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Infectious Diseases

Evaluation of new diagnostic methodology for the detection of second-line drug resistance in *Mycobacterium tuberculosis* clinical isolates.

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the **Degree of Master of Science in Medicine, by Research.**

DECLARATION

I, Yasmin Gardee, declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine (by research) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

_____

Yasmin Gardee

_____day of _____2018 in Johannesburg, South Africa.

"You are an ocean of knowledge,

hidden in a dew drop "

Jalāl ad-Dīn Muhammad Rūmī, (1207 -1273) (Rumi & Marman, 2010)

DEDICATION

In the name of God, the Most Compassionate, the Most Merciful.

I beseech the Blessings of the Almighty to

Accept my work for the benefit of all,

For “He has power over All things” *(Quran 4:85)*

CONFERENCE PRESENTATIONS

1. "Evaluation of Genotype MTBDRsl v2 line probe assay for the detection of second-line *Mycobacterium tuberculosis* resistance in culture isolates."
   Oral Abstract.
   Authors: Y Gardee, AW Dreyer, NA Ismail
   46th Union World Conference on Lung Health, 2 - 6 December 2015 - Cape Town, South Africa

2. “Prospective evaluation of Genotype MTBDRsl VER 2.0 line probe assay (LPA) for the detection of second-line *Mycobacterium tuberculosis* resistance in clinical samples.”
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RESEARCH PUBLICATIONS

1. Evaluation of the Genotype® MTBDR*sl*® VER 2.0 assay for second-line drug resistance detection of *Mycobacterium tuberculosis* isolates in South Africa. Y Gardee, AW Dreyer, H J Koornhof, S V Omar, P da Silva, Z Bhyat, NA Ismail. J Clin Microbiol 55:791–800. https://doi.org/10.1128/JCM.01865-16.

2. A Novel Molecular Strategy for Surveillance of Multidrug Resistant Tuberculosis in High Burden Settings. Halima M. Said, Nicole Kushner, Shaheed V. Omar, Andries W. Dreyer, Hendrik Koornhof, Linda Erasmus, Yasmin Gardee, Ivy Rukasha, Elena Shashkina, Natalie Beylis, Gilla Kaplan, Dorothy Fallows, Nazir A. Ismail. (2016). PLoS ONE 11(1): e0146106. doi:10.1371/journal.pone.0146106

3. Prospective evaluation of the GenoType® MTBDR*sl* VER 2.0 Line Probe Assay on clinical samples (direct sputum testing). Y Gardee, AW Dreyer, H J Koornhof, H M Said, Z Bhyat, NA Ismail.
   (Manuscript to be submitted to Journal of Clinical Microbiology for publication)

4. Evaluation of standardised Becton Dickinson® (BD) second-line DST lyophilised drug kit in comparison with the Genotype® MTBDRsl VER 2.0 LPA and in-house drug preparations for the detection of second-line drug resistance among *M. tuberculosis* clinical isolates. Y Gardee, AW Dreyer, H J Koornhof, S V Omar, Z Bhyat, NA Ismail.
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Thank you is just the beginning…

ABSTRACT

The World Health Organization (WHO) Global Report, 2017, confirms that tuberculosis (TB) is the primary cause of death globally caused by an infectious agent. Drug-resistant TB (DR-TB) is reported by the WHO to affect at least 600 000 people globally and is the primary obstacle in the fight against the elimination of TB disease.

The prompt identification and treatment of DR-TB is essential. Conventional culture-based diagnostic tests for TB require substantial laboratory capacity and result availability can take up to 3 - 4 months. The implementation of rapid molecular diagnostic tests such as line probe assays (LPA) and the Xpert MTB/RIF® (Cepheid, USA) have led to an improvement in reporting turnaround times.

GenoType® MTBDRsl VER 2.0 LPA is designed for molecular detection of second-line drug resistance-conferring mutations in genes encoding resistance to fluoroquinolones (FLQ) (*gyr*A and *gyr*B*)* and second-line injectable drugs (SLID) (*rrs* and *eis)*. This study evaluated the diagnostic performance of the Genotype® MTBDR*sl* VER 2.0 compared to phenotypic drug susceptibility testing (DST) as the gold standard, on clinical samples and *Mycobacterium tuberculosis* complex clinical isolates from South Africa.

The performance indices (sensitivity and specificity estimates) for the assay when tested on clinical samples were as follows respectively: FLQ 77.2% (95% CI, 67.2% - 85.3%); 85.4% (95% CI, 79.0% - 90.5%) and SLID 81.9% (95% CI, 75.5% - 87.2%), 91.5% (95% CI, 86.6% - 95.5%). For clinical isolates the performance indices were as follows respectively: FLQ 100% (95% CI, 95.8% - 100%); 98.9% (95% CI, 96.1% - 99.9%) and SLID 89.2% (95% CI, 79.1% - 95.6%); 98.5% (95% CI, 95.7% - 99.7).

The incorporation of the assay into the TB diagnostic algorithm will improve South Africa's TB Control Program in prompt identification and appropriate treatment of drug-resistant TB cases.

LIST OF ABBREVIATIONS AND ACRONYMS

AFB	Acid-fast bacilli
AG	Arabinogalactan
A-LYS	Lysis Buffer
AM-A	Amplification Mix A
AM-B	Amplification Mix B
AMG	Aminoglycoside
AMK	Amikacin
A-NB	Neutralization Buffer
AST	(Automated) Antibiotic Susceptibility Testing
ATCC	American Type Culture Collection
BCG	Bacillus Calmette-Guérin
BD	Becton Dickinson®
BDQ	Bedaquiline
BMRC	British Medical Research Council
BP	Base Pairs
BSC	Biological Safety Cabinets
BSL-3	Biosafety Level 3
C	Cytosine
CAP	Capreomycin
CC	Critical Concentration
CB	Clinical Breakpoint
CFZ	Clofazimine
CI	Confidence Interval
CLSI	Clinical & Laboratory Standards Institute
CON	Conjugate Concentrate
CRS	Combined Reference Standard
CTB	Centre for Tuberculosis
DEL	Delamanid
DEN	Denaturing Buffer
DNA	Deoxyribonucleic Acid
DOTS	Directly Observed Therapy Short-course
DR	Drug Resistant
DS	Drug Susceptible
DST	Drug Susceptibility Testing
EAI1_SOM	East African Indian_Somali
EMB	Ethambutol
EQA	External Quality Assessment
ETH	Ethionamide
FL	First-line
FLQ	Fluoroquinolone

G	Guanine
GC	Growth Control
GDG	Guideline Development Group
GRADE	Grading of Recommendations Assessment, Development and Evaluation
HBC	High Burden Country
HIV	Human Immunodeficiency Virus
HYB	Hybridization Buffer
IGRAs	Interferon-Gamma Release Assays
INH	Isoniazid
J Clin Microbiol	Journal of Clinical Microbiology
KAN	Kanamycin
KZN	KwaZulu-Natal
LAM	Lipoarabinomannan
LED	Light-Emitting Diode
LF	Lateral Flow
LIS	Laboratory Information System
LNZ	Linezolid
LPA	Line Probe Assay
LTBI	Latent *Mycobacterium tuberculosis* infection
MA	Mycolic Acid
MDR	Multidrug Resistant
MDR-TB	Multidrug Resistant Tuberculosis
MFX	Moxifloxacin
MGIT	Mycobacteria Growth Indicator Tube
MIRU-VNTR	Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeats
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* Complex
MUT	Mutation
NALC-NaOH	*N*-Acetyl-L-Cysteine-Sodium-Hydroxide
NDOH SA	National Department of Health of South Africa
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
NPV	Negative Predictive Value
NTM	Non-Tuberculous Mycobacteria
OADC	Oleic Acid, Albumin, Dextrose and Catalase
PAS	Para-Aminosalicylic Acid
PCR	Polymerase Chain Reaction
PDIM	Phthiocerol Dimycocerosates
PG	Peptidoglycan
POC	Point-Of-Care

PPE	Personal Protective Equipment
PPV	Positive Predictive Value
Pto	Prothionamide
PTS	Proficiency Testing Schemes
PZA	Pyrazinamide
RFLP	Restriction Fragment Length Polymorphism
RIN	Rinse Solution
RIF	Rifampicin
RR-TB	Rifampicin-Resistant Tuberculosis
SA	South Africa
SDG	Sustainable Development Goals
SIT	Spoligotype International Type
SL	Second-Line
SLID	Second-Line Injectable Drug
Spoligotyping	Spacer Oligonucleotide Typing
SRL	Supranational Laboratory
STR	Stringent Wash Solution
STRP	Streptomycin
SUB	Substrate Concentrate
TAT	Turn-Around-Time
TB	Tuberculosis
TBc ID	TBc Identification Test (Becton Dickinson®)
TDM	Trehalose Dimycolates
TDR	Totally Drug Resistant
TST	Tuberculin Skin Test
TTD	Time-To-Detection
VIO	Viomycin
WGS	Whole Genome Sequencing
WHO	World Health Organization
WT	Wild Type
XDR	Extensively Drug Resistant
ZN	Ziehl-Neelsen
κ	Cohen's Kappa

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CHAPTER 1

1. INTRODUCTION AND BACKGROUND

1.1 Tuberculosis: A historical overview

Tuberculosis (TB) has been a blight on humankind through the ages. The coexistence of *Mycobacterium tuberculosis,* the causative agent of TB and humans has been well documented. DNA-based evidence collected from bone samples in a Neolithic settlement dating back approximately 9000 years, shows the existence of a strain with a *M. tuberculosis* genetic signature similar to those of strains of today. This finding supports the concept of long-term host-pathogen coexistence (Hershkovitz et al., 2008; Rogers, 2011). Evolutionary changes in human social and communal structures in ancient settlements, therefore, encouraged the transmission of *M. tuberculosis* disease (Hershkovitz et al., 2008).

A collage of disease afflictions of man has been documented through the ages in religious texts, for example, the Holy Quran (7:131-133) and the Bible (Exodus 7:14-11:10) mention the plagues upon men and beasts (Ali, 2011). Historically TB was known as the White Plague due to the effects of TB disease on human hosts, including haemoptysis, resulting in anaemic patients. The effects of TB on breathing, an act essential to life, and wasting of the body, depicted as a worn or lost soul, lead society to view TB meta-physically.

> *"…But TB remains ignored. Today we are calling on the world to recognize that we cannot win the battle against AIDS if we do not also fight TB."*
> *(Mandela, 2004)*

This pledge was made by the late President Nelson at the WHO AIDS Conference in 2004. He contracted TB in 1988 whilst in Polsmoor Prison. Archbishop Desmond Tutu, a TB survivor, suffered from TB as a teenager. The Desmond Tutu TB Centre in Cape Town bears witness to his commitment to reach out to disadvantaged

communities to promote prevention of TB and treatment adherence. Both Nobel Peace Prize Laureates have been active in the global fight against TB.

Renowned writers, poets, artists and musicians through the ages were infected with TB. Albert Camus and Elizabeth Barrett Browning, for example, reflected in their writings in literature on the effects of TB on their lives (Enclyclopedia of World Biography, 2016). Thomas Mann, in *The Magic Mountain,* chronicled the lives of fictional characters in a sanatorium in the Swiss Alps (Enclyclopedia of World Biography, 2016; Dyer, 2010). Katherine Byrne, in *Tuberculosis and the Victorian Literary Imagination,* made commentary on pulmonary TB or "phthisis" as depicted in characters by Victorian writers such as Charles Dickens, Henry James and Bram Stoker, which "underlines the metaphorical power and the cultural impact of the disease" (Talairach-Vielmas, 2011). African writers, such as, Aluko, Achebe, Mahfouz; were all familiar with the effects of TB in their countries (Killam & Kerfoot, 2008). It is evident that the impact of TB on art, science and medicine through the medium of social commentary highlighted the significance of the effects of TB as a disease in the society. As described above, history has shown that stigmatisation and / or denial of disease is unfortunately a reflex behavioural mechanism in human society. High infection rates in impoverished societies featured prominently in the creation of socio-political divisions. The socio-environmental context of TB is as relevant today as it was over the past few centuries.

Despite the introduction of the live attenuated Bacillus Calmette-Guérin (BCG) vaccine in the 1920's, (still administered today) which prevents severe forms of TB (disseminated TB and TB meningitis) in children, an improved vaccine to prevent TB disease in adults is not yet available. At present there are 13 TB vaccines in Phase I, II or III trials for possible introduction in the future (WHO Global TB Report, 2016 & 2017).

Effective anti-TB therapy has been available for over 50 years, yet today, even as a treatable and preventable disease, TB remains a scourge to society and a challenge

to public health and social systems. There is a dire need for new therapeutic and diagnostic interventions to augment current TB control efforts.

1.2 Global management perspective of tuberculosis

The World Health Organization (WHO) administers TB prevention and control programs in collaboration with governmental healthcare organisations, private and academic institutions, that are bastions of hope for the global eradication of TB. The current global burden of TB, especially in poorly developed countries with dense populations and poor socio-economic and hygiene standards, characterises TB as a disease of considerable morbidity and significant mortality. Inadequate health seeking behaviour and non-compliance to antibiotic therapy are reported to be major obstacles to TB control programs globally. In the WHO 2016 Global TB Report a commitment to end the global TB epidemic by 2035 through Sustainable Development Goals (SDG) and the End TB Strategy, defined as “reducing the incidence of TB to fewer than 100 cases per million people” is envisaged (WHO Global TB Report, 2016 & 2017). Achievement of this goal will necessitate prompt upgrades in health care and patient management systems.

1.3 TB/HIV Co-epidemic

Prevalence of TB disease has increased in association with the worldwide HIV epidemic. Developing and resource challenged countries bare the brunt of TB disease globally. According to the WHO Global TB Report 2017; Africa, where the TB prevalence is driven by TB-HIV co-infection, reported 74% TB-HIV cases, constituting the highest burden of TB-HIV cases relative to population size globally (WHO Global TB Report, 2017). Added to this is the worldwide emergence of multidrug-resistant TB, (MDR-TB) constituting resistance to isoniazid (INH) and rifampicin (RIF) first-line anti-TB drugs, and extensively drug resistant TB (XDR-TB) defined as MDR-TB resistant to any fluoroquinolone (FLQ) and at least one second-line injectable drug

(SLID) involving the aminoglycoside drugs (AMG) amikacin (AMK), kanamycin (KAN) and the cyclic peptide, capreomycin (CAP) (WHO Guidelines, 2011). The emergence of drug resistance undermines TB control efforts, particularly in countries where HIV and TB prevalence is high (WHO Global TB Report, 2015). The problem in Africa is further exacerbated by poverty, lack of access to healthcare due to poorly managed health systems and laboratories, inferior living and social conditions, use of traditional healers, wars and civil and political instability (Cadmus, Tayeb & van Soolingen, 2012). These conditions assist in the transmission of TB disease with poor treatment outcomes resulting in the emergence of drug resistant *M. tuberculosis* strains.

In South Africa, despite the increased emphasis on TB therapy, the HIV epidemic has seriously hampered effective TB treatment and patient outcomes and increased the numbers of MDR- and XDR-TB cases (Gandhi et al., 2006; WHO Global TB Report, 2015). According to Theron et al. (2014), 80% of MDR-TB in South Africa is transmitted through person-to-person contact, rather than through selection of resistant mutants during treatment. It may be inferred that XDR-TB is transmitted in a similar manner and XDR-TB outbreaks in South Africa bear testimony to that (Gandhi et al., 2006). Studies have indicated that rapid diagnosis of TB and drug resistant (DR)-TB in particular, would lead to early appropriate and effective treatment (Theron et al., 2014). This would consequently lead to a reduction in the potential of person-to-person transmission of TB.

The advent of the global HIV epidemic in the 1980's saw resources (financial, research and capacity building) ploughed into this "new" disease phenomenon. This left resources and efforts for the development of new drugs for TB treatment lagging. The ramifications of the TB/HIV co-epidemic resulted in renewed efforts in the research into, and development of new and novel anti-TB agents and diagnostic tests. Grosset et al. (2012) reviewed evidence of newly developed anti-TB agents in an attempt to determine how they might impact on patient outcomes and TB control, especially in high burden TB countries. The authors pointed out that bedaquiline and delamanid were the first novel drugs to be developed specifically for TB treatment in 40 years.

