolic activity (Kozlov *et al.,* 2013).Rifampicin-resistant *M. tuberculosis* strains are due to mutations within *rpoB* gene and the resistance to this anti-TB drug is considered as a surrogate marker for dual resistance to MDR-TB (Veluchamy *et al.,* 2013). Furthermore, various reference laboratories that examined the 81 base pair region have reported that the sequential accumulation of mutations in *rpoB, katG* and *inhA* genes leads to the development of MDR-TB (Veluchamy *et al.,* 2013).

Generally, resistance to anti-TB drugs constitutes a serious public health concern and indicates that advances made by the global TB control programmes are undermined (Huang *et al.,* 2011). The gradual increase from single drug resistance to multiple effective drugs points to a gradual heightening in the threat of TB to human health. Development of effective strategies is essential in monitoring this continued increase in resistance to anti-TB drugs (Haung *et al.,* 2011, WHO 2014)

Molecular techniques can be used in the diagnosis of TB and its drug resistant forms (Veluchamy *et al.,* 2013). Examples of these techniques include GeneXpert (Cepheid, Sunnyvale, CA, USA) and Line probe assays (LPA) MTBDR*plus* (Hain Life Sciences, Nehren, Germany). These commonly used methods have some operational limitations which have made it difficult to detect all variants of *M. tuberculosis* thus we still rely on cumbersome phenotypic culture assays (WHO 2014).

Additionally, the ability of *M. tuberculosis* to evolve and develop new mutations has also rendered these methods less useful. Therefore new

assays for detection of *M. tuberculosis* variant mutations are constantly developed and adapted to detect both existing and emerging *M. tuberculosis* resistant strains in various geographic locations to ascertain their continued ability to detect the presence of *M. tuberculosis* mutations and variants (Pai *et al.,* 2010).

Tuberculosis strains could be diverse because of geographic area and therefore the strains that will be found in Limpopo will be compared to the already documented/identified strains at the international database by performing Spoligotyping. Spoligotyping is a rapid discriminatory test used internationally and is based on polymorphism in the chromosomal direct repeat locus (Vasconcellos *et al.,* 2014). Spoligotyping is used as a basis for the creation of the largest genotype database for *M. tuberculosis* containing a global distribution and phylogenetic analysis for worldwide genotypes (Vasconcellos *et al.,* 2014). This database assists in allocating a particular strain to a family.

Spoligotyping is a genotypic method that is used in studying the genetic diversity in subspecies identification (Lawson *et al.,* 2012). In a study carried out by Mlambo *et al.,* in 2008 there were various genotypes found in Limpopo province, namely LAM, EAI1_SOM/48, LAM4/60 and Beijing. Several studies have shown that the Beijing genotype is associated with drug resistance and was found over expressed in the Western Cape, South Africa (Streicher *et al.,* 2012, Gandhi et al., 2014). The data from urban areas of Western Cape indicated that there could be over expression of Beijing genotypes in the XDR-TB population. Spoligotyping data analysis suggested that most of the XDR-TB cases in South Africa are due to acquisition as defined by presence of a unique spoligotype within a defined geographic area (Streicher *et al.,* 2012).

The most recent assay or technique development is the Anyplex™ NTM/MTB/MDR-TB (Seegene incorporated, Korea) which has ability to

detect the presence of *M. tuberculosis* and non-tuberculosis Mycobacteria (NTM) including the mutations that are linked to MDR-TB.

Furthermore, being a newly developed technique, its quality and capacity to detect *M. tuberculosis* resistant variants has not been widely studied/tested in Africa and very few studies has been carried out in South Africa (WHO 2011-2015). Limpopo Province is constituted by rural settings and it has received little attention from researchers in documenting the new and existing circulating *M. tuberculosis* strains. This study used Anyplex assays to detect the distribution of known, new mutations and differentiate NTM from *M. tuberculosis* complex. Molecular techniques are usually designed to detect mutations which are already known and therefore the unknown mutations are usually not recognized by these techniques. The limitation of mutation-specific probes will therefore reduce the accuracy of detection hence the use of sequencing for determination of the nucleotide order to detect the unrecognized mutations however sequencing is not used routinely in low resourced setting.

1.1 **Hypothesis**

The molecular diagnostic tool (Anyplex™ Assay) will detect already documented and novel TB mutations.

CHAPTER 2

2. LITERATURE REVIEW

2.1. Classification of genus Mycobacterium

Tuberculosis is a disease commonly known to manifest in humans caused by some of the members of the mycobacterium genus. This genus has more than 130 various species and is classified under order Actinomycetalis in the family Mycobacteriaceae (Ryan *et al.,* 2010). There are pathogens which are known to cause diseases in humans and TB is the most common disease caused by *M. tuberculosis* (Ryan *et al.,* 2010). There are sub groups which were identified due to the manner in which they grow as slow or fast/rapid growers (Ryan *et al.,* 2010).The *M. tuberculosis* complex (MTBC) is included in the slow growing sub-group. The species that are forming part of the MTBC are *M. tuberculosis M. africanum, M. bovis, M. pinnipedii, M. microti, M. canetti* and *M. caprae* (Coscolla *et al.,* 2013).

Mycobacterium tuberculosis complex is responsible for causing TB however mostly due to *M. tuberculosis* organism; with more than 80% of TB cases taking the form of pulmonary TB (Alexander *et al.,* 2010). The similarities at the nucleotide level and 16s rRNA sequences are used to group the *M. tuberculosis* complex species but they differ largely in their host tropism, pathogenicity and phenotypes (Tortoli 2012).

2.2. Epidemiology

2.2.1. Burden of disease

Tuberculosis infects a significant proportion of the world's population, remaining as second most common cause of death after HIV/AIDS, (Miranda *et al.,* 2011). It is on the rise in poor disadvantaged populations around the globe with uncontrollably high mortality rates due to poor ventilations and housing. Although TB is curable globally it is estimated that approximately two billion people worldwide are infected with the TB bacilli, which remains a vast reservoir of potential TB cases (Finnie *et al.,* 2011), with about nine million new TB cases resulting in more than 1.6 million deaths annually (Parra *et al.,* 2011). About 95% of these new TB cases are in the developing world (WHO 2011/2012).

2.3 Tuberculosis pathogenesis

More than 95% TB infection cases results from the inhalation of droplet-nuclei containing tubercle bacilli into the affected site and in pulmonary TB in the alveoli of the lungs. The pathogen or tubercle bacilli get to be ingested by macrophages in the alveolar aiming to destroy them or inhibit multiplication (Ryan *et al.,* 2010). Some bacilli will multiply intracellularly and would be released once the macrophage dies. The bacilli can be spread throughout the body (including areas of the body in which TB disease is most likely to develop: regional lymph nodes, apex of the lung, kidneys, brain, and bone). The below figures (figures: 2.1, 2.2, 2.3, 2.4 and 2.5) are demonstrating the pathogenesis of the TB disease.

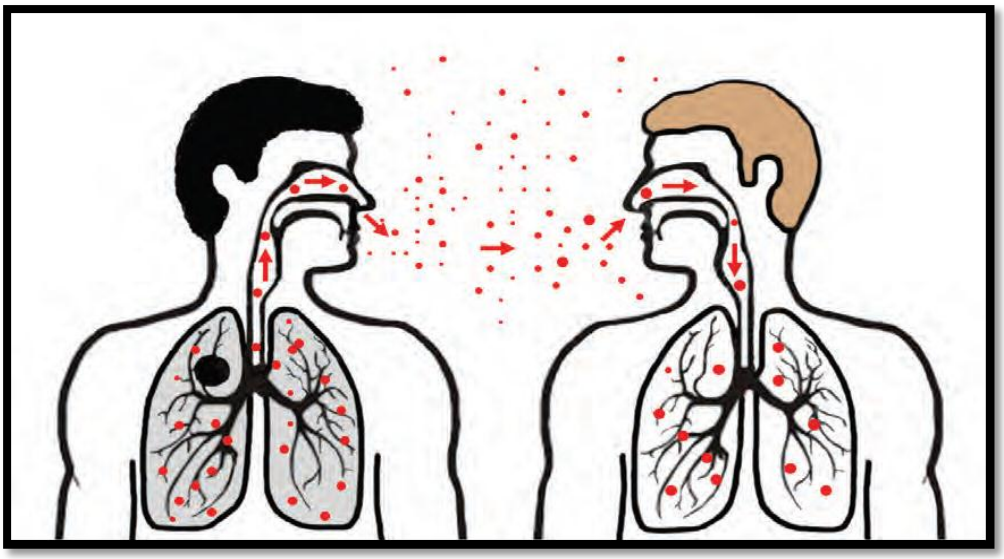


Figure 2.1: Tuberculosis Transmission**.** The aetiological agent of TB is carried in airborne particles known as the droplet nuclei. Infected individual can release nuclei droplets when engaged in a conversation, coughing and other activities. The dots represent droplet nuclei containing tubercle bacilli. Droplet nuclei are inhaled, through lungs, and lodge in the alveoli (CDC 2013).

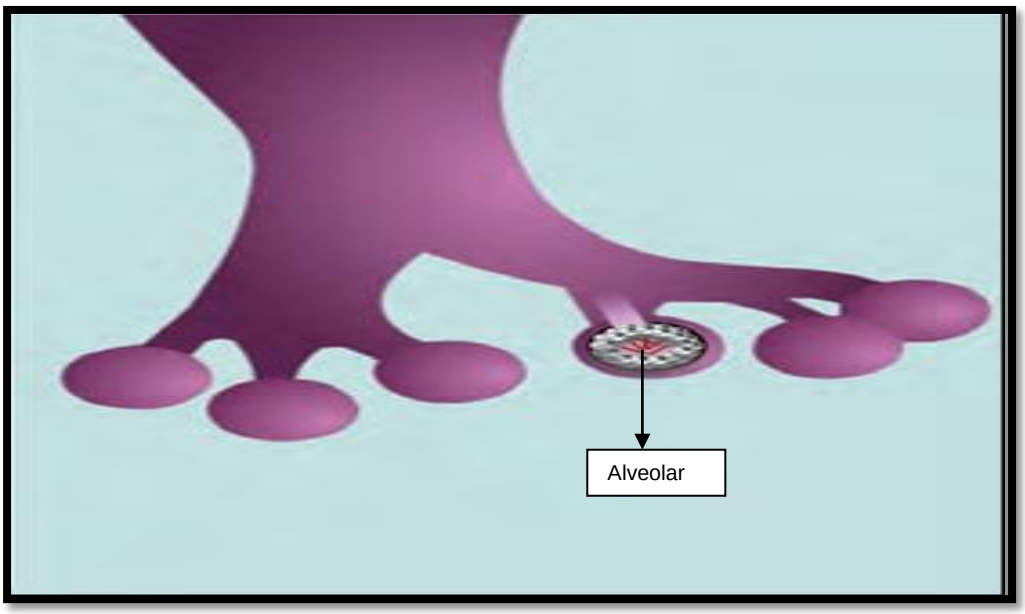


Figure 2.2: The tubercle bacilli multiply within the alveoli (CDC 2013).

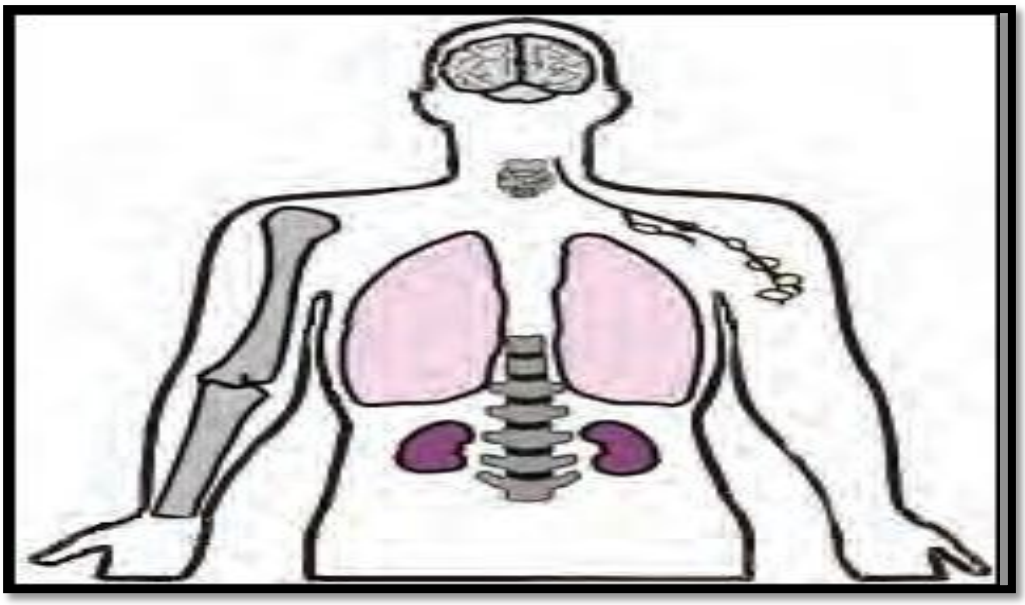


Figure 2.3: Tubercle bacilli enter the bloodstream and spread throughout the body. The development of TB disease can affect organs of the body commonly the brain, larynx, lymph node, lung, spine, bone, or kidney depending on reach of the tubercle bacilli (CDC 2013)

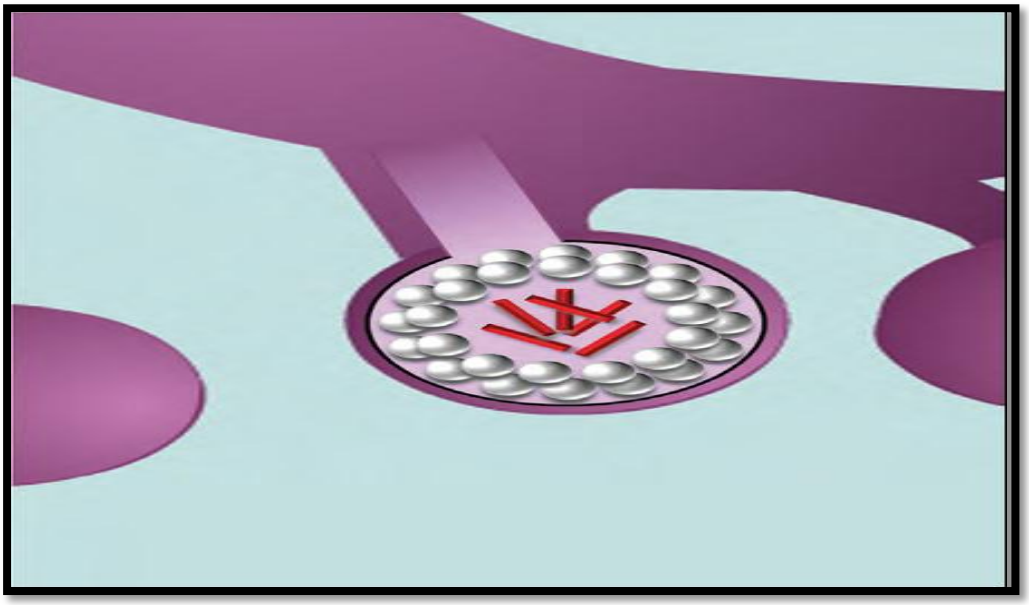


Figure 2.4: Macrophages ingest and surround the tubercle bacilli within 2-8 weeks. The macrophages form a granuloma, the barrier shell that keeps the bacilli contained and under control (LTBI) (CDC 2013)

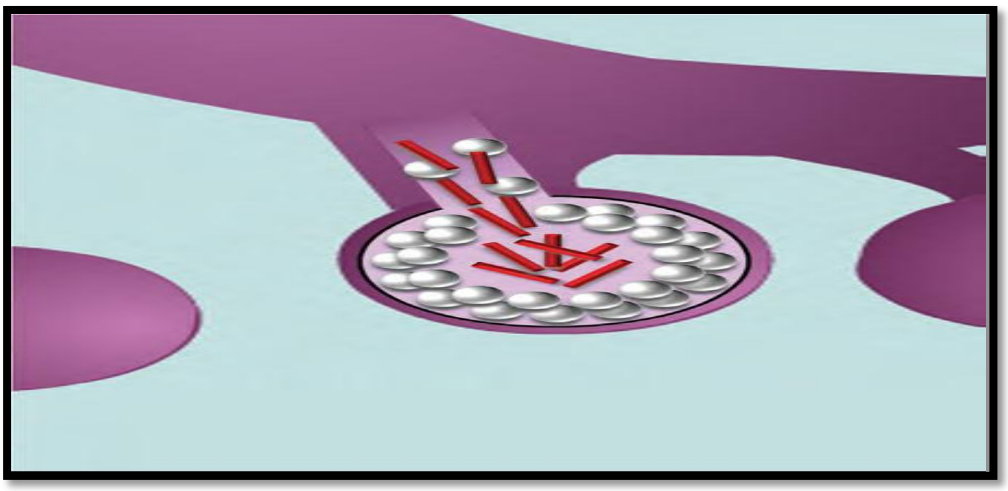


Figure 2.5: The failure to control the tubercle bacilli leads to TB disease development, at any body site the bacilli can reach and multiply rapidly (TB disease occurs if the immune system cannot keep the tubercle bacilli under control,)(CDC 2013).

2.4. General characteristics of *M. tuberculosis*

The *M. tuberculosis* is an acid fast bacillus that belongs to the family Mycobacteriaceae (Ryan *et al.,* 2010). The components of the cell wall and envelope contribute towards the acid fastness and slow growth rate of the bacteria. The bacterium is able to adapt to different conditions or environments by either allowing or inhibiting transcription to take place (Ryan *et al.,* 2010). Mycobacteria are large non-motile rod shaped bacteria which are distantly related to Actinomycetes.

The human normal flora is mostly constituted by the non-pathogenic mycobacteria which are usually found in the moist/oily and dry areas of the body (Ryan *et al.,* 2010). This rod shaped structure can be 2-4

micrometre in length and 0.2 to 0.5 um in width (Ryan *et al.,* 2010). *M. tuberculosis* are facultative-intracellular organisms usually of the macrophages and have a slow generation period of 15 to 20 hours, this characteristic may contribute to its virulence They are obligate aerobes hence in classic TB, MTBC are always found in the well-aerated upper lobes of the lungs (Ryan *et al.,* 2010).

The cell wall of *M. tuberculosis* contributes to its virulence due to the unique composition. Complex lipids and peptidoglycans are the main constituents of the cell wall composition. Lipids contribute more than half of the cell wall. The lipid fraction includes three major components namely: cord factor, mycolic acids and wax-d (Veluchamy *et al.,* 2013). Virulent strains are known to produce the cord factor. It is toxic to mammalian cells and an inhibitor to migration of polymorph nuclear (PMN) cells. Mycolic acids are hydrophobic molecules forming a lipid shell around the organism affecting the permeability properties of the cell surface.

Mycolic acids constitute up to 60% of the dry weight of the mycobacterial cell envelope. The properties, resisting to be killing by acidic and alkaline components, resistance to multiple drugs, permeability to stains or dyes, resistance to lethal oxidations and survival inside of macrophages and resistance to osmotic lyses via complement deposition have been associated with high content of lipids in the cell wall (Sarathy *et al.,* 2012).

2.5. Manifestation of TB (clinically)

The development of TB varies in each subject depending on the particular subject's immune system. Tuberculosis has several stages prior to its manifestation and each stage has different features, explained in detail below.

2.5.1. Latency

Majority of the people that are infected with *M. tuberculosis* do not show any clinical signs of TB, termed to be latently infected. The latently infected people remain as potential reservoir of the tubercle bacilli with high possibilities of latent TB developing to active TB within few years after prime infection with *M. tuberculosis* (Druszczynska *et al.,* 2012).

Studies have been conducted on elaboration about the role of bacillary virulence determinants, host genetic and immune background as well as environmental effects in latently infected patients (Druszczynska *et al.,* 2012). Although studies were conducted, TB continues to develop in more difficult forms (Druszczynska *et al.,* 2012).

2.5.2. Early primary disease

The disease at this stage is very difficult to diagnose due to non-specific signs or symptoms. The infected individual may have loss of weight, fever, frequent fatigue, non-productive cough (Druszczynska *et al.,* 2012).

2.5.3. Primary progressive disease

At this stage the sputum produced will be very much indicative of TB with more signs becoming clear. The chest radiography will be normal and diagnosis is achievable by culture (Druszczynska *et al.,* 2012.)

