DETERMINATION OF HETERORESISTANT *MYCOBACTERIUM TUBERCULOSIS* STRAINS AND THEIR ASSOCIATION WITH PATIENTS TUBERCULOSIS TREATMENT HISTORY IN LIMPOPO PROVINCE.

by

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[redacted]

RESEARCH DISSERTATION

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DECLARATION

I Matema Constance Mohatli declare that **DETERMINATION OF HETERORESISTANT *MYCOBACTERIUM TUBERCULOSIS* STRAINS AND THEIR ASSOCIATION WITH PATIENTS TUBERCULOSIS TREATMENT HISTORY IN LIMPOPO PROVINCE** is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references and that this work has not been submitted before for any other degree at any institution**.**

Full names **Signature**

_____ _____

DEDICATIONS

THIS WORK IS DEDICATED TO:

-The Lord Almighty, I believe that all this would not have been possible without his love and grace.

- My wonderful supervisor Mrs NTC Maguga-Phasha.

- My family who have guided and supported me throughout this journey.

- My daughter Oratilwe who has given me strength to keep working hard and make her proud.

- The rest of my family and friends who have given me the confidence and believing in my dream, thank you all.

SIGNATUTE **DATE**

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ABSTRACT

Background:

Tuberculosis (TB) patients may have mixed infections with both drug-susceptible and drug-resistant *Mycobacterium tuberculosis* (MTB) strains. This phenomenon termed heteroresistance presents a challenge TB management and is considered a preliminary stage to full resistance. Heteroresistance is more likely to occur in high TB incidence areas and in chronic patients as they have more opportunity to become infected with various strains of TB and has been proven to occur in new cases, treatment failure and relapse.

Methods:

Sputum samples were collected from new consulting and hospitalised patients who were on treatment for MDR TB. A total of 231 samples were run on MTBDR*plus* to determine heteroresistance of *Mycobacterium tuberculosis* to isoniazid and rifampicin. To determine heteroresistance to second-line drugs, 91 samples were run on MTBDR*sl*. Nineteen (19) samples that were heteroresistant to 2nd line drugs were subjected to spoligotyping to determine the families/lineages they belonged to.

Results:

A total of 66 were confirmed as *Mycobacterium tuberculosis* complex by the line probe assays. Out of the 66 MTBC, rifampicin resistance was found in 22 (10%) and 44 (19%) were reported susceptible. Isoniazid resistance was found in 39 (17%) and 27 (12%) were reported susceptible. Of the 66 MTBC positive samples, moxifloxacin resistance was found in 33 (16%) and 14 (7%) were reported susceptible. Kanamycin resistance was found in 17 (8%) and 30 (14%) were reported susceptible. Ethambutol resistance was found in 25 (12%) and 22 (10%) were reported susceptible. Heteroresistance was evident in 22 (10%) samples for the first-line and in 23 (11%) for the second-line drugs. Results of a total of 19 heteroresistant samples subjected to spoligotyping when compared to those in the international spolDB4 database indicated that 4 of them matched existing shared spoligotype international types, 15 were unknown (orphans). Eighteen (18) of 19 heteroresistant

samples subjected to spoligotyping were also MDR. Fourteen of the samples that were resistant to both RIF and INH were orphans. Of the 14 MDR, 3 samples belonged to clades T1, T-H_{37}RvV817 and LAM 3 with SITs: 879, 568 and 2301, respectively. One sample with SIT 1196 had an unknown clade was resistant to RIF but susceptible to INH.

Conclusion:

This study has shown that heteroresistance remains an important phenomenon in clinical tuberculosis, especially in highly endemic areas. According to the current study, heteroresistance was associated more with recurrent cases who are on initiation or continuation phase than new cases and a larger percentage of heteroresistance was reported in second-line drugs than there is in first-line drugs. The T1 genotype was found to be predominant amongst recurrent cases. The LAM3 and T-H37RvV817 lineages were found amongst the new cases. In the present study there was no significant association between heteroresistance and the patient's treatment history as indicated by a P-value of 0.473 and between heteroresistance and spoligotype families (P-value, 0.991). The predominance of orphan SITs and unknown clades followed by non-Beijing strains in the study may be due to the migration of carriers from the neighboring countries as the Limpopo Province is flanked by Botswana, Zimbabwe and Mozambique. Further studies with larger numbers of patients should focus on the prevalence to associate heteroresistance with patients' treatment history and establish the contributing MTBC strain lineages.

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ABBREVIATIONS

ACP	Acyl Carrier Protein
AFB	Acid Fast Bacilli
BCG	Bacillus Calmette Guérin
CDC	Centers for Disease Control
DNA	Deoxyribonucleic Acid
DOTS	Directly Observed Therapy Short-course
DST	Drug Susceptibility Testing
EAI	East African Indian
EMB	Ethambutol
FBP	Federal Bureau of prisons
H	Haarlem
HIV	Human Immunodeficiency Virus
INH	Isoniazid
LAM	Latin American Mediterranean
MDR	Multi drug-resistant
MTB	*Mycobacterium tuberculosis*
MREC	Medunsa Research Ethics Committee
NALC	N-acetyl L-cysteine
NHLS	National Health Laboratory Services
NTCP	National tuberculosis control programme
PCR	Polymerase chain Reaction
PZA	Pyrazinamide

RIF	Rifampicin
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
STR	Streptomycin
TB	Tuberculosis
XDR	Extremely or extensively drug resistant
WHO	World Health Organization
WT	Wild type

TERMINOLOGY

- **Amplicon**: A product of polymerase chain reaction.
- **Beijing genotype**: A virulent genotype of *Mycobacterium tuberculosis* that is associated with drug-resistant tuberculosis and was first discovered in Beijing, China.
- **Codon**: A three-nucleotide sequence in mRNA that specifies a particular amino acid or polypeptide termination signal.
- **Decontamination**: Removal of contaminants.
- **Genotype MTBDR**: A commercial and easy-to-perform assay developed for the detection of rifampicin and isoniazid resistance in tuberculosis strains.
- **H37Rv**: A *Mycobacterium tuberculosis* strain used as the "standard" or control in taxonomic studies and drug susceptibility testing.
- **Heteroresistance:** Co-existence of resistant and susceptible organism in a single clinical specimen.
- **Incidence**: The number of new and relapse of TB arising in a given time period, usually a year.
- **MDR TB**: A *Mycobacterium tuberculosis* strain that is resistant to RMP and INH which are the first line anti-TB drugs.
- **Monotherapy**: Treatment of a condition by means of a single drug.
- **Monoresistance**: Ability of microorganisms to remain unaffected by a single antimicrobial agent.
- **Mortality**: The number of deaths caused by TB in a given time period, usually a year.
- ***Mycobacterium tuberculosis* complex**: A group of species that are causative agents of human and animal tuberculosis and consists of *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium bovis BCG*, *Mycobacterium africanum*, *Mycobacterium canetii* and *Mycobacterium microti.*
- **MTBDR*plus*** assay: A line probe assay developed for the detection of *Mycobacterium tuberculosis* complex and its resistance RIF and INH resistance in strains.

- **MTBDR*sl*** assay : A line probe assay based on DNA strip technology and permits the molecular genetic identification of the *Mycobacterium tuberculosis* complex and its resistance to flouroquinolones (ofloxacin and moxifloxacin) and/or aminoglycocides/ cyclic peptides (injectable antibiotics: capreomycin, viomycin/kanamycin and amikacin) and/or ethambutol from cultivated samples or smear positive clinical specimens.
- **Non-Beijing genotype**: A genetic lineage or sublineage of *Mycobacterium tuberculosis.*
- **Polymerase Chain Reaction**: A technique used to amplify or make copies of small segments of DNA.
- **Prevalence**: The number of cases of TB at a given point in time.
- **TDR TB** : TB that shows *in vitro* resistance to all first and second line drugs tested (isoniazid, rifampicin, streptomycin, ethambutol, pyrazinamide, ethionamide, para-aminosalicylic acid, cycloserine, ofloxacin, amikacin, ciprofloxacin, capreomycin, kanamycin)
- **Treatment failure**: positive smear sputum after 5 or 8 months after onset of treatment.
- **Treatment relapse**:Positive sputum within two years after scheduled treatment cessation.
- **XDR TB**: TB that is resistant to the first-line drugs as well as a member of the quinolone family and at least one of the following second-line drugs: kanamycin, capreomycin or amikacin.

CHAPTER 1

1. INTRODUCTION

Tuberculosis (TB) remains one of the world wide health challenge and is a leading cause of death globally (Daku, 2011). The disease is spread from person to person through airborne particles when infected individuals cough, sneeze or spit. Tuberculosis primarily affects the lungs but it can also affect organs in the central nervous system, lymphatic system and circulatory system among others (Daku, 2011; WHO 2011). World Health Organization (WHO) estimated that two billion people constituting a third of the population is infected with MTB and between 6 and 8 million people die from this disease each year (Daku, 2011; CDC, 2013; WHO 2013). In 2013, countries in African continent accounted for the highest rates of cases and deaths relative to population about 280 incident cases per 100 000 on average (WHO 2014). South Africa is ranked third in the world and within a population of 50 million, approximately 400 000 cases have been reported (WHO 2003; WHO 2005). Tuberculosis is the single leading cause of mortality in people living with HIV and AIDS (WHO 2014). Since about 70% of these people live in sub-Saharan Africa, this region bears the overwhelming burden of the epidemic of human immune virus-associated TB (WHO 2014). Tuberculosis and HIV and AIDS are considered to be the main burden of infectious disease in resource limited countries and it was estimated by WHO that more than 9 million new active cases of TB and up to 2 million deaths occur per year (Pawlowski *et al.,* 2012; WHO 2014). South Africa is ranked third in the world in terms of number of TB cases. The problem is compounded by the emergence of resistant strains of *Mycobacterium tuberculosis* that are resistant to the most effective drugs that are currently available (Lange 2014). The emerging worldwide problem of multidrug-resistant tuberculosis (MDR TB) complicates the control and treatment of the tuberculosis (Lange 2014). 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). Multidrug-resistance is defined as resistance to rifampicin (RIF) and isoniazid (INH), which are the most effective drugs used in short-course TB treatment and are also called first-line drugs (WHO 2014). Multidrug-resistant tuberculosis can be acquired during the improper drug use. Thus, identification of

drug-resistant tuberculosis strains can aid in the use of more effective treatment regimen and reduce the occurrence of drug resistance (Xhiabing *et al.,* 2012).

South Africa is also burdened with another form of drug-resistant tuberculosis, extensively drug-resistant tuberculosis (XDR TB). Extensively drug-resistant tuberculosis is resistance to the first line-drugs (rifampicin and isoniazid) as well as a member of the quinolone family and at least one of the following second-line anti-TB injectable drugs: kanamycin (KAN), capreomycin (CAP) or amikacin (AMK) (Dheka *et al.,* 2010; WHO 2014). Extensively drug-resistant tuberculosis can develop due to poor management of MDR TB and therefore second line drugs also become ineffective (Dorrington *et al.,* 2006; Dheka *et al.,* 2010). In South Africa, XDR-TB was first reported in early 2006 in KwaZulu Natal, South Africa (Dorrington *et al.,* 2006; WHO 2006; Dheka *et al.,* 2010). Another form of drug resistance called "Totally Drug-resistant Tuberculosis (TDR-TB) was first described in 2009. TDR TB strains are defined as TB strains that show *in-vitro* resistance to all first line (isoniazid, rifampicin, streptomycin, ethambutol, pyrazinamide) and second line drugs (ethionamide, para-aminosalicylic acid, cycloserine, ofloxacin, amikacin, ciprofloxacin, capreomycin, kanamycin) (Velayati *et al.,* 2009). These dangerous forms of TB bacilli have been found in certain countries such as, Iran, Italy and India (Migliori *et al.,* 2007, Udwadia *et al.,* 2012). Just recently Centres for Diseases Control and Preventions (CDC) reported the first cases of TDR-TB in South Africa and they stated the disease is "virtually untreatable" and 90% of the atypical Beijing isolates had resistance mutations to 10 anti-TB drugs (Klopper *et al.,* 2013). The main mutations that confer RIF-resistance are located in the *rpoB* gene, specifically in the well-defined 81-bp core region. About 95% of RIF-resistant strains have a mutation in this region (Ramaswamy *et al.,* 1998; Varahram, 2013; Veluchamy, 2014). However, the molecular basis of resistance to isoniazid is more complex because it involves mutations in more than one gene or gene complex such as the *katG, inhA* and *kasA* genes and the intergenic region of the *oxyR-ahpC* complex (Barnard *et al.,* 2008; Varahram, 2013; Veluchamy, 2014).

The TB patients may harbour both susceptible and resistance strains, a phenomenon referred to as heteroresistance. Heteroresistance may be due to superinfection with a susceptible and a resistant strain or splitting of the same strain

into susceptible and resistant organisms (Hofmann-Thiel *et al.*, 2009). Heteroresistance could arise due to transmission of both susceptible and resistant organisms from drug-resistant patients to previously untreated cases (Robin *et al.,* 2004). It is more likely to occur in high TB incidence areas and in chronic TB patients as they have more opportunity to become infected with various populations of mycobacteria, therefore "double strains" are more common (Van Rie *et al.,* 2005). Heteroresistance has been proven to occur in new cases, treatment failure and relapses. Hoffmann-Thiel *et al.* (2009) reported rate of 20% of heteroresistance in TB in Tashkent almost similar to the rate of 17% that was reported by Rinder *et al.* (2001). Studies by de Oliveira *et al*. (2003), Makinen *et al*. (2006) and Miotto *et al*. (2006) reported significantly lower rates. The proportion of mixed infections has been reported by Dickman *et al.* (2010) to be 7.1% in Uganda and 18.5% in South Africa reported in study by Stavrum *et al*. (2009). A study conducted in Cape Town also reported mechanisms by which mixed infections can lead to changing drug-susceptibility patterns during therapy (van Rie *et al*., 2005). Kumar *et al*. (2014) in Punjab state of India reported a high rate of hetero resistance in rpoB gene at 28.8% followed by katG gene at 9.8%.

It was proven in a study by Hofmann-Thiel *et al.* (2009) that heteroresistance caused by coexistence of two different strains mostly prevailed in new cases while segregation of single strains of the Beijing genotype prevailed in treatment failure and relapses (Hofman *et al.,* 2009). Heteroresistance has been reported to be caused exclusively by Beijing genotype which was further associated with treatment failures and relapses (Hofmann-Thiel *et al.,* 2009). Whereas the study by Zheng *et al*. (2015) reported the non-Beijing spoligotype strains to be significantly associated with mixed infections more than the Beijing ones (6.62% versus 0.57%) as is the case in kwaZulu-Natal in South Africa reported in a study by Cohen *et al*. (2010).

1.2 RESEARCH PROBLEM

Drug resistance tuberculosis in the form of MDR TB, XDR and TDR presents a serious challenge in TB treatment and a burden to the health care system. Heteroresistance, which is the simultaneous presence of drug-resistant and -susceptible populations in a sample, is considered a preliminary stage to full resistance also compounds the problem. The detection of heteroresistance may help

in proper early treatment adaptation, thus its early detection may limit the development of further resistance in patients undergoing therapy. There is scarcity of data on heteroresistance and its association to treatment history in multidrug-resistant cases and MTB strain families in the Limpopo Province. As heteroresistance in MTB may influence development of full resistance there is a need to conduct the study to shed light to rate of heteroresistant strains of *Mycobacterium tuberculosis* and how its mechanisms are linked to the history of patients' treatment and the predominant lineages in these cases.

CHAPTER 2

2. LITERATURE REVIEW

Tuberculosis (TB) is a common infectious disease which attacks the lungs and is caused by various strains of *Mycobacterium tuberculosis* complex (MTBC) particularly *Mycobacterium tuberculosis* (WHO, 2011). The species included in the MTBC are: *M. tuberculosis* (the major causative agent of TB in humans), *M. bovis, M. africanum, M. canetti, M. caprae, M. microti* and *M. pinnipedii* (Grange *et al.,* 2009). All the other mycobacterium species are grouped together and called the non-tuberculous mycobacteria (NTM) (Grange *et al.,* 2009). It is an important public health problem and remains one of the most threatening curable infectious diseases, despite improvements in diagnostic and drug susceptibility testing (WHO, 2014).

2.1 Transmission

Tuberculosis can be spread from person to person through airborne particles when infected people cough, sneeze or spit. It is estimated that a single cough can generate as many as 3000 infected droplet nuclei and that less than 10 bacilli may initiate a pulmonary infection in a susceptible person (WHO, 2013). The factors attributable to the establishment are quantity and concentration of the *Mycobacterium tuberculosis* in the air and period of exposure to the infected air.

The immune status of an individual is presumed to have an effect on the infectivity of the organism. HIV positive individuals are more predisposed to tuberculosis as compared to other people (FBP, 2012). Tuberculosis is the single leading cause of mortality in people living with HIV/AIDS and 70% of these people live in the sub-Saharan Africa region, which bears the overwhelming burden of the epidemic of HIV associated TB (WHO, 2014). Tuberculosis primarily affects the lungs but it can also affect organs in the central nervous system, lymphatic system and circulatory system (WHO, 2003; WHO, 2011).