1.4 Epidemiology

According to WHO reports, approximately one third of the world's population is infected with *M. tuberculosis* and of these 10% will most likely develop active TB disease (WHO Global TB Report, 2012 & 2016). The WHO 2017 Global TB Report estimated that in 2016 there were 10.4 million incident TB cases with 1.2 million (11%) of these co-infected with HIV (WHO Global TB Report, 2016 & 2017). The decrease in TB incidence was approximately 2% annually from 2014 - 2016 which needs to decrease further by 4 - 5 % annually in order to achieve the 2020 End TB Strategy milestones. In the WHO 2017 Global TB Report an estimated 8.6 million people developed TB and 1.3 million died from TB during 2016 (WHO Global TB Report, 2016 & 2017). Furthermore, the WHO 2014 trend analysis over 2008 to 2013 showed that approximately 3.5% of new TB cases were MDR-TB. In this report South Africa is ranked 8th in the world amongst countries with the highest burden of MDR-TB (WHO Global TB Report, 2014). South Africa also experienced an increase in XDR-TB within this period, accounting for 59% of the 1 269 XDR-TB patients reported globally in 2011 (WHO Global TB Report, 2014). Currently, South Africa is listed amongst the 14 countries that appear in all three WHO 2016 -2020 revised "high burden country" (HBC) lists, figure 1.1 (WHO Global TB Report, 2016 & 2017).

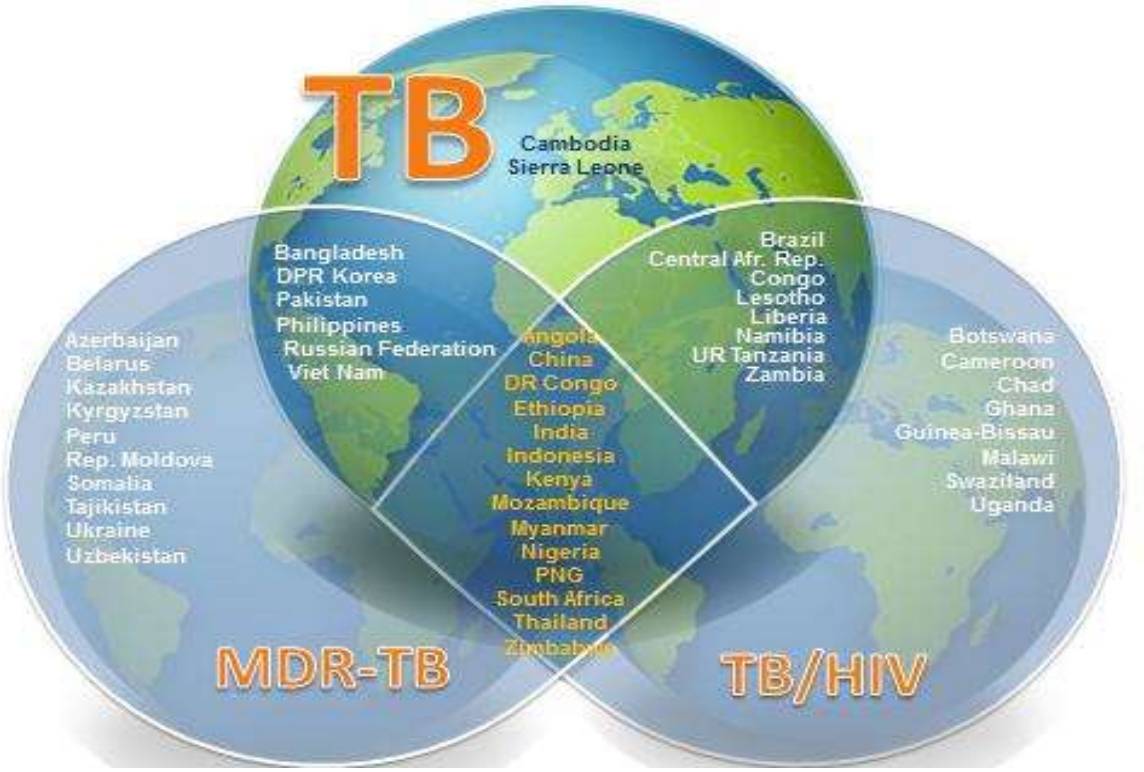


Figure 1.1: The three High Burden Country lists of 30 countries used by WHO 2016–2020 End TB Strategy (WHO Global TB Report, 2016 & 2017)

1.5 Advances in Rapid Diagnostic Testing

The necessity to develop and implement rapid diagnostic tests to enable early detection of active *M. tuberculosis* infection/disease and drug susceptibility or resistance as well as appropriate treatment is crucial. An escalation in research into new diagnostics and the development of molecular tests for the rapid detection of drug resistance is a fundamental commitment of the WHO *End TB Strategy* (2016 – 2035) targets (WHO Global TB Report, 2016 & 2017).

Conventional methods used for laboratory diagnosis of *M. tuberculosis* disease i.e. culture and phenotypic drug susceptibility testing (DST), the current reference standards, but are labour intensive and time consuming. The principle and success of these diagnostic tests is entirely dependent on the propagation of pure *M. tuberculosis* cultures from clinical samples, which may take between 2 - 4 weeks, depending on the methodology used. In addition, there may be further setbacks

during the course of the testing period, extending the time to 2 - 3 months. Such delays and test failures are related to deficient technical and resource capabilities (e.g. deficient standardisation of methodologies) as well as repeat testing due to test failure including contamination and loss of viability of cultures.

Other limitations related to phenotypic DST are the issues related to reproducibility of pyrazinamide (PZA) possibly due to the pH of testing media and the most appropriate critical concentration (100 µg/ml) (Zignol et al., 2016). Heteroresistance, which is defined as the coexistence of drug susceptible and drug-resistant strains or multiple drug –resistant strains with different resistance profiles in a single specimen (Metcalfe et al., 2017). Detection of heteroresistance or mixed infections (simultaneous infection with more than one strain with different phenotypic profiles) can potentially be masked by the majority population when using conventional DST (Cohen et al., 2012).

The promise and advantages offered by molecular assays are constrained by the limited number of drug resistance-conferring mutations for anti-TB agents that are currently known and incorporated into molecular tests. These limitations do not apply to conventional DST, making it still an effective means of detecting resistance and monitoring drug resistance trends and patient treatment outcome. Phenotypic DST provides confidence in results as all resistance mechanisms within the organism are operative during the test. Molecular assay(s) on the other hand are usually designed to target specific regions within the TB-genome relating to the mode of action of the drug being tested and identify mutations which may or may not be expressed as phenotypic resistance.

The implementation of rapid molecular assays as screening and supplementary diagnostic tools to conventional phenotypic DST, because of the therapeutic benefits and epidemiological implications (e.g. reduced transmission) of obtaining a rapid test result in the case of molecular tests, remains of paramount importance (Szumowski et al., 2013; Drobniewski, 2013; WHO Global TB Report, 2015). Molecular based assays (e.g. line probe assays (LPA) and the Xpert MTB/RIF®, (Cepheid, USA) have

significant advantages over conventional methods for optimising surveillance and control of TB management programs. They offer speed of diagnosis, standardised testing, increased test volumes, point-of-care testing and shorter turn-around-time (TAT) capabilities (Theron et al., 2014).

The evaluation of newly developed diagnostic methodologies, admittedly expensive, is essential and entails the collection of reliable data for appraisal and recommendation which may result in the redesigning of diagnostic and monitoring programs, coupled with clinical outcomes, prior to implementation.

1.6 WHO recommendation of molecular line probe assay for MDR-TB

In 2008 WHO endorsed molecular LPA testing for the diagnosis of MDR-TB to detect resistance to the first-line anti-TB drugs, INH and RIF (FL-LPA) (WHO Expert Group, 2013). FL-LPA detects mutations in the *rpo*B gene (associated with RIF resistance) and *kat*G and *inh*A promotor region (associated with INH resistance). LPAs have proven to be reliable rapid-diagnostic tests for the detection and therapeutic management of MDR-TB, as well as the prevention of transmission of drug-resistant strains (WHO Expert Group, 2013). The WHO 2017 Global TB Report confirms the endorsement of LPA tests for the detection of *Mycobacterium tuberculosis* complex (MTBC) as well as susceptibility / resistance to INH and RIF in acid-fast bacilli smear positive sputum or MTBC cultures. The FL-LPAs endorsed are GenoType MTBDRplus VER 2.0 (Hain Lifescience, Germany) and Nipro NTM+MDRTB detection kit 2 (Nipro, Japan) (WHO Global TB Report, 2016 & 2017; WHO Compendium, 2017).

The GenoType MTBDRsl Version 1 was introduced by Hain LifeScience in 2009. The intended application of this assay was the rapid detection of resistance to second-line anti-TB drugs, namely FLQ and SLID comprising AMK and KAN and the cyclic peptide CAP, together with ethambutol (EMB), in patients diagnosed with rifampicin-resistant-TB (RR-TB), defined as resistance to rifampicin with or without resistance to

other anti-TB drugs (WHO Global TB Report, 2014), or MDR-TB. The WHO Expert Group 2013 conclusions on GenoType® MTBDRsl Version 1 were that the assay cannot replace phenotypic DST but it may be used as a supplementary test to DST for FLQ and SLID resistance as a rule-in test for XDR-TB (WHO Expert group, 2013).

A meta-analysis of 21 studies using GenoType® MTBDR*sl* Version 1 done by Theron et al (2014) determined the pooled sensitivity and specificity for FLQ and SLID on indirect samples (culture isolates) and directly (sputum sediments) respectively (Table 1.1). The authors concluded that a positive MTBDR*sl* Version 1 result interpreted as resistance to FLQ, SLID or both (XDR-TB) indicates reliably that the patient "*has drug-resistant TB and further conventional drug-resistance testing is not required"* (Theron et al., 2014). GenoType® MTBDR*sl* Version 1 only targets selected *gyr*A (FLQ) and *rrs* (SLID) mutations. Mutations for FLQ and SLID that occur outside these genomic regions are missed by the assay (Theron et al., 2014).

Table 1.1: GenoType® MTBDR*sl* Version 1 - Summary of pooled results for FLQ & SLID (Theron et al., 2014)

POOLED RESULT	INDIRECT (%) SENSITIVITY	INDIRECT (%) SPECIFICITY	DIRECT (%) SENSITIVITY	DIRECT (%) SPECIFICITY
FLQ (OFX +MXF)	**83.1** (95% CI 78.7% to 86.7%	**97.7** (95% CI 94.3% to 99.1%)	**85.1** (95% CI 71.9% to 92.7%)	**98.2** (95% CI 96.8% to 99.0%)
SLID	**76.9** (95% CI 61.1% to 87.6%)	**99.5** (95% CI 97.1% to 99.9%)	**94.4** (95% CI 25.2% to 99.9%)	**98.2** (95% CI 88.9% to 99.7%)

1.7 WHO recommendation of GenoType® MTBDR*sl* VER 2.0 Line Probe Assay

The GenoType® MTBDR*sl* VER 2.0 LPA was redesigned and released in 2015 to include additional probes for the detection of FLQ resistance mutations in the *gyr*A and *gyr*B gene regions and mutations in the *rrs* and *eis* genes for resistance to SLID, that are not present in the GenoType® MTBDR*sl* VER 1.0 assay. Mutations observed

within these specific regions, however, do not presuppose that resistance to all drugs within an antibiotic group is likely to occur (WHO Policy Guidance - molecular line probe assays, 2016).

The new version of the assay was assessed by the WHO Guideline Development Group (GDG) and in May 2016, WHO recommended the use of GenoType® MTBDR*s*l VER 2.0 LPA as an initial rapid screening test for the detection of resistance to second-line anti-TB drugs (WHO Policy Guidance - molecular line probe assays, 2016; WHO Global TB Report, 2016). The assay allows for the early identification of pre- XDR-TB (resistance to INH and RIF and resistance to either a FLQ or SLID but not both) (Niehaus et al., 2015) and XDR-TB patients (resistance to both FLQ and SLID) and for patients to be placed on appropriate anti-TB drug regimens to improve their chances of treatment success. The assay is described in further detail in chapter 2.

1.8 South African National Department of Health Tuberculosis Management Guidelines 2014 - TB Diagnostic Testing Algorithm

The ambit of the National TB Management Guidelines 2014 of the National Department of Health of South Africa, (NDOH SA) as advocated by Dr Aaron Motsoaledi**,** Minister of Health, is to “reduce transmission of infection in communities, diagnose drug susceptible (DS)-TB and DR-TB early, initiate treatment in all patients diagnosed with TB early” (NDOH SA, 2014).

These guidelines (2014) describe testing algorithms for the diagnosis and management of TB based on results from laboratory tests. The recommendations are that sputum specimens from all patients presenting with symptoms of pulmonary TB are tested for bacteriological confirmation of TB disease. The introduction of rapid molecular assays such as Xpert® MTB/RIF (Cepheid, USA) and MTBDRplus VER 2.0 LPA (Hain Lifescience, Germany) as initial tests into the TB diagnostic algorithm is particularly important for the early confirmation of TB disease and early initiation of

appropriate treatment. Conventional TB culture and phenotypic DST are required to confirm MDR-TB and resistance to second line antibiotics. Smear microscopy is used for monitoring of treatment (NDOH SA, 2014). A limitation of the rapid molecular assays is that they do not distinguish between viable and non-viable TB bacilli. Their use is therefore unsuitable for monitoring treatment outcomes but of value for early diagnosis of TB disease (NDOH SA, 2014; Nikolayevskyy et al., 2015). The GeneXpert MTB/RIF and MTBDR*plus*® VER 2.0 implementation steps of the recommended TB diagnostic algorithm proposed in the National Tuberculosis Management Guidelines 2014 are illustrated in figures 1.2.a and 1.2.b.