2.5.4. Extra-pulmonary disease

Extra pulmonary TB affects other parts of the body excluding the respiratory site. This form of TB is usually progressive in immune-compromised patients. The central nervous system is mostly affected than any other extra-pulmonary sites and can lead to meningitis. In some cases the TB disseminates into the blood stream and is known to be military TB and can spread throughout the body during blood circulation. Other body parts that can be affected are the pleura, bones and many more (Ryan *et al.,* 2010).

2.6. Signs and symptoms of TB

The symptoms and signs of TB are not clearly specific and are not that different from drug resistant TB as well. Cough is the most common and at times it can be non-productive at the beginning of the illness however as the inflammation and tissue death occur sputum can be produced (Ryan *et al.,* 2010). Tuberculosis cannot be diagnosed due to a cough since most of the acute respiratory infections resolve within two weeks however it can be queried if coughing persists more than two weeks (Bark *et al.,* 2011).

Active pulmonary TB patients normally present with a chest pain and it could be due to the infiltrations of the lung parenchyma and or even the occurrence of pneumothorax (Ryan *et al.,* 2010). Tuberculosis can cause severe respiratory failure and in advanced pulmonary TB dyspnoea may arise due to the involvement of the parenchymal lungs

severe lung fibrosis or destruction and even by tuberculous bronchopneumonia (Ryan *et al.,* 2010).

There are usually systematic symptoms occurrence in active respiratory TB that include fever, tiredness, anorexia/ loss of weight (due to loss of appetite), night sweats and chills (Ryan *et al.,* 2010). The weight loss start early in the disease and progresses as the disease advances. The symptoms of extra pulmonary TB depend on the site affected (Ryan *et al.,* 2010).

2.7. Diagnosis

Though, TB and its drug resistance is common in most countries of the world and well documented in literature most patients are under diagnosed for drug resistant TB resulting from inadequate laboratory resources and rapid accurate point-of-care tests (WHO 2014).

The key component of correct and effective drug administration depends on the rapid and accurate diagnosis of the disease. The treatment of TB and the drug resistant profiles of TB are in most settings based on the epidemiological data from periodic drug resistant surveys (WHO 2014).

The slow growth of the bacterium is a potential factor affecting the diagnostic procedures, especially in culture (phenotypic) methods for direct detection in clinical specimens. Appropriate diagnosis of TB and drug resistant forms in patients is more challenging, particularly in resource poor settings like Limpopo Province (Calligaro *et al.,* 2014). In these settings, diagnosis rests on the detection of acid-fast bacilli in sputum coupled with expensive drug susceptibility tests (Calligaro *et al.,* 2014).

The diagnosis depends on detection TB bacilli in sputum or any other specimen depending on the site of infection (CDC 2013). In most of the health care facilities of poor resourced settings, routine diagnosis are based on sputum smear microscopy and culture that are associated with low sensitivity and long turnaround time. The inability to rapidly and accurately detect and treat TB leads to spread of disease in communities (Calligaro *et al.,* 2014).

2.8. Factors affecting management of TB

Tuberculosis is commonly caused by bacteria with unique characteristics that introduce difficulties in diagnosis, control and management. The low income settings have multiple challenges associated with TB management (WHO 2014). Most limitations are present at the hospitals or primary health care facilities where most patients are diagnosed and treated for TB.

Management of TB will always require excellent organisation, logistics and communication, in rural settings factors like long distance referral of specimens is a great challenge (WHO 2014). Generally, commercial methods are recommended due to better level of standardization, reproducibility and automation but they are costly and they require appropriate settings for their implementation.

2.9. Management and control of TB

Global detection and control of TB is highly constituted by slow, insensitive diagnostic methods, particularly for detection of drug resistant strains which are continuously spread in the communities (Laxminarayan *et al.,* 2013). Early detection is essential in reduction of death rate and to interrupt transmission, but the complexity and infrastructural needs of these sensitive methods limit their accessibility especially in low resourced areas.

Despite the availability of anti-TB drugs the situation is far from ideal and suggests that better therapeutic interactions are needed to reverse the current trend. The anti-TB drugs used currently seem to be ineffective thereby imposing economic burden on government efforts for effective control. The reliable and rapid detection of resistant strains is highly needed for the timely anti-TB therapy and reduction of total treatment costs for effective response to the TB cases. Universal access to high quality, patient-centred treatment for all TB patients is in demand.

2.10. Treatment of TB

2.10.1 Standard treatment regimens by WHO

This recommendation of the TB treatment is based on the mode of administration, duration of treatment and clinical efficacy trials in terms of dosage (WHO 2014). The treatment period is divided into intensive (initial) and continuation phases.

The intensive phase is a period where there is a need for rapid killing of the bacteria and it is based on all first line drugs. Patients whom were infectious rapidly change into non-infectious (usually within two weeks) and symptoms start to be reduced. The majority of patients who were initially smear positive start to produce smear negative sputum within two months. The continuation phase is a period were only few drugs are necessary but for a prolonged period of time to allow the sterilising effect of drugs to eliminate remaining bacilli.

The bacteriological proven new cases of TB treatment consists of six months of INH and RIF, for the first two months (intensive phase) there is a supplementation by PZA and EMB this regimen is given on a daily bases. Throughout the remaining four months (continuation phase) there is administration of RIF and INH three times per week.

The increased probability of drug resistance in retreatment cases poses a serious demand for effective planning in drug recommendation and administration especially in patients who received inadequate treatment. The recommended treatment regimen by WHO consists of eight months of INH, RIF, EMB and supplementation by streptomycin (STR) in the intensive period (two first months). This regimen includes the full first line TB-drugs available and has a high probability to cure patients (WHO 2014).Tuberculosis patients without treatment had shown poor quality of life and administration of treatment has proven to have a promising effect in improving the quality of life (Wells *et al.,* 2015). However treatment of TB remains as one of the most important challenges to global health because many TB control programmes failed to combat this disease and many anti-TB drugs are failing to antagonise the disease. World Health Organisation has recommended standard primary drugs for TB treatment known as first line drugs (INH, pyrazinamide (PZA), ethambutol (EMB) and RIF) administered for the first six months.

The first two months are known to be the most crucial stage of the treatment and at this point all the drugs are administered. Meanwhile the remaining four months of treatment only INH and RIF are used (WHO 2014). Streptomycin is one of the first line drugs which are usually used in the initial stage of treatment but its administration has decreased over a period of time due to the elevated levels of resistance (WHO 2014).

This is complicated by emerging resistant TB strains that cannot be cured by standard anti-TB drug regimen in several countries. The TB treatment regimen requires the intake of multiple drugs and prolonged treatment period, making it difficult to guarantee patient treatment compliance, and therefore it leads to treatment failure (Ryan *et al.,* 2010). The cure rate of these resistant strains are much less than

normal TB infection, for example XDR-TB case have a cure rate which is generally much lesser than 50% with the current available chemotherapy (WHO 2014).

Tuberculosis strains often render the first line regimen ineffective and that highlights that the drugs used are not effective in controlling that particular strain therefore the second line anti-TB drugs are to be considered. The second line drugs usually comprise of fluoroquinolone's and injectables. The number of doses ingested over a given period of is used to determine treatment. Modifications are made under special circumstances (i.e. Pregnancy, infant treatment, HIV infection or drug resistance) apart from the basic TB regimens that are broadly applicable.

2.11. Drug resistance and detection

2.11.1 Primary resistance

Primary resistance is a form of resistance that occurs in the new TB patients without any history of previously TB treatment. This describes resistance in patients who have had received TB treatment for less than a one month. The resistance in such subjects provide a measure of the degree of transmission of the *M. tuberculosis* strains.

2.11.2. Acquired resistance

The acquired drug resistance is a term used to define a drug resistant case of TB which was previously treated. The strains isolated from the cultured specimens after treatment completion that are still resistant from patients with at least one treatment episode of more than a month are referred to as retreatment cases. The term retreatment is usually used to describe the acquired drug resistant cases. Patients with acquired drug resistance have always shown a high level of resistance

than in the newly infected patients and they provide an indication of the extent to which a patient was approximately treated, like the quality of TB control.

2.11.3 Multiple drug resistance TB (MDR-TB)

Inadequate chemotherapy leads to the development of anti- TB drug resistance that arises through the selection of mutated strains (Laxminarayan *et al.,* 2013). Treatment of TB involves usage of various drugs, and if the drugs are not effective against the bacilli MDR-TB develops which is at least resistant to RIF and INH (CDC 2013).

Direct or indirect mono-chemotherapeutic agent can exert a selective pressure resulting in resistance. The *M. tuberculosis* population that has evolved resistance to the agent will continue to multiply and susceptible organism will be suppressed. Allowing the drug resistant strains to become dominant organism in the host and if mismanaged then could lead to more drug resistance (Laxminarayan et *al.,* 2013).

The appearance of MDR-TB was after the introduction of RIF in the 1966 (Laxminarayan *et al.,* 2013). The global surveillance data has recorded 150 000 MDR-TB deaths in 2008 and with relatively high estimations of MDR-TB cases (650 000) in 2010 (WHO 2011/2012). The geographic distribution of MDR-TB is highly uneven ranging from 0.7% of new cases in established market economies to about 2% in Africa (WHO 2011-2015). Thus, this global survey revealed that there is existence of variation in the magnitude and trends in drug resistant TB at both regional and national levels (WHO 2011-2015).

South Africa had high MDR-TB with more than 7000 cases diagnosed in 2010 and Limpopo Province accounted for about 126 MDR-TB cases (WHO 2011-2015). In the 2008 report of the anti-TB drug resistance surveillance global project the incidence of MDR-TB cases

ranged from 0-22.3% and the proportion of XDR-TB ranged from 0-30% globally (WHO 2011-2015). In South Africa, 5.7% of MDR-TB cases developed to XDR-TB (WHO 2011-2015). Globally there are about 100 countries that reported at least one case of XDR-TB (Falzon *et al.,* 2015)

2.11.4 Extensively drug resistant TB (XDR-TB)

Treatment to MDR-TB is challenging with lengthy process that can lead to patients non-adherence and a great potential for the development of resistance to second line drugs (Falzon *et al.,* 2015). Such strains can be amplified into being XDR-TB. The occurrence of XDR-TB in every region of the world has raised further alarm about the future of TB control (Laxminarayan *et al.,* 2013).

Researchers conducted a global drug susceptibility testing at the CDC in which 347 isolates of XDR-TB, accounted for 2% of all TB isolates surveyed and 15% of MDR-TB isolates, excluding data from African countries (CDC 2013). An incident of XDR-TB outbreak was firstly reported in one of the South African Provinces with increased drug resistant TB in 2005 (Phillips 2013). During the year 2006 there were 53 XDR-TB cases noted at a rural hospital in KwaZulu-Natal representing 24% of MDR-TB cases (Phillips 2013). The median time period from which diagnosis was made to death of XDR-TB patients was only 16 days (Klopper *et al.,* 2013).

The XDR-TB was notable in patients without TB history, were TB treatment was successfully completed and it was believed that there was a nosocomial transmission (Klopper *et al.,* 2013). Drug resistant TB strains pose difficulties in the diagnosis and treatment, with lower survival rates (Klopper *et al.,* 2013).

2.11.5 Totally drug resistant TB (TDR-TB)

The mismanagement of drug resistance continues to introduce more aggressive forms of TB. Strains isolated from the totally drug resistant (TDR-TB) are proven to show resistance to all first line and second line anti-TB drugs. In a study published in the European respiratory journal, the TB- strains isolated had transformations into adapted forms producing round or oval shaped bacilli (Zarir *et al.,* 2012). They had patients whom were treated with second line drugs and remained smear and culture positive after 18 months treatment period (Zarir *et al.,* 2012). The probability of treatment to eradicate a disease is mostly decreased with the emergence of new drug resistant strains (Caminero *et al.,* 2010). The cell wall of the TDR-TB strains is extra ordinarily thick (21-26nm) and such bacilli has never been found among susceptible and or MDR TB forms. Below in figure 2.6 is an illustration of TDR-TB.

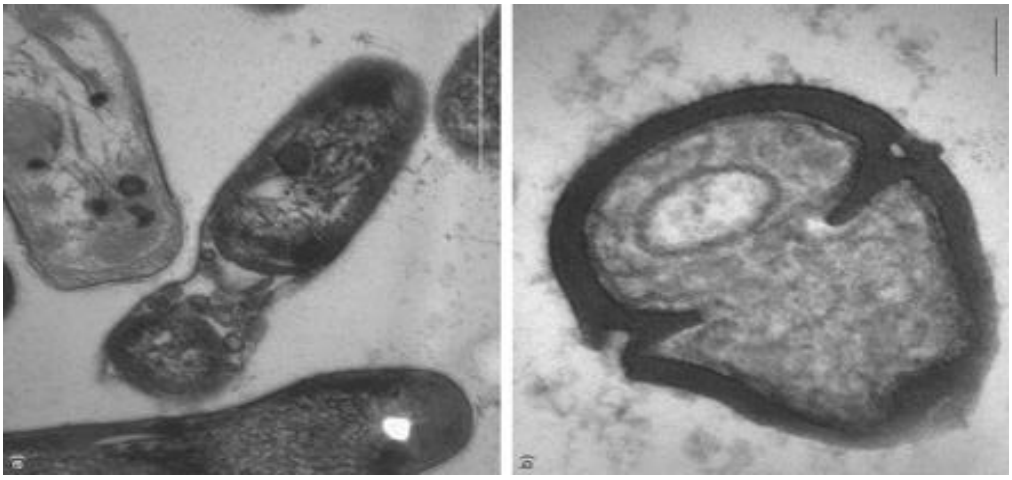


Figure: 2.6 Microscopic views of the totally drug-resistant (TDR) bacilli. a) The oval or round bodies within the TDR TB bacilli b) TDR-TB bacilli with stationary or anaerobic dormant bacilli in dividing stage (Zarir *et al*., 2009)

2.12 Mechanisms of drug resistance in TB.

Drug resistance can be classified as either primary or secondary. Primary resistance defined as infections resulting from the already resistant MTBC strains in most cases it is due to previously undocumented or unreported treatment (Palomino *et al.,* 2014). Acquired resistance is resistance that develops as a result of improper exposure of the *M. tuberculosis* complex strain to anti-tuberculosis drugs and followed by selection of resistant mutant bacilli (Palomino *et al.,* 2014).

The understanding of Mycobacterial resistance mechanisms to the anti-TB drugs will not possibly only enable the advanced development of more rapid diagnostic tests and effective drug development, but as well help in the implementation of relevant measures in preventing and managing the development of such resistance in the future (Zhang *et al.,* 2012).

Mechanisms in which resistance to drugs develops has been identified and broadly categorized: The spontaneous chromosomal mutations that affect key drug targets, enzyme production that degrades or modify compounds (rendering them useless), the formation of a layer that is rich in lipids reducing permeability to drugs and the efflux of drugs through protein pumps (Veluchamy *et al.,* 2013). Through the understanding of these mechanisms; several molecular diagnostic tests for identification of TB and its resistance to drugs have been developed.

2.13. Anti-TB drug resistance

The resistant mutant organism occurs as a result of *M. tuberculosis* undergoing spontaneous, slow and constant mutation. This is natural event which is genetically determined and differs from drug to drug. Anti-TB drugs constitute both resolution and perpetuation of resistance era; the drugs can destroy the pathogenic *M. tuberculosis* and can as well select the naturally resistant strains and those drugs are then ineffective (Marahatta 2010).

Drug resistance is due to accumulation of chromosomal mutations, in part because tubercle bacilli have no known efficient mechanism for horizontal gene transfer (Palomino *et al.,* 2014). Mutations that are evident in drug resistant strains occur at the enzyme that are involved in the activation of the drug or at the drug target region.

Drug resistant TB can be either primary that develops when an individual is initially infected with a resistant strain or secondary whereby resistance arises when inadequate treatment leads to development of mutations (Marahatta 2010). The genetic resistance to anti-tuberculosis drugs occur at a frequency of $10^{-6\ to-8}$ Mycobacterial replication (Kolyva *et al.,* 2012). In other bacteria there are mobile genetic elements that are transferred and known to assist in development of drug resistance but that has not been documented in *M. tuberculosis* resistance (Kolyva *et al.,* 2012).

Though progress has been made in reducing the global incidence of drug-susceptible TB, the emergence of MDR and XDR-TB during the past decade threatens to undermine advances made in TB control programmes across the national/international boarders (WHO 2010).

2.14. Molecular mechanisms of resistance to anti-TB

2.14.1 Rifampicin resistance

Rifampicin is a bactericidal lipophilic ansamycin that is considered as an important first line drug. It is a drug known to be actively antagonising to both growing and stationary phase bacilli with low metabolic activity correlating with its ability to shorten TB treatment from 18 months to 9 months (WHO 2010). Rifampicin resistance occurs at a frequency of 10^{-7} to 10^{-8} (Ghandi *et al.,* 2014).

The first introduction of an anti-TB drug rifampicin was in 1972 and is known for interfering with RNA synthesis. Rifampicin interferes with transcription by the DNA-dependent RNA polymerase. There are subunits (β, α, σ and β') that constitutes the RNA polymerase and RIF binds to the one known as the β-subunit hindering transcription therefore killing the organism (Valuchamy *et al.,* 2013).

Rifampicin resistant *M. tuberculosis* isolates are reported to have shown mutations within a defined region of the 81 base pair region of the *rpoB* gene. These mutations are associated with high level of resistance and it's been reported in more than 95 % of cases with frequent mutations at positions 531, 526 and 516 (Valuchamy *et al.,* 2013). However there are specific mutations (codons 511, 516, 518 and 522) that are related to low levels of RIF resistance (Palomino *et al.,* 2014).

2.14.2 Isoniazid resistance

This first line anti-TB drug consists of a chemical structure with a hydrazide and a pyridine ring (Da Silva *et al.,* 2011). Isoniazid is active only against growing tubercle bacilli and inactive under anaerobic conditions and against non-replicating bacilli (Palomino *et al.,* 2014). Isoniazid mono-resistance is frequently observed than other anti-TB drugs and occurs, at a frequency of 1 in 10^{5-6} bacilli in vitro (Varahram *et al.,* 2014).

Isoniazid, is a prodrug that requires activation by catalase/peroxidase enzyme encoded by the *katG* gene for generation of a range of highly reactive species which then attach multiple targets in *M. tuberculosis* (Palomino *et al.,* 2014).The bacterial enzyme catalase-peroxidase (*katG*) which is bi-functional converts the INH into a range of oxygenated (superoxide, peroxide, hydroxyl radical, nitric oxide) , some certain electrophilic species and other organic toxic radicals (isonicotinic-acyl radical or anions) that attack multiple targets in the mycobacterial cell (Valuchamy *et al.,* 2013). The cell wall mycolic acid is commonly characterized target of these radicals but not limited to DNA, carbohydrates, lipids and NAD metabolism. (Palomino *et al.,* 2014).

The anti-TB drug INH was found to be effective during the year 1952 and since then it has been the cornerstone of all effective regimens for active and latent TB treatment (Zhang *et al.,* 2012). Mutations in the *katG* are the main mechanisms of INH resistance (50-95% of the INH-resistant strain) and 20-35% of resistant isolates can be present in the *mabA/inhA* operon, mutations are less frequent compared to *katG* mutations and constitute the low-level of INH resistance (Sato 2012). Approximately 10 -25% of low level isoniazid-resistant strains are not

indicative of mutations in *katG* or *inhA* and may be due to new mechanisms of resistance (Sato 2012).

2.15 Laboratory diagnosis of pulmonary TB

A good quality sample is required for diagnosis of TB depending on the site of infection (i.e. good quality sputum from lung infection). The samples should always be collected aseptically in an air tight container and be transported to the laboratory at favourable conditions to prevent the multiplication of the contaminating bacteria. There are several sequential activities thus, therefore after collection samples are subjected to slide preparation to be viewed under the microscope.

2.15.1 Microscopy

This technique is used widely to identify TB either directly or indirectly due to its low cost. Microscopy has the overall sensitivity of 50-60%. Mycobacteria are composed of a rich lipid cell wall that is not easy to be stained with ordinary dyes and resist decolourisation by acidified alcohol (Zhao *et al.,* 2012). This characteristic of mycobacteria is then said to be acid-fast bacilli. Currently only two methods are used for staining namely, auramine-o flourochrome staining for florescence microscopy and Ziehl-Neelsen (ZN) for light microscopy. The auramine staining is easy to perform, requires less time even for reading thus it is more preferred than the ZN staining (Zhao *et al.,* 2012). In figure 2.7 is an illustration of acid fast bacilli under a microscope.