2.2 Prevalence

Each year WHO publishes global estimates of TB incidence, prevalence, mortality at global, regional and national levels alongside with an analysis of progress towards achievement of global targets (WHO, 2013). The World health organization (WHO) has estimated that approximately one third of the world's population is infected with *Mycobacterium tuberculosis* and between 6 and 8 million people die from tuberculosis each year (WHO, 2014). In 2009 tuberculosis was the cause of mortality in parents leaving almost 10 million children orphaned (Olson *et al.,* 2011; WHO 2003). In 2010, there were 8.8 million (range, 8.5–9.2 million) incident cases of TB, 1.1 million (range, 0.9–1.2 mil- lion) deaths from TB among HIV-negative people and an additional 0.35 million (range, 0.32–0.39 million) deaths from HIV-associated TB. According to WHO, the total number of new cases of tuberculosis and deaths was high than before (WHO, 2014).

TABLE 2.1 Estimated WHO Regional TB statistics for 2010

Region	**Incidence**	**Prevalence**	**Deaths**	**Population**
Africa	2,300,000	2,800,000	250,000	836,970,000
Americas	270,000	330,000	20,000	933,447,000
Eastern Mediterranean	650,000	1,000,000	95,000	596,747,000
Europe	420,000	560,000	61,000	896,480,000
South-east Asia	3,500,000	5,000,000	500,000	1,807,594,000
Western pacific	1,700,000	2,500,000	130,000	1,798,335,000
Grand total	**8,840,000**	**12,190,000**	**1,056,000**	**6,869,573,000**

In 2011 an estimated 12 million prevalent cases of TB have been reported (WHO, 2011). There were 1.4 million reported deaths in the world (Glaziou *et al.,* 2013; WHO, 2013). The largest estimated numbers of incident cases in 2011 were in India with 2.0-2.5 million and China with 0.9 million- 1.1 million (WHO, 2012).

In 2013, 9 million people were infected with tuberculosis around the world and around 15 million deaths caused by TB were reported by WHO. Of the 9 million estimated cases, 56% were from the South-East Asia and Western Pacific regions. The African region accounted for a quarter of the estimated cases, and also had the highest rates of cases and deaths caused by tuberculosis relative to the population. The total number of new cases of tuberculosis for India and China were 24% and 11% of total cases in the world (WHO, 2014).

South Africa has one of the highest rates of tuberculosis (TB) in the world (WHO, 2003, Dheka *et al.,* 2010). It is ranked third in the world in terms of the number of cases, and has an estimated incidence of 558 cases per 100 000 population (CDC, 2013, WHO, 2013). Within a population of 50 million people, approximately 400 000 new cases are reported each year (Daku, 2011).

World Health Organisation (WHO) reported South Africa as one of the 5 countries with the highest TB incidence, 500 000 new cases and approximately 25 000 have been documented.

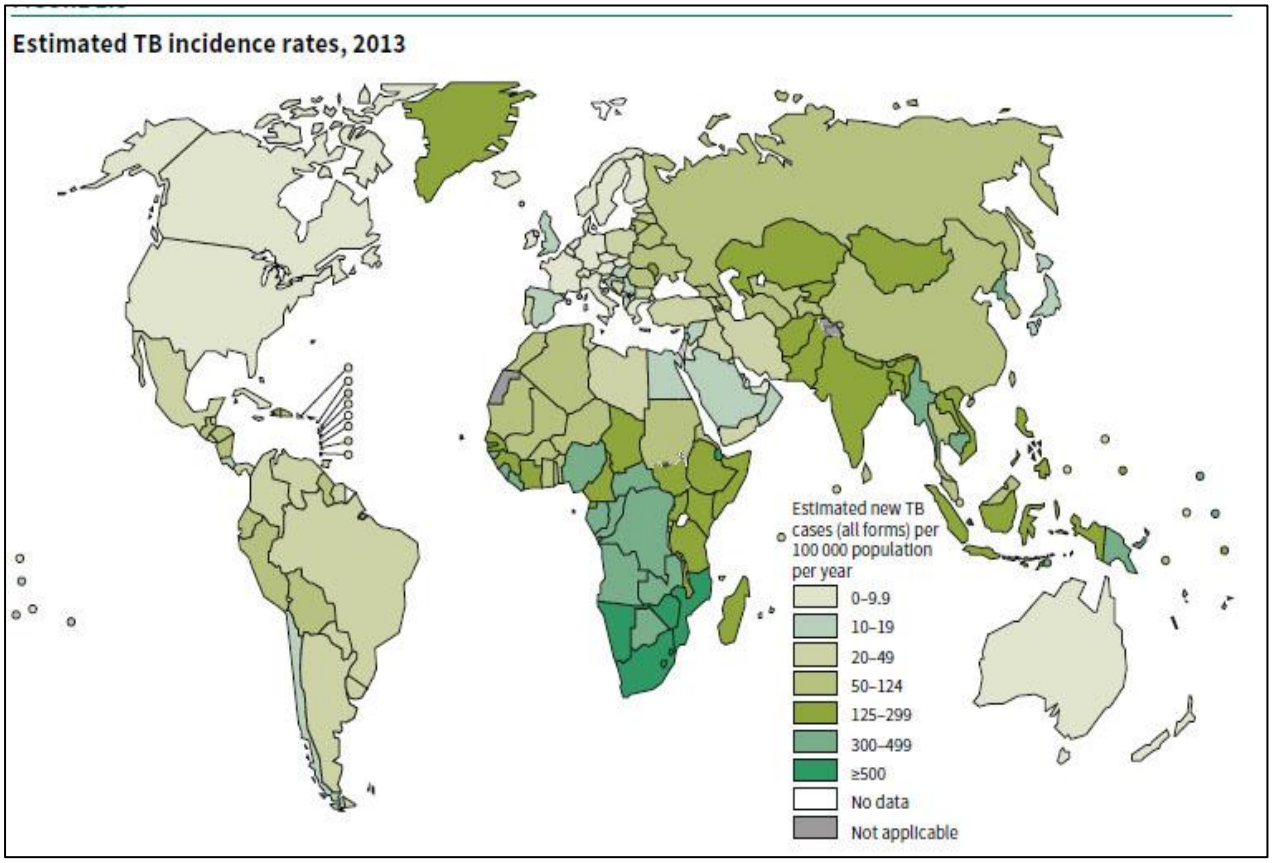


Figure 2.1 Estimated TB incident rates in 2013 (WHO, 2014)

*Figure 2.1 above presents estimated new cases of TB within a population group of 100 000. South Africa was reported with ≥ 500 new TB cases in 2013.

2.3 Symptoms

Tuberculosis usually develops slowly; symptoms might not begin until months or even years after contact with the bacterium. In some cases the bacterium infects the body but does not cause any symptoms, this is called latent TB; it is called active TB if the bacterium can cause symptoms (Management of MDR TB policy, 2010).

Symptoms of pulmonary infection may include:

- a persistent cough of more than three weeks with the presence of blood in the sputum
- breathlessness, which is usually mild to begin with and gradually gets worse
- lack of appetite and unexpected weight loss
- fever
- night sweats
- extreme tiredness or fatigue and
- chest pain

In some cases, TB can occur outside the lungs; in this case it is called extrapulmonary tuberculosis. Extrapulmonary TB is more common in people with weakened immune system, particularly people with an HIV infection. Other body parts that can be infected by tuberculosis are lymph nodes, bones and joints, digestive system, the bladder, reproductive system and nervous system (Tessema *et al.,* 2009).

2.4 Diagnosis

According to the National tuberculosis control programme (NTCP) together with the Directly Observed Therapy Short-course (DOTS) strategy, diagnosis of tuberculosis world-wide is sputum smear microscopy (developed more than 100 years ago), this is the most common method for TB diagnosis. Bacteria are observed in sputum samples examined under a microscope. In countries with more developed laboratory capacity, cases of TB may also be diagnosed via culture methods (the current gold standard) or, increasingly, using rapid molecular tests. Without treatment, mortality rates are high. In studies of the natural history of the disease among sputum smear-positive and HIV-negative cases of pulmonary TB, around 70% died within 10 years; among culture-positive (but smear-negative) cases, 20% died within 10 years (Godreuli *et al.,* 2007). Treatment using combinations of anti-TB drugs developed in the 1940s and 1950s can dramatically reduce mortality rates. In clinical trials, cure

rates of above 90% have been documented; the treatment success rate among smear-positive cases of pulmonary TB reported to WHO reached 87% at the global level in 2009 (Rapid methods of TB diagnosis, 2011).

2.5 Treatment of tuberculosis

Treatment of patients with TB is done according to the following five key components of the Directory Observed Treatment Short Course (DOTS) strategy recommended by the World Health Organisation (WHO) (Walley, 2008): government commitment, Case detection by sputum smear microscopy, standardised treatment regimen of six to eight months, a regular, uninterrupted supply of all essential anti-TB drugs and a standard recording and reporting system. Since the introduction of the DOTS strategy in the early 90s by the WHO, considerable progress has been made in the global TB control (Sterling *et al.*, 2006).

The rapid diagnosis of tuberculosis aids in the establishment of an effective treatment regimen, minimizes the risk of resistance development and limits the spread of drug-resistant *Mycobacterium tuberculosis* (CDC 2013). The four main drugs used in the treatment of tuberculosis are rifampicin (RIF), isoniazid (INH), ethambutol (EMB) and pyrazinamide (PZA), all these are first line drugs. Rifampicin is a surrogate marker for multidrug-resistant tuberculosis in many settings, WHO (2013) and is a useful predictor of treatment failure. Newly diagnosed patients are given treatment for a period of six months. All first line drugs are given in the first two months of treatment and during the last four months of treatment only rifampicin and isoniazid are given (McNerny *et al.,* 2002).

2.6 Drug-resistant tuberculosis

Drug-resistant tuberculosis has reached alarming proportions in South Africa, draining valuable resources that are needed to fight drug susceptible TB. The emerging worldwide problem of multidrug-resistant tuberculosis (MDR-TB) complicates the control and treatment of the disease (STOP TB, 2003, Espinal, 2003). Multidrug resistance is defined as resistance to rifampicin (RIF) and isoniazid (INH), these are the most effective drugs used in short-course TB treatment and are also called first line drugs (WHO 2004). It is currently estimated that 9.6% of all TB cases are MDR TB, thereby ranking South Africa as one of the highest MDR-TB

burden countries in the world. South Africa is the 5th high burden country globally or drug-resistant TB with an estimated13, 000 (WHO 2010; Streicher *et al.,* 2012).

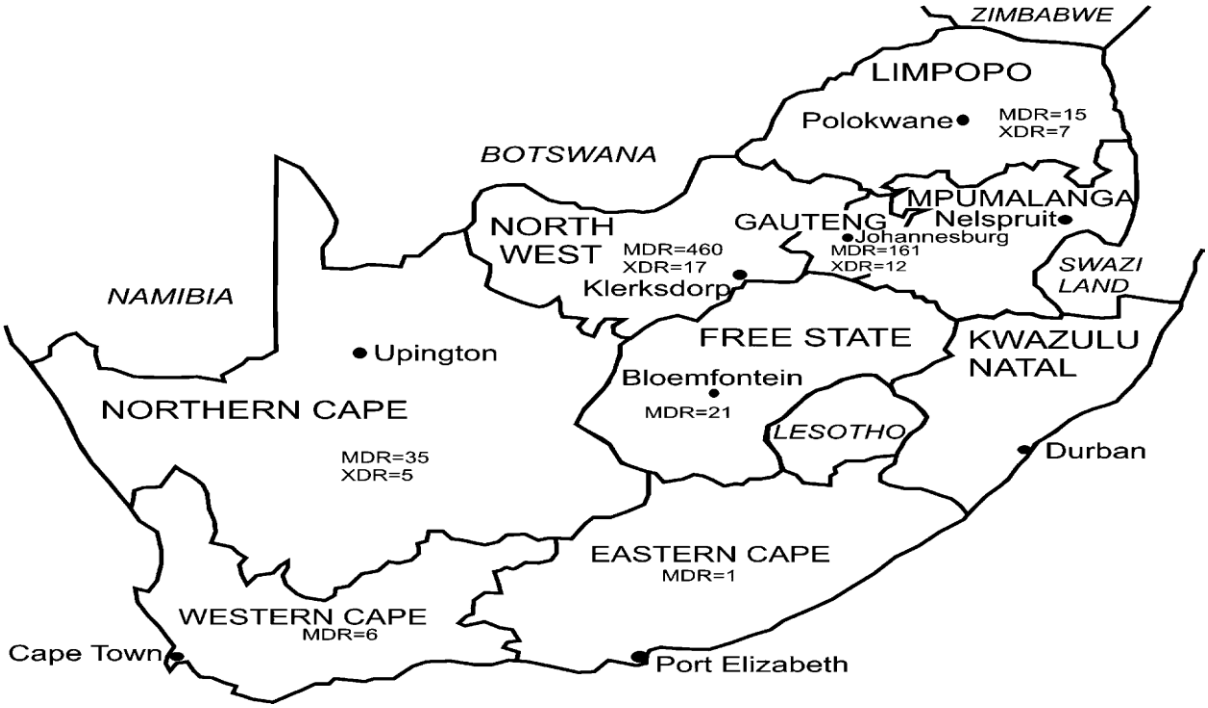


Figure 2.2. Drug resistant strains distribution in South Africa (Mlambo *et al*., 2008).
*Map of Southern Africa showing the South African provinces in which XDR-TB was found. The number of MDR-TB Isolates submitted and the number of XDR-TB patients identified in each province. XDR = extensively drug-resistant, MDR = multidrug-resistant.

In the 1990s, outbreaks of MDR-TB in North America and Europe killed more than 80% of those who contracted the disease. During a major TB outbreak in New York City in the early 1990s, one in 10 cases proved to be drug-resistant. (CDC 1991; WHO 2013). The rapid detection of drug resistance permits the establishment of an effective treatment regimen, minimizes the risk of further resistance development and limits the spread of drug-resistant *Mycobacterium tuberculosis* (Espinal , 2003). Significant high rates of multi-drug-resistant tuberculosis have been observed in some parts of the world not only in previously treated patients but also among new cases due to transmission in the community (WHO 2013). MDR TB can be acquired during the improper drug use, thus identification of drug-resistant strains can aid in the use of more effective treatments and reduce the occurrence fof drug resistance (Xhiabing *et al.,* 2012).

Globally, the proportion of new MDR-TB cases was 3.5% in the year 2013 and there have not been any changes compared with recent years. An estimated 20.5% previously treated patients had developed MDR-TB in the year 2013, that means that an estimated 480 000 people developed MDR-TB in the world in 2013 and approximately 210 000 died. Despite the progress of adequate MDR-TB detection, there is still a major diagnostic gap, there is a great number of late drug-resistant diagnosis (WHO 2013).

2.7 Extensively drug-resistant tuberculosis (XDR TB)

Extensively drug-resistant tuberculosis (XDR TB) is resistance to the first line-drugs (rifampicin and isoniazid) as well as a member of the quinolone family and at least one of the following second-line anti-TB injectable drugs: kanamycin (KAN), capreomycin (CAP) or amikacin (AMK) (Espinal, 2003). Extensively drug-resistant tuberculosis can develop due to poor management of MDR TB and therefore second line drugs also become ineffective (WHO 2004). Since XDR-TB strains have developed resistance to most of the first- and second-line drugs available to treat TB, it is potentially untreatable.

Extensively drug-resistant tuberculosis was reported in 100 countries by the end of 2013. Ten of these countries reported 10 or more cases of XDRTB in the most recent years. An estimated 9.0% of MDR-TB patients had XDRTB in 2013 (WHO, 2014). In 2011, a total of 1269 cases of XDRTB were reported in 40 countries. Of the 1269 XDRTB cases only 284 (22%) were treatment successes and a total of 438 (35%) patients died (WHO, 2014). South Africa has the highest reported prevalence of drug resistant TB in sub-Saharan Africa (WHO, 2013). In South Africa XDRTB was first reported in early 2006 in KwaZulu Natal Province. In a group of 53 patients identified with XDR-TB, all but one died within an average of 25 days from the point when drug-resistant TB was first suspected. Forty-four of the 53 patients were tested for HIV and all were found to be positive (Espinal, 2003; WHO, 2004; Ghandi *et al.,* 2006). Gandhi *et al.* (2015) reported that extensively drug-resistant (XDR) TB has increased in KwaZulu Natal Province between 2010 and 2012. The discovery of *M. tuberculosis* strains with extensive drug resistance in the KwaZulu Natal (KZN) Province of South Africa in 2005 focused global attention not only to the drug-

resistant TB problem in the country but also to global resistance (Gandhi *et al.* 2006). The strain that initially caused XDR-TB in the KZN outbreak was later identified as the F15/LAM4/KZN genotype using molecular techniques (Pillay and Sturm, 2010).

Table 2.2 Laboratory diagnosed XDR-TB patients in the 9 provinces of South Africa (Ndjeka, 2014).