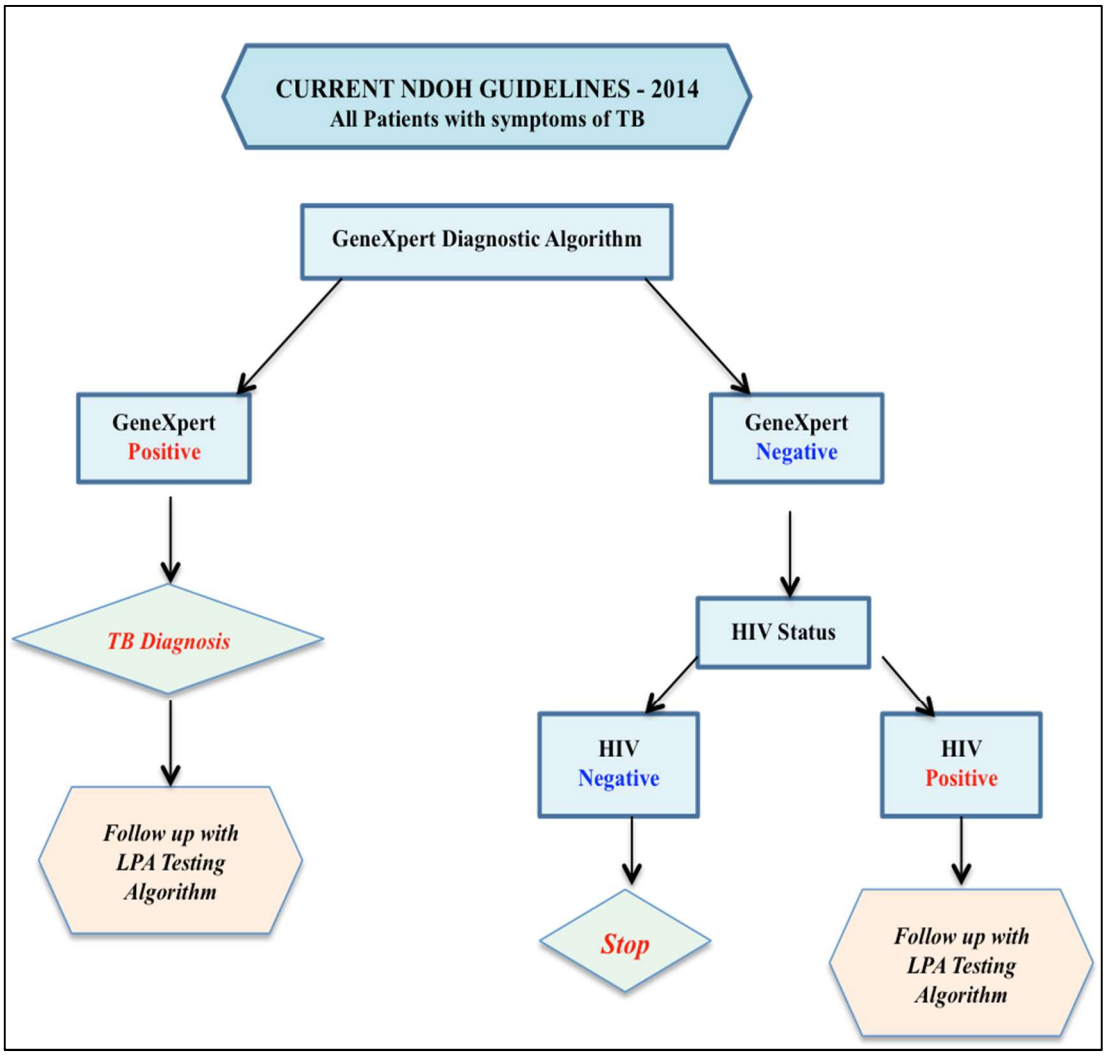


Figure 1.2.a. GeneXpert SA Diagnostic Testing Algorithm (Xpert® MTB/RIF)

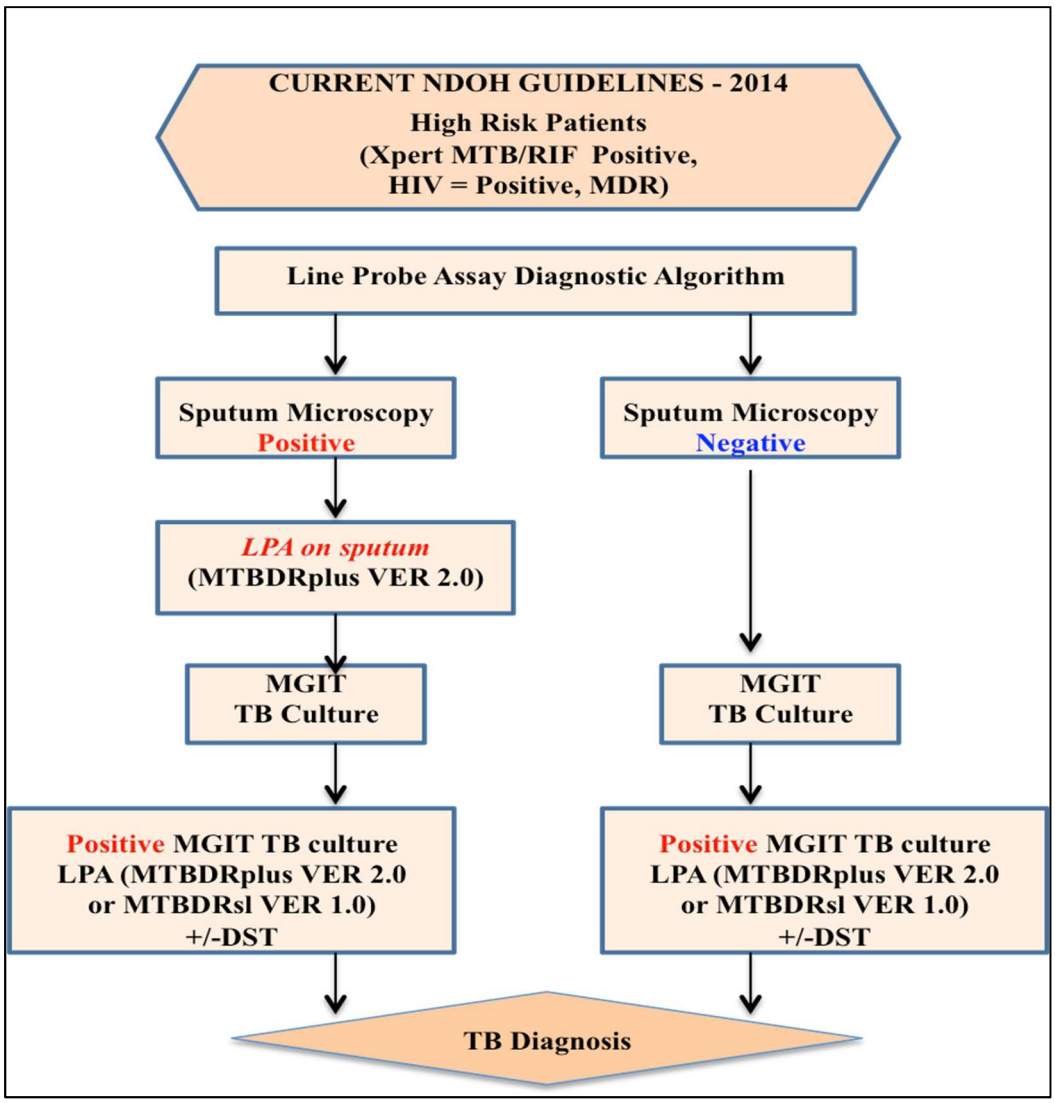


Figure 1.2.b. LPA SA Diagnostic Testing Algorithm for MTBDRplus® VER 2.0

1.9 History of *Mycobacterium tuberculosis* Complex

1.9.1 Discovery of the pathogen

Hansen (1874) discovered the first *Mycobacterium* organism, *Mycobacterium leprae,* hence leprosy was also known as Hansen's Disease (Akbar & Farni, 2012). Robert Koch, acknowledged as the founder of modern bacteriology, discovered *Mycobacterium tuberculosis* (Koch's bacillus) in 1882, which marks a critical moment in history towards understanding the disease tuberculosis (TB). Koch was awarded the Nobel Prize for Medicine in 1905 for his contribution towards research in the field of TB (Fredericks & Relman, 1996). Staining methods improved by Ziehl-Neelsen in

1883 and Ehrlich in 1887, enhanced the visualization of Koch's bacillus in sputum of TB patients, enabling diagnosis of TB disease (Akbar & Farni, 2012).

1.9.2 Taxonomic classification

The taxonomic classification of mycobacteria is illustrated in Figure 1.3. The genus Mycobacterium is the only genus of the family Mycobacteriaceae in the order of Actinomycetales. Cole et.al (1998) reported a composite genome sequence of 4 411 529 base pairs for the H37Rv *M. tuberculosis* strain. The high guanine (G) and cytosine (C) content of the genome of the *M. tuberculosis* organism comprising 65% G + C-rich DNA (Cole et al., 1998) provides stability to the mycobacterial DNA while additional support is provided by the sturdy hydrophobic cell wall of mycobacteria (Hershkovitz et al., 2008).

Mycobacterium tuberculosis complex (MTBC) consists of *M. tuberculosis, M. africanum, M.bovis, M bovis BCG, M. microtti, M. canetti, M. capre, M.mungi, M.orygis, M. suricattae* and *M pinnipedii* (Parsons et al., 2013). MTBC species are clonal in their spread with those infecting humans classified into seven spoligotypes.

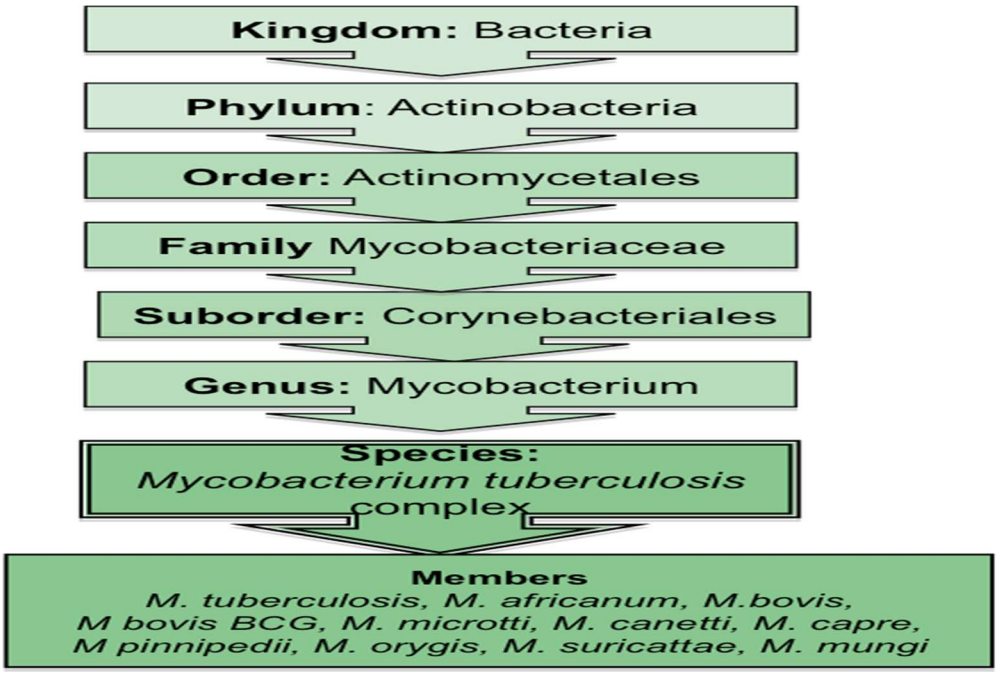


Figure 1.3: Taxonomic classification of *Mycobacterium tuberculosis* complex

1.9.3 Phylogeny of *Mycobacterium tuberculosis*

Studies using molecular techniques such as genome sequencing have shown that an ancestral progenitor of *M. tuberculosis* existed in East Africa approximately 3 million years ago and probably infected early hominids (Daniel, 2006; Brosch et al., 2002; Dye, 2015). Mycobacteria in the *Mycobacterium tuberculosis* complex group are characterized by nucleotide similarity of 99.9% and identical 16S rRNA sequences, although diverse in their pathogenicity and phenotypes (Brosch et al., 2002). Brosch et al. (2002) proposed that ancestral *M. tuberculosis* strains were all human pathogens as opposed to the theory that *M. tuberculosis* evolved from an adaptation of the animal pathogen *M. bovis* to a human pathogen. As reported by Brosch et al. (2002) the *M. tuberculosis* specific deletion 1 genomic region, 'TbD1', grouped *M. tuberculosis* into "ancient" and "modern" strains. The 'TbD1' genomic region is deleted in all 'modern' *M. tuberculosis* lineages.

As illustrated in Figure 1.4, there are seven phylogenetically distinct lineages corresponding to the different spoligotype families of *M. tuberculosis* complex, which are proposed to have evolved as a human pathogen in Africa (Brosch et al., 2002). Africa is the only continent where all seven lineages of *M. tuberculosis* complex are known to occur. Human migration across continents has accounted for the global distribution of *M. tuberculosis* strains, in particular, the spread of the modern Beijing and other *M. tuberculosis* strains (Dye, 2015). The dissemination of MDR-TB in Eurasia is proposed to be driven by the Beijing / East Asian lineage strains which are reported to have selective advantages for resistance acquisition, increased transmissibility and compensatory mutations for fitness cost of resistance conferring mutations (Merker et al., 2015).

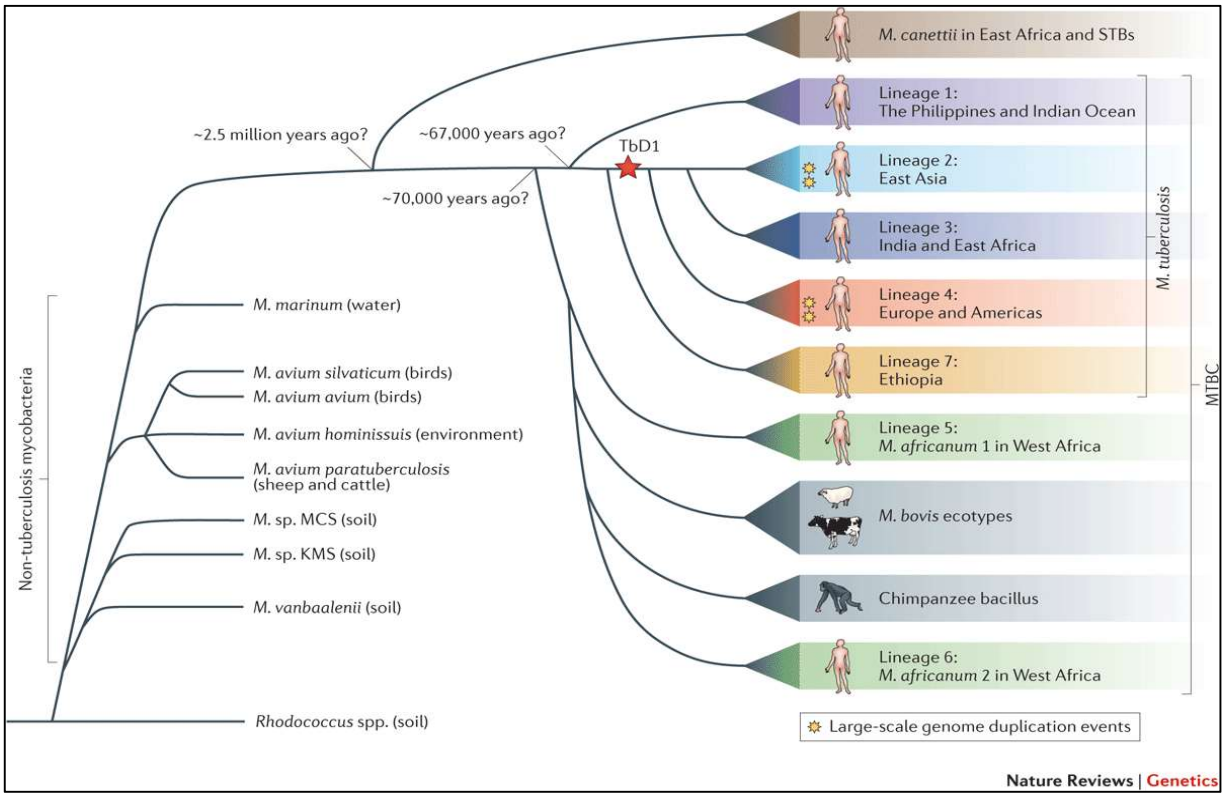


Figure 1.4. Evolutionary relationship between *M. tuberculosis* complex and selected mycobacteria (Galagan, 2014)

1.9.4 Molecular epidemiology of tuberculosis

Epidemiological analysis of transmission, reinfection and outbreak events, which are important for TB control and management programs, have been elucidated by the introduction of molecular epidemiological techniques. Several molecular methods are used for epidemiological studies and these have provided an understanding of the evolution, epidemiology and pathogenesis of TB disease. Implementing the most suitable method or combination of methods is essential for answering questions relating to transmission and phylogenetics (Mathema et al., 2006; Jagielski et al., 2014; Nikolayevskyy et al., 2016). The main molecular typing techniques currently in use are: (Table 1.2)

a. Insertion sequence based IS*6110* restriction fragment length polymorphism (RFLP) typing with high discriminatory power and reproducibility is considered the gold standard for *M. tuberculosis* typing.

b. Spoligotyping (spacer oligonucleotide typing), a hybridisation assay, is based on the detection of 43 interspersed spacer sequences (polymorphism) in the direct locus (DR) region of MTBC strains.
c. 24-loci mycobacterial interspersed repetitive unit-variable number tandem repeats (MIRU-VNTR) typing, determines the size and number of tandem repetitive DNA sequences.
d. Whole genome sequencing (WGS) single nucleotide polymorphism (SNP) analysis. SNP genotyping surveys the whole genome allowing for a higher discriminatory power. Typing, identification and antibiotic resistance data can be analysed from the data obtained.
(Mathema et al., 2006; Jagielski et al., 2014; Nikolayevskyy et al., 2016)

Table 1.2: Most Common Molecular Typing Techniques – advantages and disadvantages (Mathema et al., 2006; Jagielski et al., 2014; Nikolayevskyy et al., 2016)

Technique	Advantages	Disadvantages
IS*6110* RFLP	High discriminatory power, reproducible considered the gold standard for *M. tuberculosis* typing Mixed infection detection Extensively used, large amount of data available	Labour intensive and time consuming with long TAT Uses culture isolates Requires large volume of high quality DNA
Spoligotyping	Most uncomplicated technique for MTBC typing, commercial reagent kits available Small volume of DNA required Culture isolates and clinical samples can tested	Less discriminatory than IS*6110* RFLP and MIRU-VNTR Mixed infections not detected
MIRU-VNTR - 24-loci	Fast, high result output, automated analysis possible, commercial reagent kits used Small volume of DNA required Better discrimination than spoligotyping Detects mixed infections	Equal in discriminatory power to IS*6110* RFLP
SNP	High resolution Offers precise information on strains based on sequencing, automated analysis possible	Extensive sequencing required Major obstacle data standardization and integration

WGS may be most favourable option that would enable the investigation of epidemiology, transmission and resistance profiles in a single application. Unfortunately, WGS is still an expensive technique and outside the budgetary constraints of routine TB surveillance and management programs (Jagielski et al., 2014). Implementing PCR based MIRU-VNTR 24-loci in combination with spoligotyping offers has a discriminatory power equal to IS6110-RFLP, is less labour intensive with internationally standardised result analysis is suitable for routine high volume laboratory testing and currently more cost effective than WGS (Jagielski et al., 2014).