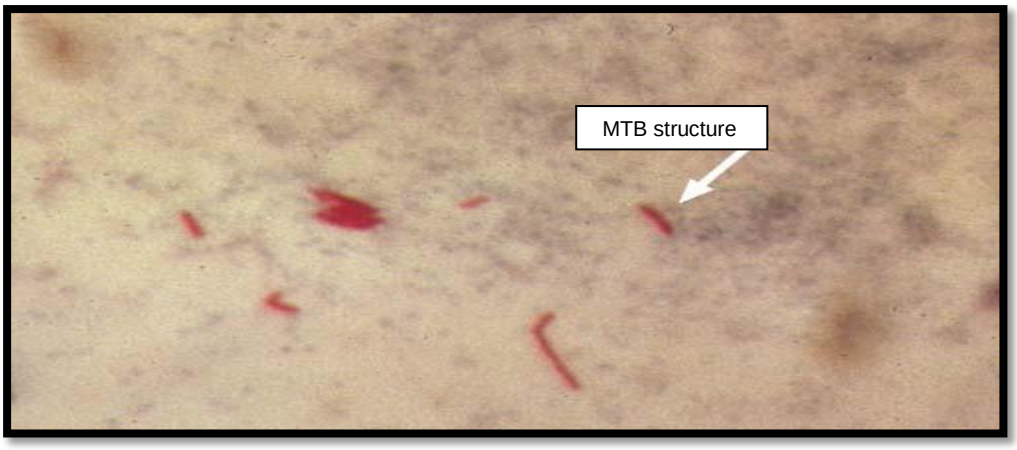


Figure 2.7: The acid fast TB bacilli visualised under a bright field microscope (CDC 2013).

2.15.2 Culture

Culture is referred to as the gold standard for diagnosis of TB regard less of the form of media used (solid or liquid). Cultures used have the ability to differentiate *M. tuberculosis* from the rest of mycobacteria due to particular enzyme that is not found in any but only MTB strain (Palomino *et al.,* 2014). The specimen is pre-treated by decontamination to prevent other bacteria that can disrupt the identification of *M. tuberculosis*. Although solid media are still used due to the low cost, liquid media is advised due to its ability of producing growth of the bacilli rapidly (Zhao *et al.,* 2012). A Lowenstein-Jensen medium which is egg-based and Middle brook media (agar-based) is the commonly used below in figure 2.8 is the picture of *M. tuberculosis* on LJ media (Palomino *et al.,* 2008).

Figure 2.8 Colonies of *M. tuberculosis* on Lowenstein-Jensen medium (CDC 2013)

2.16. Methods of diagnosis

The rapid and accurate diagnosis of TB is the cornerstone of global strategies for TB control (McNerney *et al.,* 2012). The use of molecular technology is beneficial to TB diagnostics due to its short turnaround time. Tuberculosis is currently diagnosed by total assessment of symptoms, clinical signs, and results from diagnostic methods (chest radiography, microscopy, culture and molecular diagnostics) (Van Deun *et al.,* 2011). Together with culture as the gold standard, molecular diagnostic methods based on PCR are broadly used for the early diagnosis of TB (WHO 2012/2015).

World Health Organization encourages the implementation of these assays due to their reliability, accuracy and reproducibility (WHO 2012/2015). New assays are developed to keep tract with new mutations and minimize the time of diagnosis. The newly developed technologies are highly specific and sensitive for detection of TB and

the drug resistant strains thereby simplifying management of TB (WHO 2012/2015)

Newly developed assays such as Anyplex™ MTB/NTM screening test can contribute greatly to future molecular diagnosis of TB.

2.17. Drug susceptibility testing (DST)

Drug susceptibility testing (DST) is the test used to determine the strain susceptibility to drugs utilized in therapy: a “sensitive” indicates high probability of treatment success the patient with that strain has, while a “resistant” result translates that there are high chances of treatment failure and highlights a need for therapy to be changed.

The amplification of drug mono-resistance to multiple drug resistant is on the rise and is indicated by exponential occurrence of the MDR and XDR-TB strains which are undermining the advancement made in the control and management of TB indicating that there is a need for accurate drug susceptibility testing.

2.18. Conventional (phenotypic) DST

The phenotypic DST is based on the detection of drugs effects on the bacterial multiplication in comparison to the controls which are not exposed to any drug (van Deun *et al.,* 2011). These conventional methods are probably more accurate, less costly and are more appropriate for surveillance but they are very slow to produce results (van Deun *et al.,* 2011). Therefore various laboratories use different methods that are inclusive of medium based, slide DST, absolute concentration, micro-colony, colorimetric redox indicator and automated MGIT 460/960/940 methods. The turnaround time for these methods contributes to delay in clinical intervention. There are colorimetric methods that are rapid but not reliable and the

macrophage based method (fast plague assay) that can give results within two days, but due to lack of accuracy these assays are not widely used (van Deun *et al.,* 2011).

2.19. Genotypic methods

The molecular DST methods depend on the identification of resistance-conferring mutations of the bacillary genome (Abebe *et al*., 2011). These techniques do not require viable mycobacteria but always requires extraction of DNA. Genotypic DST methods create a capacity for early treatment of MDR-TB cases with them requiring less expensive infrastructure for their implementation (Huyen *et al.,* 2010).

Safety of personnel performing these methods is secured than in conventional techniques. The techniques can be easily centralised and their specificity and sensitivity for RIF which is a surrogate predictor of MDR-TB is proven to be excellent (Abebe *et al.,* 2011).

However that does not rule out the fact that even the genotypic methods have limitations. The most problematic issue with the techniques (excluding sequencing) is their limited ability to detect known mutations in a defined region and are not adequately sensitive to detect unknown mutations due to their probe design (Abebe *et al.,* 2011). Numerous commercially available molecular tests endorsed by WHO can only detect resistance to RIF and INH; although rare contamination can easily be introduced in these methods (Abebe *et al.,* 2011).

The molecular techniques to be performed can be very demanding looking at the logistic issues with reagents having to be stored and transported at specific conditions. Most of the consumables are shipped (short expiry dates) and that could be a great challenge to low income settings considering the fact that even the instrumentation used

have to be serviced on a regular basis (Abebe *et al.,* 2011). Their operation also requires well trained personnel and modern infrastructure. They are namely GeneXpert, MTBDR*plus* and Anyplex assays.

2.19.1 GeneXpert MTB/RIF

Currently PCR-based assays are used in clinical laboratories providing an opportunity for early diagnosis often as part of routine diagnostic work-up (Lawn *et al*., 2011) The GeneXpert (Cepheid, Sunnyvale, CA, USA) is an automated real time PCR system that requires minimal technical expertise and has been validated for clinical use in pulmonary and extra-pulmonary clinical samples (Lawn *et al.,* 2011). This is a diagnostic test that is rapid and can simultaneously detect TB and RIF resistance (Helb *et al.,* 2010). The assay can produce results within two hours. The WHO has endorsed the assay for the diagnosis of pulmonary TB and is an integral part of its effort to strengthen TB diagnostics in clinical laboratories across the world (WHO 2011). Several studies have demonstrated the robust diagnostic performance of GeneXpert MTB/RIF assay in patients with both pulmonary and extra-pulmonary TB (Hillemann *et al* 2011, Tortoli *et al.,* 2012 and Omnari *et al.,* 2014).

The GeneXpert MTB/RIF assay uses a molecular beacon technology that has five different nucleic acid hybridization probes that are utilized in the same multiplex reaction (Lawn *et al.,* 2011). The five overlapping molecular probes are responsible to detect *M. tuberculosis* collectively in the entire 81 base pair core region the molecular probes are each assigned or rather complementary to a particular target sequence within the *rpoB* gene of RIF-susceptible *M. tuberculosis* and it is indicated by a different colored fluorophore (Lawn *et al.,* 2011). The *M. tuberculosis* is detected when at least two from the five molecular

probes give a positive signal with a cycle threshold value of less or equal to 38 cycles (Helb *et al.,* 2010). The results are interpreted depending on the occurence or absence of *M. tuberculosis* and the presence or absence of RIF resistance and semi-qualitative estimates of the concentration of bacilli as defined by the CT range (high, <16; Medium, 16–22; low, 22–28; very low, >28). The RIF resistance is reflected as detected when a mutation in the *rpoB* gene has been detected that falls within the valid delta Ct setting, Indeterminate if the *M. tuberculosis* concentration was below detection and resistance could not be determined and not detected in a case were by there is no mutation in the *rpoB* gene. The invalid results are indicated only in a case that assays are negative for *M. tuberculosis* and for the internal control- *B. globigii* (Lawn *et al.,* 2011)

2.19.2 Line Probe Assay (MTBDR*plus*)

The assay is a hybridization based on the interactions between two complimentary strands of the double helix DNA molecule (Abebe *et al.,* 2011). Probes are bound to a strip (solid support) hence is known as DNA-strip based test that hybridize the labelled PCR amplicons. Initially labelled primers are used to amplify the targeted resistance regions of the clinical strain.

Therefore the resulting amplicon is hybridized with the immobilized probe and visualised by an enzymatic colour reaction (Crudu *et al.,* 2012). The genotype MTBDR*plus* assay detects *M. tuberculosis* and its resistance to RIF and INH drugs (Huyen *et al.,* 2010). Genotypic line probe assay was identified as one of the technologies that can offer great promise in the detection of MDR-TB and better care due to the reliability and rapidity in detecting *M. tuberculosis* complex and resistance to RIF and INH (Crudu *et al.,* 2012). Therefore there was a highlighted indication that there is a need for operational research

evaluating test performance in real world settings (WHO 2014). Initially MTBDR*plus* version one (Hain Life Science, Nehren, Germany) was evaluated and endorsed by the WHO which detected *M. tuberculosis* complex and resistance to RIF as well as INH only in culture-positive specimens and smear positive samples (Crudu *et al.,* 2012). The sensitivity in microscopy is known to be low in Mycobacteria detection and thus a substantial number of bacilli are not detected and culture methods have to be used to overcome the detection of bacteria in the smear negative samples (Crudu *et al.,* 2012). Therefore genotype MTBDR*plus* version 2 (Hain Life Science, Nehren, Germany) was designed to overcome the limitations, by detecting MTB complex and resistance to RIF and INH in smear positive, smear negative and in culture-positive samples (Crudu *et al.,* 2012).

It has been proven in multiple studies that genotypic line probe assay performs better than conventional DST (Crudu *et al.,* 2012). The accuracy of this test is evident by the short turnaround time, ease of use, the specificity and sensitivity. Several investigations indicated the accuracy of this assay in detecting RIF resistance by 91.7% to 100% and 34.6% to 94.6% in INH resistance, as well as a high range of 92% to 100% accuracy of MDR-TB detection (Parsons *et al*., 2011). The evaluation of MTBDR*plus* 2.0 was modified by improving DNA extraction methods using Genolyse solutions (Crudu *et al.,* 2012).The former soviet country (Georgia) was one of the 27 countries with high burden of MDR-TB before the use of this molecular technique and since 2008, has become one of the resource limited countries to achieve universal access to diagnosis and treatment of MDR TB (WHO 2011, Tukvadze *et al.,* 2012).

2.19.3 Anyplex™ Assay

The Seegene™ innovative Multiplex Real-time PCR method, READ (Real Amplicon Detection), assay is rapid and does not require changes in the laboratory structures or highly qualified individuals to perform and interpret results. Anyplex™ MDR-TB Screening Test an assay that is based on two technologies which are PCR related. The Dual priming oligonucleotide that optimizes PCR, provide freedom on primer design and further maximizing the PCR sensitivity and specificity by fundamentally blocking the non-specific priming (Perry *et al.,* 2014). The real amplicons detection is a new concept that installs the real high-throughput multiplexing in to real time PCR platform. Single test is completed within three hours. There are fifteen probes designed to detect for RIF resistance and six for INH resistance.

2.20. Differences and similarities of molecular assays

Molecular assays are the promising future in the TB and DR-TB diagnosis and are mostly applicable for the laboratory-based diagnosis and their accuracy on respiratory specimens is generally similar (Kontsevaya *et al.,* 2011). Most of the molecular assays are used due to several factors of easy to use, rapidity of sample preparations/test completion, infrastructure requirement and their cost efficacy. Both the MTBDR*plus* and Anyplex™ assays are designed to simultaneously detect MTB and its resistance to RIF and INH whereas the GeneXpert MTB/RIF is only designed to diagnose TB and resistance to only one first line drug, RIF. The turnaround times vary greatly as in GeneXpert results are expected within 2 hours, in Anyplex assay is 3.5 hours and the MTBDR*plus* assays can only take 2 days. The MTBD*Rplus* assay has particular probes that pick different mutations both in RIF and INH resistant strains and that also applies in the Anyplex™ assay. The GeneXpert technology uses molecular beacons to detect various RIF-

resistant conferring mutations. The differences and similarities are summarised in table 2.1 below.

Table 2.1: The *rpoB* mutations detected by various diagnostic tools.

Anyplex(15 mutations,1 region)		MTBDR*plus* (4 mutations)		GeneXpert (5regions/probes)
L511P	H526Y	WT 1/F505L /T508A/S509T	WT 7/H526Y (MUT2A)H 526D (MUT 2B)/H526R/ H526P/H52 6Q H526N/H52 6L H526S/H52 6C	PROBE A 507-513 (WT1-3) PROBE B 513-518 (WT3-5) PROBE C (WT4-6) 518-522 PROBE D (WT7-8) 526-531 PROBE E (WT 8) 531-533
Q513L	S531L S531W	WT2 L511P		
D516G D516V D516Y	L533P	WT3 Q513L/Q513P/ 514-516		
S522L	513-516	WT3/4,D516V(MUT1)/D516Y/ del 515	WT 8 S531L (MUT3) S531 Q S531W L533P	
H526D H526L H526N H526Y H526R		WT 4/5 del 518 N518I/WT 5/6 S522L S522Q		

The elaborations of various diagnostic tools that can detect mutations resulting in RIF resistance.

2.21. Spoligotyping

Genotyping is the most useful tool in the understanding of the existing TB genotypes and their circulation within a particular population (Miranda *et al.,* 2011). Spoligotyping has been internationally accepted as a rapid discriminatory test for *M. tuberculosis* complex creation, containing a global distribution and phylogenetic analysis for the world-wide spoligotyping (Miranda *et al*., 2011). *M. tuberculosis* complex characterization can be performed by the application of spoligotyping as it is based on a polymorphism in the chromosomal direct repeat locus (Pang *et al.,* 2012). Molecular epidemiological application has indicated certain *M. tuberculosis* strains, which induce severe forms of TB and have shown higher treatment failures/relapse (Miranda *et al.,* 2011).

Spoligotyping is a PCR based technique that detects and type the M>tuberculosis simultaneously on the difference in the direct repeat locus of bacilli, occurring by one of three mechanisms-homologous recombination between neighbouring or distant direct variable repeats, IS-mediated transposition, and DNA replication slippage (Pang *et al.,* 2012). The method is useful for tracking TB epidemics, determining new outbreaks, and selectively documenting high-risk populations to focus prevention strategies (Pang *et al.,* 2012). Spoligotyping can be regarded to constitute a potential tool for global TB epidemiology, population genetics, and phylogeny, although it cannot be depended on without another independent genotyping method in many settings to prove clonality (Pang *et al.,* 2012).

2.22. Sequencing

Sequencing of the DNA visualizes the complete nucleotide sequence of the target DNA thus referred to as the reference molecular method. This is the most reliable and direct assays for studying mutations and permits detection of both previously recognised and unrecognised mutation. The performance of sequencing requires expertise and it is costly, which makes it difficult to perform in routine laboratory diagnosis especially in developing countries, where less complex cost effective DST is needed. Therefore, sequencing is not easily applicable for routine use in identifying drug resistant mutations as it is in identification of mycobacteria species (Abebe *et al.,* 2011).

The mutations that could be recognised by sequencing may be associated to drug resistance or while other mutations could be silent or just represent DNA alterations not related to drug resistance (Abebe *et al.,* 2011).

CHAPTER 3

3 PURPOSE OF STUDY

Limpopo province is basically rural in setting and has received little attention from researchers. The dearth of information on the circulating resistant *M. tuberculosis* strains in the province may constitute a clinical problem in the treatment and management of TB and to the national anti-TB programme. This study used the most recent technique to detect known and unknown drug resistant *M. tuberculosis* mutations in the province. The availability of this information will greatly enhance the knowledge of clinicians and help in treatment and or management of TB and its drug resistant strains in the Province.

3.1 AIM

To determine drug-resistant *Mycobacterium tuberculosis* mutations using Anyplex™ MTB/NTM/MDR-TB real time assay and characterise the drug-resistant strains.

3.2 OBJECTIVES

- To screen for RIF and INH resistant conferring mutations using Anyplex™ plus (Seegene Incorporated) and GenoType® MTBDR*plus* 2.0 (Hain life Science) assays.
- To determine the sensitivity and specificity of molecular techniques in detection of RIF, INH and MDR-TB.
- To perform DNA sequencing on sample of interest and compare generated sequenced data with reference strains from GenBank
- To identify circulating TB families in detected MDR-TB samples using Spoligotyping.

CHAPTER 4

4 RESEARCH METHODOLOGY

4.1 Ethical Considerations

The ethics approval was obtained from the Medunsa Research and Ethics Committee (MREC) (MREC/HS/89/2013: PG). The department of Health was contacted for approval before the study commenced. The NHLS has a solid agreement with the hospital for performance of a series of investigations that include research that assists with quantification of the findings in modifying the laboratory methods which do not require patient identity. Should there have been any situation that required a patient to be identified then the researcher and NHLS would have engaged in a binding agreement for confidentiality purposes. Culture isolates stored at 4 degrees were selected. The samples formed part of the FIND project that was previously approved by the Research, Ethics and Publication Committee of Medical Research Council. There was no need to draw blood from the patients. No information by which samples could be traced back to patients was released or published. The samples collected at Modimolle MDR hospital were given for research purpose, no consent was required.

4.2. Research design

This was a diagnostic evaluation study of novel Anyplex assays for the detection of TB, RIF-R, INH-R and MDR-TB. The Anyplex assays performance was compared with respective “gold standard’’ in both prospective and retrospective phases of the study.

4.3 Sample size

According to the NHLS and ETR.net data, during 2011, 1114 tuberculosis case were reported and among them 126 were MDR cases. Thus the prevalence of MDR may be estimated to be 126/1114 (or 11.3%).

$$N = \frac{z^2 p (1-p)}{d^2}$$ (Naing *et al,* 2006)

N= [(1.96)2 (0.113) (1-0.113)]/ (0.05)2

N = 157

The present study needed a minimum of 157 sputum samples.