Province	2006	2007	2008	2009	2010	2011	2012	Total	(%)
EC	61	108	175	123	320	377	477	**1662**	**27**
FS	3	4	3	3	7	18	31	**76**	**1**
GP	19	33	30	65	37	91	50	**344**	**6**
KZN	336	241	181	254	201	758	754	**3011**	**48**
LP	5	2	2	6	6	18	3	**44**	**1**
MP	-	12	14	18	5	24	3	**76**	**1**
NC	3	7	19	40	39	111	72	**305**	**5**
NW	9	4	4	13	14	14	10	**74**	**1**
WC	28	42	60	72	112	163	145	**650**	**10**
Total	**464**	**453**	**488**	**594**	**741**	**1574**	**1545**	**6242**	**100**

*__Table 2.2__ above presents the number of patients diagnosed with XDR-TB from 2006-2012 in 9 provinces of South Africa. The province with the most number of diagnosed XDR-TB patients in 2006 was the KwaZulu-Natal Province with 336 patients and the province with the least number of diagnosed XDR-TB patients was the Mpumalanga province with 0 patients. In 2012, the KwaZulu-Natal Province had the highest number of the diagnosed XDR-TB cases with 754 and the Limpopo and Mpumalanga Provinces had the least numbers of diagnosed XDR-TB in South Africa with 3 patients each.

2.8 Totally Drug-resistant tuberculosis (TDR TB).

Totally Drug-Resistant Tuberculosis (TDR-TB) for TB is defined as strains that showed in-vitro resistance to all first and second line drugs tested (isoniazid, rifampicin, streptomycin, ethambutol, pyrazinamide, ethionamide, para-aminosalicylic acid, cycloserine, ofloxacin, amikacin, ciprofloxacin, capreomycin, kanamycin) (Velayati *et al.,* 2009). Within a year of the first reports of extensively drug-resistant TB (XDR-TB) in 2006, two patients were reported from Italy with strains having resistance to all first and second line anti-TB drugs (Migliori *et al.,* 2007). In 2009, 15

TB patients in Iran were reported to be resistant to all anti-TB drugs tested (Velayati *et al.,* 2009). In December 2011, clinicians in Mumbai, India described four patients with “TDR-TB” (Udwadia *et al.,* 2012). A few weeks later, the Times of India reported another eight cases in Mumbai. The report by the Centres for Diseases Control and Preventions (CDC) on the first cases of TDR-TB in South Africa stated that the disease is “virtually untreatable” and 90% of the atypical Beijing isolates had resistance mutations to 10 anti-TB drugs is alarming (Klopper *et al.,* 2013).

2.9. Mutations of first line drugs

Multidrug-resistant tuberculosis develops due to mutations in the genes of the mycobacteria bacilli. The mutation is largely the consequence of errors in prescription of chemotherapy e.g. monotherapy, patient management, patient adherence, and management of drug supply (Saurez *et al.,* 2002; Vladyslav *et al.,* 2009; WHO 2003). Resistance to first line drugs is linked to at least 10 genes; *katG, inhA, ahpC, kasA and ndh* for INH resistance; *rpoB* for RIF resistance where it replaces amino acids: phenyl-alanine, tryptophan and tyrosine with non-aromatic amino acids resulting in poor bonding between RIF and RNA polymerase (Vladyslav *et al.,* 2009), *embB* for ethambutol (EMB) resistance, *pncA* for pyrazinamide (PZA) resistance and *rpsL* and *rrs* for streptomycin (STR) resistance (Jacobs, 1994; Cole 1994).

2.9.1. Rifampicin

Resistance to RIF occurs at a frequency of 1 out of 10^7 to 10^8 bacterial cells. Most RIF-resistant strains show one mutation in the gene. Two to four mutations are rarely reported (Jacobs, 1994; Mani *et al.,* 2001). The most prevalent mutations (81%) affecting codons 531 and 526 and usually lead to a high level of phenotypical resistance (Cole,1994; CDC 1991). Mutations in *rpoB* generally result in high level resistance and cross-resistance to all rifamycin (Zhang *et al.,* 2009).

Mode of action of rifampicin

Rifampicin interferes with RNA synthesis by binding to the β subunit of the RNA polymerase. The RNA polymerase is an oligomer consisting of a core enzyme formed by four chains α2ββ′ in association with the σ subunit to specifically initiate transcription from promoter (Zhang *et al.,* 2009). The RIF-binding site is located upstream of the catalytic centre and physically blocks the elongation of the RNA chain. As in other bacteria, mutations in a defined region of the 81 base pair (bp) region of the *rpoB* are found in about 96% of RIF-resistant *Mycobacterium tuberculosis* isolates (CDC, 1991).

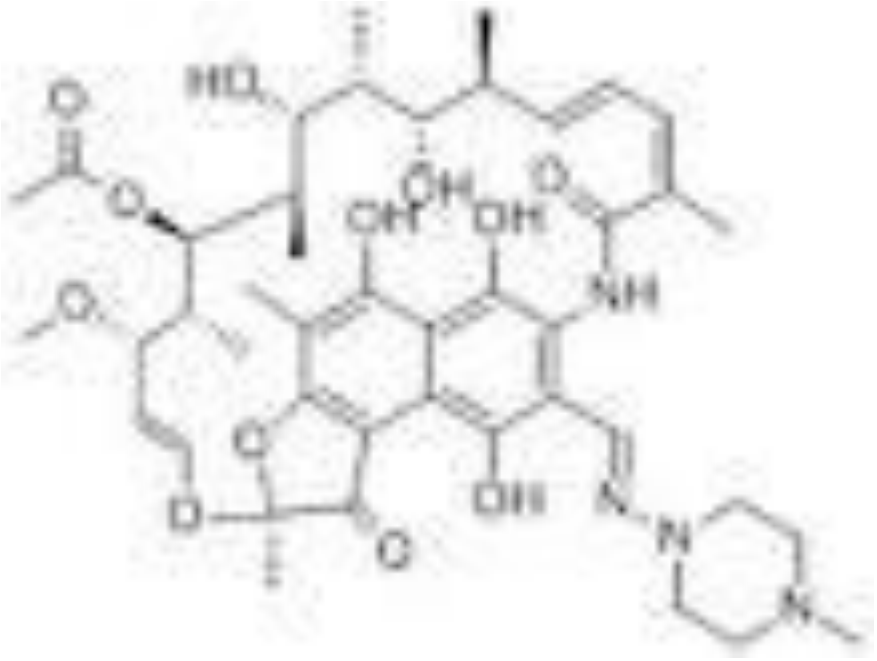


Figure 2.3 Molecular structure of rifampicin (Kolyva *et al*., 2012)

2.9.2. Isoniazid

Isoniazid was synthesized in the early 1900s but its anti-TB action was first detected in 1951 (Mani *et al.,* 2001; Riska *et al.,* 1999; Zhang *et al.,* 2009).

Mode of action of isoniazid

This toxic substance subsequently affects intracellular targets such as mycolic acid biosynthesis which are an important component of the cell wall. A lack of mycolic acid synthesis eventually results in loss of cellular integrity and death of the bacteria

(Heym *et al.* 1994; Slayden *et al.,* 2000) .Subsequently, genetic studies demonstrated that transformation of INH-resistant *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* strains with a functional *katG* gene restored INH susceptibility and that *katG* deletions give rise to INH resistance (Sterling *et al.,* 2006; Walley 2008).

Most mutations are found between codons 138 and 328 with the most commonly observed gene alteration being at codon 315 of the *katG* gene (CDC, 1991). The Ser315Thr substitution is estimated to occur in 30–60% of INH resistant isolates (Julio *et al.,* 2001; Management of drug-resistant TB 2010; Sterling *et al.,* 2006). One of the targets for activated INH is the protein encoded by the *inhA* locus. The gene *InhA* is an enoyl–acyl carrier protein (ACP) reductase which is proposed to be the primary target for resistance to INH and ethionamide (ETH) (McNerny *et al.,* 2002). Ethionamide, a second line drug, is a structural analogue of INH that is also thought to inhibit mycolic acid biosynthesis and several studies have suggested that low-level INH resistance is correlated with resistance to ETH. To date approximately 70–80% of INH resistance in clinical isolates of *Mycobacterium tuberculosis* can be attributed to mutations in the *katG* and *inhA* genes (Xhiabing *et al.,* 2012).

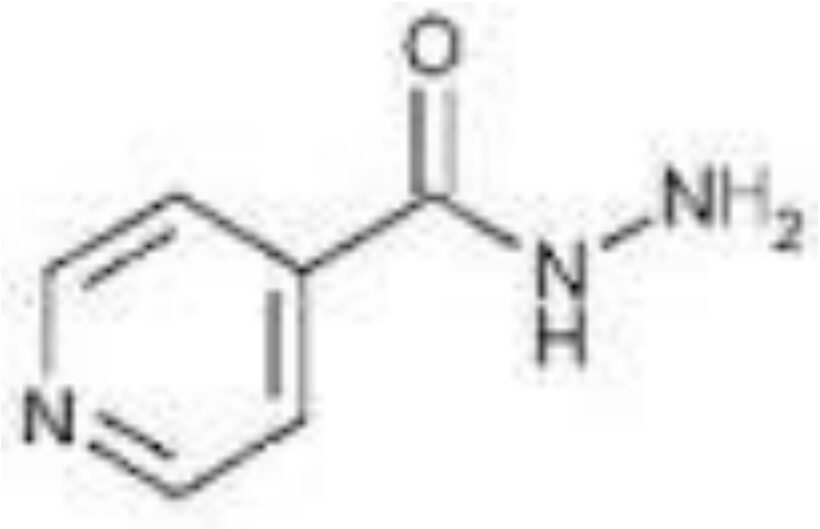


Figure 2.4 Molecular structure of Isoniazid (Kolyva *et al*., 2012)

2.10 Mutations of second line drugs

2.10.1 Fluoroquinolone: Moxifloxacin

Fluoroquinolones are important second line drugs for TB and one of the main tools to fight resistant TB strains (Blumperg *et al*., 2003). Currently levofloxacin (LFX) and moxifloxacin (MFX) are recommended by WHO for treatment of MDR-TB (WHO 2010). The target of quinolones is the enzyme DNA gyrase which is involved in DNA supercoiling and bacterial replication (Xu *et al.,* 1996). Quinolones exert their antibacterial activity by interfering with the enzymatic reaction cycle, binding to the enzyme-DNA binary complex, thereby stabilizing the covalent linkage enzyme-DNA. The resulting ternary complex blocks DNA replication and leads to cell death (Hawkey *et al.,* 2003). The increased use of quinolones in TB has led to decreased quinolone susceptibility in *Mycobacterium tuberculosis*.

Quinolone resistance is generally due to point mutations in *gyrA* and *gyrB* genes, in particular in quinolone resistance determining regions (QRDRs), which are involved in quinolone binding (Espinal 2003; Julio *et al.,* 2001; Stop TB 2003; WHO 2004; WHO, 2005; WHO 2013). Additional fluoroquinolones are the only class of antimicrobial agents in clinical use that are direct inhibitors of bacterial DNA synthesis. Fuoroquinolone resistance in *Mycobacterium tuberculosis* mutations in Ala-90 and Asp-94 of *gyrA* have been the most commonly found (Sun *et al.,* 2008; Lica *et al.,* 1997).

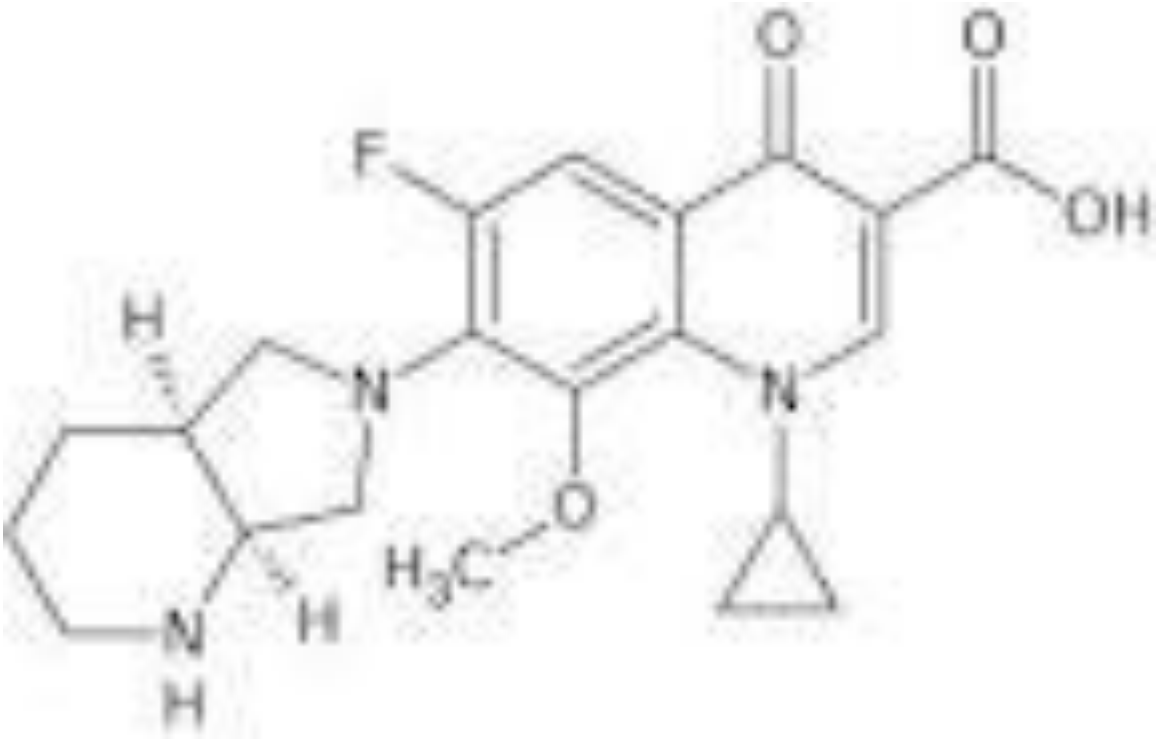


Figure 2.5. Molecular structure of moxifloxacin (Kolyva *et al*., 2012)

2.10.2. Kanamycin

Kanamycin is one of the key second-line drugs for the treatment of tuberculosis. Patients who are suffering from tuberculosis caused by multidrug-resistant strains with resistance to the first-line antituberculosis drugs such as rifampin, isoniazid, ethambutol, streptomycin, or pyrazinamide have a poor prognosis (Dheka *et al.,* 2010). For such patients, kanamycin is one of the best choices for treatment.

In bacteria, resistance to kanamycin is attributed to three mechanisms. One mechanism involves an aminoglycoside-modifying enzyme carried by transposons (Kapur *et al.,* 1994). The second mechanism is specific methylation of rRNA. Modification of the rRNA at position 1405 or 1408 was responsible for kanamycin resistance (Bastian *et al.,* 2000). The third mechanism involves nucleotide changes in the 3′ part of the 16S rRNA gene (*rrs*). The structural and functional organization of rRNA is highly conserved among bacteria, so it is reasonable to consider that the same mutation results in resistance to kanamycin in mycobacteria, as was seen in other bacteria.

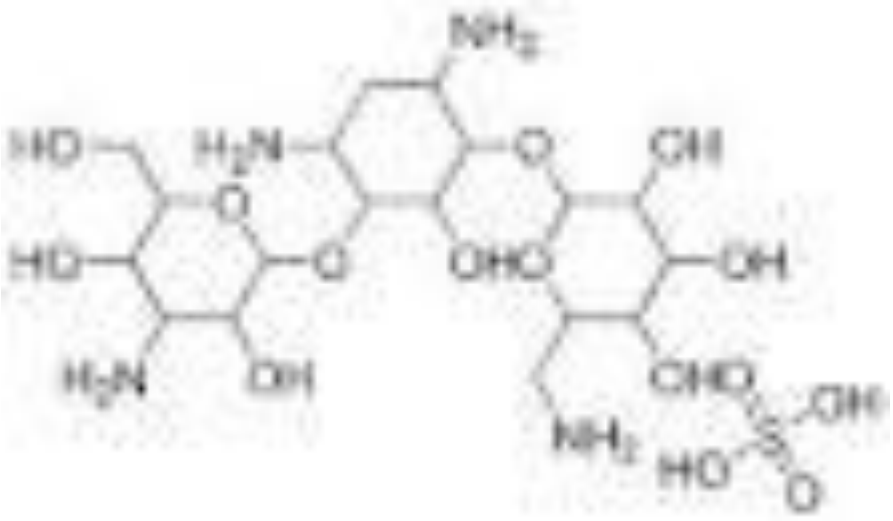


Figure 2.6 Molecular structure of kanamycin (Kolyva *et al*., 2012)

2.11. Treatment of MDR TB

The limited number of available second-line drugs imposes obvious limitations on the design of adequate MDR-TB treatment regimens. The most successful treatment regimens are those that include multiple drugs, which the patient had not previously received (Espinal *et al.,* 2000; Blumberg *et al.,* 2003). A standardised MDR-TB regimen is recommended and this is based on the country-specific profiles of drug resistance and previous drug use of second-line drugs (Heym *et al.,* 1994; Hawkey 2003).