1.10 Mycobacteria - General characteristics

1.10.1 Mycobacterial Cell Structure

The rigid structure of the organism is advantageous in physiologically stressful conditions, for example, osmotic shock, and plays a role in conferring drug resistance to *M. tuberculosis* strains (Cole et al., 1998).

The mycobacterial cell envelope has a uniquely complex composition and is essential for mycobacterial physiology. The cell envelope is composed of an outermost layer, a cell wall and plasma membrane (Chiaradia et al., 2017). The cell wall is composed of an outer lipo-protein layer, the so- called “mycomembrane” and an inner layer consisting of peptidoglycan (PG), lipoarabinomannan (LAM), arabinogalactan (AG), and mycolic acids (MA), which are constructed by means of novel biosynthetic pathways and some are reported to provide characteristics such as mycobacterial longevity and virulence, elicit inflammatory host reactions and aid in pathogenesis (Cole et al., 1998). The cytoplasm of the *M. tuberculosis* cell is surrounded by the plasma membrane. This structure forms the inner layer of the *M. tuberculosis* cell wall which contains a peptidoglycan layer covered by an outer membrane. The cell wall contains various complex lipids such as, trehalose dimycolates (TDM) and phthiocerol dimycocerosates (PDIM) which are highly apolar and hydrophobic and have a role in cell wall architecture, permeability and virulence (Forrellad et al., 2013).

The high mycolic acid / lipid content of the cell wall makes the cell surface hydrophobic and impermeable to stains rendering acid-fast properties to mycobacteria (Akbar & Farni, 2012; Cole et al., 1998; Todar, 2008; Smith, 2003). The disruption of the virtually impregnable cell wall allows the influx of hydrophilic and hydrophobic molecules, forming the basis of the mechanism of action of some anti-TB drugs including INH which interferes with mycolic acid synthesis and is a major drug for the treatment of TB.

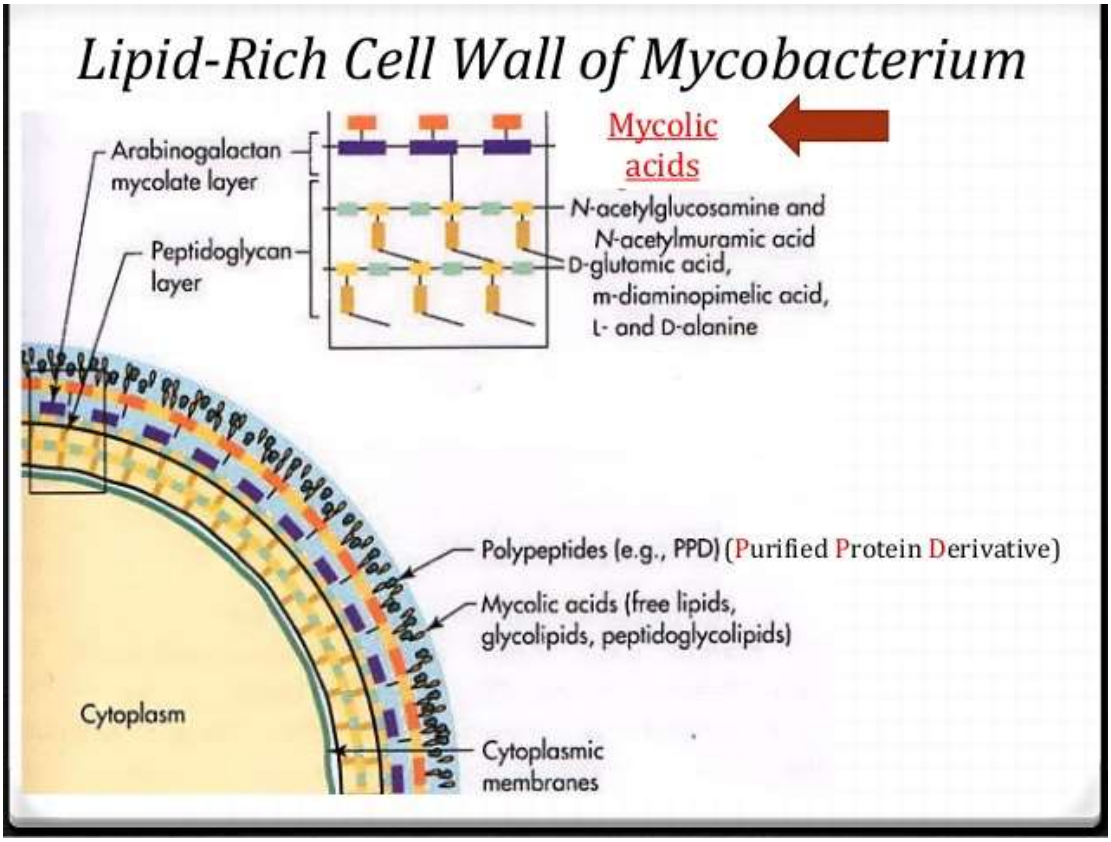


Figure1.5 Cell wall structure of *Mycobacterium* (Yadav, 2014)

1.10.2 Morphology

Mycobacteria are classified as acid-fast due to their impermeability to certain dyes. The Ziehl-Neelsen (ZN) stain for acid-fastness is the classic staining method for demonstrating this property of mycobacteria. It was developed in 1883 and is still the basic technique used to visualise *M. tuberculosis* morphology using light microscopy.

Bacilli are stained with carbol-fuchsin and appear as red curved rods or fluorescent auramine-rhodamine stained organisms for visualisation of fluorescent bacilli using fluorescent or light emitting diode (LED) microscopy. They may vary in shape and size; from coccobacilli to long rods of 1- 10 μm in length (Akbar & Farni, 2012; Todar, 2008).

M, tuberculosis bacilli are pleomorphic and when present within macrophages denote pathogenic potential. On culture, they can assume filamentous forms which are classically seen in older cultures where they can also be short and have an ovoid appearance during starvation. They may produce branches or buds in extensively drug resistant (XDR) strains (Akbar & Farni, 2012; Babady & Wengenack, 2012).

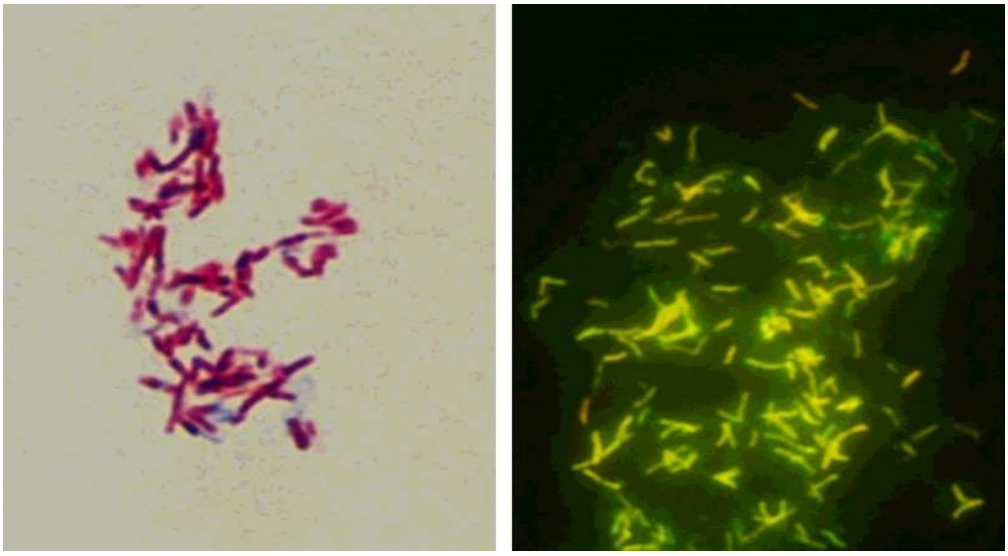


Figure 1.6. Acid-fast bacilli stains. Mycobacteria are stained with carbol fuchsin-based Kinyoun stain (left) and fluorescent auramine-rhodamine stain (right) (Caulfield & Wengenack, 2016)

1.10.3 Transmission and pathophysiology

M. tuberculosis is a slow growing microorganism with a generation time of 15-20 hours and is a resilient and adaptable organism. In laboratory culture it is an obligate aerobe, which is basic to its existence as a facultative intracellular pathogen in host

patients. *M. tuberculosis* is transmitted by infectious airborne particles (droplet nuclei), 1-5 microns in diameter (Cole et al., 1998). Mucus entrapment of the bacilli is the human body's first physical defence in preventing infection. TB bacilli that reach the alveoli on inhalation are engulfed by macrophages in the next defence mechanism of the body. Complement protein, C3, plays a role in phagocytosis of TB bacilli by binding to the cell wall and increasing the recognition by mycobacteria to the host macrophages (opsonisation) (Knechel, 2009). In progressive TB disease most bacilli are found in the caseum, a term used for necrotic material present in the core of a granuloma which is also called a tuberculoma. This is formed in response to invasion by tubercle bacilli and their cell wall constituents, including cord factor, which produce the necrotic core of the granuloma (Knechel, 2009).

There are 5 stages of TB pathology described (Shi & Sugawara, 2013):

- Stage 1 – phagocytosis by alveolar macrophages,
- Stage 2 – logarithmic growth within immature non-activated macrophages,
- Stage 3 – first stage of caseous necrosis with activation of T-lymphocytes and macrophages by T-cell specific antigens,
- Stage 4 – formation of granuloma characterised by caseous necrosis which eventually leads to active disease or to a latency stage in most infected hosts,
- Stage 5 – Host immunity fails in active TB cases with liquefaction of granuloma and multiplication of bacilli extracellularly.

Transmission results when the inhaled bacilli travel through the upper respiratory tract and reach the alveoli. This results in several outcomes (a) clearance by alveolar macrophages, (b) latent infection, (c) active or primary disease (d) reactivated disease (e) extra-pulmonary disease (Shi & Sugawara, 2013).

Primary TB classically begins in the lung where bacilli replicate intracellularly in alveolar macrophages and spreads to the hilar lymph nodes. Secondary TB occurs in patients who have developed sufficient cell-mediated immunity which through delayed hypersensitivity mechanisms cause TB granulomas in patients. These pulmonary lesions occur as a result of reinfection or reactivation of a primary focus in the lung.

Secondary TB involves the apex of one or both lungs and is characterised by cavitation (Shi & Sugawara, 2013). Immunocompromised patients due to HIV coinfection, immunosuppressive drugs, cancer, or other co-morbidities tend to develop primary tuberculosis which may be aggressive and culminate in disseminated TB including TB meningitis. Thus, HIV-infected patients, depending on their cell-mediated immune status may develop primary TB manifestations or a mixture of primary and secondary disease (Hunter et al., 2006).

1.10.4 Clinical presentation

Classic presentation of pulmonary TB typically includes chronic cough, sputum production, appetite loss, weight loss, fever, night sweats, and haemoptysis. Extra-pulmonary TB may affect any organ and has diverse clinical presentations, and therefore requires clinical follow-up for definitive diagnosis of underlying disease. TB/HIV co-infection raises further challenges to the management of TB disease with the absence of classic symptoms and the presentation of disseminated TB with a spectrum of clinical presentations, including initially that of an asymptomatic, nonspecific chronic illness (Zumla et al. 2013; WHO 2015). Therefore, screening patients for active TB infection is essential.

1.10.5 Latent tuberculosis Infection

Latent *M. tuberculosis* infection (LTBI) is defined by the WHO as “a state of persistent immune response to stimulation by *M. tuberculosis* antigens without evidence of clinically manifested active TB”, figure 1.7 (WHO LTBI, 2014). Viable organisms are able to survive in necrotic material for years, and when the immune system becomes compromised, the disease can be reactivated.

Tests used to detect LTBI are Tuberculin Skin Test (TST) and Interferon-Gamma Release Assays (IGRAs). An inherent limitation of these tests is that they cannot distinguish between active TB with viable mycobacteria, LTBI or healed infections,

neither can they predict who will develop active TB (WHO LTBI, 2014). The risk of reactivation of TB in confirmed LTBI is reported to be 5 – 10% during an infected persons' lifetime. The WHO recommends treatment for LTBI to prevent advancement to active disease (WHO LTBI, 2014).

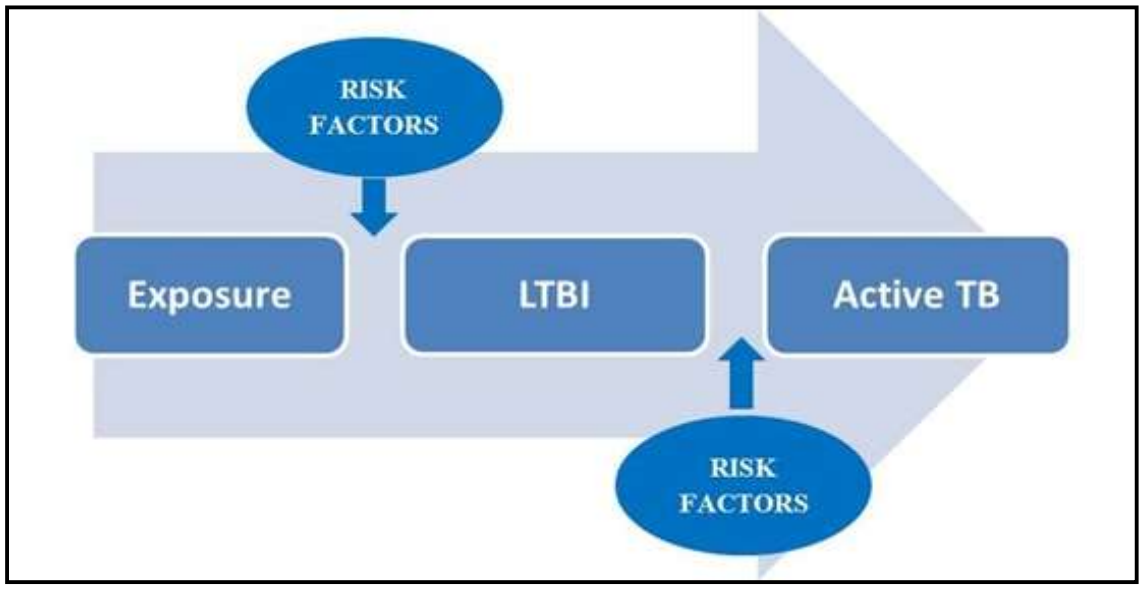


Figure 1.7. *Mycobacterium tuberculosis:* Exposure, Latent & Active TB (WHO LTBI, 2014)

1.11 Laboratory diagnosis

Tests for laboratory diagnosis of TB disease recommended by the WHO include sputum smear microscopy, rapid molecular tests, such as, Xpert MTB/RIF® and line probe assays to first-line and second-line anti-TB drugs, TB culture and phenotypic DST, the latter two culture-based tests being the current reference methods (WHO Global TB Report, 2017; WHO Compendium, 2017).