4.4 Prospective collection

4.4.1. Sputum collection

A total of 204 sputum samples were collected from TB patients attending Modimolle MDR-TB reference hospital in Limpopo Province. The samples were collected from patients as a third container following the samples collected upon admission. The samples were transported to South African Medical Research Council (SAMRC) TB laboratory in Pretoria in ambient temperature were and upon arrival, were processed according to standardised standard operating procedures (SOPs). The quality and quantity of the samples was analysed and recorded. The sputum samples with a greater or equal to 3ml and of good quality were used (i.e. mucoid, purulent and or muco-purulent). The decontaminated samples were then used to inoculate the LJ for future references. Therefore DNA was extracted using the genolyse kit from Hain Life Sciences and stored in -20^{o} C until required for use. The GenoType® MTBDR*plus* assay was used as a gold standard for TB,

RIF and INH resistance detection. Screening for *M. tuberculosis* organism was done with MTBDR*plus*, and upon a positive diagnosis; Anyplex™ MTB/NTM/MDR-TB assays were performed. Spoligotyping was performed on selected few samples with Anyplex™ assay resistance patterns. Below in figure 4.1 is a detailed specimen flow:

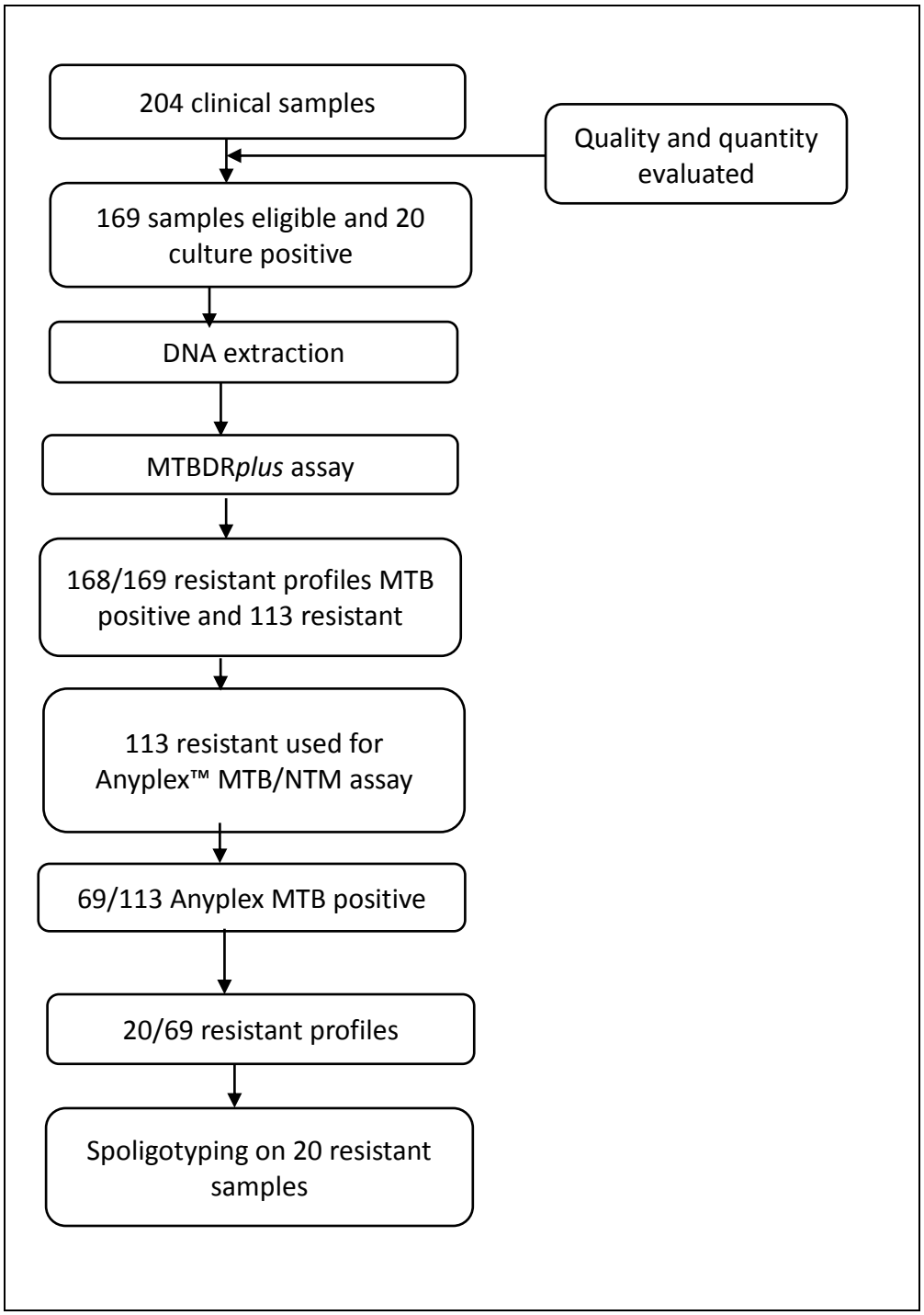


Figure4.1. Overview of the procedures done in the processing of clinical samples. Of the 204 clinical samples only 169 samples were found to be eligible for study.

4.4.2 **Decontamination procedure**.

The clinical samples were selected to be eligible for the study if the quality (saliva and watery ruled out) of the sputum and quantity (less than 3ml excluded) were satisfactory without any leakage during transportation. Sputum samples were collected and decontaminated in BSL3 laboratory under a bio-safety cabinet.

The samples were decontaminated using the BBL Mycroprep kit (BD diagnostics system, sparks, MD) performed according to manufacturer s instructions. Briefly the BBL™phosphate buffer was prepared by pouring the provided contents of one packet into a 500ml volumetric flask and filled up to the line with purified water. Then the solution was transferred into a screw cap bottle and autoclaved at 121 ° C for 15 minutes. The Mycroprep reagent bottle had an ampule (containing NALC) within its container that had to be broken and mixed to resuspend the NALC. Equal amounts of both sputum sample and solution from the Mycroprep reagent bottle were put into a sterile 50ml tube (sigm-aldrich) and vortexed to assure that specimen was liquefied. Mixture was left at room temperature for 15 minutes with occasional shaking. The prepared Phosphate buffer was added up to the 50ml mark and centrifuged at 3000xg for 20 minutes. The supernatant was discarded and 0.8ml of phosphate buffer was added to re-suspend the pellet which was then stored at -20°C until further processing with MTBDR*plus* and Anyplex™ MTB/NTM assays.

4.4.3 **Culture (LJ)**

Lowenstein Jensen media was inoculated using the decontaminated sputum. A sterile loop was used to inoculate and streak down the media with the decontaminated sputum. Incubate at 37°C for 4 to 6 weeks with constant monitoring.

4.4.4 DNA extraction directly from clinical samples.

The DNA was extracted by Genolyse (Hain Life Science, Germany) and performed according to the manufacturer's instructions. A volume of 500µl of decontaminated sputum was centrifuged at 10000 rpms for 15 minutes and supernatant discarded. Then the pellet was resusopended by 100µl of Alcalic-lysis buffer solution and incubated at 95 ° C for 5 minutes. Thereafter 100µl of neutralization buffer was added to lysate, vortexed and centrifuged at 13000rpms for five minutes. The supernatant of 50 µl was transferred to a new 1.5 micro centrifuge tube and stored at -20° C until further processing.

4.4.5 GenoType® MTBDR*plus* Assay.

Principle of the assay

The Line Probe Assay (MTBDR*plus* version 2) is a DNA strip based test with increased sensitivity (100 cells per millilitre) based on three stages the DNA extraction (i.e. which can be performed on pulmonary patient specimen and the cultured material), the multiplex amplification with the primers that are biotinylated and the reverse hybridization. The polymerase and primers are included in the amplification mixtures A and B and are optimized for this procedure. There are specific complimentary DNA strands that are incorporated into the membrane strip. The probes reliably discriminate several sequence variations in the gene regions excluded. The alkaline phosphate is streptavudin-conjugated thereby allowing the binding to the amplicons biotin through the streptavidin moiety. The substrate added is transformed into a dye by the alkaline phosphate which becomes visible on the membrane strips as a colored precipitate. This procedure was performed in a unilateral flow which was performed in a PCR laboratory (sequence of movement)

Master Mix preparation and DNA amplification

The master mix reagents were already provided by the manufacturer (Hain Life Science, Germany). Each tube contained 45µl (35µl of the amplification mix B and 10 µl of the amplification mix A) of master mix and the volumes were calculated according to the number of samples to be amplified. A volume of 45µl was transferred into each tube and transported to the DNA addition room. In the negative control tube 5µl of distilled water was added and 5 µl of H37Rv was added to the positive control tube. In each 45µl of master mix there was amplification mix A that contains the nucleotides and taq polymerase and the amplification mix B the buffer as well as the additives. Only 5 µl of the DNA template was added to make a final volume of 50 µl in each tube except the negative control.

Then the tubes were subjected to amplification in the PCR room by the use of a thermo cycler (Vereti, Life Technologies) with specific protocols loaded on the machine. The amplification process in table 4.1 included the following steps below.

Table 4.1: Amplification conditions

PCR programme	Clinical specimens(50 cycles)	Culture samples(30 cycles)
15 minutes at 95ºC	1 cycle	1 cycle
30 seconds at 95ºC 2minutes at 65ºC	20 cycles	10 cycles
25 seconds at 95ºC 40 seconds at 50ºC 40 seconds at 70ºC	30 cycles	20 cycles
8 minutes at 70ºC	1 cycle	1 cycle

Reverse hybridization

Reverse hybridisation was done on the automated GT blot machine (Hain Life Science, Germany). Initially 20µl of both amplicons and denaturation solution were added manually at corner of the well, mixed and incubated for 5 minutes at room temperature to allow for chemical denaturation. The strips were labelled in correspondence with the samples while the double stranded DNA was separated into two single stranded DNA. Then the tray was loaded into the GT blot (Hain Life science, Germany) machine and subjected to the relevant protocol below.

The machine added 1ml of hybridization buffer (green in colour) with constant shaking to maximise the spread and maintain a homogenous colour and the labelled strips were then loaded into the corresponding

sample and incubated for 30 minutes at 45 ºC allowing the single stranded DNA to bind into the complimentary probes on the strip. The hybridization buffer in the tray was then aspirated and the non-specific base pairs were washed away by addition of 1ml of stringent wash solution (red in colour) to each well containing a strip and incubated for 15 minutes at 45 ºC with constant shaking. The stringent wash solution (clear solution) was then poured out from the GT blot completely and strips were rinsed with 1ml of rinse solution at room temperature for 1 minute with a continuous shaking. After 1 minute the rinse solution was aspirated and 1ml of conjugate (orange in colour) was added and incubated for 30 minutes with shaking. Following the conjugate aspiration 1ml of Rinse solution was added again to wash the strip twice removing the unbound conjugate. A volume of 1ml of distilled water was added following the rinse solution aspiration to stop the reaction. The water was aspirated after the incubation period of 1minute at room temperature. The substrate (brownish in colour) with a volume of 1ml was added to bind to the conjugate and was incubated for 15 minutes away from light. Distilled water was used to stop the reaction and therefore the reading card was used to interpret the results. Master Mix controls, negative control as well as the positive control were included in every run. The results were interpreted according to manufacturer's protocol.

4.4.6 Anyplex™ Assays

Principle of the assays

The Anyplex™ plus MTB/NTM/MDR-TB Detection (Seegene Incorporated, Korea) is a multiplex real-time PCR assay that permits the detection of *M. tuberculosis* and non-tuberculosis mycobacteria

(NTM), and the simultaneous amplification and detection of RIF and INH-resistance.

The Anyplex is based on PCR; the amplified products are detected by monitoring the fluorescence intensities during the cycles of PCR. There are two outstanding PCR related technologies which are Dual Priming Oligonucleotide (DPO) technology that allows the freedom in designing primers and PCR optimization while maximizing/optimizing PCR sensitivity and specificity by blocking non-specific priming fundamentally and the Real Amplicons Detection (READ) technology which is the new concept of methodology that allows real high-throughput multiplexing onto a real-time PCR platform. The assay was performed according to the package insert compiled by the manufacturer.

Mastermix preparation and Real Time PCR (RT-PCR)

The Anyplex™ MTB/NTM procedure was performed according to the manufacturer (Seegene Inc, Korea). According to the number of reactions then a master mix reaction tube was prepared. In each tube 15 µl of the master mix there was 2µl of 10x MTB/NTM OM, 3 µl of RNase-free water and 10 µl of 2x Anyplex PCR Master mix (with Uracil-DNA Glycosylase-UDG).There were specific tubes used for this procedure with caps. Therefore the tubes were taken to the DNA addition room whereby 5 µl of the template DNA was added to a final volume of 20 µl in each tube.

The protocol was carried out with provided controls every time it was performed The tubes were loaded into the CFX96 Real time PCR system (Bio-Rad, Hercules, California U.S) and results were generated within two hours. The temperatures for spoligotyping amplification process are briefly described in table 4.2.Results were interpreted

according to manufacturer's instructions. The interpretation of results is presented as MTB, NTM, invalid and not detected (negative). Below in figures 4.2a, b and table 4.3 are possible resulting cases.

Table 4 .2: The thermal profile for PCR is defined according to four stages

segment	No of cycles	Temperature ºC	Duration
1	1	50 ºC	5 minutes
2	1	95ºC	15 minutes
3	45	95ºC	30 seconds
		60ºC	1 minute
4			

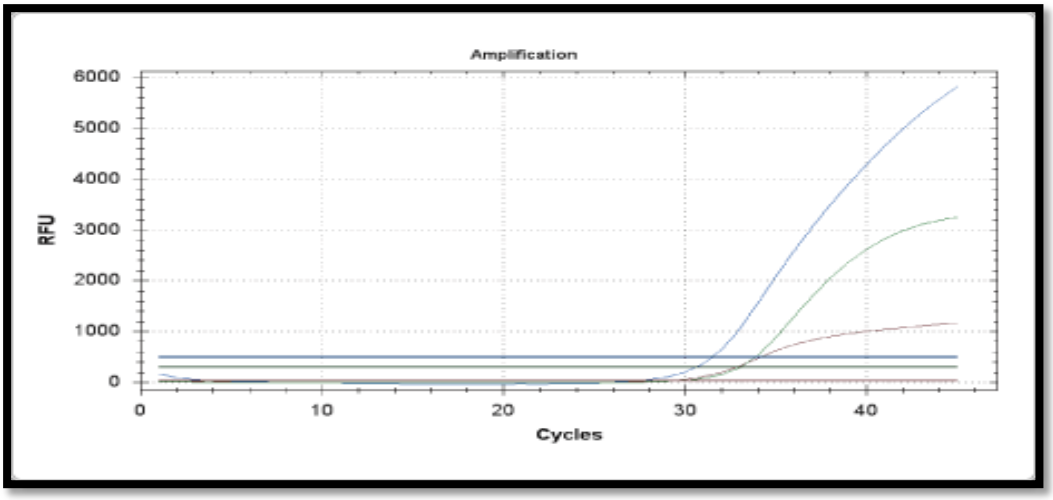


Figure 4.2a

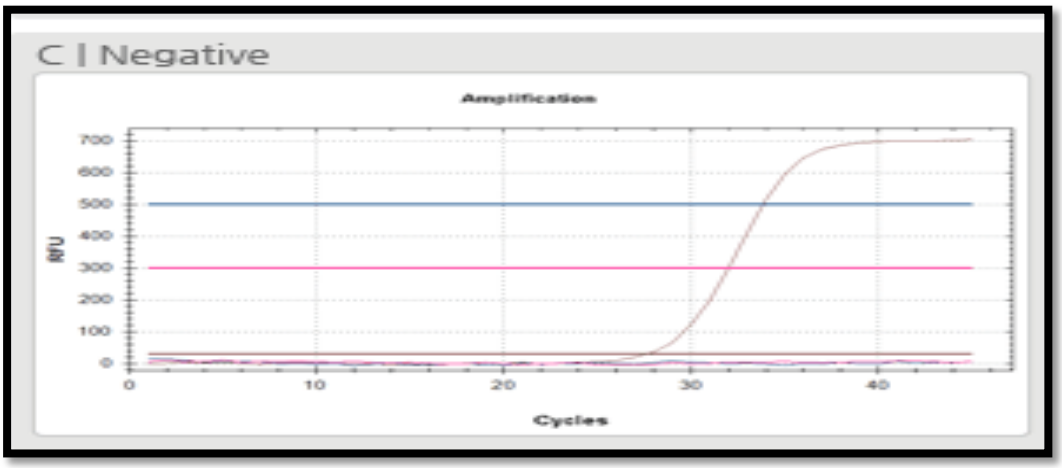


Figure 4.2b

Figure 4.2. A-Illustrations on the CT value of the positive results (4.2a) and B represent an illustration of negative results (4.2b).

Table 4.3: Results interpretation for the Anyplex™ MTB/NTM assay.

Case	RESULTS			Interpretation
	Internal control(quasar 670)	MTB(fam)	Mycobacterium (hex)	
1	Positive	+	+	MTB
2		+	_	MTB
3		_	+	NTM
4		_	_	Not-detected
5	Negative	+	+	MTB
6		+	_	Invalid
7		_	+	Invalid
8		_	_	Invalid

The (-) sign indicates negative and (+) means positive

Anyplex™ MDR Assay

The performance of this procedure was only done on the samples that were positive for MTB by the Anyplex™ MBT/NTM assay. The Anyplex™ MDR assay was performed according to the manufacturer (Seegene inc, Korea)

Master Mix preparation and RT PCR

The master mix was prepared using the number of MTB positives detected by Anyplex™ MBT/NTM and in each tube there was 4µl of the 5xMDR-tb plus DOM, 10µl of 2x detection mix and 5µl of RNase-free water. The final volume of the reagents was 19 and only one micro litre of a positive sample from the MTB/NTM PCR product was added as

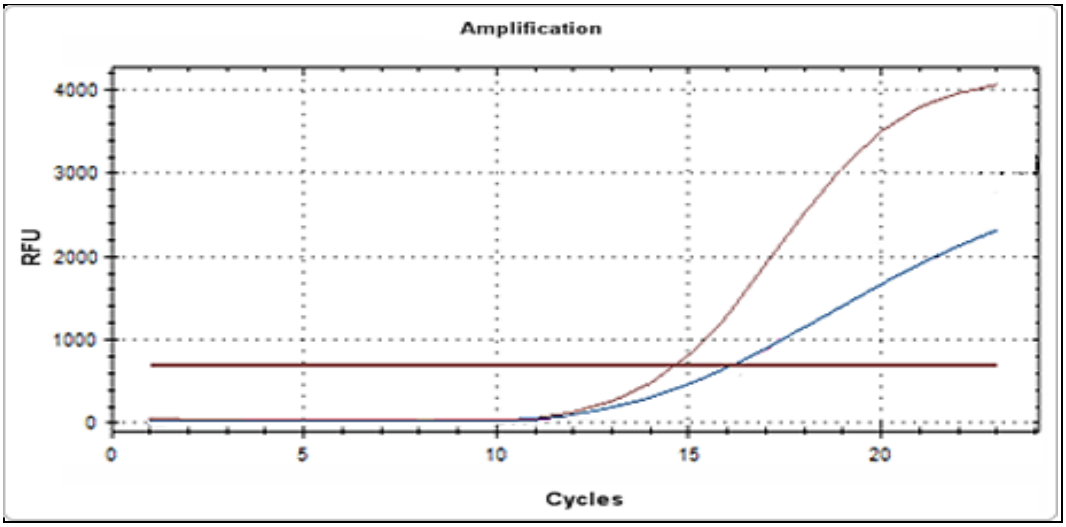
the amplicons. The PCR protocol had the following stages described in table 4.4 and the fluorescence in this protocol was detected at 75 ºC.

Table 4.4: Anyplex MDR TB amplification protocol

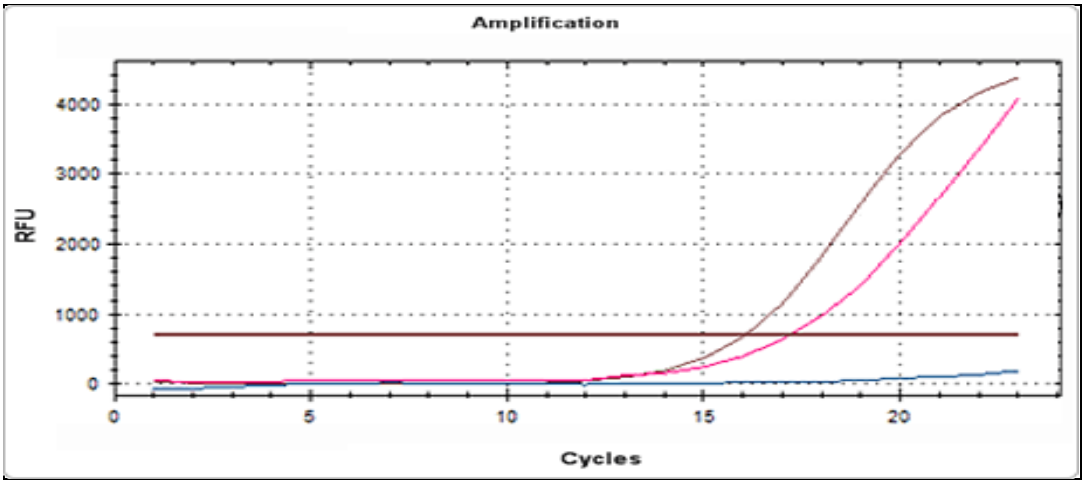
Segment	N0 of cycles	Temperature	Duration
1	1	95 ºC	2minutes
2	23	95 ºC	20 seconds
3	23	64 ºC	30 seconds
4	23	75ºC	10 seconds

Results Interpretation

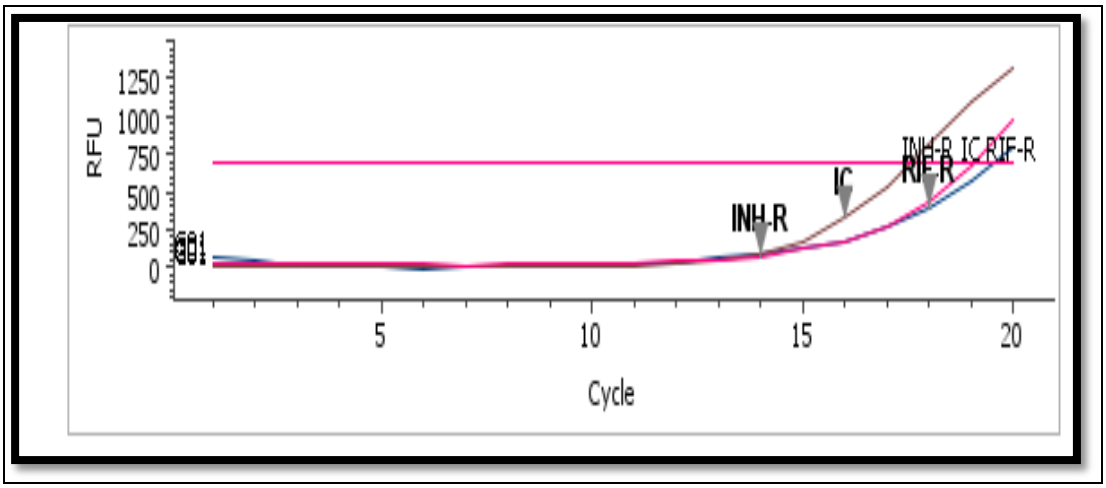
There are fluorophores that are assigned for different analytes, the Anyplex™ MDR assay is a based on the detection of resistance to two most effective first line drugs namely the Rif and INH, therefore the fluorophore assigned for Rif and INH resistance are FAM, and CAL RED 610, respectively and a fluorophore assigned for internal control is known as the Quasar 670. The CT value for all these fluorophores are less or equal to twenty and anything more or nothing is regarded as negative. The illustrations of RIF, INH and MDR positives are elaborated on figures 4.3 a, b and c respectively. The results are further interpreted briefly on table 4.5.