The standardised regimen of MDR TB at Modimolle MDR TB unit in Limpopo Province, South Africa is based on two phases of treatment: Initiation phase followed by a continuation phase. Initiation phase consists of at least six months intensive phase treatment with five injectable drugs: Kanamycin and capreomycin taken at least six times per week. A continuation phase treatment consists of four drugs moxifloxacin, ofloxacine, terizidone and terivaldine.

Treatment of MDR and XDR-TB requires 2 years or more of daily, directly observed treatment with drugs that are less potent, more toxic, and much more expensive than those used to treat drug-susceptible TB. A treatment based on isoniazid and rifampicin cannot be expected to cue or substantially improve tuberculosis in patients infected with MDR TB should ineffective treatment reduce the transmission of MDR-TB. Despite the challenges, aggressive treatment with second-line drugs has

produced successful outcomes in MDR and XDR -TB patients. However, TDR -TB is a growing threat. (Keshavjee *et al*, 2008).

2.12 Heteroresistance

Heteroresistance of *Mycobacterium tuberculosis* is understood as mixed populations of drug sensitive and drug-resistant organisms in a single sputum specimen or clinical isolate (Tolani *et al.,* 2012). The vast majority of resistances are caused by point mutations in a limited number of genes whose products are either the target of or activate the anti-tuberculosis drug (Xhang *et al.,* 1992). Heteroresistance could arise due to transmission of both susceptible and resistant bacterial populations from drug-resistant patients to previously untreated cases (Hofmann-Thiel *et al.,* 2009). It is more likely to occur in high TB incidence areas and in cultures isolated chronic patients as they have more opportunity to become infected with various populations of *Mycobacteria* therefore "double strains" were more common (Robin *et al.,* 2004). Heteroresistance is considered a preliminary stage of full resistance and provides the bacteria an opportunity to explore the possibilities of growth in the presence of antibiotics (Morand *et al.,* 2007). Heteroresistance has been identified in strains with resistance to isoniazid, rifampicin, ethambutol and streptomycin (Adjers-Kaskelia *et al.,* 2003; Cullen *et al.,* 2003; Karahan *et al.,* 2005). The clinical significance of heteroresistance is unclear but it is still an obstacle to both molecular drug resistance and successful therapy.

Two proven mechanisms of heteroresistance namely, coexistence of two different *Mycobacterium tuberculosis* strains which is theoretically explained by superinfection of a patient, infected with one *Mycobacterium tuberculosis* strains with an additional one and segregation of single strains into resistant and susceptible organisms under insufficient anti-TB therapy. They have been proven to occur in new cases, treatment failures and relapses. It was proven by Hofmann-Thiel *et al.* (2009) that coexistence of two different strains prevailed in new cases while segregation of single strains prevailed in treatment failures and relapses (Hofmann-Thiel *et al.,* 2009).

2.13 Prevalence of heteroresistance

The rate of heteroresistance is related to the level of clinical isolates of TB prevalence in certain regions. Commonly, heteroresistance in tuberculosis is detected in 9 to 20% of clinical isolates (Rinder *et al.,* 2001; Chakravorty 2011). It has been detected in 25, 8% of clinical isolates with resistance to INH and RIF in Samara Russia, where a high incidence of TB can be found (Nikolayevskyy *et al.,* 2009). Xhiang *et al,* (2012) showed that heteroresistance emerged in 23% of isolates with the majority due to infection with a single strain. In addition, the high frequency of heteroresistance that occurred in those MDR TB isolates revealed the improper use of anti-TB drugs leading to drug resistance and would also increase the possibilities of development of XDR TB (Xhiang *et al.,* 2012). A study conducted in Vietnam reported heteroresistance in 20% of the cases (Duong *et al.,* 2009). A study conducted by Hofmann *et al*. (2009) at Uzbekistan 20% heteroresistance, while a study by Farooqi *et al*. (2012) in Pakistan reported 4% heteroresistance. The highest heteroresistance was reported by Tolani *et al*. (2012) from Mumbai, India at 34%.

In five of those cases, heteroresistance was caused by two different strains and in two by a single strain of the Beijing genotype. The latter cases had a history of relapses of their TB. A study conducted in Cape Town, South Africa reported 19% of cases of patients infected with multiple different strains (Van Rie *et al.,* 2005). The cases were not heteroresistant although the possibilities could not be avoided. Multiple infections were more frequent in retreatment cases (23%) as compared with new cases (17%). Van Rie *et al*. (2005) suggested that drug susceptibility strain population may be reduced during first-line antibiotics, while allowing drug-resistant strain population to grow, thus converting the patient from an apparently drug-susceptible to an MDR-TB case. Another study also conducted in Cape Town reported 9% mixed infection cases (Robin *et al.,* 2004).

2.14 Beijing and non-Beijing strains

One clade of *Mycobacterium tuberculosis*, the Beijing genotype family, first described in 1995 by Van Soolingen, is one of the most successful clades in the current worldwide tuberculosis epidemic (Musser *et al.,* 1996). The Beijing genotype

is now found in many different countries, especially in Asia, the former Soviet republics and (South) Africa, but also Northern American cities (Banerjee *et al.,* 1994; Rinder *et al.,* 2001; Van Soolingen *et al.,* 1995; Bifani *et al.,* 2002). The Beijing genotype clade consists of at least two major subgroupings; typical and atypical Beijing strains, which differ in their distribution in different countries and age groups (Brudery *et al.,* 2006; Glynn *et al.,* 2002; Abebe *et al.,* 2006; Kremer *et al.,* 2004). Typical (modern) Beijing strains including W strains, exhibit highly similar multicopy IS 6110 RFLP patterns, the atypical (ancestral) Beijing strains more closely resemble the common ancestor of the Beijing clade. The W-Beijing family of strains of *Mycobacterium tuberculosis* is globally distributed (Banerjee *et al.,* 1994) and documented as a cause of outbreaks of infection that often involve multidrug-resistant organisms (Kurepina *et al.,* 1998). The W strain caused an outbreak of drug resistance in New York city in 1991 (Kremer *et al.,* 2004). W-Beijing strains have also been associated with extra-thoracic disease (Kremer *et al.,* 2009) and human immune virus (HIV) infection (Mokrousov *et al.,* 2002)

Many studies have been conducted to investigate why the Beijing genotype has spread globally in a relatively limited time period. Different animal models have shown that Beijing strains are more virulent, causing more histopathology, higher outgrowth and increased mortality. Studies in human patients found higher rates of drug resistance among Beijing strains in some, but not all geographic areas (Van Soolingen *et al.,* 1995). Some studies found a relation between the Beijing strains and the BCG vaccination, suggesting that Beijing strain is an ‘escape variant’. A study in Vietnam in the year 2000 discovered that BCG vaccine is less protective against infection with Beijing strains, a higher proportion of Beijing strains were found among BCG vaccinated subjects compared to non-vaccinated individuals (Anh *et al.,* 2000). A few studies linked the Beijing strains with treatment failure.

Although Beijing family strains were assumed to be the predominant strains causing active tuberculosis in China, different sublineages of non-Beijing strains have been detected in the countries and regions adjacent to mainland China (Anh *et al.,* 2000; Kong *et al.,* 2007; Arora *et al.,* 2009). In Taiwan, strains in the Haarlem (H) sublineage, East African-Indian (EAI) and EAI-like sublineages, and Latin American-Mediterranean (LAM) sublineage are also prevalent (Caminero *et al.,* 2001; Singh *et*

al., 2007). Three of the most hypervirulent clinical strains isolated in a study in Shanghai and Hong Kong belonged to families of non-Beijing strains (Caminero *et al.,* 2001).

2.15 Association of heteroresistance and Beijing genotype.

Studies done in Vietnam and Singapore reported that heteroresistance has been caused exclusively by Beijing genotype which was further associated with treatment failures and relapses. These areas reported high rates while others reported lower rates, this is because the group of TB patients and study site influence the rate of heteroresistance (Hofmann-Thiel *et al.,* 2009). Another study conducted in Vietnam to investigate the association of Beijing genotype to treatment relapse or failures also reported that Beijing genotype is a risk factor for treatment failures and relapses (Lan *et al.,* 2003).

2.16. Diagnosis for drug-resistant tuberculosis

2.16.1 MTBDR*plus* assay

The MTBDR*plus* is a commercially available line probe assay and easy-to-perform assay developed for the detection of RIF and INH resistance in TB strains. The test is based on reverse hybridization between amplicons derived from a multiplex PCR and nitrocellulose-bound wild-type and mutated probes for the mutations of interest. This test has been evaluated mainly on cultures and clinical specimens in various settings, demonstrating excellent specificity and good concordance with phenotypical drug susceptibility testing (DST) results (Douglas *et al.,* 2003; Dou *et al*., 2008; Wong *et al.,* 2007).

The MTBDR*plus* strip contains 27 probes, including hybridization (CC) and amplification (AC) controls. *Mycobacterium tuberculosis* was detected in a sample by the use of the *Mycobacterium tuberculosis* complex-specific (TUB) probe. The *rpoB*, *katG*, and *inhA*-specific regions are detected by *rpoB*, *katG*, and *inhA* control probes, respectively. The activities of an amplified fragment with the 8 *rpoB* wild-type probes (*rpoB* WT1 through *rpoB* WT8), 2 *inhA* wild-type probes (*inhA*WT1 and *inhA*WT2),

and 1 *katG* wild-type probe are used to detect the mutations that lead to RIF and INH resistance in *Mycobacterium tuberculosis* (Hilleman *et al.,* 2007).

2.16.2. MTBDR*s*/ assay

MTB DR*s*/ line probe assay is based on the DNA strip technology and permits the molecular genetic identification of the *Mycobacterium tuberculosis* complex and its resistance to flouroquinolones (ofloxacin and moxifloxacin) and/or amicoglycocides/ cyclic peptides (injectable antibiotics: capreomycin, viomycin/kanamycin and amikacin) and/or ethambutol from cultivated samples or smear positive clinical specimens. The identification of resistant flouroquinolones is enabled by the detection of the most significant mutations of the *gyrA* gene (coding for DNA gyrase). For the detection of genes *embA* and *embC* (coding for arabinosyl transferase) are examined.

2.17. Spoligotyping

Spoligotyping is a PCR based technique developed for identification and differentiation of strains of *Mycobacterium tuberculosis*. The test is based on PCR amplification of highly polymorphic direct repeat locus in the *Mycobacterium tuberculosis* genome. Spoligotyping is a rapid and efficient way of fingerprinting the *Mycobacterium tuberculosis* complex, and it can be performed directly on sputum samples, which makes it practical in acute clinical settings (Kamerbeek *et al*., 1997). Results can be obtained from *Mycobacterium tuberculosis* culture within one day thus; the clinical usefulness of spoligotyping is determined by its rapidity, both in detecting the causative bacteria and in providing epidemiologic information on strain identities (Driscoll, 2009). The principle of spoligotyping is that amplification of the spacers is accomplished by using the primers (direct repeats) DRa and DRb, which enable to amplify the whole DR region. Only a very small amount of template DNA is required. Typically the PCR is performed on 10 ng purified chromosomal mycobacterial DNA but, with minor adaptations, DNA extracts from clinical samples or lysed bacteria (from freezer or Löwenstein) can also serve as template. The PCR products are labelled with biotin, because primer DRa is biotinylated (Hermans *et al.,* 1995).

CHAPTER 3

3. PURPOSE OF THE STUDY

3.1 RESEARCH QUESTIONS:

- What is the rate of heteroresistance in MDR TB strains of the Limpopo Province?
- Is there an association between heteroresistance and the treatment history of the cases harbouring the strains?
- Which MTB strain lineages are predominant in cases with heteroresistance?

3.2 AIM OF THE STUDY:

- To determine the prevalence and nature of heteroresistance occurring in patients with multidrug-resistant tuberculosis.

3.3 OBJECTIVES:

- To detect mutations in the *rpoB* gene for RIF and *inhA* and *katG* genes for INH using MTBDR*plus*.
- To detect mutations in *gyrA* and *gyrB* for moxifloxacin, *rrs* gene for Kanamycin and *embB* gene for ethambutol using MTBDR*sl*.
- To assess the association of heteroresistance and patients treatment history.
- To genotype heteroresistant *Mycobacterium tuberculosis* samples by spoligotyping in order to classify them into either the Beijing or non-Beijing families.

CHAPTER 4

METHODOLOGY

4.1 Research method

This is cross sectional study because it involves data collection at a defined time and often used to assess the prevalence of acute or chronic infections. In this study three consecutive sputum samples were collected by health practitioners, two were kept at the MDR TB unit for diagnostic purposes and one sample was collected for the research without follow up. The samples were assessed for presence of mutations associated with resistance to first-line drugs, RIF and INH using the MTBDR*plus* Version 2 line probe assay (LPA). The same samples were further run on MTBDR*sl* V.2 to detect mutations in genes coding for the targeted second line drugs (moxifloxacin, kanamycin and ethambutol). The assays detected the presence of mixed strains that are resistant and susceptible to the targeted drugs in the same clinical specimen indicating heteroresistance. The specimens with heteroresistance were further associated with the patients' treatment status, whether they are new, retreatment or relapse cases. DNA was extracted from some of the samples for spoligotyping to determine which MTB strain families they belong to and how they may have influenced the development of heteroresistance.

4.2. Study population

Samples were collected at Modimolle MDR-TB unit from hospitalised and outpatient cases diagnosed with MDR-TB. Modimolle MDR-TB unit is a referral MDR-TB unit for all hospitals in the Limpopo province. Limpopo Province covers 123 910 km^2 with an estimated population of 5.8 million. The population of Limpopo contributes to 11.3% of South Africa's population of 47.9 million, according to STATS SA (2007). This makes Limpopo the 4th most populated province in the country.

4.3. Sampling and data collection

4. 3.1. Sample collection

Samples collected from Modimolle MDR-TB unit were form patients who had not started with treatment (new cases) and patients who were on the initiation or continuation phase of treatment (recurrent cases) for MDR-TB.

Table 4.1: Clinical data of MDR TB cases and patients' treatment history and stages

Clinical data	Patients number (%)	Treatment history	
		Before sputum collection	After sputum collection
New cases	5 (2%)	No treatment	Initiation phase (kanamycin and capreomycin)
Recurrent cases	226 (98%)	Initiation phase (kanamycin and capreomycin)	Continuation phase (moxifloxacin, ofloxacin, terizidone and terivaldine).

Table 4.1 above presents clinical data of patients. There were 5 (2%) new cases that had not started with treatment before sputum collection and 226 (98%) were recurrent cases that were on initiation phase of treatment during sputum collection.

The standardised regimen given to the MDR TB patients at Modimolle MDR-TB unit in Limpopo Province South Africa is based on two phases of treatment: Initiation phase followed by a continuation phase. Initiation phase consists of at least six months intensive phase treatment with five injectable drugs: Kanamycin and capreomycin taken at least six times per week. A continuation phase treatment consists of four drugs moxifloxacin, ofloxacin, terizidone and terivalidin.

4.3.2. Sample size

Sample size was calculated using the Daryle W Morgan formula (Morgan, 1970).

$s = X^2 NP (1-P)/ d^2 (N – 1) + X^2 P (1 – P)$.

s = required sample size

X^2 = the table value of chi-square for the 1 degree of freedom at the desired +confidence level (3.841).

N = the total number of drug resistance TB patient in Limpopo province reported in 2011 by NHLS and NICD which is 151 (NHLS and NICD, 2011).

P = the population proportion (assumed to be 0.50 since this would provide the maximum sample size).

d = the degree of accuracy expressed as a proportion (0.05)

$s = (3.841) (151) (0.5) (1-0.5)/ (0.05)^2 (151-1) + (3.841) (0.5) (1- 0.5)$

s = 135

A minimum of 135 samples were to be collected.

4.3.3 Data collection

(a) Patients treatment history.....(Appendix 1)

Patients treatment history was collected from hospitalised and outpatient MDR TB patients files during specimen collection at the Modimolle MDR-TB unit. The treatment history (new cases, recurrent cases) was needed to be associated with the occurrence of heteroresistance.

(b) Lab procedures

(i) All samples were decontaminated by the modified petroff method (NALC-NAOH)…. (Appendix 2).

Sputum samples were collected from Modimolle MDR-TB unit and subjected to the following procedures: The working solution was prepared by dissolving 2g of: N-acetyl L-cysteine (NaLC) in 200ml of distilled water, 2.9% sodium citrate and mixed with an equal amount of 4% NaOH. Then, a 2X volume of the solution was added to the specimen and mixed by vortex. Therefore, the mixture was left at room temperature for 15minutes. When 15 minutes has elapsed, 5ml of the mixture was transferred into a 15ml centrifuge tube and 10 ml of phosphate buffer (pH6.8) was added to the 15ml tube and centrifuged at 3000xg for 15 minutes. Then, the supernatant was discarded into a disinfectant container and +/- 3ml left in the tube for MGIT procedure and DNA extraction.

(ii) All the samples were ran on *MTBDRplus* to detect mutations in the *rpoB* and *inhA* and *katG* of RIF and INH, respectively at NHLS IN Polokwane ….(Appendix 3).

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 included the following steps below.

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 in the mean time when 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.