Sputum microscopy and culture on solid media or in liquid medium with subsequent phenotypic DST are currently recommended as standard methods for diagnosing active TB (Zumla et al. 2013; WHO 2015; WHO Global TB Report 2016). In 2009, the

WHO endorsed the implementation of the more sensitive and faster LED fluorescent microscopy, using auramine O and/ or rhodamine B stains as an alternative to light microscopy using ZN staining (WHO Global TB Report, 2015). The WHO 2016 recommendation was “one positive smear microscopy result for the diagnosis of pulmonary TB” (WHO Global TB Report, 2016). Automated liquid culture systems, such as the Bactec MGIT^{TM} 960 system from Becton Dickinson, USA (Figure 1.8), enable the recovery of mycobacteria over periods as short as 5 – 7 days.

Rajaram et al. reported a new molecular pathway that utilises LAM-receptor activation to regulate macrophage inflammatory response by limiting phagosome-lysosome fusion, thus aiding in TB pathogenesis (Rajaram et al., 2010). Rapid point-of-care (POC) assays for TB based on the detection of LAM in urine have been developed. The lateral flow (LF)-LAM assay detects LAM antigen in urine which is released from the mycobacterial cell walls of metabolically active or degenerating mycobacterial cells. LAM antigen appears to be present in patients with active TB disease. LF-LAM assay is reported to have improved sensitivity in TB-HIV co-infected individuals (WHO Global TB Report, 2017). Using a urine-based POC assay for TB detection has several advantages over sputum as urine specimens are easier to collect and have limited infection control risk when compared to sputum collection and testing. WHO recommends the use of LF-LAM for the diagnosis of TB in HIV-positive adults (WHO Global TB Report, 2017; WHO Compendium, 2017; WHO Global TB Program - LF-LAM, 2015).

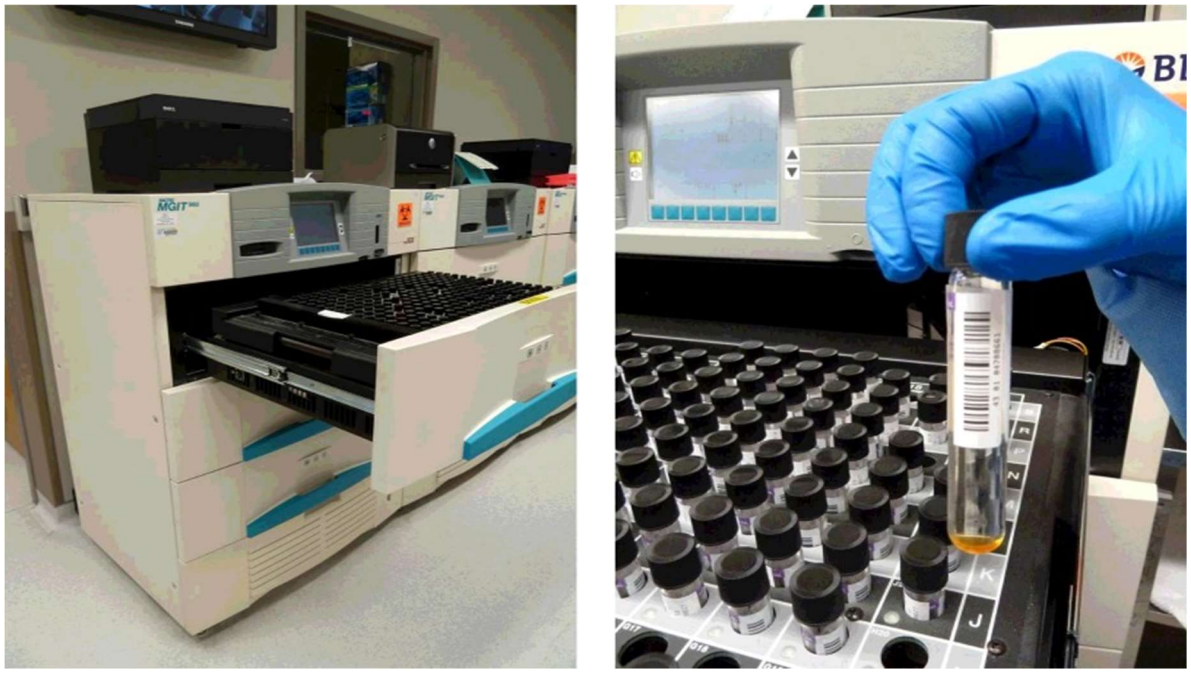


Figure 1.8. BACTEC MGIT™ 960 instrument (Becton Dickinson) & culture tubes - broth-based mycobacterial growth ((Siddiqi & Ruesch-Gerdes, 2007; Becton Dickinson 2016)

1.11.1 Limits of Detection – microscopy, culture & molecular (LPA/GXP)

The limit of detection is defined as the smallest quantity or lowest concentration of an analyte / component that can be reliably detected by an assay under standard stated conditions (Shrivastava & Gupta, 2011).

- The limit of detection for any microscopic diagnostic method is between 10^4 - 10^6 bacilli /ml of sputum sample (Babady & Wengenack, 2012).
- Culture of sputum samples is more sensitive than the microscopic examination method. Conventional TB culture detects 10 - 100 viable bacilli /ml of sputum (Caulfield & Wengenack, 2016).
- Xpert MTB/RIF® has an established limit of detection of 131 colony forming units/ml (CFU/ml) (Blakemore et al., 2010)
- The GenoType MTBDRplus VER 2.0 and GenoType MTBDR*sl* VER2.0 line probe assays have reported limits of detection for clinical samples of 150 - 160 bacteria/ml and 1.6 x 10^4 - 10^5 bacteria/ml for culture isolates (Hain Lifescience, 2015).

The availability of new diagnostic tools and laboratories with the capacity to provide LED microscopy, culture, phenotypic and genotypic DST and rapid molecular testing is paramount to effectively control the transmission of TB, especially MDR and XDR TB (WHO Global TB Report, 2016; Cadmus, Tayeb & van Soolingen, 2012).

1.12 Biosafety

Laboratory biosafety is dependent on the level of risk laboratory procedures generate. Infection from *M. tuberculosis* occurs when infectious aerosols are inhaled. Thus the most significant biohazard risk within a TB laboratory is the production of infectious aerosols during testing procedures (WHO TB Biosafety, 2012). Appropriate risk assessment for all processes involving sample and culture manipulation with particular care where the operation involves aerosol generation should be conducted (Babady & Wengenack, 2012; WHO TB Biosafety, 2012).

The WHO Safety Expert Committee 2012 recommendations for a laboratory conducting manipulation of TB isolates for procedures such as culture, identification, phenotypic DST and LPA is “high risk” (TB-containment) (WHO TB Biosafety, 2012). Where laboratories test isolates that are MDR-TB, XDR-TB or, with the advent of strains that are resistant to all anti-TB drugs currently in use, totally drug resistant (TDR) TB strains, it is recommended that these laboratories should function according to the safety specifications of a TB-containment Biosafety Level 3 laboratory (WHO TB Biosafety, 2012).

1.13 Timeline of anti-TB chemotherapy

Since the discovery of the first anti-TB drug, streptomycin (STRP) in 1943, 70 years later, there are more reported TB cases globally than ever before with an increasing number of cases of DR-TB. The WHO 2017 Global TB Report estimated 600 000 new

RR-TB cases globally in 2016, of which 490 000 were estimated to be MDR-TB cases that were reported globally (WHO Global TB Report, 2017).

As research and history have shown, *M. tuberculosis* is a resilient and adaptable organism. When the combination of anti-TB drugs is not optimal or the treatment is incorrect or incomplete the risk of drug resistance developing is amplified. As can be inferred from Table 1.2, which shows a timeline of the development of anti-TB drugs, the global challenge of DR-TB persists and is far from being conquered.

Table 1.3: Timeline of Anti-TB Drug Discovery and Resistance Indication
(Dyer, 2010; Boire, NA et al., 2013; WHO Global TB Report, 2016)

Year of Discovery	Event
1943 Streptomycin (STRP)	1944 - First patient successfully treated with streptomycin. Resistance first documented in 1948 by British Medical Research Council (BMRC)
1948 para-aminosalicylic acid (PAS)	STRP & PAS administered as combined therapy
1951 Isoniazid (INH)	1951 -1953 BMRC clinical trials – introduced combined therapy INH +STRP +/- PAS
1952 - 1956 pyrazinamide (PZA) cycloserine ethionamide (ETH)	First used in 1957, revised in 1959 as "secondary" treatment with PAS & PZA for INH resistant TB
1957 rifampicin (RIF)	1963 - Introduced as short course therapy
1962 ethambutol (EMB)	Introduced into combined oral TB therapy
1969 clofazimine (CFZ)	CFZ – Primarily used for leprosy, used for DR-TB in 1970's
1970s ↓ 1990s	MDR-TB first described globally Directly Observed Therapy Short-course (DOTS) launched by WHO
1960s – 2000 quinolones/fluoroquinolones	Introduced as anti-TB therapy in 1990s after emergence of MDR-TB
1995 – 2002 FDA approval linezolid (LNZ)	2008 - LNZ used to treat MDR-TB
1997 - 2004 → 2013 Bedaquiline (BDQ)	2004 – First new anti-TB drug in 40 years announced 2013 - WHO approval to use BDQ for treatment of MDR-TB
2011 Delamanid (DEL)	2014 - WHO approval to use DEL for treatment of MDR-TB in adults

1.14 Treatment of TB

1.14.1 Treatment of drug susceptible TB

The recommended NDOH SA treatment guidelines for susceptible TB disease in adults and children >8 years of age include first-line anti-TB drugs (INH, RIF, PZA, EMB) in an intensive Phase of 2 months. This is followed by a continuation Phase of 4 months with INH and RIF for patients who had converted to smear negative status (NDOH SA, 2014).

Combination therapy is necessary for the treatment of all forms of TB disease since both drug susceptible (DS) and DR populations are known to co-exist (Kaplan et al., 2003). Treatment with a single anti-TB drug leads to the selection of DR-populations resulting in treatment failure and poor patient outcome (Zhang & Yew, 2009). The diagnosis and prompt treatment of DS-TB is therefore essential to the management and control of TB disease and the prevention of the development of all forms of DR-TB.

1.14.2 Treatment of drug-resistant tuberculosis

The treatment of confirmed cases of DR-TB is more expensive than treatment of DS-TB with first-line anti-TB drugs. Furthermore, the toxic side effects of second-line drugs are more severe. The WHO recommended second-line anti-TB drugs for treatment of DR-TB are FLQ and SLID (AMK, KAN and CAP) (WHO Global TB Report, 2017; WHO Compendium, 2017). A shorter MDR-TB treatment regimen of 7 drugs with a treatment duration of 9 -12 months was recommended by WHO in May 2016. The regimen composition is KAN, moxifloxacin (MXF), prothionamide (Pto), clofazimine (CFZ), pyrazinamide (PZA), high-dose isoniazid (INH) and EMB for 4 – 6 months, for the intensive phase and MXF, CFZ, PZA and ETH (ethionamide) for a further 5 months, for the continuation phase (WHO Guidelines, 2016; WHO Global TB Report, 2017; WHO Compendium, 2017). Individualised treatment regimens for patients not showing progress with the shorter regimen is recommended by the WHO (WHO Guidelines, 2016; WHO Global TB Report, 2017; WHO Compendium, 2017).

1.14.3 Drug resistance

The phenomenon of drug resistance for *M. tuberculosis* was first reported by the British Medical Research Council (BMRC) in 1944 and involved resistance to streptomycin (Boire, NA et al., 2013). The genetic basis for resistance in mycobacteria occurs as a result of selection of spontaneous chromosomal mutations encoding resistance at a frequency of 1 in $10^6 – 10^8$ replications. The chances of resistance emerging are magnified due to increased selective pressure when inappropriate drug regimens are used and there is poor compliance with treatment by patients (Zhang & Yew, 2009).

Drug resistance can be divided into 'primary' and 'acquired' categories. The status of a drug-resistant strain that is isolated from a patient who has not received treatment with anti-TB drugs is defined as primary drug resistance. Acquired resistance occurs when a patient is infected with a drug-susceptible strain which during treatment develops resistance to the administered drug due to selection of a resistant mutant. The development of drug resistance is illustrated in Figure 1.9 (Zhang & Yew, 2009).

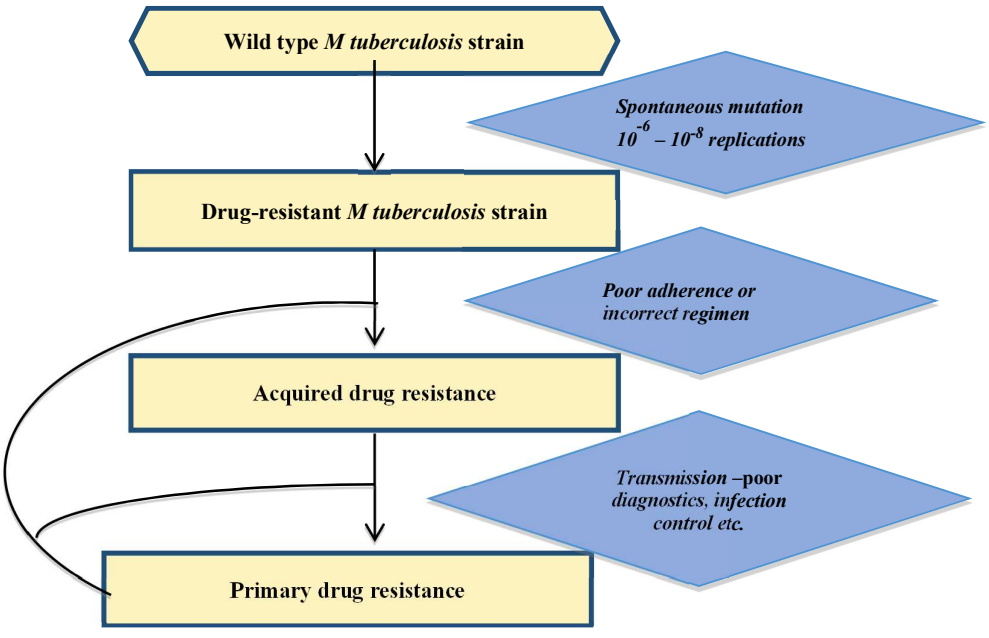


Figure 1.9 Development of drug resistance in *M. tuberculosis* (modified) (Zhang & Yew, 2009)

The antibiotic selective pressure is enhanced by inadequate or incorrect treatment and / or poor patient compliance to treatment (Zhang & Yew, 2009). In a setting where there is a high prevalence of DR-TB, the risk of transmission of DR-TB strains is amplified. The directly observed treatment (DOTS) program was introduced in the 1990s by the WHO to facilitate better management of patient compliance to treatment and is viewed as a "highly efficient and cost effective strategy" (WHO Global TB Report, 2012).

1.14.4 Fitness cost

Mutations encoding drug resistance are associated with a fitness cost in the absence of antibiotic pressure. When a mutation encoding drug resistance occurs it is likely that the mutation would affect the fitness of the resistant clone, resulting in a strain that may be less virulent or affect the organism's capacity for continued survival, replication and transmission. This is known as 'fitness cost'. Studies have reported that some drug-resistant *M. tuberculosis* strains have the ability to compensate their 'fitness burden' and exhibit fitness comparable to wild-type strains. Compensatory mutations are known to curtail the effects of fitness cost associated with drug-resistance (Vos et al., 2013).

Studies conducted by Vos et al., (2013) have shown that rifampicin resistance (RR) is associated with a fitness cost. These reported compensatory mutations in *rpo*C region which alleviate the fitness cost in *M. tuberculosis* strains as a result of rifampicin resistance-conferring mutations in the *rpoB* region were described amongst RR-isolates in a high burden setting in South Africa (Vos et al., 2013). These *rpo*C mutations in the RR-TB isolates were 'strongly associated with transmission of RR-strains' (Vos et al., 2013).