4.3. a Anyplex RIF resistance



4.3. b Anyplex INH resistance



4.3. c Anyplex MDR case

Figure 4.3: The illustrations above are demonstrations of RIF resistant strain (4.4 a), INH resistant (4.4b) and MDR strain (4.4 c) analysed by the CFX 96 instrument.

Table 4. 5: Anyplex MDR assay results interpretation

CASE	RESULTS			INTERPRETATION
	Internal control(QUASAR 670)	RIF-R(FAM)	INH-R(CAL RED 610)	
1	Positive	+	+	MDR(INH-R&RIF-R)
2		+	_	RIF-R
3		_	+	INH-R
4		_	_	NOT DETECTED
5	Negative	+	+	NDR(INH-R&RIF-R)
6		+	_	INVALID
7		_	+	INVALID
8		_	_	INVALID

4.4.7 Spoligotyping.

Master Mix preparation

The chromosomal DNA of *M .tuberculosis* strain H37Rv and *M. bovis* BCG P3 were included as positive controls and water used as a negative control. The reaction mixture was prepared (2µl template DNA extracted as above in section 4.8.4., 2µl of both DRa and DRb (0.2µmol/µl) 6.5 µl water and 12.50µl of ready mix) The tubes with a total volume of 25µl were then placed in the thermo cycler subjected to the protocol on table 4.6 for amplification.

Table 4.6: Amplification protocol

Period	Temperature	Cycles
3minutes	95ºC	1 cycle
1minute	94ºC	30 cycles
1 minute	55ºC	
30 seconds	72ºC	
10 minutes	72ºC	1 cycle

Hybridization

There are immobilized spacer oligos on the membrane that represent spacers of known sequence. The biotin labelled PCR products are hybridized to the immobilized spacers on the membrane. The presence of spacers is then visualized on a film as black squares after incubation with streptavidin–peroxidase and ECL-detection.

Procedure

The buffers (2xSaline Sodium Phosphate EDTA/0.1% Sodium dodecyl sulphate at 42ºC, 2xSSPE/0.5 %,SDS at 60ºC, 2xSSPE/0,5% at 42ºc and 2xSSPE, at room temperature) were all pre warmed to desired temperatures before use. A volume of 25µl of PCR product was added to 150µl of 2xSSPE/0.1 % SDS and heat denatured at 99ºC for 10 minutes. Then was cooled on ice immediately. The membrane was washed for 5 minutes at 60ºC using 250ml of 2xSSPE/0.1% SDS.

The membrane together with the support cushion was placed on the miniblotter in such a way that slots are perpendicular to the line pattern of applied oligonucleotides. Residual fluids were removed from the

miniblotter by aspiration. The slots were filled with diluted samples avoiding bubble formation and then hybridized for 1 hour at 60ºC on a horizontal surface without shaking. The samples were removed aseptically avoiding contamination by aspiration and the membrane was removed using forceps. The membrane was washed twice in 250 ml of 2xSSDE/ 0.5% SDS for 10 minutes at 60 ºC. The membrane was then placed in a rolling bottle and left to cool. Total of 7, 5µl of streptavidin-poxidase conjugate (500U/ml) was added to 10ml of 2xSSDE0.5% SDS. The solution was mixed and poured into the rolling bottle; the membrane was incubated in that solution for 50 minutes at 42 ºC.A volume of 250 ml of 2xSSPE0.5% was used to wash the membrane twice 10 minutes at 42 ºC. The membrane was rinsed twice with 250ml of 2xSSPE for 5 minutes at room temperature.

The following steps were performed in the dark room away from light. Then for the chemiluminiscent detection of hybridizing DNA, the membrane was incubated for 1 minute in 20ml ECL (10ml reagent 1 and 10 ml reagent 2) detection liquid. The membrane was covered with a transparent plastic sheet and exposed a light sensitive film to the membrane for 1 minute. The strength of the signal was adjusted accordingly, if too week or strong the time was adjusted using a different film for an extended or reduced time.

4.5 Retrospective collection

The retrospective phase of the study used a total of 104 culture positive specimens stored at the SAMRC-TB Biobank. The isolates were collected in four major provinces of South Africa namely Gauteng, KwaZulu-Natal, Western Cape and Northern Cape from a previous study conducted in 2008. The phenotypic resistant patterns to RIF and INH were already available for this population.

4.5.1 Culture Selection

A total of 104 culture positive specimens with known resistance to RIF and INH drugs stored at SAMRC TB laboratory Biobank were used in this phase of the study. The samples were collected from four provinces in South Africa namely Gauteng, Western Cape, Kwazulu-Natal and Northern Cape already processed for MGIT DST and MTBDR*plus* version 1 with their DST results stored in a database. DNA was extracted and used as a template in performing both MTBDR*plus* (version 2) and Anyplex ™MTB/NTM/MDR-TB assay. In case of *M. tuberculosis* organism detection by Anyplex™ MTB/NTM assay, a further analysis by Anyplex™ MDR-TB was performed according to manufacturer's protocol. The detection of RIF and INH susceptibilities in both MTBDR*plus* and Anyplex™ assays was compared with the gold standard MGIT DST; In case of discordance between MGIT DST and MTBDR*plus*, Anyplex™ MDR-TB assay was used to resolve some disagreements. However in the case unresolved disagreement further analysis by sequencing was done. Below is a detailed specimen flow:

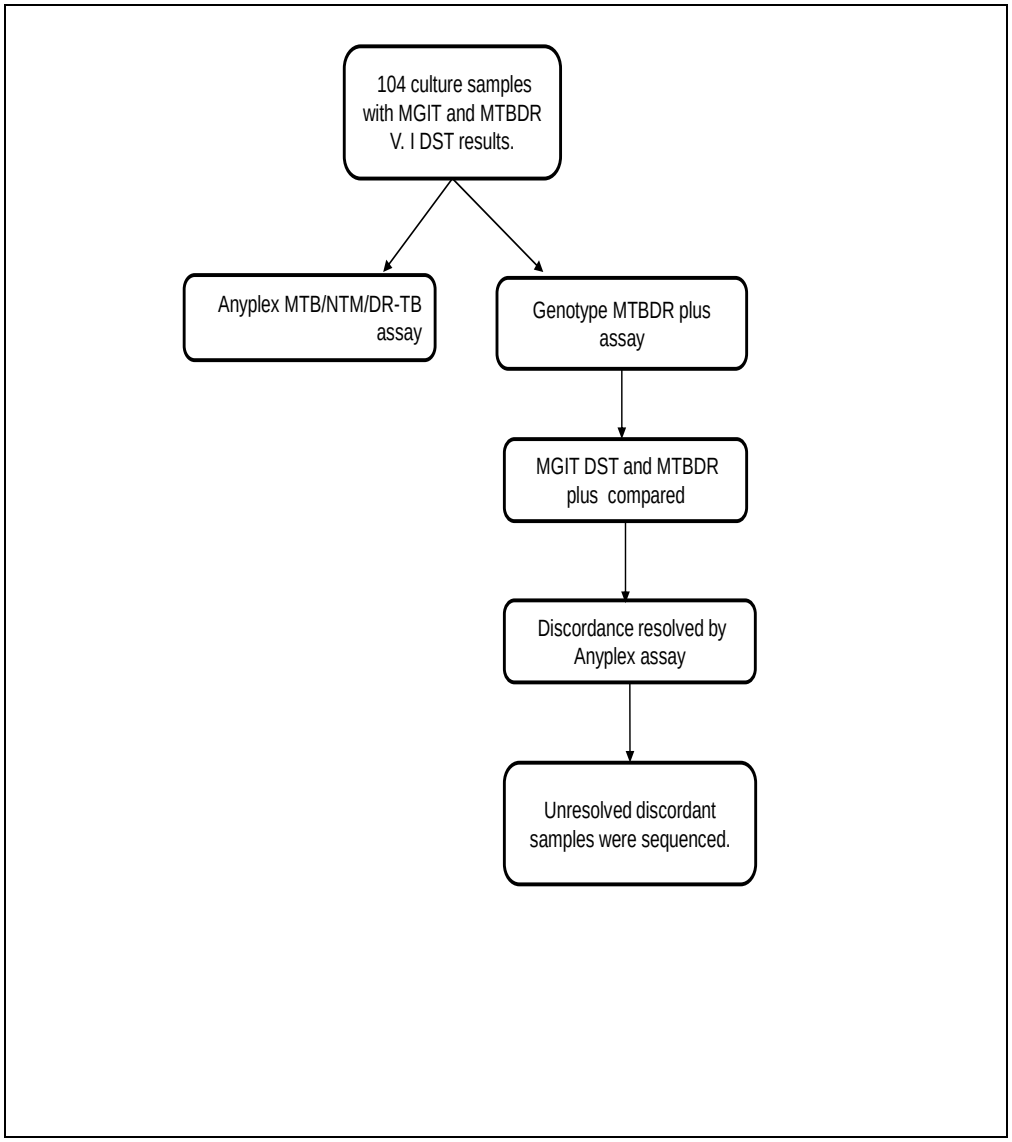


Figure 4.4: The flowchart of laboratory methods performed on the culture samples.

4.5.2 DNA extraction for culture isolates

A volume of 1000µl of culture broth was centrifuged at 10000 rpms for 15 minutes and supernatant discarded. Then the pellet was resuspended by 100µl of distilled water and incubated at 95 ° C for 20 minutes. Thereafter it was sonicated for 15 minutes in a waterbath. After sonication the tube was vortexed and centrifuged at 13000rpms

for five minutes. The supernatant of 50 µl was transferred to a new 1.5 micro centrifuge tube and stored at -20° C until further processing.

4.5.3 Molecular assays

Genotype MTBDRplus

The assay was performed and results analysed according to manufacturer's instruction as described above in section 4.4.5.

Anyplex assays

The novel multiplex technique, the Anyplex MTB/NTM/MDR assay was performed as described above (4.4.6). The results were analysed according to the manufacturer s instructions described in section 4.4.6.

4.5.4 Conventional PCR assay

Amplification of *rpoB, katG* and *inhA* genes was performed on all samples discordant between MGIT, MTBDR*plus* and Anyplex™ MDR-TB. A protocol previously described by Campbell *et al.,* 2010 was used. Genes associated with RIF and INH mechanisms of resistance were targeted using primers in table 4.7 to determine genetic bases of resistance.

Table 4.7 The used primers

Region	Forward/reverse	size	Primer
rpoB	Reverse	20	ATCTCGTCGCTAACCACGCC
	Forward	20	CTTGCACGAGGGTCAGACCA
katG	Reverse	20	GCGAACTCGTCGGCCAATTC
	Forward	20	AACGACGTCGAAACAGCGGC
InhA	Reverse	20	ATGAGGAATGCGTCCGCGGA
	Forward	20	TGCCCAGAAAGGGATCCGTCATG

Master Mix preparation

The master mix for each reaction tube was to a final volume of 25 and it included, 12.5 micro litres of the hotstart *Taq* master mix (Qiagen, Benelux B.V), 3µl of 5µM primers (1, 5µl for both forward and reverse), 8.5µl of distilled water and 1µl of DNA template.

DNA amplification

The amplification program was performed as follows: The initial denaturation stage for fifteen minutes at 95ºC, followed by 35 cycles of denaturation for 30 seconds at 95 ºC, with annealing stage at 60 ºC for thirty seconds and followed by elongation for 30 seconds at 72 ºC, with final elongation period of seven minutes at 72 ºC. The primers used (table 4.7) were all designed by Inqaba Biotechnical Industries Pretoria, South Africa.

Detection of PCR products

The amplified products were analysed on a 2% agarose gel stored with ethidium bromide was used to allow band visualisation and verified PCR products were used as templates DNA for sequencing. After electrophoresis the DNA bands can be read directly from the positions of the bands in the gel using a molecular weight marker.

4.5.5 Sanger Sequencing

The PCR positive samples were sequenced at Inqaba biotechnological industries (pty) ltd Pretoria using ABI 3130xi genetic analysers. The sequencing assay was based on the principle that single-stranded DNA molecules that differ in length even by single nucleotide can be separated from each other using polyacrylamide gel electrophoresis (Sanger et al 1977). Sequenced data was analysed with bioinformatics software (Clustal W, Bioedit).

4.6 Data analysis

The data was analysed using the Epi Info version 3.5 (percentages and frequencies) were used to explain proportions. Initially a set of variables were defined and entered as data to create a number of cases. Therefore each case was defined by values stated in the variable. Following analysis the programme presented variables in the form of suitable charts.

The researcher was responsible for the interpretation of the results. Microsoft excel, excess and Epi info (95% confidence interval) were also used for comparative analysis of the phenotypic and molecular techniques. The sequenced samples were analysed depending on the program version used by Inqaba Diagnostics and therefore Chromaspro (version 1.45) was used in the analysis of the sequencing results. The generated data from the spoligotyping was analysed by SpolBD4 and SITVITWEB database.

4.7 Significance of the study

The study will inform us about the circulating *M. tuberculosis* strain families in the Limpopo Province.

CHAPTER 5

5 RESULTS

5.1 Detection of *M. tuberculosis*, rifampicin and isoniazid resistance on clinical isolates

Of 204 clinical specimens collected, 169/204 (83%) were eligible for this phase of the study based on the quantity and quality scores. The 169 samples cultured on LJ for *M. tuberculosis* growth 136/169 (80%) of the samples failed to grow, while 13/169 (8%) were contaminated (i.e. cloudy media) and 20/169 (12 %) had positive growth identified as *M. tuberculosis* based on morphological characteristics (cream to golden colour, buffy, rough and tough granular colonies). On parallel 169 samples were DNA extracted and screened for *M. tuberculosis,* RIF, INH and MDR-TB resistance. GenoType® MTBDR*plus* assay detected 168/169 (99%), 11/168 (7%), 4/168 (2%) and 98/168 (58%) samples for *M. tuberculosis*, RIF-mono, INH-mono, and MDR-TB, respectively. Upon a positive *M. tuberculosis* resistant case detected by MTBDR*plus* both Anyplex™ MTB/NTM and Anyplex™ MDR-TB were performed. The Anyplex™ MTB/NTM assay detected 69/111(62%) cases of *M. tuberculosis* and while Anyplex™ MDR-TB detected 16/69 (23%), 2/69 (3%) and 2/69 (3%) cases of RIF, INH, MDR-TB, respectively (A detailed flow diagram is attached below, figure 4.1). The proportion of *M. tuberculosis*, RIF, INH, and MDR-TB detected by Anyplex™ MTB/NTM and Anyplex™ MDR-TB in relation to MTBDR*plus* was determined (Tables 5.1, 5.2, 5.3). Furthermore 20 cases detected by Anyplex™ MDR-TB were assigned into families using spoligotyping assay (Kamerbeek *et al,.* 1997) and data was analysed using SPOLDB4 database (Demay *et al.,* 2012).

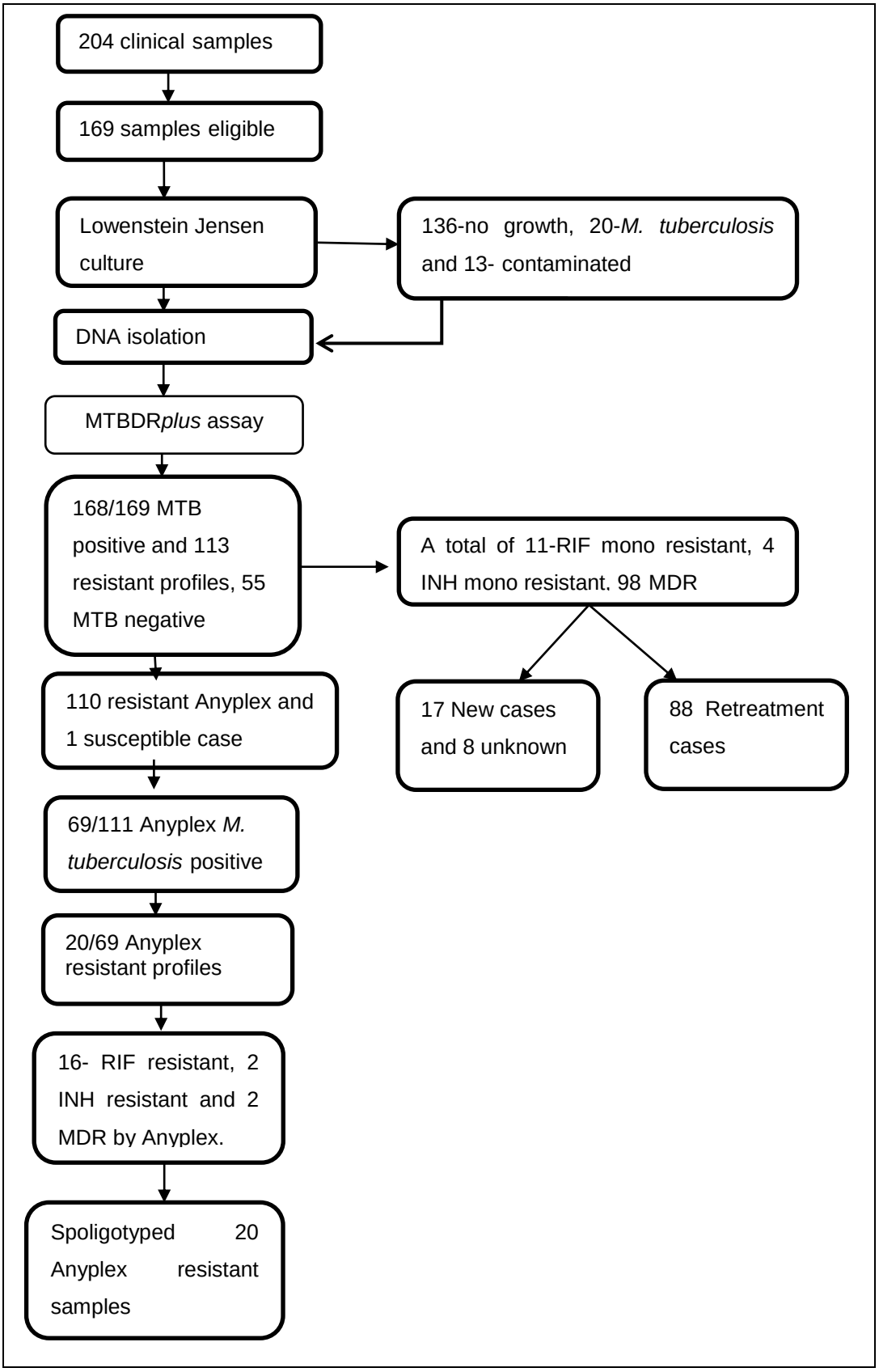


Figure 5.1: Laboratory processing on clinical samples.

5.1.1 The *M. tuberculosis* detection

The GenoType® MTBDR*plus* assay was able to give almost 100% valid results. It detected *M. tuberculosis* strains in 168/169 (99%) with only one negative test. The results interpretation for *M. tuberculosis* detection was done according to manufacturer's instruction. The Anyplex™ MTB/NTM assay was able to detect *M. tuberculosis* in 69/ 111 (62 %) samples with 100% interpretable results. The remaining 39/111 (38%) samples were found to be negative and three were invalid even after being repeated twice. Below in table 5.1 are a summary of TB detected using both molecular assays on clinical specimens.