(iii) All the samples were ran the GenoType®MTBDRsl assay on to detect mutations in the *rpoB* and *inhA* and *katG* of RIF and INH, respectively at NHLS at MRC in Pretoria….(Appendix 4)

In this study The MTBDR*sl* assay was performed on extracted DNA samples . The MTBDRsl*plus* assay was performed according to the instructions provided by the manufacture (Hain Life Science GmbH, Nehren, Germany). Briefly, the amplification

mixture contained 35 µl of the primer nucleotide mix, 5 µl of 10 x polymerase incubation buffer, 5 µl of 25 Mm $MgCl_2$, 1 µl of AmpliTaq Gold polymerase (5 µl; AppliedBiosystems), and 5 µl of the supernatant of the cell lysate, for a final volume of 50 µl. The amplification protocol consists of 5 min of denaturation at 95°C, followed by 10 cycles comprising 30 s at 95°C and 2 min at 58°C, an additional 20 cycles comprising 25 s at 95°C, 40 s at 53°C, and 40 s at 70°C, and then a final extension at 70°C for 8 min. Hybridization and detection was performed with a TwinCubator (Hain Lifescience GmbH, Nehren, Germany). The hybridization procedure includes the following steps: chemical denaturation of the amplification products at room temperature for 5 min, hybridization of the single-stranded biotin-labeled amplicons to membrane-bound probes at 45°C for 30 min, stringent washes, addition of a streptavidin–alkaline phosphatase (AP) conjugate at room temperature for 30 min, and an AP staining reaction to detect colorimetric bands.

The GenoType MTBDR*sl* DNA strip is coated with 22 probes. Briefly resistance to fluroquinolones is based on the use of 3 wild-type probes covering GyrA codons 85 to 97. The presence of the most frequently observed mutations are confirmed by positive hybridization with 6 mutant probes (A90V, S91P, D94A, D94G and D94H). For aminoglycoside and cyclic peptide resistance, two wild-type probes cover nucleotides 1401G and 1402 and 1484, and two mutant probes specifically detect the A1401G and G1484T exchages. For ethambutol resistance, one wild-type probe covers codon 306 and the presence of the most frequently observed mutations, M306V and M3061, is confirmed by positive hybridization with two probes. The amplication reactions and the GenoType MTBDRsl will be performed as recommended by the manufacturer, using 5 µl ot the extracted DNA.

(iv) Genotyping of sputum samples was performed by spoligotyping at MRC Pretoria... (Appendix 5).

The reaction mixture was prepared: 2 µl template DNA, 3 µl primer DRa (0.2 µmol/µl), 3 µl primer DRb (0.2 µmol/µl), 20 µl 2×TaqPCR MasterMix and 12 µl MQ water (to a final volume of 40 µl). The tubes were placed in a PCR-apparatus for

amplification, and performed the following temperature cycling: 3 min 94°C 1 Cycle, 1 min 94°C, 1 min 55°C, 30 sec 72°C 25 Cycles, 7 min 72°C 1 Cycle and ∞ 4°C

Hybridization with PCR product and detection

Purpose: Hybridization of the biotin-labelled PCR products to the immobilized spacer-oligos that represent spacers of known sequence. The presence of spacers is visualized on film as black squares after incubation with streptavidin-peroxidase and ECL detection.

All buffers were prewarmed before use. The following buffers were prepared from concentrated stocks, using demineralised water for dilution (quantities for one membrane):

250ml 2×SSPE/0.1% SDS, 42 °C,

250ml 2×SSPE/0.5% SDS, 60 °C,

250ml 2×SSPE/0.5% SDS, 42 °C,

250ml 2×SSPE, room temperature,

A volume of 25µl of the PCR products was added to 150µl 2×SSPE/0.1 % SDS. The diluted PCR product was heat-denatured for 10 min at 100°C and cooled on ice immediately. The membrane was washed for 5 min at 42°C in 250 ml 2×SSPE/0.1 % SDS. The membrane was placed and supported cushion into the miniblotter, in such a way that the slots were perpendicular to the line pattern of the applied oligonucleotide. The residual fluid was removed from the slots of the miniblotter by aspiration. The slots were filled with the diluted PCR product (avoiding air bubbles!) and hybridize for 60 min at 60 °C on a horizontal surface (no shaking!). Avoid contamination of neighbouring slots. The samples were removed from the miniblotter by aspiration and took the membrane from the miniblotter using forceps. The membrane was washed twice in 250 ml 2×SSPE/0.5 % SDS for 5 min at 60°C. The membrane was placed in a rolling bottle and allowed to cool down to prevent inactivation of the peroxidase in the next step. 5 µl streptavidin-peroxidase conjugate (500 U/ml) was added to 14 ml of 2×SSPE/0.5% SDS, and incubated the membrane in this solution for 60 min at 4°C in the rolling bottle. The membrane was washed twice in 250 ml of 2×SSPE/0.5 % SDS for 10 min at 42°C. The membrane was rinsed twice with 250 ml of 2×SSPE for 5 min at room temperature. For chemiluminiscent detection of hybridizing DNA, the membrane was incubated for 1

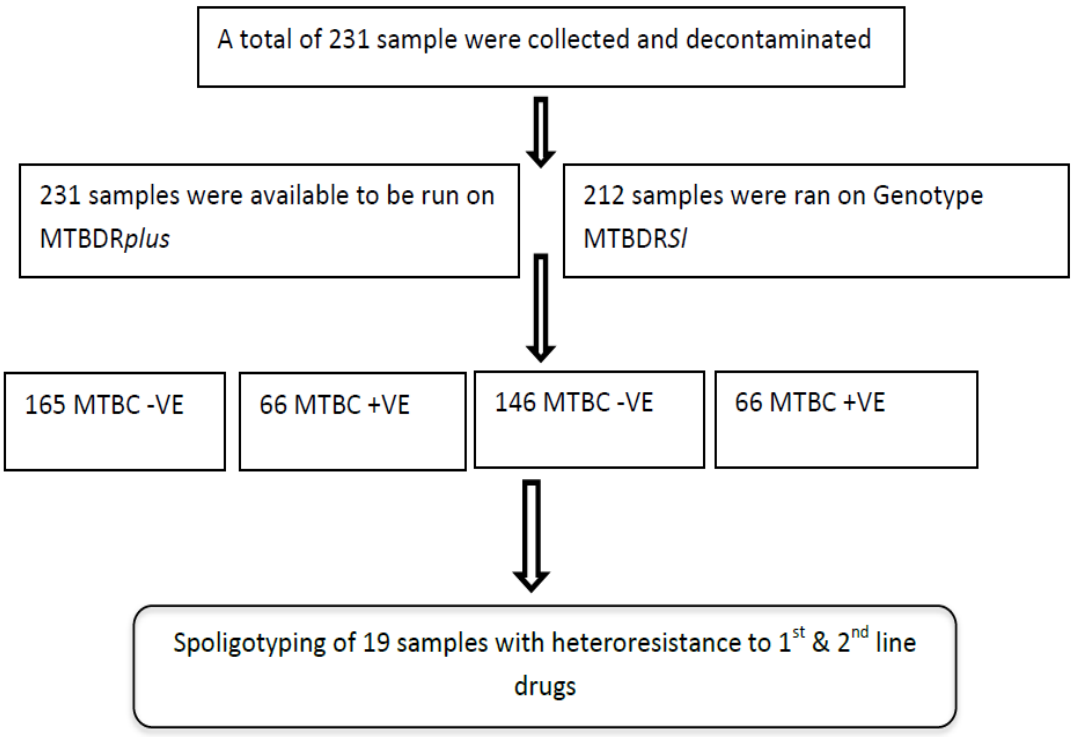
min in 16 ml ECL detection liquid. The membrane was covered with a transparent plastic sheet or Saran-wrap and exposed a light sensitive film to the membrane for 20 min. If the signal was too weak or too strong the membrane could be used again directly to expose another film for a shorter or longer period.

Regeneration of the membrane

Purpose: The hybridized PCR product is dissociated from the membrane in order to regenerate the membrane for the next hybridization. Note; a membrane can be regenerated for at least 10 times.

The membrane was washed twice by incubation in 1 % SDS at 80 °C for 30 min. The membrane was washed in 20 mM EDTA pH 8, for 15 min at room temperature. The membrane was then stored at 4 °C until use (sealed in plastic or wrapped in Saran-wrap, to avoid dehydration of the membrane).

Methodological Flow chart



4.4 Statistical analysis

- Data for the mutational analysis was first entered in MS Excel then imported to SPSS version 20.
- Software SPSS v.20 was used to analyse data.
- Data collected from MTBDR*plus* and MTBDR*sl* assays was interpreted as having drug resistance or not having drug resistance to RIF and INH and MOX, KAN and EMB, respectively.
- The association between heteroresistance and patients treatment history was identified using the Pearson Chi-square.
- A P value of ≤ 0.05 is generally considered to be statistically significant.
- The spoligotyping results were compared with those in the international SpolDB4 data base and further analysed. Thereafter the spoligotypes were assigned to families and subfamilies.
- Chi Square test was also used to assess the association of heteroresistance with the MTB genotypes

4.5 Ethical Considerations Ethical Considerations

The ethics approval was obtained from the Medunsa Research and Ethics Committee (MREC) (**MREC/HS/283/2013: PG**). Permission to collect samples and approval to conduct the study was issued by the Department of Health of the Limpopo Province. 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. 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.

4.6 Significance of the study

The present study will contribute towards development and improving molecular diagnostic assays that will detect heteroresistance which is considered a preliminary stage to development of full resistance. Regular testing of patients during treatment will improve rapid interventions and control measures in clinical infections resulting from MDR *M. tuberculosis*. Moreover the study will add information about the circulating *M. tuberculosis* strain families in the Limpopo Province.

CHAPTER 5

RESULTS

Line probe assay drug susceptibility testing

Genotype MTBDR*plus* was applied directly to 231 decontaminated sputum samples. Of the 231, 5 (2%) were new cases (before the start of treatment) and 226 (98%) were recurrent cases (after the start of treatment). In 165 samples there were no TUB zones thus, the tested sputum samples did not contain *M. tuberculosis* complex strains and were not considered for evaluation. A total of 66 were confirmed as *Mycobacterium tuberculosis* complex by the line probe assay. Out of the 66 MTBC, rifampicin resistance was found in 22 (10%) and 44 (19%) were reported susceptible. Isoniazid resistance was found in 39 (17%) and 27 (12%) were reported susceptible. Heteroresistance was evident in 22 (10%) samples (Table 5.1).

MTBDR*sl* was applied to 212 decontaminated sputum samples. Of the 212, 1(1%) was a new case (before the start of treatment) and 211 (99%) were recurrent cases (after the start of treatment). In 146 samples there were no TUB zones thus, the tested sputum samples did not contain *M. tuberculosis* strains. Of the 66 MTBC positive samples, moxifloxacin resistance was found in 33 (16%) and 14 (7%) were reported susceptible. Kanamycin resistance was found in 17 (8%) and 30 (14%) were reported susceptible. Ethambutol resistance was found in 25 (12%) and 22 (10%) were reported susceptible. Heteroresistance was evident in 23 (11%) samples (Table 5.1).

Table 5.1. Demographics of the MDR-TB study population.

Description			Number	%
MTBDR*plus*	Sputum (n=231)	New TB cases	5	2
		Recurrent TB cases	226	98
	Sputum specimens collected (n=231)	Before start of treatment	5	2
		After start of treatment	226	98
	Results	No TUB	165	71
		RIF resistant, *rpoB* pos.	22	10
		RIF susceptible	44	19
		INH resistant, *katG* positive, *inhA* positive	39	17
		INH susceptible	27	12
		Heteroresistance, *rpoB* band, *katG* band, *inhA* band + WT positive (all 3 loci)	22	10
MTBDRs*l*	Sputum (n=212)	New TB cases	1	1
		Recurrent TB cases	211	99
	Sputum specimens collected (n=212)	Before start of treatment	1	1
		After start of treatment	211	99
	Results	No TUB	146	69
		MOX resistant, *gyrA* band, *gyrB* band.	33	16
		MOX susceptible	14	7
		KAN resistant, *rrs* band.	17	8
		KAN susceptible	30	14
		EMB resistant, *embB* band.	25	12
		EMB susceptible	22	10
		Invalid	20	9
		Heteroresistance mutant + WT positive	23	11

Table 5.1 above presents the susceptibility patterns and patient treatment history. RIF=rifampicin, INH=isoniazid, MOX=moxifloxacin, KAN=kanamycin, EMB=ethambutol, No TUB= no *M. tuberculosis* complex strains.

Table 5.2 Resistance profiles of the DR-TB study population.

Drug resistance status as per MTBDR*plus* or MTBDR*sl* (n=231)	No. WT negative	No. WT positive	TOTAL %
Not resistant (WT positive all 3 loci)	0	23	10
RIF+INH	36	18	23
RIF+INH+KAN	0	0	0
RIF+INH+MOX	3	3	3
RIF+ INH+EMB	0	0	0
RIF+INH +MOX+KAN	1	1	1
RIF+INH+MOX+KAN+EMB	1	1	1
All other combinations	3	3	3
RIF monoresistant	2	0	1
INH monoresistant	1	0	0.4
KAN monoresistant	0	0	0
MOX monoresistant	1	1	1
ALL (total)	48	50	42.4

Table 5.2 presents resistance profiles of MDR-TB study population with resistance from MTBDR*plus* and MTBDR*sl.* *WT (Wild-type) negative = at least one locus band negative; WT positive =All 3 loci. RIF= *rpoB* pos; INH= *katG* or *inhA* pos; KAN/AMK= *gyrA* or *gyrB* pos; FLQ= *rrs* pos. RIF=rifampicin; INH=isoniazid; MOX=moxifloxacin; KAN=kanamycin; EMB= ethambutol.

Mutation analysis and heteroresistance

The total number of MDRTB resistance profiles was calculated from the total samples that had TUB. For the purpose of the study, the outcome of the TUB was ignored. Samples that had RIF with bands in the *rpoB* and INH resistance with bands in the *katG* gene (MDR-TB) were 36. Of the 36 MDR-TB sputum samples, 23 (64%) were WT positive (heteroresistant). There were no samples with a combination resistance to RIF, INH and KAN, RIF, INH and EMB and KAN monoresistance. There were 3 samples that had a combination resistance to RIF (*rpoB*), INH (*katG*) and MOX *(gyrA*), all 3 (100%) also showed heteroresistance with WT bands in all

loci. There was one sample that had a combination resistance to RIF+INH+MOX+KAN showing bands in the *rpoB, katG. gyrA and rrs genes, respectively* and RIF+INH+MOX+KAN+EMB showing bands in the *rpoB, katG. gyrA rrs and embB* genes, respectively and MOX monoresistance and also showed heteroresistance.

Table 5.3. Association of heteroresistance with patients' treatment history.

HETERORESISTANCE * TREATMENT HISTORY Cross tabulation				
Count				
		TREATMENT HISTORY		Total
		Recurrent	New case	
Resistance type	H, R-RIF, R-INH	14	4	18
	H, R-RIF, S-INH	2	0	2
	H, S-RIF, R-INH	1	0	1
	H, S-RIF, S-INH	1	0	1
	H, R-MOX, R-KAN, R-EMB	6	0	6
	H, R-MOX, S-KAN, R-EMB	8	0	8
	H, R-MOX, S-KAN, S-EMB	8	0	8
	H, R-MOX, R-KAN, S-EMB	1	0	1
Total		41	4	45

The table shows resistance types and heteroresistance and treatment history. H=heteroresistance, R= resistance, RIF=rifampicin, INH=isoniazid, MOX=moxifloxacin, KAN-kanamycin, EMB=ethambutol. There were a total of 45 heteroresistant samples. 41 cases were recurrent cases and 4 were new cases.

Heteroresistance and treatment history

Heteroresistance was evident in 45 of 231 (19%) of MDR-TB patients. There was a total of 45 heteroresistant samples analysed on MTBDRplus and MTBDR*sl*. Of the 45, 18 (40%) had heteroresistance with MDR-TB. Of the 18, 14 were recurrent cases and 4 were new cases. There was heteroresistance in 2 (4%) cases that were resistant to rifampicin but susceptible to isoniazid, both samples were recurrent cases (Table 5.3).

There was heteroresistance to one sample (2%) that was also susceptible to rifampicin and resistant to isoniazid and another one (2%) susceptible to both rifampicin and isoniazid. Both samples were recurrent cases.

The association between heteroresistance and patients treatment history was analysed using the Pearson Chi-square (P- value).

A P value of ≤ 0.05 is generally considered to be statistically significant.

The P value for association of heteroresistance with patients' treatment history was 0.473.

Table 5.4 Association of heteroresistance with spoligotype families

MTBDR*plus* * SPOLIGOTYPING (SIT) Crosstabulation							
Count							
		SPOLIGOTYPING CLADE (SIT)					Total
		Orphan	T1 (879)	UNKNOWN (1196)	T-H_{37}RvV817 (568)	LAM 3 (2301)	
Resistance type	H, R-RIF, R-INH	14	1	1	1	1	18
	H, R-RIF, S-INH	1	0	0	0	0	1
Total		15	1	1	1	1	19

The table presents 19 heteroresistant samples that were subjected to spoligotyping to determine what families they belonged to.