1.14.5 Extremely drug-resistant tuberculosis strains in South Africa

The outbreaks of XDR-TB in KwaZulu-Natal (KZN) highlighted the adverse impact drug-resistant TB can have on patient mortality, especially in areas where high rates of TB/HIV co-infection are prevalent. Genotyping of these XDR isolates showed that >85% were from the KZN-family of *M. tuberculosis* strains and that

transmission of these XDR strains most probably occurred between individual patients (Gandhi et al., 2006; Ioerger et al., 2009).

1.15 Evaluation of new diagnostic assays

Integral to implementing a new or redesigned assay is the process of validation and verification. Validation is defined as “confirmation through objective evidence that the requirements for a specific intended use have been fulfilled”, i.e. capability of the procedure or application to consistently deliver the required quality and intended results (ISO 15189:2012, 2015). A verification process “shall confirm through objective evidence that the performance claims of the examination procedure or application have been met”, i.e. procedure or application is in reality consistently achieving the required results and quality (ISO 15189:2012, 2015).

The Grading of Recommendations Assessment, Development and Evaluation (GRADE) system is the WHO process used for evaluating new assays and technologies for recommendation as illustrated in figure 1.10 (WHO Global TB Report, 2015).

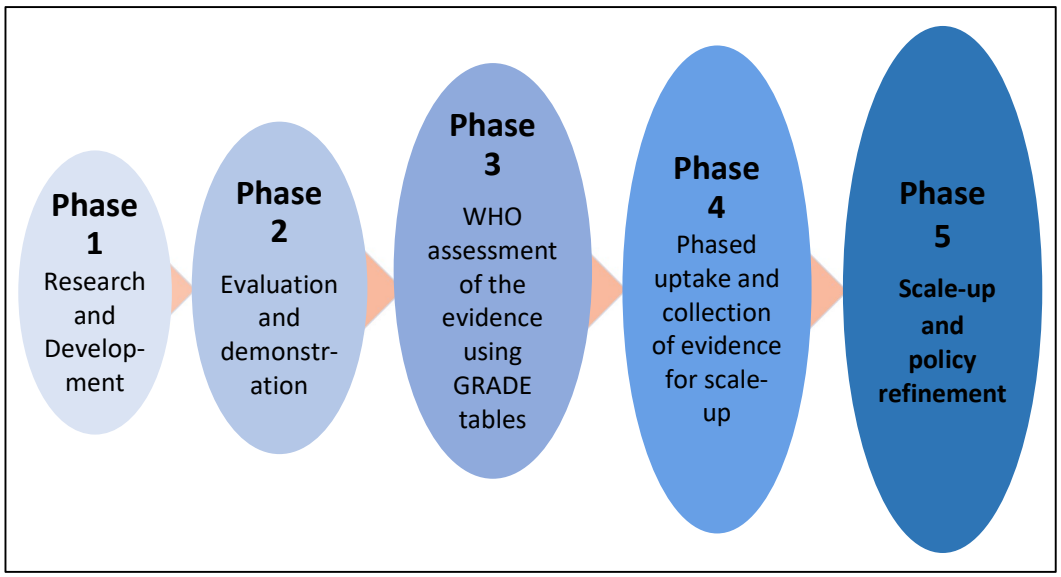


Figure 1.10 The phases of TB diagnostics development and assessment for WHO recommendation using the GRADE (Grading of Recommendations Assessment, Development and Evaluation) process (WHO Global TB Report, 2015)

The GRADE system using guiding 'PICO' questions proposed and agreed to by the WHO GDG in 2015, was applied to determine the quality of data and information provided on the efficacy and recommendations for the GenoType® MTBDRsl VER 2.0 from independent evaluations conducted (WHO Policy Guidance - molecular line probe assays, 2016).

The WHO acronym for 'PICO" refers to four components within the questions used for the assessment of the evidence obtained from evaluation studies. These are:

- **P** indicating the population or intervention/test(s) of interest (when applied to systematic reviews of diagnostic test accuracy);
- **I**ntervention / index test (the new test of interest whose performance is being evaluated);
- **C**omparator / Reference test(s) against which the index test is compared;
- **O**utcome – the primary outcome of interest obtained from a systematic review of test accuracy is the data for contingency tables, usually relating to sensitivity and specificity. (WHO Policy Guidance - molecular line probe assays, 2016; Bossuyt et al., 2015)

An assessment of the performance of the newly developed GenoType® MTBDRsl VER 2.0 LPA (Hain Lifescience, Germany) to detect second-line drug resistance in *M. tuberculosis* clinical samples and isolates would be a first step towards possible incorporation, and offer significant improvement to the TB diagnostic algorithm for South Africa's TB Control Program. Implementation of the assay will potentially reduce transmission of DR-TB as well as allow for appropriate intervention, quality of care and treatment outcomes.

1.16 Study Aim and Objectives

1.16.1 Aim

Evaluation of the performance of the GenoType® MTBDR*sl* VER 2.0 LPA for the detection of resistance to second-line *M. tuberculosis* drugs in clinical samples and culture isolates.

1.16.2 Study Objectives

a. To determine the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of GenoType MTBDRsl VER 2.0 LPA
b. To compare GenoType MTBDR*sl* VER 2.0 LPA with sequencing results on characterised *M. tuberculosis* strains
c. To evaluate the performance of GenoType MTBDR*sl* VER 2.0 LPA on clinical samples (direct sputum DNA testing)
d. To compare the GenoType MTBDR*sl* VER 2.0 LPA with the phenotypic BACTEC MGIT™ 960 using standardized second-line DST

CHAPTER 2

2 MATERIAL AND METHODS

2.1 Study Design

The technical aspects of this study were conducted as a laboratory-based evaluation of the GenoType® MTBDRsl VER 2.0 LPA (index test) in comparison with the Bactec MGIT™ 960 phenotypic DST method (Gold standard).

2.2 Study Site

The evaluation of the GenoType® MTBDRsl VER 2.0 was conducted at the Centre for Tuberculosis, National Institute for Communicable Diseases, National Health Laboratory Service (CTB, NICD/ NHLS), Johannesburg, South Africa. The laboratory is a designated WHO Supranational Laboratory (SRL) accredited to ISO 15189:2012 (2015).

2.3 Overview of the process

The study was structured with three inter-related components involving:
Phase 1: Validation of the assay using characterised *M. tuberculosis* complex isolates, including their drug resistance profiles, at the primary site, Centre for Tuberculosis (CTB),
Phase 2: Verification and reproducibility assessment using a subset of these isolates tested by selected TB culture laboratories in South Africa, as secondary sites,
Phase 3: A prospective evaluation of the GenoType® MTBDRsl VER 2.0 LPA on residual Genolyse® DNA extracts of acid fast bacilli (AFB) present in smear-positive sputum sediments with known rifampicin resistance (RR) or MDR status and the corresponding culture isolates obtained from CTB and selected routine TB laboratories for this purpose (Appendix D).

The objectives for phases 1, 2 and 3 were accomplished by:

- Collecting well characterized *M. tuberculosis* strains with 1) good representation of phenotypic resistance and 2) well distributed genetic mutations encoding resistance to all test-related second-line anti-TB agents,
- Proficiency testing panel strain selection, sub-culturing, distribution, result analysis and evaluation of participant TB laboratories,
- Performing the GenoType® MTBDRsl VER 2.0 LPA on all isolates and comparing the findings with results obtained from the established phenotypic second-line DST method using the Bactec MGIT™ 960 (Gold standard).
- Performing prospective testing on residual DNA from smear-positive decontaminated sputum samples with known RR profiles, detected by the Xpert® MTB/ RIF assay or MDR status, tested with Genotype® MTBDRplus VER 2.0. These results were compared with second-line LPA and phenotypic DST results that were available.
- Performing susceptibility testing on standardised Becton Dickinson® (BD) second-line DST lyophilised drug kit using WHO critical concentrations. Comparison of these findings with the results obtained from in-house drug preparations for second-line phenotypic DST and the Genotype® MTBDR*sl* VER 2.0 LPA.
- Discordant results - Analysis of discordant results will be resolved in conjunction with results obtained from routine testing for genotyping and whole genome sequencing performed at the CTB, NICD.

The following study processes were conducted by the student:

- Selection, sub-culturing, confirmation of purity and identification of *M. tuberculosis* isolates (characterised reference strains and laboratory isolates),
- Proficiency testing panel strain selection and design, distribution, result analysis and evaluation of participant TB laboratories,
- The GenoType® MTBDRsl VER 2.0 LPA at CTB on all isolates and sediments selected for the study,
- Collation and data analysis of all results from selected TB culture laboratories and CTB

Phenotypic DST, WGS and genotyping data was sourced from the routine testing and research activities on routine and surveillance samples tested at the CTB.

The workflow of the phases of the study is illustrated in figures 2.1a and 2.1b.

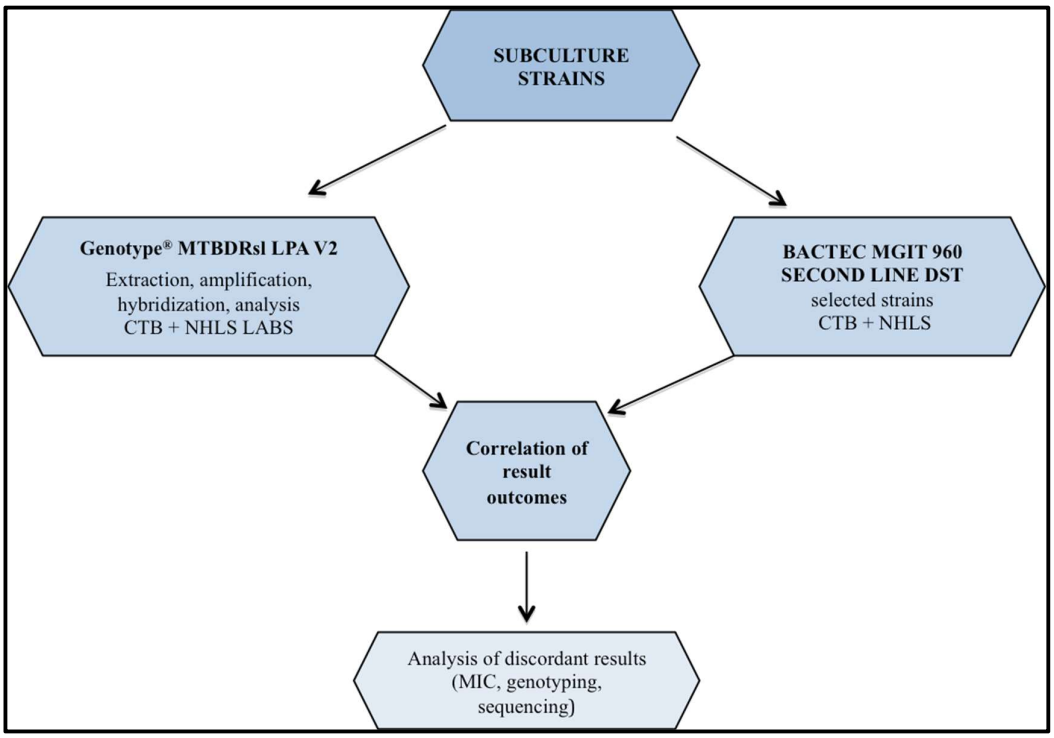


Figure 2.1a: Phase 1 & 2: Validation & Verification of characterised MTBC strains

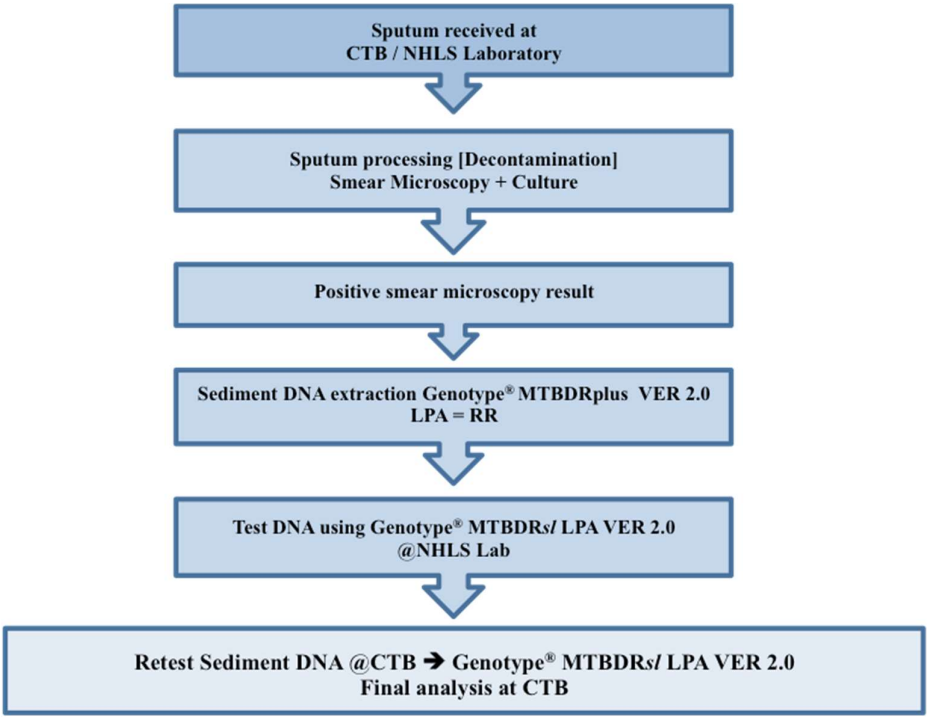


Figure 2.1b: Phase 3: Prospective Validation on Sputum Sediments

2.4 Inclusion Criteria

- Well-characterized *M. tuberculosis* strains from the CTB culture collection tested against all second-line anti-TB agents.
- Direct sputum sediment DNA with known RR or MDR status.

2.5 Exclusion Criteria

M. tuberculosis strains and sputum sediments susceptible to all first-line anti-TB agents.

2.6 Ethics Approval

This laboratory based diagnostic evaluation used stored *M. tuberculosis* complex culture isolates and residual sputum sediments, following the sputum decontamination-concentration procedure, without any patient identifiers on the stored isolates or DNA samples used for the assays. Unique alphanumeric identifiers from the NHLS laboratory information system (LIS) were used to identify samples. The University of Witwatersrand Human Subjects Research Ethics Committee (MEDICAL) approved the study protocol. Ethics clearance certificate number M150752 (Appendix A), approved on 17/08/2015 is valid for 5 years.

2.7 Biosafety Precautions and Laboratory set-up

The CTB laboratory, a high-risk TB laboratory, is a Biosafety Level 3 (BSL-3) facility with Biological Safety Cabinets (BSCs), negative air pressure and a ventilation system appropriate for working with mycobacteria, according to the WHO Laboratory Safety recommendations (WHO TB Biosafety, 2012). As illustrated in figure 2.2, the segregated laboratory structure has designated sections for the decontamination and culture of sputum samples and /or manipulation of *M. tuberculosis* complex isolates and a separate molecular laboratory section (Barnard et al., 2012). All laboratory personnel are provided with personal protective equipment (PPE) such as the appropriate respirators, gloves and gowns as per the NICD / NHLS Safety protocols.