Table 5.1: The *M. tuberculosis* case detection from patient sputum samples

M. tuberculosis detection	
MTBDR*plus* result	
MTB+	168
MTB-	1
Total	169
Anyplex™ MTB/NTM result	
MTB+	69
NTM	0
Negative	39
Invalids	3
Total	111 [b]

[b] only the MTBDR*plus* resistant profiles were analysed. The MTBDR*plus* assay detected TB in 168/ 169 (95%) samples and Anyplex assay detected 69/113 (60%) of MTBDR*plus* resistant profiles.

5.1.2 Rifampicin resistance detection

The case detection of RIF resistance was done on both GenoType® MTBDR*plus* and the novel Anyplex™ MDR-TB assays. The RIF resistance detection is done by presence of probes complementary DNA sequences found on MTBDR*plus* and Anyplex™ MDR-TB assay. Initially MTBDR*plus* assay was used in this phase to determine the proportion of RIF resistance detected by presence of mutations within *rpoB* gene 11/168 (7%) and 98/168 (58%) sample were resistant to RIF *in* combination to INH resistant as well. The Anyplex™ MDR-TB detection assay was performed on the samples that were *M tuberculosis* positive on Anyplex™ MTB/NTM assay. The probes in this assay are designed to detect fifteen mutations using multiplexing technology associated with RIF resistance in the *rpoB* gene. RIF resistance detection performed on 69 *M. tuberculosis* positive samples, 16/69 (23 %) were detected with *rpoB* mutations.

Table 5.2: Rifampicin resistance case detection

RIF resistant case detection	
MTBDR*plus* results	
RIF resistant	109
RIF susceptible	4
Total	113
Anyplex™ MDR results	
RIF resistant	16
RIF susceptible	51
Invalid	3
Total	69 [b]

[b] only positive by Anyplex MTB/NTM assay. Of the 113 samples only 109 were found to be RIF resistance using the MTBDR*plus* assay. Anyplex assay detected 16 of 69 tested for RIF resistance.

5.1.3 Isoniazid resistance case detection

Mutations within *inhA* and *katG* regions of *M. tuberculosis* genome are used to detect INH resistance. The GenoType® MTBDR*plus* assay also targets the same genes and in this study to detected four samples with INH mutations that were resistant to isoniazid, The novel Anyplex™ MDR assay with six mutations profile, detected in two samples. Below in table 5.3 is a summary of results.

Table 5.3: Isoniazid resistance case detection

INH	MTBDR*plus*	Anyplex™ MDR
Resistant	102	4
Susceptible	11	63
Total	113	67 [b]

[b] excluded 2 invalid results and only positive by Anyplex™ MTB/NTM assay. A total of 102/113 (90%) were INH resistant by MTBDRplus assay. The Anyplex assays detected only about 4/67 (6%) of INH resistance.

5.1.4 MDR-TB resistance detection

The GenoType® MTBDR*plus* assay detected MDR-TB in 98/113 (87%). The Anyplex MDR-TB assay was able to detect two MDR samples while 47 samples being sensitive to both RIF and INH. Only two samples remained invalid after being repeated. The detected MDR TB cases are briefly mentioned in table 5.4 below.

Table 5.4: MDR case detection

	MTBDR*plus*	Anyplex MDR
MDR	98	2
Non-MDR	15	65
Total	113	67 [b]

[b] excluded 2 invalid results and only positive by Anyplex™ MTB/NTM assay. The MTBDR*plus* assay detected 98/113 (87%) MDR cases. There were only 2/67 (3%) of MDR cases that were detected by Anyplex assay.

5.1.5 Spoligotyping

A proportion of 20/69 (29%) sample resistant to either one or two drugs by Anyplex MDR-TB assay were considered for the spoligotyping. The Anyplex™ MDR-TB assay was able to detect 16, 2 and 2 of RIF-mono, INH-mono and MDR-TB, respectively. There were 19 hetero-resistant samples and according to MTBDR*plus* a total of 20 samples were spoligotyped. These resistant patterns detected by Anyplex™ MDR-TB that was considered for spoligotyping to determine the predominant families and mixed infections in these samples. The results are summarised in table 5.6.

Table 5.5: The spoligotyped sputum samples

Sample	Octal number	Family	Clade	MTBDR*plus*	ANYPLEX ™MDR TB	Treatment status	MTBDR*plus* ID
AINF9862	773377677761671		Orphan	RR	RIF resistant	Retreatment	Hetero resistant
AINF9861	777777777763771		54	RR	RIF resistant	Retreatment	Hetero resistance
AINF9852	777376777760601	X2	439	RR	RIF resistant	Retreatment	Hetero resistant
AIUA9618	775377437665671		Orphan	RR	INH resistant	New case	Hetero resistant
AIAU9616	000000000000000	Atypical	2669	RS	RIF-resistant	New case	Hetero resistant
AIUA9610	777767477760671		Orphan	RR	RIF resistant	New case	Hetero resistant
AIUA9608	777377777760731	T2	2188	RR	Rif resistant	New case	Hetero resistant
AIUA9607	777363677760671		Orphan	RR	MDR	New case	Hetero resistant
AIUA9604	776167607760671	LAM3	2301	RR	RIF resistant	New case	Hetero resistant
AIAU9879	771357677760771		Orphan	RR	INH resistant	Retreatment	Hetero resistant
AIUA9957	77534767770671		Orphan	RR	RIF resistant	Retreatment	Hetero resistant
AINF9878	777777477760771	H37Rv	451	RR	RIF resistant	Retreatment	Hetero resistant
AINF9871	777777677761771		Orphan	RR	RIF resistant	Retreatment	Hetero resistant
AINS1380	775767677762771		Orphan	RR	MDR	Retreatment	Hetero resistant
AINS1372	773377600001771		Orphan	RR	RIF resistant	Retreatment	Hetero resistant
AIBT6647	777777777760771	T1	53	RR	RIF resistant	New case	Hetero resistant
AJJU978	777767777443771		Orphan	RS	RIF resistance	Retreatment	Normal resistance
AINS1375	777777777763771	Manu2-S	54	RR	RIF resistant	Retreatment	Hetero resistant
AINF9616	703777740003771	Cas1.Delhl	26	RS	RIF resistant	New cases	Hetero resistant
AINF9892	777777777763771	Manu2-S	54	RS	RIF resistant	Retreatment	Hetero resist

RIF- rifampicin, INH- Isoniazid, MDR-Multiple drug resistant, RR-resistance to rifampicin and isoniazid, RS- resistant to rifampicin and susceptible to isoniazid.

5.2 Detection of *M. tuberculosis,* rifampicin and isoniazid on culture isolates

The study utilized 104 culture positive isolates with resistant profiles from the MRC bio bank and electronic database. Samples with enough MGIT broth material were selected and DNA was extracted using the Genolyse (Hain Life Sciences). Both GenoType® MTBDR*plus* as well as the Anyplex™ MTB/NTM followed by Anyplex MDR-TB assays were performed concurrently on the same DNA isolate. The MGIT DST (gold standard) was compared to GenoType® MTBDR*plus,* Anyplex MDR-TB and performance characteristics (sensitivity and specificity) determined. The Anyplex™ MDR-TB assay was used secondary molecular assay to resolve the discordance between the gold standard and the WHO endorsed MTBDR*plus* assay. The discordance was found in 45/104 (43%) of the samples .The Anyplex™ MDR-TB with an extended mutational profile resolved 23/45 (51%) cases for RIF and INH, respectively. The 22/45 (49%) unresolved discordant cases were PCR amplified and sequenced to detect for novel or confirm mutations detected by Anyplex™ MDR-TB. Summarized in figure 5.2 below.

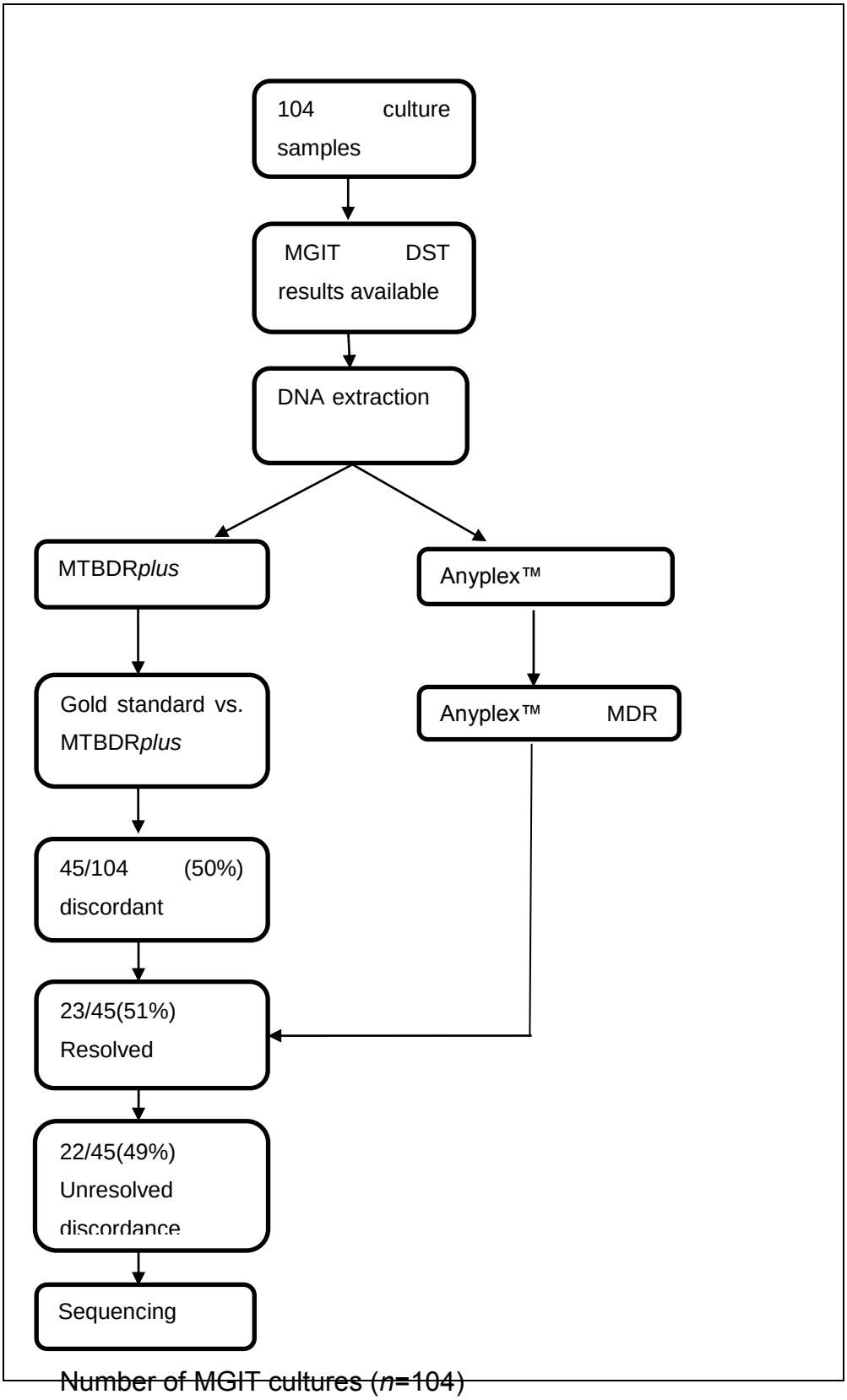


Figure 5.2: Laboratory methods performed on culture samples.

Number of MGIT cultures (*n*=104)	
MTBDR*plus* result	
MTB+	82 (79%)
MTB-	22 (21%)
Total	104
Anyplex™ MTB/NTM result	
MTB+	100(96%)
MTB-*	4 (4%)

5.2.1 The detection *M. tuberculosis*

The phenotypic MGIT cultures were used in this study phase for retrospective evaluation. The MTBDR*plus* assay was able to generate 79% valid results for *M. tuberculosis* detection in 104 samples used in this investigation. Anyplex™ MTB/NTM assay detected *M. tuberculosis* strain in 100/104 (96%) samples and identified Non *M. tuberculosis* in four (4%) of the remaining samples.

Table 5.6: The detection of *M. tuberculosis*

Number of MGIT cultures (n=104)	
MTBDR*plus* result	
MTB+	82 (79%)
MTB-	22 (21%)
Total	104
Anyplex™ MTB/NTM result	
MTB+	100(96%)
MTB-*	4 (4%)
Total	104

The detection of TB in culture isolates by MTBDR*plus* assay was successful in 82/104(79%) samples. The Novel assay (Anyplex) detected 100/104 (96%). n- total sum.

5.2.2 Rifampicin resistance

The antibiotic resistance testing for each isolate was performed using three different methods, MGIT, MTBDR*plus* and Anyplex™ assays. The Bactec MGIT 960 system was used to detect RIF resistance and there were 70/104 (67%) samples that indicated to harbour the mutations within *rpoB* gene. GenoType® MTBDR*plus* and Anyplex™

MDR-TB assay detected 49/104 (47%) and 48/97 (49%), respectively. The sensitivity and specificity are calculated below.

Table 5.7: Detection of RIF resistance.

MGIT RIF (n=104)							
MTBDR*plus*	R	S	Total	SS (%)	SP (%)	PPV (%)	NPV (%)
R	46	3	49	96	91	96	94
S	2	31	33				
MTB-	22	0	22				
Total	70	34	104				
MGIT RIF (n=104)							
Anyplex™	R	S	Total [a]	SS	SP	PPV	NPV
R	32	16	48	67	59	67	55
S	14	17	31				
MTB +	18	0	18				
Total	64	33	97[b]				

n- total sum, R- resistant, S- susceptible, SS- sensitivity, SP- specificity, PPV, positive predictive value, NPV-negative predictive value, [a]-according to MTBDR*plus* valid results and [b] excluded 3 invalid results.

5.2.3 Isoniazid resistance detection

The Phenotypic DST had resistance to INH in 92/104 (88%) samples utilised. The GenoType® MTBDR*plus* assay was able to detect alterations in the *katG* and in the *InhA* promoter in 56/104 (54%) samples, either specifically through a mutation probe or through the absence of a hybridisation signal for one of the wild type probes. The novel diagnostic assay, the Anyplex™ MDR-TB assay was able to detect only 10/104 (10%) INH resistant samples. Sensitivity and specificity are calculated below in table 5.9.

Table 5.8: The detection of INH resistance

MGIT INH (n=104)							
MTBDR*plus*	R	S	Total	SS	SP	PPV	NPV
R	55	1	56	78	92	98	42
S	15	11	11				
MTB-	22	0	22				
Total	92	12	104				
MGIT INH (n=104)							
Anyplex™	R	S	Total [a]	SS	SP	PPV	NPV
R	10	0	10	15%	100%	100%	17%
S	57	12	69				
MTB+	18	0	18				
Total	85	12	97[b]				

n- total sum, R- resistant, S- susceptible, SS- sensitivity, SP- specificity, PPV, positive predictive value, NPV-negative predictive value [a] according to MTBDR*plus* valid results and [b] excluded 3 invalid results.

5.2.4 Multi drug resistance (MDR)

The phenotypic MGIT DST had resistance in 68/104 (65%) samples classified as MDR-TB. The molecular based MTBDR*plus* technique was able to detect drug resistance to both RIF and INH drugs 42/104 (40%) samples as MDR-TB. The novel Anyplex™ assay was able to detect up to 12/104 (12 %) samples that had the MDR-TB phenotype

Table 5.9: MDR detection

MGIT MDR (n=104)							
MTBDR*plus*	R	S	Total	SS	SP	PPV	NPV
R	41	1	42	89%	97%	98%	87%
S	5	35	40				
MTB-	22	0	22				
Total	68	36	104				
MGIT MDR (n=104)							
Anyplex™	R	S	Total[a]	SS	SP	PPV	NPV
R	10	2	12	23%	94%	83%	49%
S	34	33	67				
MTB +	18	0	18				
Total	62	35	97[b]				

n- total sum, R- resistant, S- susceptible, SS- sensitivity, SP- specificity, PPV, positive predictive value, NPV-negative predictive value ,[a]according to MTBDR*plus* valid results and [b] excluded 3 invalid results.

5.2.5 Sequencing

A proportion of samples with molecular and phenotypic results were PCR amplified and sequenced. There were 45/104 (43%) samples that indicated discordance between the conventional MGIT and the genotypic MTBDR*plus* assay. The Anyplex™ MDR-TB assay was used to resolve the discordance between the diagnostic methods (MGIT and MTBDR*plus*), 23/45 (51%) samples were resolved (Table 5.10) and the remaining 22/45 (49%) samples were still unresolved. The samples that were unresolved by Anyplex™ MDR-TB were then sequenced together with few resolved cases to identify the mutations responsible for resistance and even those which are not involved in drug resistance.

Table 5.10: The resolved profiles

Groups	Total cases	MGIT		MTBDR*plus*		Anyplex	
		RIF	INH	RIF	INH	RIF	INH
A	8	R	R	I	I	R	S
B	6	R	R	I	I	S	S
C	4	R	R	I	I	NTM	NTM
D	1	R	R	I	I	S	R
E	1	R	R	I	I	R	R
F	1	R	R	S	R	R	S
G	1	S	R	S	S	S	R
H	1	R	S	S	S	R	R

R- Resistance, S- Susceptible and I –Invalid, NTM-non-tuberculosis mycobacteria, RIF-rifampicin, INH- isoniazid and MGIT-Mycobacteria growth Indicator tube.

The regions that were sequenced were amplified and loaded on a gel to visualize the presence of DNA. Below are the individual regions visualized on 2% agarose gel.

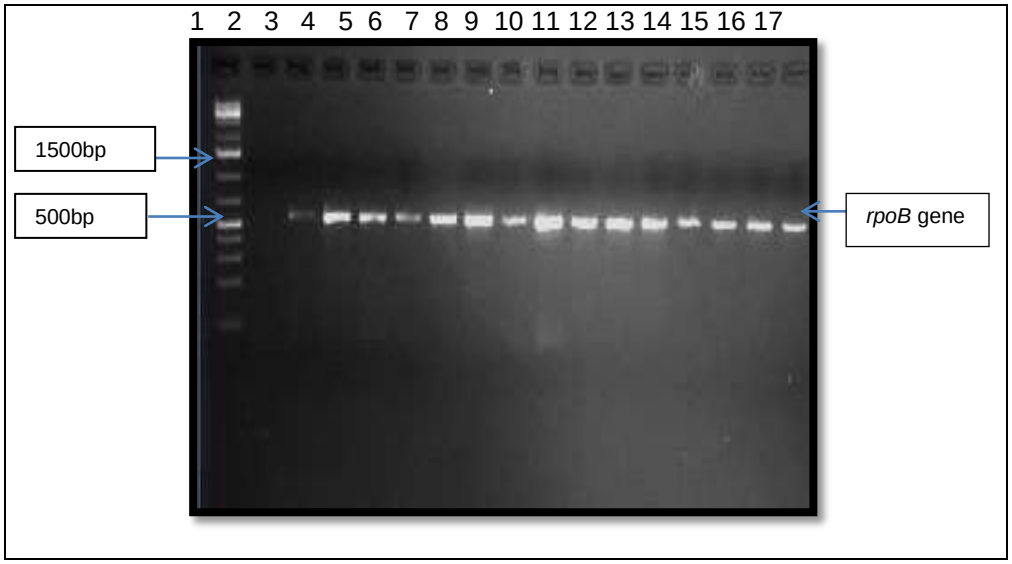


Figure 5.3: Amplification of the *rpoB* gene in the DNA samples. The first lane is the 100 base pair ladder. Lane 2 is negative control and from lane 3 to 17 are the DNA isolates.

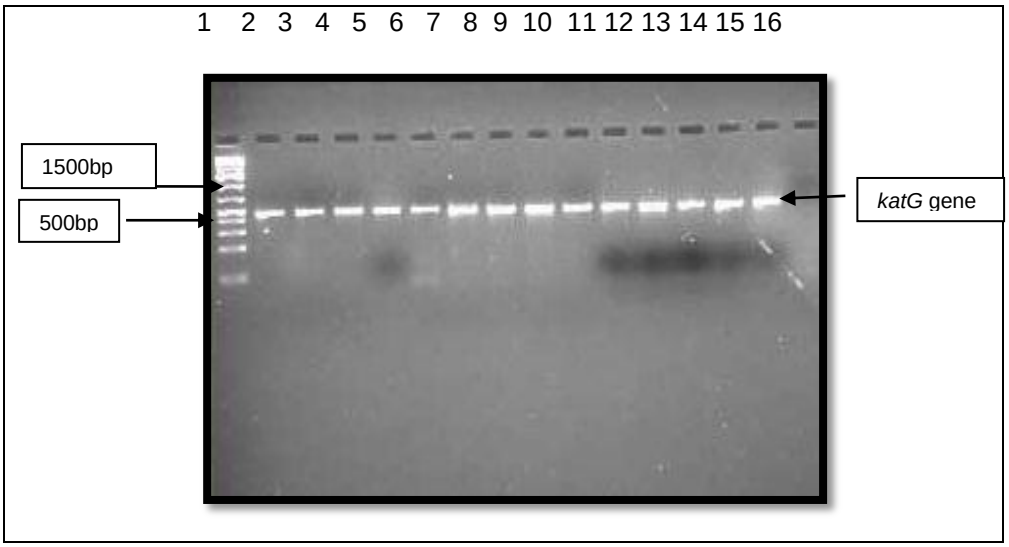


Figure 5.4. Amplification of the *katG* gene in DNA samples. Lane 1 is the molecular marker (100 bp ladder) and 2-15: DNA isolates. Lane 16 is the negative control.