Diversity of heteroresistant MTB samples by spoligotyping

Eighteen (18) of 19 heteroresistant samples subjected to spoligotyping were also MDR. Fourteen of the samples that were resistant to both RIF and INH were orphans, that is those particular strains are unknown. Of the 14 MDR, 3 samples belonged to clades T1, T-H_{37}RvV817 and LAM 3 with SITs: 879, 568 and 2301, respectively. One sample with SIT 1196 had an unknown clade, was resistant to RIF but susceptible to INH. (Table 5.4). The association between heteroresistance and spoligotype families was analysed using the Pearson Chi-square (P- value). P value = 0.991.

When spoligotyping results were compared to those in the international spolDB4 database, 4 of them matched existing shared spoligotype international types, 15 were unknown (orphans) and only 3 were identified (Table 5.5). Of the 4 samples 1 was not identified by the SpolDB4 database. Of the 15 recurrent cases only two (13%): Samples AINF 9865 and AINF 9874 had identified lineages which were 879 and 1196, respectively. Sample AINF 9865 belonged to the T1 family. Of the 4 new cases 2 (50%) samples: AINF 9875 and AIAU 9609 were identified with SITs 568 and 2301, respectively. Sample AINF 9875 belongs to T-H37RvV817 family and sample AIAU9609 belonged to the clade LAM3 (Table 5.5).

Table 5.5 Spoligotyping results of heteroresistant samples and their treatment history.

Strain	**Spoligotype43**	**SIT**	**CLADE**	**MIT**	**VIT**	**Clinical data**
AINS9410	771347637741671	Orphan	Unknown	Orphan	Orphan	Recurrent
AINF9865	777767777760671	879	T1	Orphan	Orphan	Recurrent
AINS1379	751047604740671	Orphan	Unknown	Orphan	Orphan	Recurrent
AINF9858	777347677761671	Orphan	Unknown	Orphan	Orphan	New case
AINS1391	777357777761671	Orphan	Unknown	Orphan	Orphan	Recurrent
AIWJ9414	771357607761671	Orphan	Unknown	Orphan	Orphan	Recurrent
AINF9869	777367477761771	Orphan	Unknown	Orphan	Orphan	Recurrent
AINF9860	771347677761671	Orphan	Unknown	Orphan	Orphan	Recurrent
AINF9874	777777777761771	1196	Unknown	Orphan	Orphan	Recurrent
AIAU9612	777367477761671	Orphan	Unknown	Orphan	Orphan	Recurrent
AINF9875	777767477760771	568	T-H_{37}RvV817	Orphan	Orphan	New case
AIAU9617	777767677761671	Orphan	Unknown	Orphan	Orphan	Recurrent
AINS9428	753367607761771	Orphan	Unknown	Orphan	Orphan	Recurrent
AINS1368	777767677761671	Orphan	Unknown	Orphan	Orphan	Recurrent
AINF9883	777377777763771	Orphan	Unknown	Orphan	Orphan	Recurrent
AINF9868	777367677761771	Orphan	Unknown	Orphan	Orphan	Recurrent
AINF9881	777767677761671	Orphan	Unknown	Orphan	Orphan	Recurrent
AINF9887	773377677761671	Orphan	Unknown	Orphan	Orphan	New case
AIUA9609	776167607760671	2301	LAM3	Orphan	Orphan	New case

*SIT designation in SITVIT2 database.** clade designation according to the SITVIT2 using revised SpolDB4 rules. 19 samples that were heteroresistant to first- & second- line drugs were subjected to spoligotyping to determine what families/lineages they belonged to. The samples whose SITs were orphans mean that the particular strain has not been identified in the SpolDB4 database. The samples which are represented as unknown are orphans. Three samples had different identified strains which are T1, H-$_{37}$RvV81 and LAM3, The fourth sample with SIT 1196 had an unknown clade.

CHAPTER 6

DISCUSSION

Heteroresistance has been proven to occur mostly in high TB incidence locations and may be influenced by certain factors such as: Geographical location/ study site, group of patients used for the particular study and prevalence of tuberculosis in the area. South Africa having one of the highest rates of TB in the world is at risk of this type of drug resistance. Heteroresistance could influence development of resistance of *Mycobacterium tuberculosis* strains of patients undergoing therapy, thus this study was conducted.

The current study aimed to investigate the heteroresistant strains for any link between the occurrence of heteroresistance, patients' treatment history and *Mycobacterium tuberculosis* strain families. Thus molecular investigations were applied to identify heteroresistance in the *Mycobacterium tuberculosis* complex. The line probe assays MTBDR*plus* for the first-line and MTBDRs*l* for the second-line were applied directly to decontaminated sputum samples. The significant finding of the study calculated from the total samples that had TUB, was a rate of 10% heteroresistance in the *rpoB*, *katG* and *inhA* regions for the first-line drugs and 11% heteroresistance in the *gryA*, *gyrB*, *rrs* and *embB* regions for the second-line drugs. The heteroresistance rate in the current study is much lower than the rates reported by Rinder *et al*. (2001) of 17%, Kumaar *et al*. (2014) of 28% in Punjab state of India, 20% by Hofmann-Thiel *et al.* (2009) in Tashkent and the highest rate of 34% by Tolani *et al.* (2012) in Mumbai India, could be due to the fact that heteroresistance is influenced by geographical locations and the amount of drug resistance. This study identified Heteroresistance in 23/66 (64%) of the MDR TB sputum samples. Three samples had a combination of *rpoB*, *katG* and *gyrA* bands with WT bands in all loci, indicating heteroresistance, while one sample with bands in the *rpoB*, katG, *gyrA* and *rrs* genes also showed heteroresistance. The average 11% heteroresistance in the present study is lower than the 12.6% reported by Zhang *et al*. (2013) and slightly higher than the 8.6% reported in amikacin-resistant isolates by Streicher *et al*. (2012) in South Africa. Heteroresistance was evident in 18 (40%) of the 45 MDR-TB cases 41 of which were recurrent cases and 4 new cases. Two (4%) with heteroresistance, that were rifampicin-resistant but susceptible to isoniazid were recurrent cases.

There was heteroresistance to one sample susceptible to rifampicin and resistant to isoniazid and another one susceptible to both rifampicin and isoniazid both from recurrent cases. The findings of the current study with higher heteroresistance in recurrent cases in comparison to new cases can be supported by findings of Mekonnen *et al*. (2015) stating that heteroresistant populations of MTB during a chronic infection occurring as numerous subpopulations with different susceptibility profiles may be found in the same patient, during treatment or by mixed or superinfection with susceptible and resistant strains.

This study, to our knowledge is the first in our location (Limpopo Province) to associate heteroresistance with treatment history in multidrug-resistant cases, and the finding of heteroresistance mostly in recurrent cases supports the notion that drug susceptible mycobacteria in new cases gradually develop resistance during therapy and the susceptible population that is not completely eliminated will be detected with the resistant population.

When spoligotyping results from this study were compared to those in the international spolDB4 database, only 4 of them matched existing shared spoligotype international types, 15 were unknown (orphans) and only 3 were identified. Of the 15 recurrent cases only two (13%): Samples AINF 9865 and AINF 9874 had identified lineages which were 879 and 1196, respectively. Sample AINF 9865 belongs to the T1 family which is predominately found in locations such as Argentina, Mexico, USA, Tanzania and a few others and sample AINF 9874 belongs to an unknown clade. Other studies done in other parts of South Africa have reported a number of distinct clades circulating in their provinces. A study done in Port Elizabeth, South Africa reported clades such as Beijing, LAM4, LAM3, LAM9, X1, X3 and T1 (Nqini, 2012). Beijing was the most dominant (71%) family in their study.

Of the 4 new cases 2 (50%) samples: AINF 9875 and AIAU 9609 were identified with SITs 568 and 2301, respectively. Sample AINF 9875 belongs to T-H37RvV817 family which is predominantly found in locations such as Thailand and USA and sample AIAU9609 belongs to the clade LAM3 which is predominantly found in locations such as Argentina and ZAF and in South Africa as reported by Filliol *et al*. (2002) ; Streicher *et al*. (2004) and Victor *et al*. (2004).

Another study done in the Western Cape Province and Johannesburg (Gauteng province) area of South Africa by Mlambo, 2011 reported EIA and X families which were predominant in Johannesburg area. The family with the highest dominance in their study was the Beijing genotype (Mlambo 2011). Several studies reported that heteroresistance was caused exclusively by Beijing family linked to the history of relapse and this family has been known as a risk factor for further resistance development (Lan *et al*., 2003; Sun *et al*., 2006; Hofmann-Thiel *et al*., 2009). In the current study, there were more non-Bejing genotypes than those reported in a number of areas in the African continent (Nqini 2012; Mlambo 2011; Warren *et al.,* 2004; Homolka *et al.,* 2008, Kibikio *et al* ., 2007, Githui *et al*, 2007, Glynn *et al* 2005) who reported more cases of Beijing genotype which is normally documented in new TB cases.

Thus the study has highlighted that there are moderately elevated rates of heteroresistance in retreatment and relapse patients and the predominant clades implicated are unknown or Non-Beijing in the Limpopo Province. The patients comprise of the recurrent cases consulting at the Modimolle TB unit which serves a referral hospital for the province.

Limitations of the study

There was no information on treatment stages of patients. This information on how long the treatment was taken was not available to the researcher at the time of samples collection.

Due to limited resources and reagents, only 212 samples were ran on MTBDR*sl* instead of 231 that were ran on MTBDR*plus*. Only 19 of the 45 heteroresistant samples were spoligotyped due to limited resources and reagents.

Conclusions were based on MTBDR*plus* and MTBDR*sl* and spoligotyping of the 19 samples.

Heteroresistance was recorded as reported by MTBDR*plus* and MTBD*sl*.

CONCLUSION

To date heteroresistance is an underestimated phenomenon in tuberculosis, especially in highly endemic areas. The study has reported a larger percentage of heteroresistance in second-line drugs than there is in first-line drugs in Limpopo Province, South Africa. The T1 genotype was found to be predominant amongst recurrent cases. The LAM3 and T-H37RvV817 lineages were found amongst the new cases. However, as only 19 of the 45 heteroresistant samples were subjected to spoligotyping, we cannot confirm that LAM3 is the most frequent lineage as reported. The same limitation including lack of information on treatment stages of patients may have contributed to the failure of the present study to find a significant association between heteroresistance and the patient's treatment history as indicated by a P-value of 0.473 and between heteroresistance and spoligotype families (P-value, 0.991). Despite the limitations, according to the current study, heteroresistance was associated more with recurrent cases who are on initiation or continuation phase than the new cases. This finding highlights the clinical significance and therapeutic implications for patients with both drug-susceptible and drug-resistant strains in the same sputum. The predominance of orphan SITs and unknown clades followed by non-Beijing strains in the study may be due to the location of the Limpopo Province. Limpopo Province is South Africa's far north province flanked by the countries of Botswana to the west, Zimbabwe to the north, and Mozambique to the east. This prevalence of the orphans in the heteroresistant samples may be partly due to carriers of these unique strains coming from the neighbouring countries and other African countries as they migrate to work at the mines in this province.

It is therefore recommended that further studies with larger numbers of patients be conducted to determine the prevalence and to associate heteroresistance with patients' treatment history and establish the contributing MTBC strain lineages.

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APPENDICES

APPENDIX 1

Data presentation of the collected samples

SAMPLE ID	Resistance type (MTBDR*plus*)	Resistance type (MTBDR*sl*)	TREATMENT HISTORY	(SIT)	CLADE
AINS9410	H, R-RIF, R-INH	Unavailable	Recurrent	Orphan	Unknown
AINF9865	H, R-RIF, R-INH	Unavailable	Recurrent	879	T1
AINS1379	H, R-RIF, R-INH	Unavailable	Recurrent	Orphan	Unknown
AINF9858	H, R-RIF, R-INH	Unavailable	New case	Orphan	Unknown
AINS1391	H, R-RIF, R-INH	Unavailable	Recurrent	Orphan	Unknown
AIWJ9414	H, R-RIF, R-INH	Unavailable	Recurrent	Orphan	Unknown
AINF9869	H, R-RIF, R-INH	Unavailable	Recurrent	Orphan	Unknown
AINF9860	H, R-RIF, R-INH	Unavailable	Recurrent	Orphan	Unknown
AINF9874	H, R-RIF, R-INH	Unavailable	Recurrent	1196	Unknown
AIAU9612	H, R-RIF, R-INH	Unavailable	Recurrent	Orphan	Unknown
AINF9875	H, R-RIF, R-INH	Unavailable	New case	568	T-H_{37}RvV817
AIAU9617	H, R-RIF, R-INH	Unavailable	Recurrent	Orphan	Unknown
AINS9428	H, R-RIF, R-INH	Unavailable	Reccurent	Orphan	Unknown
AINS1368	H, R-RIF, S-INH	Unavailable	Reccurent	Orphan	Unknown
AINF9883	H, R-RIF, R-INH	Unavailable	Reccurent	Orphan	Unknown
AINF9868	H, R-RIF, R-INH	Unavailable	Reccurent	Orphan	Unknown
AINF9881	H, R-RIF, R-INH	Unavailable	Reccurent	Orphan	Unknown
AINF9887	H, R-RIF, R-INH	Unavailable	New case	Orphan	Unknown
AIUA9609	H, R-RIF, R-INH	Unavailable	New case	2301	LAM3
1	S-RIF, S-INH	H,R-MOX, R-KAN,R-EMB	Reccurent		
2	NO TUB	NO TUB	Reccurent		
3	NO TUB	NO TUB	Reccurent		
4	NO TUB	NO TUB	Reccurent		
5	NO TUB	NO TUB	Reccurent		
6	NO TUB	NO TUB	Reccurent		
7	NO TUB	NO TUB	Reccurent		
8	NO TUB	NO TUB	Reccurent		
9	NO TUB	NO TUB	Reccurent		
10	R-RIF, S-INH	H,R-MOX, R-KAN,R-EMB	Reccurent		
11	R-RIF, R-INH	H, R-MOX, S-KAN, R-EMB	Reccurent		
12	S-RIF, R-INH	H, R-MOX, S-KAN, R-EMB	Reccurent		
13	R-RIF, R-INH	H, R-MOX, R-KAN,R-EMB	Reccurent		

14	NO TUB	NO TUB	Reccurent		
15	NO TUB	NO TUB	Reccurent		
16	R-RIF, S-INH	H,R-MOX, S-KAN,S-EMB	Reccurent		
17	NO TUB	NO TUB	Reccurent		
18	NO TUB	NO TUB	Reccurent		
19	NO TUB	NO TUB	Reccurent		
20	NO TUB	NO TUB	Reccurent		
21	NO TUB	NO TUB	Reccurent		
22	S-RIF, S-INH	S-MOX, S-KAN, S-EMB	Reccurent		
23	NO TUB	NO TUB	Reccurent		
24	R-RIF, R-INH	S-MOX, S-KAN, S-EMB	New case		
25	R-RIF, R-INH	H, R-MOX, S-KAN, R-EMB	Recurrent		
26	NO TUB	NO TUB	Recurrent		
27	NO TUB	NO TUB	Recurrent		
28	NO TUB	NO TUB	Recurrent		
29	NO TUB	NO TUB	Recurrent		
30	NO TUB	NO TUB	Recurrent		
31	NO TUB	NO TUB	Recurrent		
32	NO TUB	NO TUB	Recurrent		
33	NO TUB	NO TUB	Recurrent		
34	NO TUB	NO TUB	Recurrent		
35	S-RIF,R-INH	H, R-MOX, S-KAN, S-EMB	Recurrent		
36	R-RIF, R-INH	S-MOX, S-KAN, S-EMB	Recurrent		
37	NO TUB	NO TUB	Recurrent		
38	NO TUB	NO TUB	Recurrent		
39	NO TUB	NO TUB	Recurrent		
40	NO TUB	NO TUB	Recurrent		
41	S-RIF, R-INH	H,R-MOX, S-KAN, R-EMB	Recurrent		
42	NO TUB	NO TUB	Recurrent		
43	NO TUB	NO TUB	Recurrent		
44	NO TUB	NO TUB	Recurrent		
45	S-RIF, S-INH	H, R-MOX, S-KAN, R-EMB	Recurrent		
46	S-RIF, R-INH	S-MOX, S-KAN, S-EMB	Recurrent		
47	NO TUB	NO TUB	Recurrent		
48	S-RIF, S-INH	S-MOX, S-KAN, S-EMB	Recurrent		