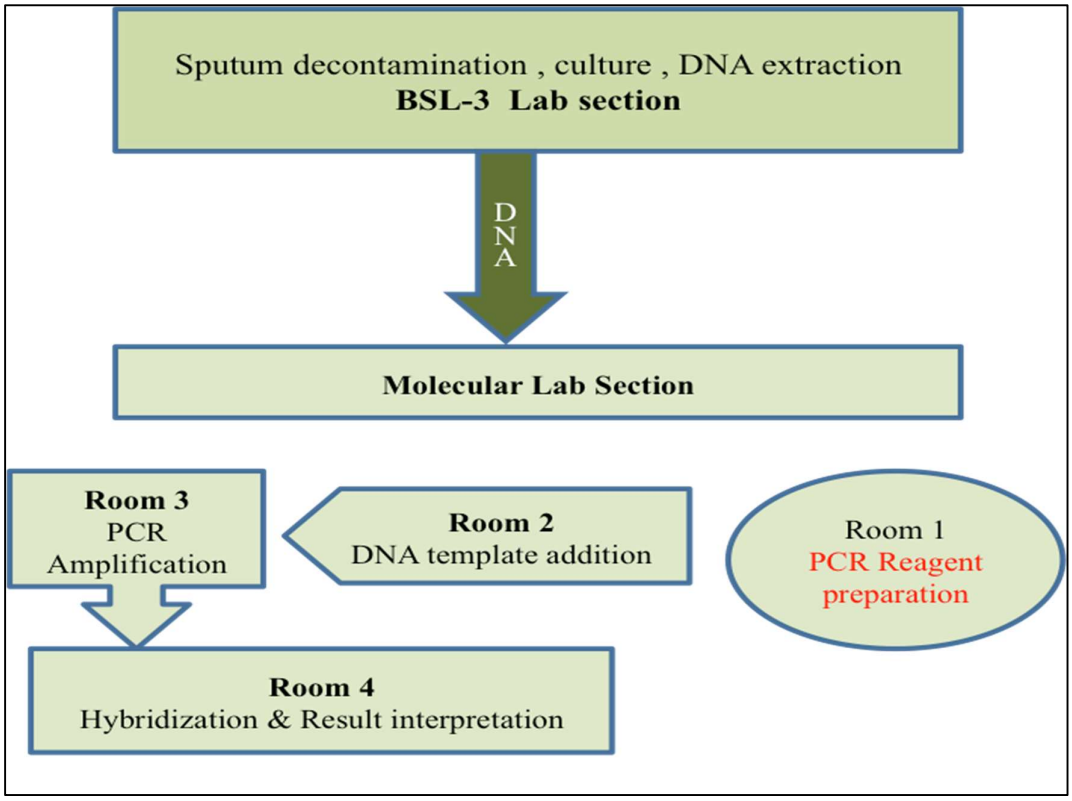


Figure 2.2: Segregated Laboratory Structure

2.8 Sputum decontamination, Microscopy and Culture

Demonstration of the presence of AFB in a sputum smear offers a presumptive diagnosis of mycobacterial disease and the culture of *M. tuberculosis* complex or other non-tuberculous mycobacteria (NTM) is a definitive diagnosis of tubercular or mycobacterial disease. The recommended BACTEC MGIT™ 960 decontamination procedure with a final concentration of 1% *N*-Acetyl-L-Cysteine-sodium-hydroxide (NALC-NaOH) was used by all study sites (Siddiqi & Ruesch-Gerdes, 2007). The concentrated sputum sediment was re-suspended in 2ml phosphate buffer (pH 6.8) and was used to make smears for AFB microscopy, MGIT medium inoculation for TB culture and DNA extraction for LPA (Siddiqi & Ruesch-Gerdes, 2007).

2.9 Sub-culturing of MGIT *M. tuberculosis* complex culture isolates

M. tuberculosis complex isolates retrieved from the CTB sample repository were freshly sub-cultured into Bactec™ MGIT media (modified Middlebrook 7H9 broth). 0.8 ml MGIT OADC (Oleic acid, Albumin, Dextrose and Catalase) enrichment broth and 0.1ml aliquot of repository isolate or one (1) storage micro-bead coated with *M. tuberculosis* complex was aseptically added to each of the uniquely labelled MGIT culture tubes. All inoculated MGIT tubes were mixed by gently inverting the tubes. Aerosols were allowed to settle for 15 -20 minutes prior to incubation in a BACTEC MGIT™ 960 instrument. Each uniquely bar-coded tube was scanned into a BACTEC MGIT™ 960 instrument, where incubation was maintained at a constant 37ºC ± 1ºC, as per manufacturer's recommendations. The standard time-to-detection (TTD) for *M. tuberculosis* complex is 6 - 10 days (Siddiqi & Ruesch-Gerdes, 2007).

2.10 Identification of MGIT Culture Isolates

All positive *M. tuberculosis* complex culture isolates were checked for purity. A drop of MGIT culture was aseptically streaked on blood agar plates, incubated at 37ºC ± 1ºC for 48 - 72 hours and observed for growth of contaminating organisms or non-tuberculous mycobacteria (NTMs) (Siddiqi & Ruesch-Gerdes, 2007).

The BD MGIT TBc identification test (TBc ID) (Becton Dickinson, Sparks, USA) was used to identify all positive cultures. This is an immuno-chromatography based rapid diagnostic assay that tests for the presence of MPT64 antigen. MPT64, a mycobacterial protein is secreted by *M. tuberculosis* complex isolates and is used to differentiate *M. tuberculosis* complex from NTMs (Becton Dickinson, 2015; Said et al., 2011). The test was performed according to manufacturer's instructions, with a positive test determined by the colour development of the control and lines within 15 minutes (Becton Dickinson, 2015).

2.11 Quality Control

American Type Culture Collection (ATCC®) *M. tuberculosis,* H37Rv ATCC 27294 (ATCC, 2006) is recommended as quality control (QC) organism (Siddiqi & Ruesch-Gerdes, 2007). The reference strain was used for this purpose in the study for both LPA and phenotypic DST testing. The Growth Control (GC) MGIT tube without any anti-TB drug added was used to represent a negative resistance / susceptibility QC for phenotypic DST (Siddiqi & Ruesch-Gerdes, 2007). Molecular biology grade water was used as negative QC for LPA (Hain Lifescience, 2015). *M. kansasii,* ATCC 35775 (ATCC, 2016), NTM isolate was included as a QC strain to serve as confirmation of analytical specificity of strains not detectable by the GenoType® MTBDRsl VER 2.0 assay (Hain Lifescience, 2015). All control cultures/reagents were processed according to their respective LPA and phenotypic DST protocols (Siddiqi & Ruesch-Gerdes, 2007; Hain Lifescience, 2015; ISO 15189:2012, 2012).

2.12 Line Probe Assay

The GenoType® MTBDRsl VER 2.0, a qualitative assay based on DNA-STRIP technology which identifies *M. tuberculosis* complex as well as resistance to FLQs and SLID (KAN, AMK, CAP) was performed according to manufacturer's instructions (Hain Lifescience, 2015). DNA extraction, PCR amplification and reverse hybridisation of amplicons are integral processes in LPA methodology. All reagents required for LPA were supplied in the GenoType® MTBDRsl VER 2.0 kit. To avoid cross-contamination / carry-over contamination during processing which may have resulted in erroneous results, aseptic technique was followed throughout the LPA procedures.

2.12.1 DNA Extraction

All residual AFB smear positive sputum sediments and *M. tuberculosis* complex cultures used in the study, were processed in the designated BSL-3 DNA extraction laboratory. DNA extraction from decontaminated smear positive sediments (direct testing) and *M. tuberculosis* culture isolates from solid or liquid media (indirect testing) was done using Genolyse® kit (Hain Lifescience, 2015). Only extracted DNA was processed in the molecular sections of the laboratory.

The Genolyse® Extraction Procedure using Genolyse reagents, Lysis Buffer (A-LYS) and Neutralization Buffer (A-NB) (Hain Lifescience, 2015) was used for all DNA extraction for the study (figure 2.3). The samples used; 500 µl of decontaminated sputum sediment or 1000 µl of liquid culture was transferred into a labelled 1.5 ml O-ring screw cap tube and centrifuged for 15 minutes at 10 000 x g. The supernatant was aspirated using a pipette with a filtered tip and discarded in a biohazard waste container. The concentrated pellet was re-suspended in 100 µl A-LYS. The chemical lysis process breaks down the bacterial cells to release the DNA into solution. All samples were incubated for 5 min at 95°C in a heating block for heat inactivation of the bacteria. 100 µl A-NB was added to lysate and the samples were vortexed or mixed for a few seconds. The A-NB stops the lysis reaction and prevents further degradation of the extracted DNA. The final lysate was separated by centrifugation for 5 minutes at full speed into extracted DNA, which is in solution in the supernatant, and cellular debris in the pellet. A volume of 150 µl DNA solution was transferred into a uniquely labelled 1.5 ml O-ring screw cap tubes for extended storage at -20ºC for further testing.

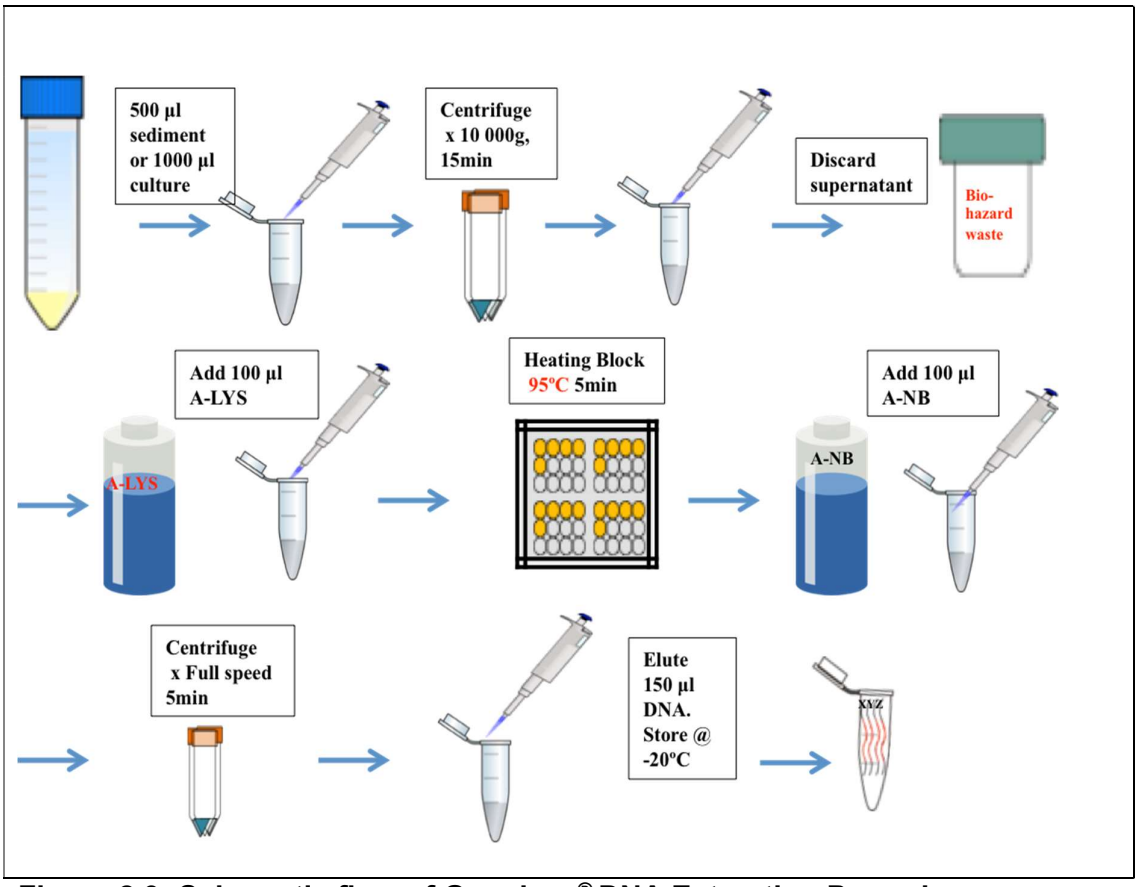
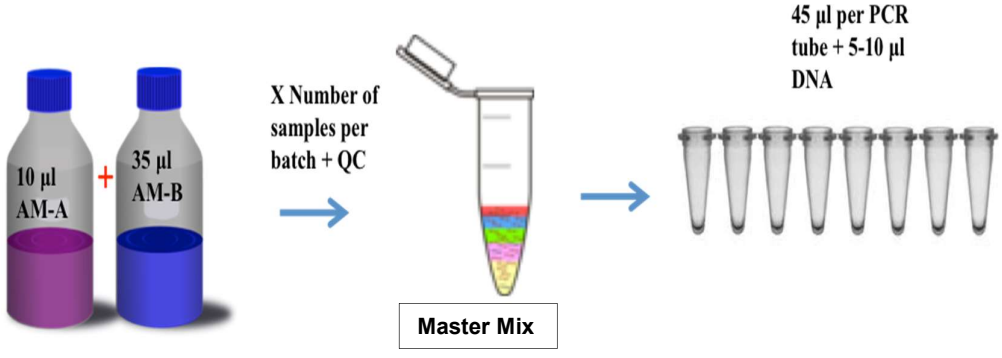


Figure 2.3: Schematic flow of Genolyse® DNA Extraction Procedure

2.12.2 Master Mix Preparation and Amplification

All reagents required for PCR are provided in the GenoType® MTBDRsl VER 2.0 kit as Amplification Mixes A and B (AM-A, AM-B) and stored optimally at -20ºC until required. The Master Mix was prepared as per manufacturer's instructions, by adding into a labelled tube 10µl AM-A to 35µl AM-B with a final volume of 45 µl per sample. The Master Mix was made in the designated PCR Reagent Preparation room and DNA template addition and amplification was conducted in the laboratory areas as illustrated in figure 2.2.

Figure 2.4: Master Mix + DNA required for PCR



Master Mix required for each sample:

10 µl AM-A + 35 µl AM-B **+** 5 µl – 10 µl DNA template **=** Final volume: 50 - 55 µl

2.12.3 Multiplex PCR Amplification Protocol

The amplification of *M. tuberculosis* complex DNA was performed using a programmed thermal cycler for the various cycles of denaturation, annealing, elongation and extension of the DNA copies. The recommended manufacturer's amplification protocol, using the recommended ramp rate (speed of temperature change between cycles) of ≤2.2ºC/sec and storage of DNA amplicons at -20ºC was used for sediment and culture PCR amplification as follows (Hain Lifescience, 2015):

Sediment DNA

15 minutes at 95˚C (Denaturation)	→	1 cycle
30 seconds at 95˚C (Denaturation)	→	20 cycles
2 minutes at 65˚C (Elongation)		
25 seconds at 95˚C (Denaturation)	→	30 cycles
40 seconds at 50˚C (Annealing)		
40 seconds at 70˚C (Elongation)		
8 minutes at 70˚C (Extension)	→	1 cycle
Heating rate (ramp rate)		≤2.2ºC/sec

Culture Isolate DNA

15 minutes at 95˚C (Denaturation)	→	1 cycle
30 seconds at 95˚C (Denaturation)	→	10 cycles
2 minutes at 65˚C (Elongation)		
25 seconds at 95˚C (Denaturation)		
40 seconds at 50˚C (Annealing)	→	20 cycles
40 seconds at 70˚C (Elongation)		
8 minutes at 70˚C (Extension)	→	1 cycle
Heating rate (ramp rate)		≤2.2ºC/sec

2.12.4 Hybridisation of PCR amplicons

Reverse hybridisation of DNA amplicons to specific complementary probes pre-hybridised on a nitrocellulose membrane strip was performed using the GT-Fast program on the automated Hain GT Blot® system as per recommended protocol (Hain Lifescience, 2015).

Reagents required for hybridisation are denaturing buffer (DEN), hybridization buffer (HYB), stringent wash solution (STR), rinse solution (RIN), conjugate concentrate (CON) and substrate concentrate (SUB). Single stranded DNA is required for the reverse hybridisation process; 20 µl of double stranded amplicon DNA was denatured by the addition of 20 µl of denaturing buffer (DEN) into each sample well of the hybridization tray (maximum 48 samples) and incubated at room temperature for 5 minutes. A 1:100 dilution working solution for CON and SUB was freshly prepared and placed into the appropriate colour-coded position on the instrument. The hybridization tray was loaded onto the GT Blot® instrument and 1 ml hybridization buffer was dispensed by the GT Blot® into each well. Nitrocellulose strips were labelled with the correct sample number and added to the corresponding sample well on the instrument when the GT Blot® indicated “ADD AMPLICON”.

The GT Blot® automatically dispensed and aspirated all reagents according to the program settings until the completion of the hybridization process.

2.12.5 Interpretation of Line Probe Assay Strips

Analysis and interpretation of the developed hybridised strips (figure 2.5) was performed manually and by using the automated Genoscan® with the Blotrix software program (Barnard, et al., 2012; Hain Lifescience, 2015).