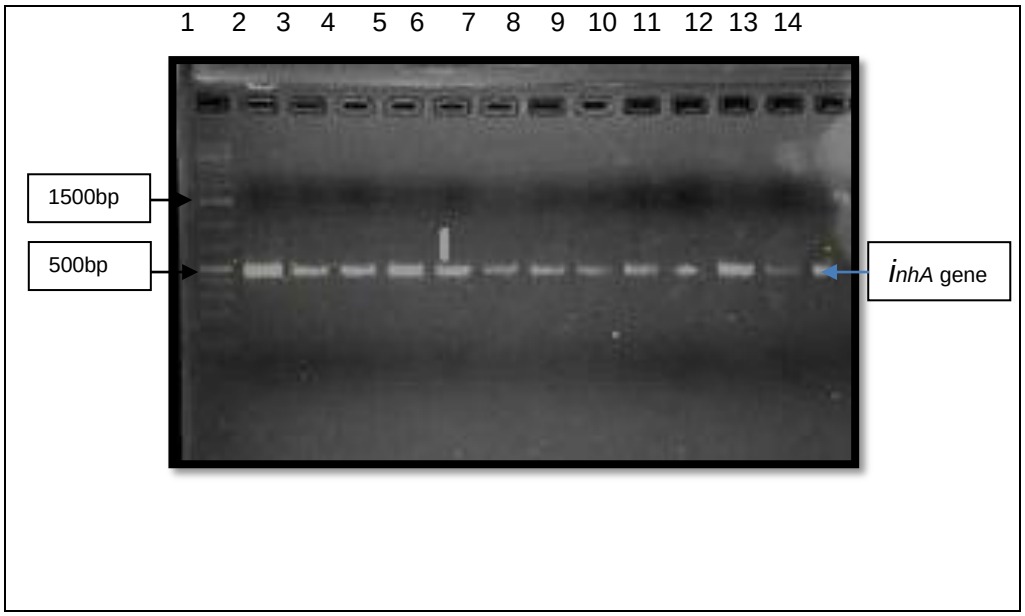


Figure 5.5. The Amplification of *inhA* gene in the DNA samples. Lane 2 to 14 is the DNA isolates. The initial (number1) lane is the 100 base pair ladder.

5.2.5.1 The *rpoB* gene sequencing

There are more than 90% of RIF resistant strains that harbour mutations in the RRDR of the *rpoB* gene and in this study the RRDR region was sequenced. A total of 24 samples were found to be discordant between MGIT RIF and MTBDR*plus* assay. The sample were amplifies and 9 samples that failed PCR were excluded for sequencing therefore only 15 samples. Below is table 5.12 with summarised results.

Table 5.11: Sequenced *rpoB* regions

SAMPLE NO	MGIT	MTBDR*plus*	ANYPLEX™	SEQUENCING	E. Coli NUMBERING
SGT2037864	S	S (WT)	R	WT	WT
SGT2028516	S	R(WT4 missing/513-519)	R	D435Y	D516Y
SGT2153044	S	R	R	L430P	L511P
SGT1924823	S	R(WT2 MISSING/510-513)	R	L430P	L511P
SGT2026505	S	R(WT2MISSING/510-513)	R	L430P	L511P
SGT2189942	S	R	S	L430P	L511P
SGT1942547	S	S	R	D435Y	D516Y
SGT2115749	S	R(WT2 MISSING/510-513)	R	WT	WT
SGT2145141	S	R(WT2MISSING/510-513)	S	WT	WT
PKI3394980	S	S(WT)	S	L452P	L533P
SGT2130328	S	S (WT)	R	WT	WT
SGT2000929	S	R(WT4 MISSING/513-519)	S	L452P	L533P
SGT1945722	S	R (MUT3)	R	L452P	L533P
SGT2102878	S	R (WT1&2 MISSING)	R	L430P	L511P
SGT1980552	S	R(WT3&4 MISSING)	R	D435Y	D516Y

No- number, S- susceptible, R- resistant, WT- wild-type, MGIT- Mycobacteria growth indicator tube, D-Aspartic, Y-Tyrosine, L-Leucine, P-Proline.

5.2.5.2. The sequencing of *katG* and *inhA*

A total of 29 samples identified to be discordant when compared to the gold standard MGIT INH results. Samples were amplified and run on agarose gel. Positive results were recorded for only 14 samples and 15 samples failed PCR. The 14 samples were then sequenced and below are the result in table 5.13.

Table 5.12: Sequenced INH regions

SAMPLE NO	MGIT	MTBDR*plus*	ANYPLEX™ assay	SEQUENCING	
				katG	*InhA*
PKI3391572	R	S(WT)	S	WT	WT
SGT1901090	R	S(WT)	S	WT	WT
TTB2064531	R	S(WT)	S	R385W	WT
SGT2023820		S(WT)	S	WT	WT
SGT1879139	R	S(WT)	S	WT	WT
SGT2189942	R	S (WT)	S	WT	WT
SGT2153581	R	S(WT)	S	WT	WT
SGT2115749	S	S(WT)	R	WT	WT
SGT2091778	R	S(WT)	S	WT	WT
SGT2107315	R	S(WT)	INVALID	WT	WT
SGT2130328	R	S(WT)	S	WT	WT
PKI3398908	R	S(WT)	S	WT	WT
SGT2176491	R	S(WT)	S	WT	WT
SGT2123498	R	S (WT)	S	WT	WT
SGT1980552	R	R (WT)	S	WT	WT

MGIT- Mycobacteria Growth Indicator Tube, no- number, R- resistant-susceptible, WT- Wild type, R-Arginine-Tryptophan.

CHAPTER 6

6 DISCUSSION

6.1 Overview of the study

The public health problems are on an exponential rise and TB epidemic continues to advance in challenging the management strategies in most parts of the world (Scott *et al*., 2011). The TB eradication efforts have been disrupted by the emergence of drug resistant TB forms such as MDR-TB posing serious challenges mostly in developing countries (Mishra *et al.,* 2014). The circulating MDR-TB strains are reaching high levels and thus pose threats in management of patients on available chemotherapy. Throughout the world MDR-TB incidents caused by RIF and INH drug resistance are recorded and this is due to the unusual combination of mutations within *rpoB, katG*, *inhA* and other genes (Casali *et al.,* 2012).

The worst documented epidemics of MDR-TB in the world were in South Africa during the year 2008 (WHO TB 2010). South Africa was positioned fifth in terms of absolute numbers regarding the MDR-TB burden following Pakistan, Russian Federation, India and China, respectively (Naidoo *et al.,* 2014). During the year 2012 there were estimates that 450000 people developed MDR-TB world widely and 15,419 cases were reported in South Africa (Naidoo *et al.,* 2014). Furthermore the latest national Drug Resistance Survey revealed that the burden of MDR-TB cases in South Africa was approximately 13,000 (Bantubani *et al*., 2014).

The public health control systems in South Africa is not optimal to overcome the continuous rise of TB and its resistant forms especially in resource limited settings such as Limpopo Province (Brust *et al*., 2010)

The prompt diagnosis of MDR-TB is an essential task for effective and rapid treatment thus limiting the spread of the disease in the community (Dheda *et al.,* 2013). The early and correct diagnosis coupled with effective treatment is a step in the right direction for MDR-TB cure (Steingart *et al.,* 2013).The introduction of GeneXpert and MTBDR*plus* in the health care system was a significant milestone in public health; however it is still not feasible at a primary health care level. The increased level of MDR-TB indicates that there is a crucial need for development of new diagnostic tools with high mutation profile such as Anyplex MDR-TB assay. In this study we have evaluated Anyplex assays for rapid diagnosis of TB and MDR-TB in clinical and culture specimen. Furthermore we investigated mutations causing discordance between molecular and phenotypic assays.

6.2 Detection of *M. tuberculosis* by Anyplex ™ MTB/NTM assay in clinical and culture isolates

The performance of Anyplex MTB/NTM assay was evaluated on clinical and culture isolates in detecting *M. tuberculosis* and discrimination to NTM organism. The Anyplex™ MTB/NTM assay was performed on clinical samples the sensitivity and specificity were 90% and 39%, respectively using smear microscopy as a gold standard. Similarly in an investigation done by Lim and co-workers a sensitivity of 87% by Anyplex MTB/NTM assay was detected in both smear positive and negative specimens (Lim *et al.*, 2014). Although our specificity was low, most of our study isolates were mostly smear negative (85/111) 76% and culture negative. Anyplex™ MTB/NTM assays was also evaluated on prospective sputum specimens in the United Kingdom with a sensitivity of 74% using smear microscopy as a gold standard (Perry *et al.,* 2014). A high proportion of smear negative is common in our settings, especially with a high HIV prevalence.

The performance of Anyplex ™ MTB/NTM assay on cultured isolates was good. The assay detected *M. tuberculosis* in 96% on retrospective isolates. A study conducted by Perry and co-workers had a sensitivity of 86% in culture isolates which were 10% lower than the results derived from this study (Perry *et al.,* 2014). Although our study had none of the cultures confirmed as NTM, we were able to detect four NTM cases. Contamination on cultured isolates is a common phenomenon and Anyplex MTB/NTM is highly sensitive in discriminating between *M. tuberculosis* and NTM organisms.

The MTBDR*plus* version 2 assays is dedicated to more convenience, since it has a master mix that is ready to use after a combination of two components provided with the kit. In this study, MTBDR*plus* version 2 assay detected *M. tuberculosis* in (99%) 168/169 clinical samples, while one was negative. Using 104 *M. tuberculosis* culture positive samples, a detection rate of 82/104 (79%) was observed, while the remaining samples were negative or inconclusive. Bernard and group reported a comparative sensitivity of 73% and 81% on smear microscopy isolates, respectively in culture isolates (Bernard *et al.,* 2012). Genotype MTBDR*plus* assay is easy to perform and reliable in the detection of TB both in clinical specimens and culture isolates. Although faint TUB bands were mostly visualized on the clinical specimens than in culture isolates. The faint TUB band might be due to the mixed infection in the tested specimens that contains both *M. tuberculosis* complex strain and a non-tuberculous *mycobacterium.* In rare cases the TUB band could be absent due to competition of the single amplified reactions during PCR (Dorman *et al.,* 2012).

6.3 Molecular detection of RIF and INH resistance by MTBDR*plus* and Anyplex™ MDR-TB assays

The MTBDR*plus* assay version 2 is advanced and has more probes designed to detect INH resistance specifically the mutations responsible for low level resistance and further more with additional three probes for RIF resistance detection (Dorman *et al.,* 2012). The GenoType MTBDR*plus* version 2 assay detected RIF resistance in clinical isolates with a sensitivity and specificity 96% and. The study has same results as other studies that used clinical strains and recorded 92% sensitivity (Lacoma *et al.*, 2008).

Of 69 *M. tuberculosis* detected by Anyplex MTB/NTM, a further investigation on Anyplex MDR-TB assay was done. Anyplex MDR-TB assay detected isolates that harbour the RIF resistance in 16/69 (23%) clinical isolates. So far our study is the first one to report the performance of Anyplex MDR-TB assay performance. Although the performance was low (23% vs 96%) as compared to MTBDR*plus* assay, occurrence of heteroresistance was also high which could affect the reporting dyes of Anyplex MDR-TB. Heteroresistance is not easily detected by molecular assays in suspensions with a low proportion of resistant bacteria probably due to the very low concentration of the amplified fragments that contain mutations (Folkvardsen *et al*, 2013). This could be the case in Anyplex MDR-TB which uses multiplexing technology and reporter system for diagnosis.

The performance of MTBDR*plus* on RIF detection on culture isolates in terms of sensitivity, specificity, PPV and NPV were 96%, 91%, 96%, 94%, respectively. The sensitivity of detection of RIF resistance was in agreement with those recorded worldwide ranging from 92 to 100% (Hayen *et al*, 2010). The RIF resistance that was detected by the novel Anyplex MDR-TB assay with a specificity, sensitivity, PPV and NPV of 67%, 59%, 67%, 55%,

respectively. The principle of Anyplex assay can be compared to that of the Cepheid GeneXpert RT-PCR however sensitivity of both assays is different. The Anyplex assay has low sensitivity compared to GeneXpert with usually a pooled sensitivity of 80 to 99% in low resourced areas and 86 to 97% in developed areas.

The detection of INH-resistance by MTBDR*plus* assay is entirely dependent at codon 315 of the *katG* gene and in the *inhA* promoter region (Huyen *et al.,* 2010). In this study, the GenoType MTBDR*plus* assay identified INH resistance in 102 clinical specimens consisting of both mono resistant and those resistant to RIF as well (inclusive of MDR cases). The sensitivity of MTBDR*plus* assay in the detection of INH in a study conducted by Lacoma and co-worker was found to be 89% in culture isolates. The Anyplex assay detected INH resistance in only 4 clinical specimens. Anyplex MDR-TB assay for the detection of INH resistance was found also to be low with 15% but with a total specificity of 100%.

The MTBDR*plus* assay has been used in several studies with both clinical and culture samples, it is useful due to its accuracy and specificity, as well its ease of use and the short turnaround time (Huang *et al.,* 2009). The MTBDR*plus* test had sensitivity of 78% for detecting INH unlike in the study conducted by Huang and co-workers recorded an 82% in INH resistance. The studies carried out earlier had recorded sensitivities of the GenoType MTBDR*plus* assay for the detection of INH ranging from 42.1–99.6% and as compared to what was found in this study it is in concordance with other finding in others studies (Felkel *et al.,* 2013). The MTBDR*plus* assay sensitivity in the current study is in range compared to the sensitivities recorded earlier in other studies (Felkel *et al.,* 2013, Yadav *et al.,* 2013)

6.4 Identification of mutations within *rpoB*, *katG* and *inhA* genes by sequencing.

Rifampicin resistance is commonly expressed by changes in the *rpoB* gene however resistance can be due to mutations outside the target region of analysis or changes that might have occurred in genes whose product participate in antibiotic metabolism or permeability (Veluchamy *et al.,* 2013). According to a study done by Talenti and co-workers in 1993 about 3 to 5 percent of the RIF resistant isolates did not harbour a mutation within the 81bp hotspot region and this was supported by reports from India on mutations that are outside the RRDR (Talenti *et al.,* 1993, Mani *et al*., 2001 and Veluchamy *et al.,* 2013).

Fifteen specimens that were RIF resistant by Anyplex MDR-TB and susceptible by MGIT assay were sequenced for *rpoB* regions. A total of 11/15 (73%) harboured mutations within *rpoB*. The fifteen samples were phenotypically susceptible to RIF on MGIT DST. The four strains that had no mutations, two lacked WT 2 probe while the remaining had all WT probe present but were Anyplex MDR-TB resistant. The resistant samples were sequenced to reveal the following mutations namely: D516Y, L511P and L533P.

The L511P mutation was found in the (5/15) 33% of the sample and was the most common among the recorded mutations. The L511P mutations was shown confer low-level resistance with minimum inhibitory concentration (MIC) of 1 mg/L (Ocheretina *et al.,* 2014). The appearance of this mutation was recorded in a study done in Haiti were by isolates with a single L511P mutation had MIC values ranging from 0.125 to 0.25 µg/ml and in combination with one more mutation their MIC was 0.25 to 0.5 µg/ (Ocheretina *et al.,* 2014). The L511P mutation is usually found in the RIF susceptible strains and was recorded in several studies (Hillemann *et al.,* 2007, Hauck *et al.,* 2009 and Jamieson *et al.,* 2014)

The L533P mutation is known to confer weak resistance or have no effect, whereas for other codons the resistance phenotype depends on the type of amino acid substitution at codons 511, 516 and 526 (Hillemann D *et al.,* 2007). The L533P mutation was found in (3/15) 20% of the samples, with two of them resistant by MTBDR*plus* assay. Hillemann *et al*. reported misidentification of a L533P mutation in a RIF-susceptible strain (Hillemann *et al.,* 2007). According to reports the L533P is the disputed mutation that is commonly easier to detect, occurring at a rather constant frequency of 3 to 6% (Jou *et al.,* 2005). Recently GeneXpert-MTB/Rif produced false RIF susceptibility results due to presence of mutation L533P in clinical specimens with different bacterial loads (Somoskovi *et al*, 2013).

The D516Y mutation was detected in 3/15 (20%) samples, 2 had WT 3/ 4 missing (Codon 513-516) and the remaining isolate was only identified to be susceptible to RIF by MTBDR*plus* assay. The D516Y mutation was described in one isolate detected in China (Hauck et *al.,* 2009). The deletion involving codons 513– 516 (amino acid 514 to 515) was observed in five strains from the Republic of Djibouti with very similar genotypes, and was previously described in other studies (Kapur *et al.,* 1994, Hauck *et al.,* 2009).

Of the 11/15 (73%) samples sequenced the mutations found were: D516Y, L511P and L533P .Therefore excluding the samples with these mutations we were able to increase Anyplex specificity to 94%. These mutations should be interpreted with caution and their introduction must be well explained in future molecular assays. However studies conducted both in Bangladesh and Kinshasa indicated that these discussed mutations (L511P, L533P and D516Y) were found to contribute about only 10 % of all *rpoB* mutations among both failure and relapse cases (Van Deun *et al.,* 2011). Moreover phenotypic DST easily misses the detection of

disputed mutations: D516Y and L511P mutations and report them as susceptible (van Deun *et al.,* 2011).

The resistance to INH is associated with mutations dispersed in the gene loci including the *katG* (Catalase-peroxidase), the promoter region of *aphC* (alkyl hydroperoxidase), *inhA* (enoyl-acyl reductase), *kasA* (beta ketoacyl ACP synthase), *mabA* (3-ketoacyl reductase), and *ndh* (NADH dehydrogenase) in other investigations (Ducasse-Cabanot *et al*., 2004). The loss of *katG* function in resistant strains is compensated by mutations in the *OXyR-aphC* intrinsic region, where the putative promoter of *aphC* is located (Veluchamy *et al.,* 2013). Our study focused only on *inhA* and *katG* mutations.

About 15 samples sequenced for both *inhA* and *katG* in INH resistant samples. There was only a single strain that indicated INH resistance on the *katG* region with a novel mutation, R385W amongst all the 15 samples. The remaining *inhA* regions were all found not to have any sequence changes.

The fact that other regions (i.e *aphC*) conferring resistance to INH were never studied therefore might explain why there were no mutations responsible to the resistance and not resolving the discordance. The design of these molecular assays is done in such a way that only particular mutations are detected hence lot more genotypic resistance cannot be explained by such assays. Sequencing of PCR-amplified products of *rpoB* and *katG* has become the commonly used genotypic technique for drug resistant *M. tuberculosis* detection. It is accurate and reliable and regarded as the reference standard for mutation detection. However other genes must be considered to have a full understanding of resistance to particular drugs in future molecular assays.