49	NO TUB	NO TUB	Recurrent		
50	NO TUB	NO TUB	Recurrent		
51	NO TUB	NO TUB	Recurrent		
52	NO TUB	H, R-MOX, R-KAN, R-E	Recurrent		
53	NO TUB	H, R-M, R,K, R-EMB	Recurrent		
54	NO TUB	H, R-MOX, R-KAN, R-EMB	Recurrent		
55	NO TUB	NO TUB	Recurrent		
56	NO TUB	NO TUB	Recurrent		
57	R-RIF,S-INH	H, R-MOX, S-KAN, R-EMB	Recurrent		
58	S-RIF, S-INH	S-MOX, S-KAN, S-EMB	Recurrent		
59	R-RIF, S-INH	S-MOX, S-KAN, S-EMB	Recurrent		
60	R-RIF, R-INH	S-MOX, S-KAN, S-EMB	Recurrent		
61	R-RIF, R-INH	S-MOX, S-KAN, S-EMB	Recurrent		
62	NO TUB	NO TUB	Recurrent		
63	S-RIF,-INH	H, R-MOX, S-KAN, R-EMB	Recurrent		
64	NO TUB	NO TUB	Recurrent		
65	NO TUB	NO TUB	Recurrent		
66	NO TUB	NO TUB	Recurrent		
67	NO TUB	NO TUB	Recurrent		
68	NO TUB	NO TUB	Recurrent		
69	NO TUB	NO TUB	Recurrent		
70	NO TUB	NO TUB	Recurrent		
71	R-RIF, R-INH	H, R-MOX, S-KAN, S-EMB	Recurrent		
72	R-RIF, S-INH	H, R-MOX, S-KAN, S-EMB	Recurrent		
73	NO TUB	NO TUB	Recurrent		
74	NO TUB	NO TUB	Recurrent		
75	R-RIF, R-INH	H, R-MOX, S-KAN, S-EMB	Recurrent		
76	NO TUB	NO TUB	Recurrent		
77	NO TUB	NO TUB	Recurrent		
78	NO TUB	NO TUB	Recurrent		
79	NO TUB	NO TUB	Recurrent		
80	NO TUB	NO TUB	Recurrent		
81	NO TUB	NO TUB	Recurrent		

82	R-RIF, R-INH	H, R-MOX, S-KAN, S-EMB	Recurrent		
83	H,S-RIF, R-INH	S-MOX, S-KAN, S-EMB	Recurrent		
84	NO TUB	NO TUB	Recurrent		
85	NO TUB	NO TUB	Recurrent		
86	NO TUB	NO TUB	Recurrent		
87	NO TUB	NO TUB	Recurrent		
88	R-RIF, R-INH	H, R-MOX, S-KAN, R-EMB	Recurrent		
89	NO TUB	NO TUB	Recurrent		
90	NO TUB	NO TUB	Recurrent		
91	NO TUB	NO TUB	Recurrent		
92	R-RIF, S-INH	H, R-MOX, S-KAN, S-EMB	Recurrent		
93	NO TUB	NO TUB	Recurrent		
94	NO TUB	NO TUB	Recurrent		
95	R-RIF, S-INH	H, R-MOX, S-KAN, R-EMB	Recurrent		
96	NO TUB	NO TUB	Recurrent		
97	NO TUB	NO TUB	Recurrent		
98	NO TUB	NO TUB	Recurrent		
99	NO TUB	NO TUB	Recurrent		
100	NO TUB	NO TUB	Recurrent		
101	NO TUB	NO TUB	Recurrent		
102	NO TUB	NO TUB	Recurrent		
103	NO TUB	NO TUB	Recurrent		
104	NO TUB	NO TUB	Recurrent		
105	NO TUB	NO TUB	Recurrent		
106	NO TUB	NO TUB	Recurrent		
107	NO TUB	NO TUB	Recurrent		
108	NO TUB	NO TUB	Recurrent		
109	NO TUB	NO TUB	Recurrent		
110	NO TUB	NO TUB	Recurrent		
111	NO TUB	NO TUB	Recurrent		
112	NO TUB	NO TUB	Recurrent		
113	NO TUB	NO TUB	Recurrent		
114	NO TUB	NO TUB	Recurrent		
115	NO TUB	NO TUB	Recurrent		
116	NO TUB	NO TUB	Recurrent		
117	NO TUB	NO TUB	Recurrent		
118	NO TUB	NO TUB	Recurrent		
19	NO TUB	NO TUB	Recurrent		

120	NO TUB	NO TUB	Recurrent		
121	NO TUB	NO TUB	Recurrent		
122	NO TUB	NO TUB	Recurrent		
123	NO TUB	NO TUB	Recurrent		
124	NO TUB	NO TUB	Recurrent		
125	NO TUB	NO TUB	Recurrent		
126	NO TUB	NO TUB	Recurrent		
127	NO TUB	NO TUB	Recurrent		
128	NO TUB	NO TUB	Recurrent		
129	NO TUB	NO TUB	Recurrent		
130	NO TUB	NO TUB	Recurrent		
131	R-RIF, S-INH	S-MOX, S-KAN, S-EMB	Recurrent		
132	S-RIF, S-INH	S-MOX, S-KAN, S-EMB	Recurrent		
133	NO TUB	NO TUB	Recurrent		
134	NO TUB	NO TUB	Recurrent		
135	NO TUB	NO TUB	Recurrent		
136	NO TUB	NO TUB	Recurrent		
137	R-RIF, R-INH	S-MOX, S-KAN, S-EMB	Recurrent		
138	NO TUB	NO TUB	Recurrent		
139	NO TUB	NO TUB	Recurrent		
140	NO TUB	NO TUB	Recurrent		
141	NO TUB	NO TUB	Recurrent		
142	NO TUB	NO TUB	Recurrent		
143	NO TUB	NO TUB	Recurrent		
144	NO TUB	NO TUB	Recurrent		
145	NO TUB	NO TUB	Recurrent		
146	NO TUB	NO TUB	Recurrent		
147	NO TUB	NO TUB	Recurrent		
148	NO TUB	NO TUB	Recurrent		
149	NO TUB	NO TUB	Recurrent		
150	NO TUB	NO TUB	Recurrent		
151	NO TUB	NO TUB	Recurrent		
152	NO TUB	NO TUB	Recurrent		
153	NO TUB	NO TUB	Recurrent		
154	NO TUB	NO TUB	Recurrent		
155	NO TUB	NO TUB	Recurrent		
156	R-RIF,R-INH	H, R-MOX, R-KAN, S-EMB	Recurrent		
157	R-RIF, R-INH	S-MOX, S-KAN, S-EMB	Recurrent		
158	NO TUB	NO TUB	Recurrent		

159	NO TUB	NO TUB	Recurrent		
160	NO TUB	NO TUB	Recurrent		
161	NO TUB	NO TUB	Recurrent		
162	NO TUB	NO TUB	Recurrent		
163	NO TUB	NO TUB	Recurrent		
164	NO TUB	NO TUB	Recurrent		
165	NO TUB	NO TUB	Recurrent		
166	NO TUB	NO TUB	Recurrent		
167	NO TUB	NO TUB	Recurrent		
168	NO TUB	NO TUB	Recurrent		
169	NO TUB	NO TUB	Recurrent		
170	NO TUB	NO TUB	Recurrent		
171	NO TUB	NO TUB	Recurrent		
172	NO TUB	NO TUB	Recurrent		
173	NO TUB	NO TUB	Recurrent		
174	NO TUB	NO TUB	Recurrent		
175	S-RIF,S-INH	INVALID	Recurrent		
176	NO TUB	NO TUB	Recurrent		
177	R-RIF, R-INH	INVALID	Recurrent		
178	NO TUB	NO TUB	Recurrent		
179	H, R-RIF, S-INH	INVALID	Recurrent		
180	R-RIF, R-INH	INVALID	Recurrent		
181	NO TUB	NO TUB	Recurrent		
182	NO TUB	NO TUB	Recurrent		
183	NO TUB	NO TUB	Recurrent		
184	NO TUB	NO TUB	Recurrent		
185	NO TUB	NO TUB	Recurrent		
186	S-RIF,S-INH	INVALID	Recurrent		
187	NO TUB	NO TUB	Recurrent		
188	NO TUB	NO TUB	Recurrent		
189	NO TUB	NO TUB	Recurrent		
190	NO TUB	NO TUB	Recurrent		
191	S-RIF, R-INH	INVALID	Recurrent		
192	R-RIF, S-INH	INVALID	Recurrent		
193	NO TUB	NO TUB	Recurrent		
194	NO TUB	NO TUB	Recurrent		
195	NO TUB	NO TUB	Recurrent		
196	NO TUB	NO TUB	Recurrent		
197	NO TUB	NO TUB	Recurrent		
198	NO TUB	NO TUB	Recurrent		
199	NO TUB	INVALID	Recurrent		
200	NO TUB	INVALID	Recurrent		

201	NO TUB	INVALID	Recurrent		
202	NO TUB	INVALID	Recurrent		
203	NO TUB	INVALID	Recurrent		
204	H, S-RIF, S-INH	INVALID	Recurrent		
205	NO TUB	INVALID	Recurrent		
206	NO TUB	INVALID	Recurrent		
207	R-RIF, R-INH	INVALID	Recurrent		
208	R-RIF, R-INH	INVALID	Recurrent		
209	R-RIF, S-INH	INVALID	Recurrent		
210	S-RIF, S-INH	INVALID	Recurrent		
211	S-RIF,S-INH	INVALID	Recurrent		
212	S-RIF, S-INH	INVALID	Recurrent		

The table presents data on the MTBDR*plus* and MTBDR*sl* drug resistance results; treatment history and spoligotypes. NoTUB means no *Mycobacterium tuberculosis* complex; H-heteroresistance;INH, isoniazid resistance; RIF, rifampicin resistance; KAN, kanamycin resistance; MOX, moxifloxacin resistance. R, resistant; S, susceptible.

APPENDIX 2- Sputum decontamination

Principle

NALC is a mucolytic agent that at concentrations of 0.5 to 2.0% can rapidly digest even tenacious sputa from cystic fibrosis patients within 2 minutes. Decontamination is achieved by the addition of sodium hydroxide. NAOH digests sputum debris, normal flora and other infectious organisms and the remainder will be highly resistant organisms such as mycobacteria. Mycobacteria have high quality of mycolic acids which restrict entry of sodium hydroxide.

NAOH - NALC decontamination solution

Ingredients:

NaOH - Na citrate stock solution

- 8 g sodium hydroxide, 5.8g sodium citrate, 400ml distilled water

Procedure:

- Dissolve 8 g NAOH in 200ml of sterile distilled water
- Dissolve 5.8g Na citrate in 200ml sterile distilled water.
- Combine the NAOH and Na citrate solutions (equal volumes)
- Mix and autoclave at 121-124ºC for 15 minutes.
- Store in refrigerator for up to one month

Decontamination procedure

1. Place 2ml of sputum samples in 15 ml centrifuge tube (if less than 2ml, make up to 2ml mark with phosphate buffer)
2. Add 2ml NAOH-NALC solution
3. Cap the tube tightly and vortex for 20 seconds, invert the centrifuge tube to ensure NAOH-NALC solution contains the entire interior surface of the tube and lid

4. Let stand for minimum of 15 minutes at room temperature.
5. Fill the tube to the 14ml mark with phosphate buffer (pH 6.8) to neutralize alkali and terminate the decontamination process, and mix well by inverting the tube 4 times
6. Centrifuge at 3000g for 15 minutes
7. The supernatant was carefully discarded into a liquid waste container with 10% sodium hypochlorite
8. The sample was incubated overnight (24 hours) at 37˚C.

APPENDIX 3 - MTBDR*plus*

Principle

- MTBDR*plus* assay was performed according to manufacturer's instruction, this test was used directly on decontaminated sputum samples and is based on reverse hybridization between amplicons derived from a multiplex PCR and nitrocellulose bound wild-type and mutated probes for the mutations of interest.

DNA extraction method:

DNA was extracted directly from sputum samples

- Sputum specimens should be decontaminated according to specimen.
- Following suspension of the pellet in phosphate buffer, use a disposable Pasteur pipette to pipette 500µl of decontaminated sample to a 1.5ml micro centrifuge tube with screw cap.
- Centrifuge for 15 minutes at 10,000 x g.
- Discard supernatant and suspend pellet in 100ul molecular grade water.
- Incubate for 20 minutes at 95°C in the hot plate.
- Incubate for 15 minutes in the ultrasonic bath.
- Centrifuge for 5 minutes at 10,000 x g.
- Tubes should be loaded in the centrifuge and unloaded in the same tube alignment to make visualization of pellet easier for removing the supernatant.
- Carefully carry the tubes to the BSC. Uncap tubes one at a time, and carefully transfer 40-80ul of supernatant to a clean micro centrifuge tube using a P100 pipette.
- Careful attention must be paid to avoid touching the pipette tip to the pellet. If this is done, and pellet is drawn into the pipette tip, a small amount of liquid can be expelled back into the tube until only clear supernatant remains.
- Add 5µl of DNA supernatant to corresponding PCR tubes

Polymerase Chain Reaction (Amplification)

- A 35 µl of primer-nucleotide mix (provided with the kit), amplification buffer containing 2.5 mM MgCl2, 1.25 U of Fast Start *Taq* polymerase and 5 µl of supernatant in a final volume of 50 µl will be used.
- The amplification protocol consists of 10 min of denaturing at 95°C; 10 cycles of 30 s at 95°C and120 s at 58°C; 20 additional cycles of 25 s at 95°C, 40 s at 53°C, and 40 s at 70°C; and a final extension at 70°C for 8 min.
- PCR products was analyzed in 1.5%agarose gel for the control of incomplete or marginal amplification
- The biotinylated PCR products was then be denatured and hybridized to a strip with specific oligonucleotide probes.
- Hybridization and detection was performed in an automated washing and shaking device (TwinCubator; Hain Lifescience GmbH,Nehren, Germany).
- The program was started after 20 µl of the amplification products was mixed with 20 µl of denaturing reagent (provided with the kit) for5 min in separate troughs of a plastic well.
- Following the addition of 1 ml of pre-warmed hybridization buffer, membrane strips was placed in each trough.
- The hybridization procedure is performed at 45°C for 0.5 h and is followed by two washing steps.
- For colorimetric detection of hybridized amplicons, streptavidin conjugated with alkaline phosphatase and substrate buffer will be added.
- After final washing, strips was air dried
- Each strip contains 17 probes, including amplification and hybridization controls.
- *Mycobacterium tuberculosis* was detected in a sample by the use of the *Mycobacterium tuberculosis* complex-specific probe.
- The *rpoB*- and *katG*-specific regions was detected by *rpoB* and *katG* control probes, respectively.

APPENDIX 4 - MTBDR*s*/

Principle:

MTBDR*s*/ test is based on the DNA•STRIP technology. The whole procedure is divided into three steps: (i) DNA extraction from decontaminated sputum specimens or cultured material (solid/liquid medium) – the necessary reagents are not included in the kit, (ii) a multiplex amplification with biotinylated primers, and (iii) a reverse hybridization. All reagents needed for amplification, such as polymerase and primers, are included in the Amplification Mixes A and B (AM-A and AM-B) and are optimized for this test. The membrane strips are coated with specific probes complementary to the amplified nucleic acids. After chemical denaturation, the single- stranded amplicons bind to the probes (hybridization). Highly specific binding of complementary DNA strands is ensured by stringent conditions which result from the combination of buffer composition and a certain temperature. Thus the probes reliably discriminate several sequence variations in the gene regions examined. The streptavidin-conjugated alkaline phosphatase binds to the amplicons' biotin via the streptavidin moiety. Finally, the alkaline phosphatase transforms an added substrate into a dye which becomes visible on the membrane strips as a colored precipitate. A template ensures the easy and fast interpretation of the banding pattern obtained.

Quality control

In order to control the correct performance of the test and the proper functioning of kit constituents, each strip includes 6 control zones: – a Conjugate Control zone (CC) to check the binding of the conjugate on the strip and a correct chromogenic reaction - an Amplification Control zone (AC) to check for a successful amplification reaction – our Locus Control zones (gyrA, gyrB, rrs, and eis) checking the optimal sensitivity of the reaction for each of the tested gene loci

Observe the usual precautions for amplification setup. It is essential that all materials (such as pipette tips) coming in contact with the reagents are free from DNases. Do not interchange or pool Amplification Mixes or membrane strips from different kits unless the lots are identical. A negative control sample for detection of possible contamination events containing water (molecular biology grade) instead of DNA should be part of each test run; the respective test strip should show the CC and AC bands only.

DNA extraction

The same DNA extracted from decontaminated sputum samples for use on MTBDR*plus* were used. The GenoLyse® kit was used for DNA extraction from decontaminated sputum samples. The method described above was used for performance evaluation of the MTBDR*sl* test. Each DNA extracted from sputum or a cultivated sample using the GenoLyse® kit was used for amplification with the MTBDR*sl* VER 2.0 kit (e.g. subsequent to the GenoType MTBDR*plus* VER 2.0).

Amplification

All reagents needed for amplification such as polymerase and primers are included in the Amplification Mixes A and B (AM-A and AM-B) and are optimized for this test. AM-A and AM-B were spun briefly and mixed carefully by pipetting up and down. AM-A and AM-B pippeted in a DNA free area. The DNA solution was added in a separate working area. A volume of 10 µl of AM-A , 35 µl AM-B and 5 µl DNA solution were mixed into tubes to make a final volume of 50 µl. A master mix containing AM-A and AM-B was prepared and mixed carefully but thoroughly. This lead to 0.68 ml master mix for 12 amplification reactions (12 tests kit) or, respectively, 4x 1.35 ml for 4x 24 amplification reactions (96 tests kit). A volume of 45 µl was added into each of the prepared PCR tubes plus 5 µl water (molecular biology grade) to one aliquot (negative control). In a separate working area, 5 µl DNA solution was added to each aliquot (except for negative control).

APPENDIX 5 - SPOLIGOTYPING

Principle

- This test is based on DNA polymorphism present at one particular chromosomal locus, the "Direct Repeat" (DR) region, which is uniquely present in *Mycobacterium tuberculosis* complex bacteria. This locus was first described by Hermans *et al., 1995* who sequenced this region in *Mycobacterium bovis* BCG, the strain used worldwide tovaccinate against tuberculosis. The DR region in *Mycobacterium bovis* BCG consists of directly repeated sequences of 36 base pairs, which are interspersed by non-repetitive DNA spacers, each 35 to41 base pairs in length. The number of copies of the DR sequence in *Mycobacterium bovis* BCG was determined to be 49. In other *M. tuberculosis* complex strains the number of DR elements was found to vary significantly. The vast majority of the *Mycobacterium tuberculosis* strains contain one or more IS*6110* elements in the DR region.

Procedure

In vitro amplification of spacer DNA by PCR

1. Chromosomal DNA of *Mycobacterium tuberculosis* strain H37RV and *mycobacterium bovis* BCG P3 were be used as positive controls.
2. Reaction (master) mixture was prepared as follows for 1 sample:

- 25µl Hotstat Taq Polymerase.
- 2 µl primer DRa
- 2 µl primer DRb
- 19 µl MQ water

3. A volume of 48 µl of the reaction mix was added to PCR tubes
4. A volume of 2µl DNA was added to the reaction mix in PCR tubes to make a total volume of 50 µl.
5. The tubes were placed in a PCR thermocycler for amplification
6. The following temperature cycling were performed :
    - 3 min 94ºC 1 cycle
    - 1 min 94ºC
    - 1 min 55ºC

- 30 sec 72ºC 25 cycles
- 7 min 72ºC 1 cycle
- ∞ 4ºC

Hybridization with PCR product and detection

Purpose

Hybridization of the biotin-labelled PCR products to the immobilized spacer-oligos that represent spacers of known sequence. The presence of the spacers is visualized on the film as black spacers after incubation with streptavidin-peroxidase and ECL-detection

1. All buffers were prewarmed before use. The following buffers from concentrated stocks, using demineralized water for dilution
   - 2×SSPE/0.1% SDS, 42ºC
   - 2×SSPE/0.5% SDS, 60ºC
   - 2×SSPE/0.5% SDS, 42ºC
   - 2×SSPE, room temperature
2. Add 20µl of the PCR products to 150 µl 2×SPPE/0.1% SDS
3. Heat-denature the diluted PCR product for 10 min at 100ºC and cool on ice immediately
4. Wash the membrane for 5 min at 42ºC in 250ml 2×SPPE/0.1% SDS
5. Place the membrane and a support cushion into the miniblotter, in such a way that the slots are perpendicular to the line pattern of the applied oligonucleotides
6. Remove residual fluid from the slots of the miniblotter by aspiration
7. Fill the slots with diluted PCR product (avoid air bubbles) and hybridize for 60 min at 60ºC on a horizontal surface (no shaking), avoid contamination of neighbouring slots
8. Remove the samples from the miniblotter by aspiration and take the membrane from the miniblotter using forceps
9. Wash the membrane twice in 250ml 2×sspe/0.5% SDS for 5 min at 60ºC
10. Place the membrane in a rolling bottle and allow it to cool down to prevent inactivation of the peroxidase in the next step

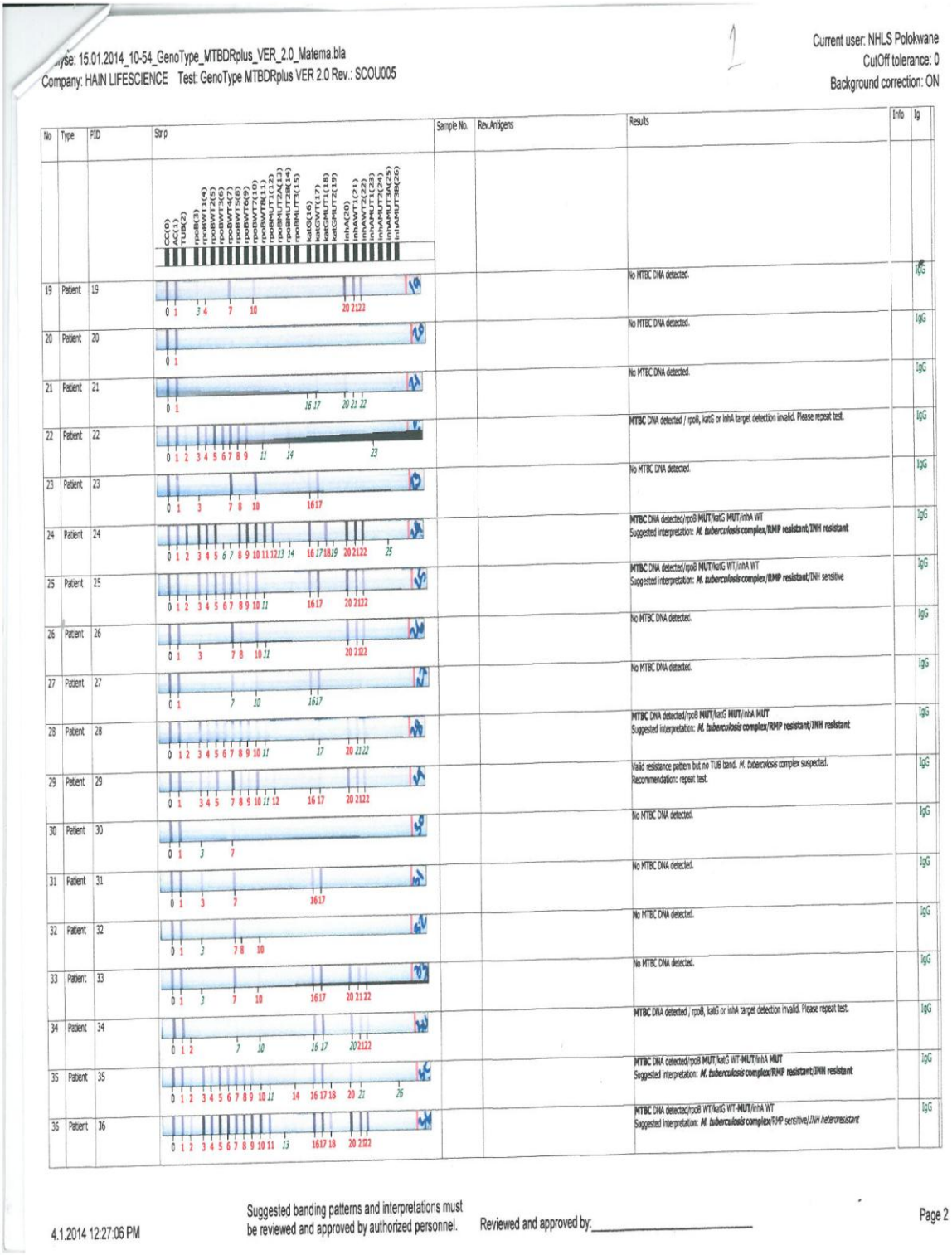
11. Add 5µl streptavidin-peroxidase conjugate (500U/ml) to 14ml of 2×SSPE/0.5% SDS, and incubate the membrane in this solution for 60 min at 4ºC in the boiling bottle
12. Wash the membrane twice in 250ml of 2×SSPE/0.5%SDS for 10 min at 42ºC
13. Rinse the membrane twice with 250ml of 2×SSPE for 5 min at room temperature
14. For chemiluminiscent detection of hybridizing DNA , incubate the membrane for 1 min in 16ml ECL detection liquid
15. Cover the membrane with a transparent plastic sheet or Saran-wrap and expose a light sensitive film to the membrane for 20 min
16. If the signal is too weak or too strong it can be used again directly to expose another film for a shorter or longer period

Regeneration of the membrane

The hybridized PCR product is dissociated from the membrane in order to regenerate the membrane for the next hybridization. A membrane can be regenerated for at least 10 times

1. Wash the membrane twice by incubation in 1% SDS at 80ºC for 30 min
2. Wash the membrane in 20mM EDTA pH 8, for 15 min at room temperature
3. Store the membrane at 4ºC until use (sealed in plastic or wrapped in Saran-wrap, to avoid dehydration of the membrane)

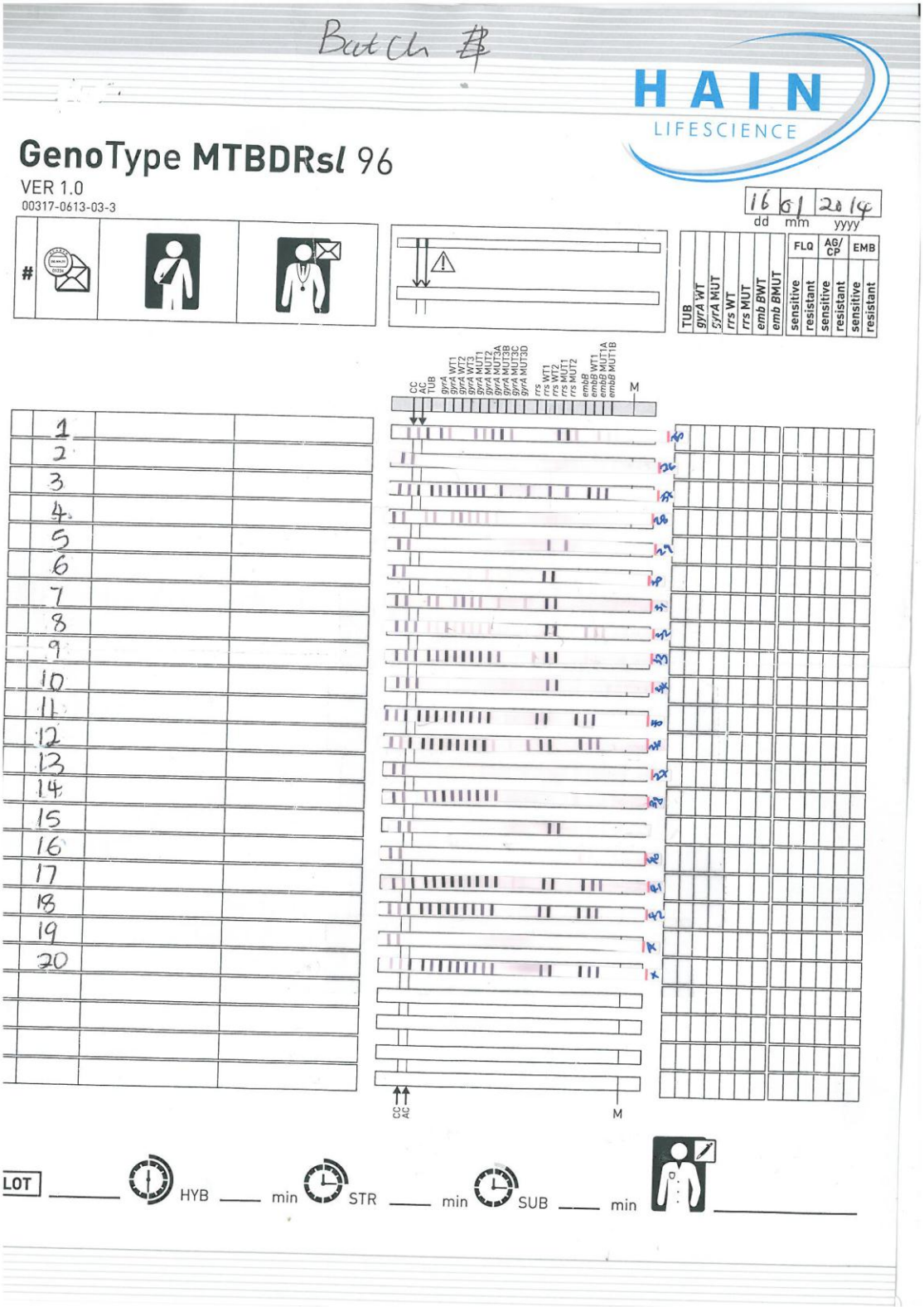
APPENDIX 6 - MTBDR*plus* results



The figure presents MTBDR*plus* results. Each strip consists of 27 reaction zones (bands), including 6 controls (conjugate, amplification, *Mycobacterium tuberculosis* complex, rpoB, katG, and i*nhA* controls), 8 *rpoB* wild-type [WT1-WT8 (506-509, 510-513, 513-517, 516-519, 518-522, 521-525, 526-529 and 530-533)] and 4 mutants [MUT1, 2A, 2B and 3 (D516V, H526Y, H526D and S531L)], 1 *katG* WT (315) and 2 mutants [MUT1 and 2 (S315T1 and

S315T2)] and 2 i*nhA* WT [WT1 and 2 (-15/-16 and -8)] and 4 mutants [MUT1, 2, 3A and 3B (C15T, A16G, T8C and T8A)]. In general for the 3 loci, a pattern comprising only WT bands was interpreted as sensitive. Resistance was interpreted as: i) absence of 1/more WT bands ii) presence of mutant bands with or iii) without the simultaneous absence of the complementary WT. The simultaneous presence of WT and corresponding mutant bands was referred to as a mixed pattern indicating heteroresistance.

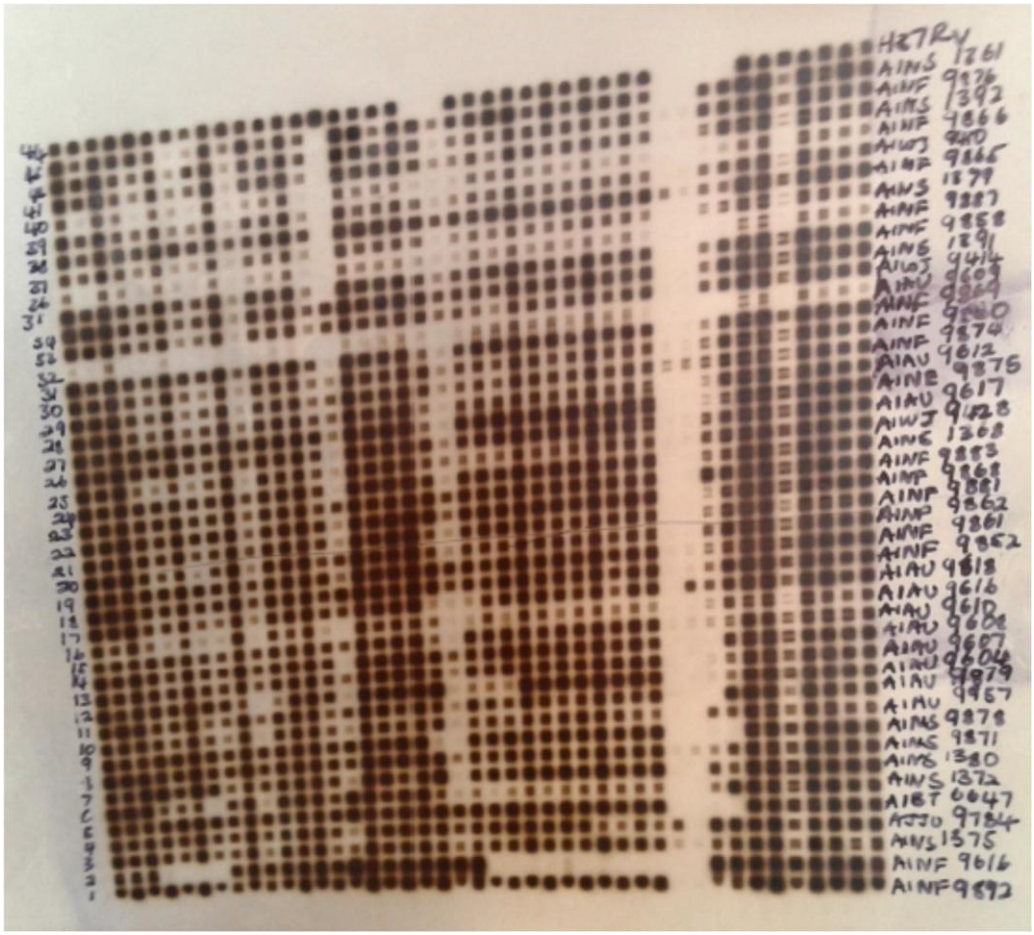
APPENDIX 7- MTBDR*sl* results.



The figure represents MTBDR*sl* results. MTBDR*sl* strip showing absence of bands for wild-type/ susceptible strains and presence of bands for mutant/ resistant isolates in the *gyrA* and *gyrB* genes for moxifloxacin, *rrs* gene for kanamycin and *embB* gene for ethambutol.

Heteroresistance to either kanamycin, moxifloxacin and ethambutol is shown in samples 5, 14,17 and 18.

APPENDIX 8 Spoligotyping results



The figure shows the film of samples subjected to spoligotyping.