6.5 Investigation of heteroresistance by spoligotyping on Anyplex MDR-TB resistant isolates.

A total of 12/20 (60%) and 8/20 (40%) were retreatment and new cases, respectively with or without heteroresistance according to MTBDR*plus* were spoligotyped. Amongst the spoligotyped samples the following were detected: The 4/20 (20%)-Euro-American samples (Latin American and Mediterranean (LAM 3), a European family X2, and a default family T1/2), 1/20 (5%)-East-African Indian of cas1 Delhl, 3/20 (15%)-Manu 2, 1/20 (5%) H37Rv and 1/20 (5%) atypical family. There were 10/20 (50%) samples identified as orphans by spoligotyping therefore the remaining were not classified into lineages. The LAM3 strain is widely distributed in the whole world and has been isolated in the TB epidemic in South Africa and most particularly in the Eastern Cape Province (Hove *et al.,* 2012). This genotype is been identified to be of major concern regarding its success in contributing to the TB problem in South Africa (Streicher *et al.,* 2007)

The T2 genotype is defined by the absence of spacer 40 in addition to the absence of 33–36 group of spacers and it is distributed world widely as well (Sebban *et al.,* 2002). The study conducted in Madagascar had predominantly shown isolates of the Cas-1 Delhl which are also found in Tanzania (Brudey et *al.,* 2006). The T1 family has been widely isolated in populations in all continents and corresponds to about 30% of all entries in the international database (Brudey *et al.,* 2006) The X family is referred to as the super family that is divided into at least 3 distinct families(X1, X2 and X3)(Sabben *et al.,* 2002). The family is linked to an Anglo-Saxon ancestry, because it has been discovered in the UK and English-colonized areas such as the USA, Australia, South Africa, the Caribbean (Sabben *et al.,* 2002)

Heteroresistance is an underestimated concept in TB disease, especially in highly endemic areas and neglected concept in the low

incident areas (Rinder *et al.,* 2001). Heteroresistance (appearance both of wild type and mutation) was recorded as in our study and could be an obstacle against the performance of the assay regarding the DST and even successive therapy (Rinder *et al.,* 2001). Heteroresistance was found in 79/113 (69%) within *rpoB* while i*nhA* mutations were responsible for 68/113 (60%) and lastly *katG* with 4/113 (3%) samples. This phenomenon is thought to be an early stage in the development of drug resistant TB and it provides bacteria with an opportunity to explore the possibility of growth in the presence of anti-TB drugs (Hofmann-Thiel *et al.,* 2009).

Heteroresistance arises when there is coexistence of two different *M. tuberculosis* strains and segregation of single strain into resistant and susceptible bacteria (Hofmann-Thiel *et al.,* 2009). There is a lack of information regarding the prevalence of heteroresistance and is presumably dependent on the local resistance epidemiology (Folkvardsen *et al.,* 2013). The finding of such cases is usually accidental and therefore more sensitive methods are needed for the detection of heteroresistance cases (Hingley-Wilson *et al.,* 2013)

The mechanisms of heteroresistance are linked to the clinical entities relapses, new cases and treatment failures (Hofmann-Thiel *et al.,* 2009). Theoretically, heteroresistance due to infection with two different strains is explained by super infection of a patient already infected with one *M. tuberculosis* with an additional one (Hofmann-Theil *et al.,* 2009). Heteroresistance resulting from an infection with a single strain is most probably explained by segregation into susceptible and resistant organisms under selective pressure of insufficient anti-TB therapy (Hofmann-Thiel *et al.,* 2009). However the occurrence of 10/ 20 (50%) orphan strains could indicate mixture of strains. More studies with higher discriminatory power than spoligotyping (i.e. MIRU VNTR) should be done on heteroresistant cases.

Heteroresistance in clinical isolates present a difficulty to directly detecting it from MGIT DST and can lead to inconsistency with DST results (Rinder *et al.,* 2001). In this study 19/20 (95%) isolates had been identified as heteroresistant with only 1/20 (5%) retreatment case that had a normal resistance. The heteroresistant samples were comprised of 8/19 (42%) new cases and 11/19 (58%) retreatment cases. The orphans were comprised of 7/10(70%) retreatment cases and 3/10 (30%) new cases. In a study conducted by Zhang and co-workers, heteroresistance was commonly detected in patients who had received TB-treatment before (Zhang *et al.,* 2012). Our study had a higher portion of retreatment cases which could contribute to heteroresistance.

Limpopo Province was found to have the Beijing genotype which is associated with drug resistance, as the most predominant genotype (Said *et al.,* 2012). In a contrast a study by Stavrum and co-workers indicated that the mostly isolated genotype in Limpopo was the super family X during the period 2001 to 2002 (Stavrum *et al.,* 2009). However in this study the major family isolated were of European American lineage. This represents a continued evolution of *M. tuberculosis* in this setting which will have a negative impact on molecular diagnostic assays due to dominance of certain mutations in different families.

6.6 Conclusion

- The Anyplex MTB/NTM assay performance in detecting *M. tuberculosis* in both clinical and culture isolates was good. The assay successfully resolved MTBDR*plus* discordant cases and thus should be considered as alternative assay for MTB/NTM detection in culture cases. However the detection of *M. tuberculosis* cases in clinical cases was average as compared to MTBDR*plus* assay. Furthermore MTBDR*plus* reported heteroresistance which complicates diagnosis.
- The Anyplex MDR-TB assay performance in detecting RIF resistance was good (67%) as compared to INH resistance detection which was poor (15%) in culture isolate. Anyplex MDR-TB uses multiplex technology on a RT PCR platform and can have a negative effect in detecting resistance. However a combination of molecular assays (MTBDR*plus* and Anyplex MDR-TB) successfully resolved 51% of discordant cases between phenotypic and molecular assays.
- To resolve discordant cases between molecular and phenotypic assays we performed Sanger sequencing. Phenotypically susceptible isolates that were RIF resistant by both Anyplex and MTBDR*plus* assay revealed L511P, L533P and D516Y. A high number of heteroresistant cases were detected in the study. Although molecular assays are not ideal for MDR-TB diagnosis in retreatment cases, is important to determine their impact on treatment outcomes of patients. Moreover the detection of diverse lineages and orphan strains is problematic to successful treatment of these patients.

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6.7 Limitations

- We used the smear microscopy results for performance on Anyplex MTB/NTN *M. tuberculosis* detection. Moreover most of the specimens were culture negative (45 days incubation) and smear negative which is a common phenomenon in HIV positive patients.
- A common extraction method was used for all molecular assays (Anyplex and MTBDR*plus*). This could have a negative impact on Anyplex assay performance based on RT-PCR.

6.8 Recommendations

- Further evaluation on Anyplex assay is needed especially in smear negative and HIV positive patients. Since HIV positive patients have low bacterial load in sputum specimens which are often culture negative.
- The existence of L511P, L533P and D516Y mutations in molecular assays needs further investigation, since these mutations are mostly phenotypically susceptible. Molecular studies with treatment outcomes of these mutations are needed to warrant their inclusion in future molecular assays.
- Heteroresistance prevalence in retreatment and new cases of MDR-TB must be investigated. Moreover increased sensitivity in phenotypic assays like microscopic observed drug-susceptibility (MODS) should be considered especially in high HIV/TB prevalent settings.

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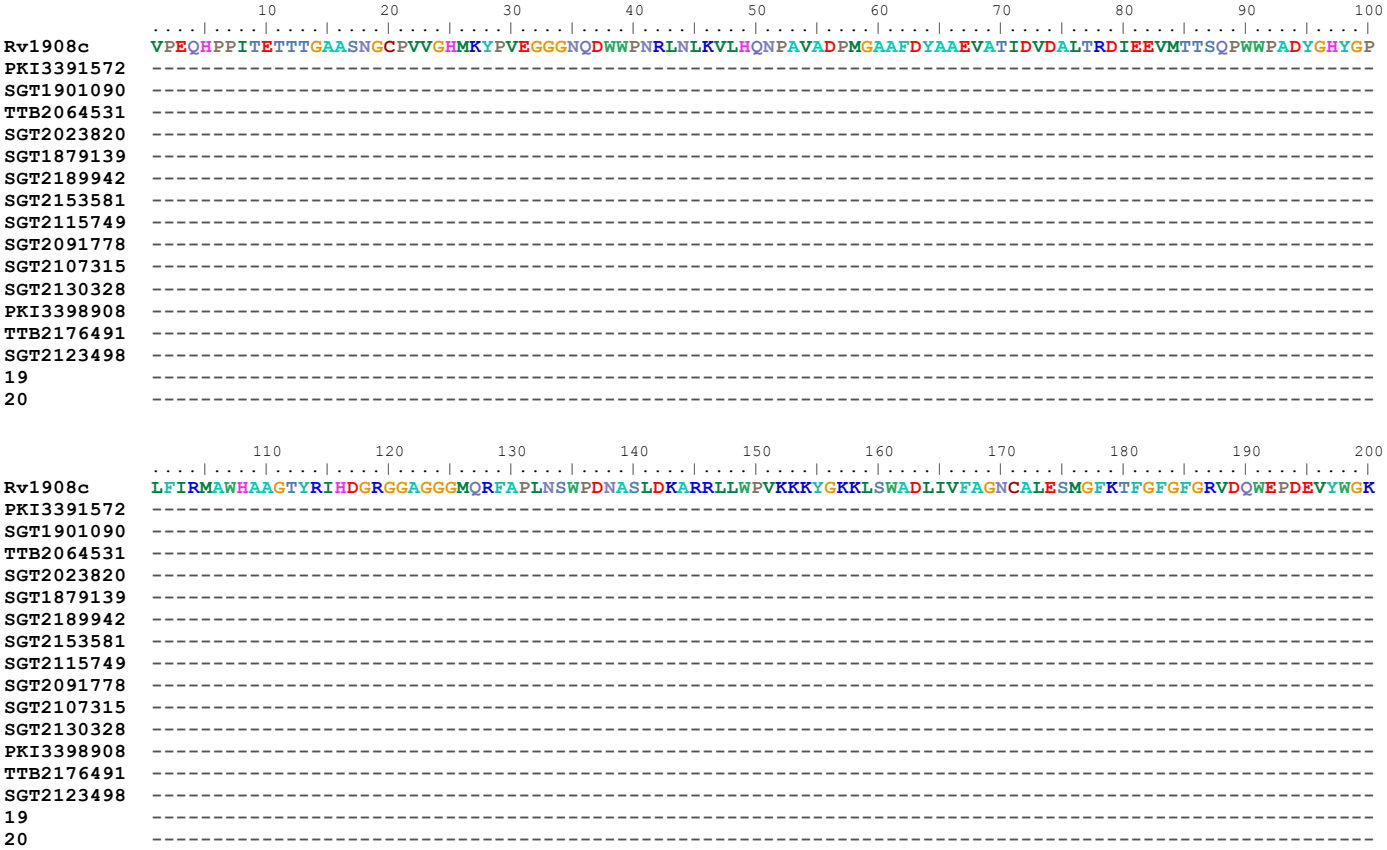
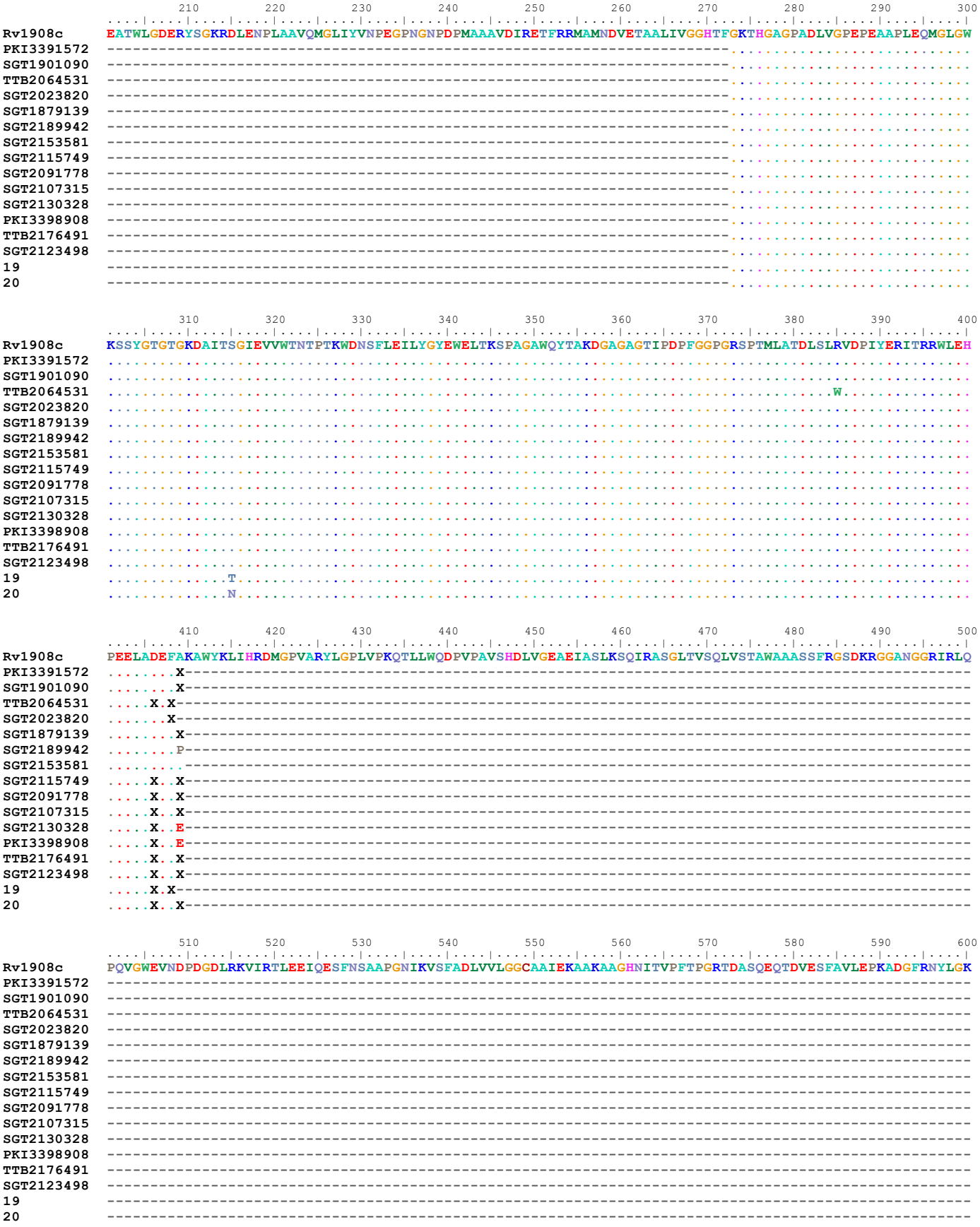
APPENDIX

KatG gene

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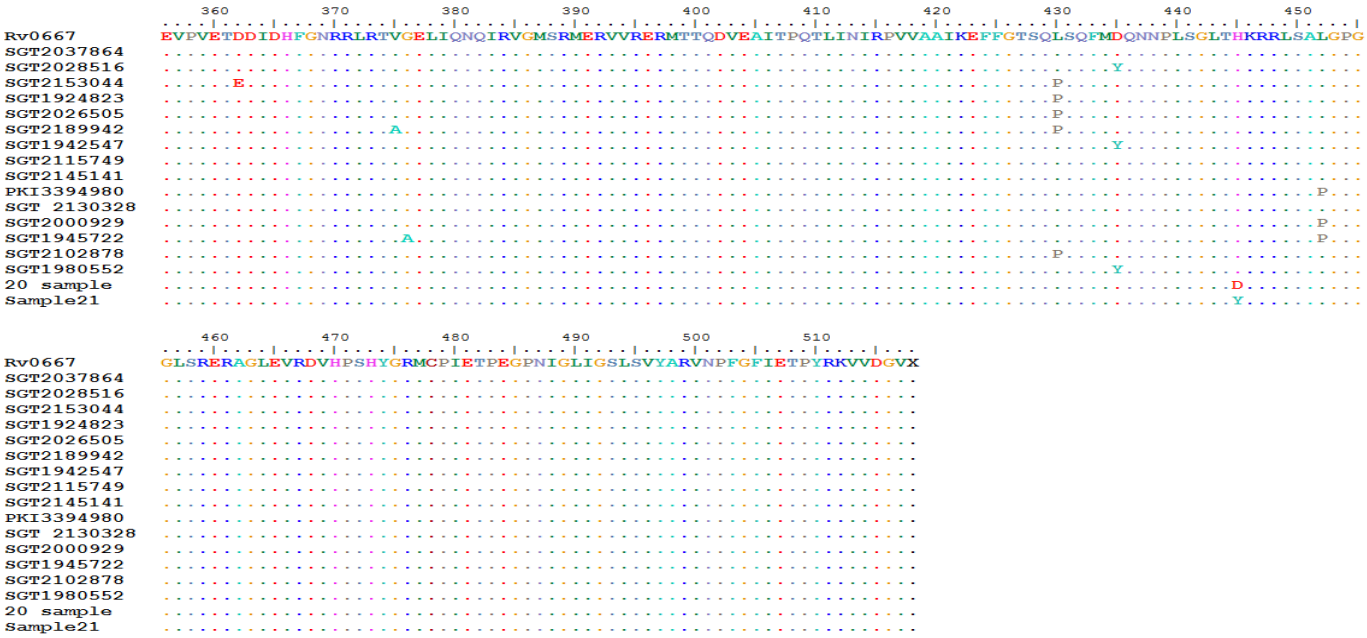
rpoB gene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20 sample
Sample21
460 470 480 490 500 510
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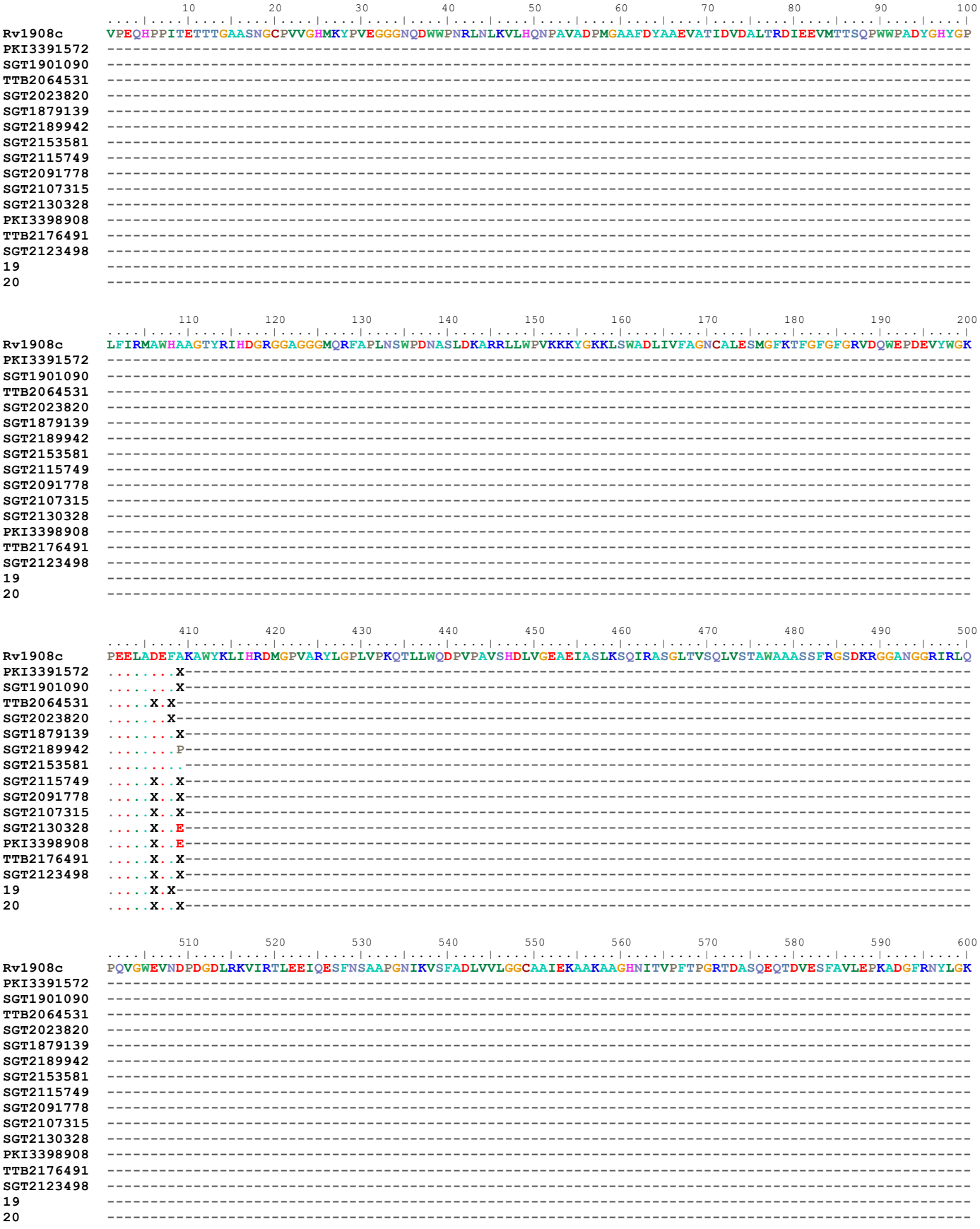
20 sample
Sample21

inhA gene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SGT2130328
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TTB2176491
SGT2123498
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20
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SGT1879139 X
SGT2189942 ....P
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SGT2091778 .....X..X
SGT2107315 .....X..X
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