The presence of all wild type bands and absence of mutation bands indicated susceptibility. The development of specific mutation bands (defined mutation) or the absence of wild-type bands (undefined mutation) (Daum et al., 2014) related to a specific gene on the hybridization strip was interpreted as resistance to the respective drug (Hain Lifescience, 2015). Heteroresistance was indicated by the presence of both wild type and mutation band(s) and was regarded as resistant for interpretation purposes. The specific complementary probes used in the design of the strips are shown in Table 2.1. Each strip contained 27 reaction zones with probes for all specific targeted regions. Seven probes for *gyr*A (A90V, S91P, D94A, D94N/Y, D94G, and D94H) and 2 probes for *gyr*B (N538D, E540V) were used to detect FLQ resistance. SLID resistance was detected by selected *rrs* (A1401G, C1402T and G1484T) and *eis* (C-14T and C-12T) probes (Hain Lifescience, 2015).

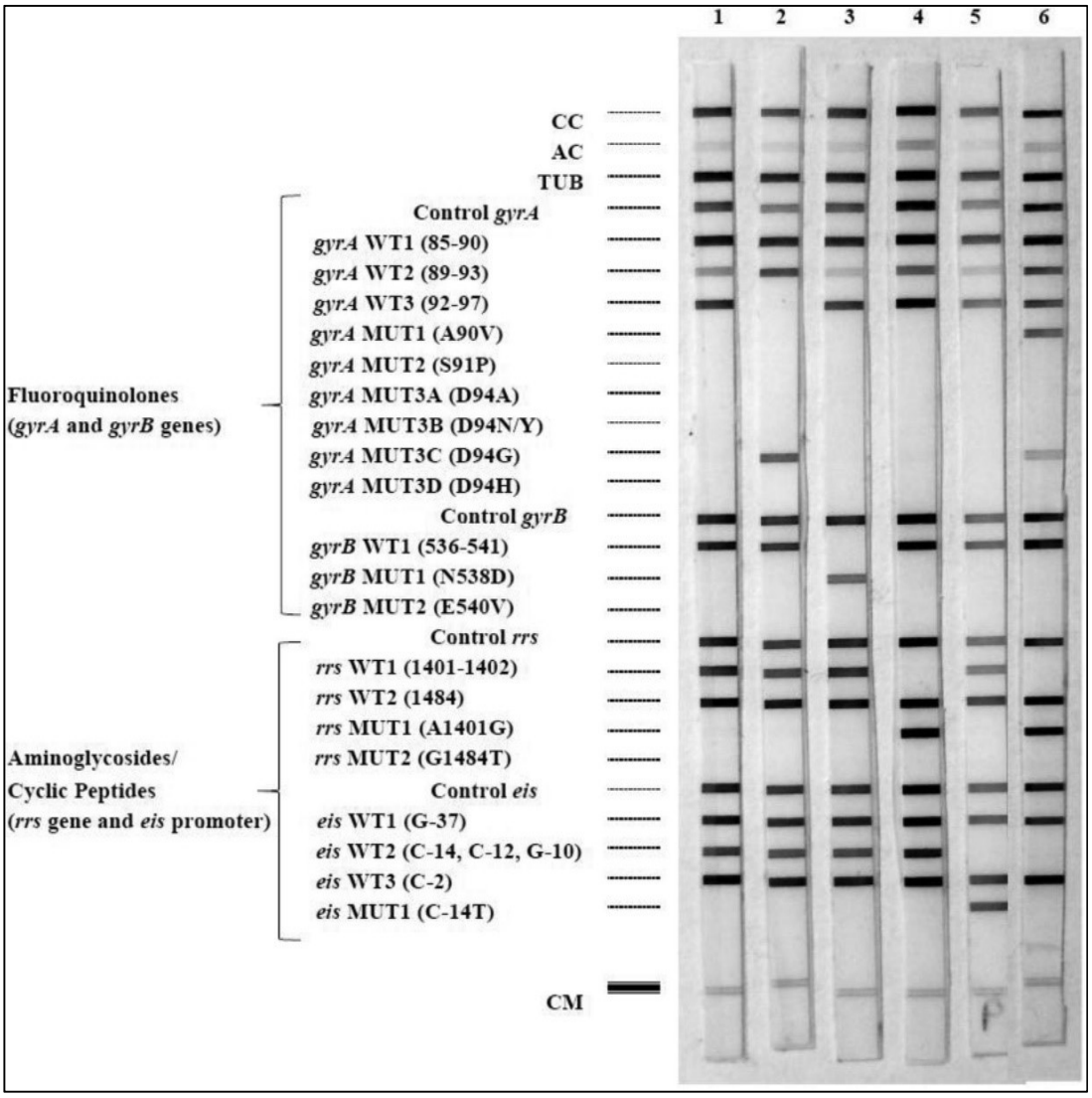


Figure 2.5: Genotype® MTBDRsl VER 2.0 Strips (Brossier et al. 2016)

Table 2.1: Genotype® MTBDRsl VER 2.0 kit - Mutations in genes & corresponding wild type & mutation bands (Hain Lifescience, 2015)

PHENOTYPIC RESISTANCE	WILD TYPE (WT)	CODONS ANALYSED	MUTATION BAND	MUTATION
FLQ	*gyr*A WT1	85 – 90	-	G88A G88C
	*gyr*A WT2	89 – 93	*gyr*A MUT1 *gyr*A MUT2	A90V S91P
	*gyr*A WT3	92 - 97	*gyr*A MUT3A *gyr*A MUT3B *gyr*A MUT3C *gyr*A MUT3D	D94A D94N / D94Y D94G D94H *
	*gyr*B WT	536 - 541	*gyr*B MUT1 *gyr*B MUT2	N538D E540V **(N538K N538S N538T T539N T539IT539P E540D – in β-Version only)
CAP AMK KAN CAP VIO*** KAN CAP VIO AMK KAN	*rrs* WT1	1401	*rrs* MUT1	A1401G
		1402	-	C1402T
	rrs WT2	1484	*rrs* MUT2	G1484T
Low-level KAN	*eis* WT1	-37	-	G-37T
	eis WT2	-14, -12, -10	*eis* MUT1	C-14T C-12T G-10A
	eis WT3	-2	-	C-2A

*(RARE MUTATION)

** Genotype® MTBDRsl β-version kit for evaluation only
***Viomycin

2.13 Phenotypic Drug Susceptibility Testing

The performance of BACTEC MGIT™ 960 second-line DST lyophilised drug kit developed for five anti-TB drugs (Becton Dickinson, USA), as shown in Table 2.2, (WHO Companion handbook, 2014) was compared with the available MGIT DST protocol for second-line drugs prepared by an in-house supplier using WHO recommended critical concentrations (WHO Companion Handbook, 2014).

BACTEC MGIT™ 960 first-line DST procedure is recommended for conducting second-line DST. Confirmed *M. tuberculosis* complex - positive MGIT cultures

were used. DST was performed by direct inoculation of 0.1ml of a Day 1 or Day 2 positive MGIT culture or 0.1ml of 1:5 dilution of 3, 4 or 5 day old positive MGIT culture into a labelled drug-containing MGIT tube (Siddiqi & Ruesch-Gerdes, 2007). According to Siddiqi (2014), preparation of the second-line drug solutions, MGIT Instrument / Epicentre test protocol and result interpretation is specific for second-line drugs as described in the MGIT™ Procedure Manual – FIND (Siddiqi & Ruesch-Gerdes, 2007; Siddiqi, 2014).

Table 2.2: WHO 2013 guidelines for critical concentrations (WHO Companion Handbook, 2014)

ANTIBIOTIC GROUP	ANTI-TB AGENT	WHO CRITICAL CONCENTRATIONS FOR BACTEC MGIT 960 SYSTEM
Fluoroquinolones	Ofloxacin (OFX)	2.0 µg/ml
	Moxifloxacin (MXF)	0.5 µg/ml & 2.0 µg/ml
Aminoglycosides	Amikacin (AMK)	1.0 µg/ml
	Kanamycin (KAN)	2.5 µg/ml
Cyclic Peptides	Capreomycin (CAP)	2.5 µg/ml

2.14 Whole Genome Sequencing

The whole genome sequencing (WGS) protocol was performed using the Nextera reagent kit and MiSeq platform (Illumina, San Diego, CA). The protocol involved the following processes;

i. DNA extraction on the Nuclisens® easyMag (bioMérieux, USA)
ii. 1 Nanogram of sample DNA was used for library preparation with the Illumina Nextera XT® kit and sequencing reaction using the MiSeq® version 3 cartridge (2 x 300bp).
iii. Variant detection was performed using the resequencing module with the reference strain *M. tuberculosis* H37Rv (NC_000962) on CLC Genomics Workbench (v7.5.1).

2.15 Genotyping

Genotyping of MTBC strains was performed using two methods, spacer oligonucleotide typing (spoligotyping) and 24-loci mycobacterial interspersed repetitive units-variable number tandem repeats (MIRU-VNTR).

2.15.1 Spoligotyping

A commercial kit from Ocimum Biosolution (Hyderabad, India) was used to perform spoligotyping according to the manufacturer's recommended procedure (Sharma & Gupta, 2015).

i. DNA extraction from heat inactivated culture isolates was done on the Nuclisens® easyMag (bioMérieux, USA). PCR was performed using the reaction mixture provided in the kit. The recommended amplification protocol was used:

    3 min 96°C
    1 min 96°C ┐
    1 min 55°C ├ }
    30 sec 72°C ┘
    5 min 72°C

ii. Hybridization of the biotin-labelled PCR products onto the membrane provided in the kit with immobilized spacer-oligos that represent spacers of known sequence.

iii. The presence of spacers is visualized on film as black squares after incubation with streptavidin-peroxidase and ECL-detection.

2.15.2 MIRU-VNTR

The 24-loci MIRU-VNTR was performed according to the manufacturer's recommended procedure using the quadruplex version of the GenoScreen MIRU typing kit (Genoscreen, Lille, France, 2012).

i. A 96-well plate was loaded with 8 µl per well of quadruplex mixes supplied as per PCR spreadsheet provided. 2 µl DNA, positive and negative controls were added. The plate was sealed and centrifuged.

ii. PCR was performed according to the recommended amplification protocol:

15 min	95°C	
1 min	94°C	40 cycles
30s	59°C	
1 min 30s	72°C	
10 min	72°C	
∞	4°C	

iii. A 96-well sequencer plate was loaded with 10 µl of formamide-GeneScan™ 1200 LIZ® mix in each well. 2 µl of PCR product was added to the wells according to the PCR spreadsheet. After denaturation at 95°C for 5 min and cooling on ice, the sequencer plate was loaded on the sequencer.

iv. Analysis of spoligotype patterns was done using the binary codes in the SpolDB4 database (http://www.pasteur-guadeloupe.fr/tb/bd_myco.html Pasteur Institute of Guadeloupe). Two or more strains with an identical pattern was defined as a cluster. Analysis of MIRU-VNTR PCR products and assignment of corresponding alleles was performed using GeneMapper software 4.0 (Applied Biosystems,USA) (Said et al. 2016).

2.16 Data Analysis

The results obtained from phases 1, 2 and 3 for LPA and DST testing were analysed by means of contingency tables to determine sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV) and 95% confidence intervals (95% CIs) (Sauerbrei and Blettner, 2009) and were calculated for both the antibiotic class and individual drugs. Agreement between the gold standard and index assay was calculated using Cohen's Kappa (**κ**). The **κ** statistic measures the degree of interrater agreement, a measure of test reliability and was interpreted using the following scale: <0.20 =poor; 0.21 – 0.40 = fair; 0.41 – 0.60 = moderate; 0.61 -0.80 = good; 0.81 -1.0 = very good (Kwiecien et al., 2011).

Statistical analyses were performed using Stata: Release 13 (Stata Press 2013). An overview of the data analysis process is detailed in Appendix B.

CHAPTER 3

3 RESULTS FOR PHASE 1 and 2 [1]

3.1 *"Evaluation of the GenoType® MTBDRsl VER 2.0 assay for second-line drug resistance detection of Mycobacterium tuberculosis isolates in South Africa"*

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This chapter addresses the following objectives of the study as described in chapter 1.16.2:

a. To determine the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) performance indices of the GenoType® MTBDR*sl* VER 2.0 LPA.
b. To compare the GenoType® MTBDR*sl* VER 2.0 LPA with sequencing results on characterised *M. tuberculosis* strains.

[1] Editorial format for Journal of Clinical Microbiology was applied to this published chapter

Full title: Evaluation of the Genotype® MTBDR*sl*® VER 2.0 assay for second-line drug resistance detection of *Mycobacterium tuberculosis* isolates in South Africa

Short title: EVALUATION OF MTBDR*sl* VER 2.0: SOUTH AFRICAN STUDY

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ABSTRACT

Early detection of resistance to second-line anti-tuberculosis drugs is important for the management of multidrug-resistant (MDR)-TB. The Genotype® MTBDR*sl* VERSION 2.0 (VER 2.0) line probe assay has been redesigned for molecular detection of resistance-conferring mutations of fluoroquinolones (FLQ) (*gyr*A and *gyr*B genes) and second-line injectable drugs (SLID) (*rrs* and *eis* genes). The study evaluated the diagnostic performance of MTBDR*sl* VER 2.0 for the detection of second-line drug resistance compared with phenotypic drug susceptibility testing (DST), using the Bactec™ MGIT 960 system on *Mycobacterium tuberculosis* complex isolates from South Africa. A total of 268 repository isolates collected between 2012 and 2014, with rifampicin -mono-resistant (RR) or MDR based on DST were selected. MTBDR*sl* VER 2.0 testing was performed on these isolates and results analysed. The MTBDR*sl* VER 2.0 sensitivity and specificity indices for culture isolates were; FLQ 100% (95% CI, 95.8 - 100%); 98.9% (95% CI, 96.1 - 99.9%) and SLID 89.2% (95% CI, 79.1 - 95.6%); 98.5% (95% CI, 95.7 - 99.7%). The sensitivity and specificity observed for individual SLID were: amikacin 93.8% (95% CI, 79.2 - 99.2%); 98.5% (95% CI, 95.5 - 99.7%), kanamycin 89.2% (95% CI, 79.1 - 95.6%); 98.5% (95% CI, 95.5 - 99.7%) and capreomycin 86.2% (95% CI, 68.3 - 96.1%); 95.9% (95% CI, 92.2 - 98.2%). An inter-operator reproducibility of 100% and an overall inter-laboratory performance of 93 % - 96% were found. The overall improvement in sensitivity and specificity with excellent reproducibility makes the Genotype® MTBDR*sl* VER 2.0 a highly suitable tool for rapid screening of clinical isolates for second-line drug resistance for use in high burden TB/HIV settings.

KEY WORDS: Genotype® MTBDR*sl* VERSION 2.0; Drug Resistant (DR)-TB; MDR-TB; XDR-TB, line probe assay, LPA, fluoroquinolones, second-line injectable drugs, molecular diagnostic testing, phenotypic drug susceptibility testing

INTRODUCTION

The World Health Organization (WHO) Global Tuberculosis Report 2015 notes that approximately 3.3% of new cases of tuberculosis (TB) and 20% of previously treated TB cases in 2014 were multidrug-resistant TB (MDR-TB) defined as resistance to isoniazid (INH) and rifampicin (RIF). South Africa is listed amongst the 14 countries that appear in all three WHO 2016 -2020 revised "high burden country" (HBC) lists (1). South Africa with a high burden of MDR-TB, also experienced an increase in extensively drug resistant TB (XDR-TB), accounting for 59% of XDR-TB patients reported globally in 2011 (1, 2). XDR-TB is defined as MDR-TB with additional resistance to any fluoroquinolone (FLQ) and at least one second-line injectable drug (SLID) amongst the aminoglycoside drugs (AG) amikacin (AMK), kanamycin (KAN) and the cyclic peptide, capreomycin (CAP) (2–5). Drug resistance in *Mycobacterium tuberculosis* isolates occurs at a low frequency due to spontaneous chromosomal mutations (6) which are selected through the improper use of anti-TB agents and low patient compliance with treatment, exerting selective pressure for the emergence of drug resistant mutants (acquired resistance) (7).

In South Africa, despite the emphasis on TB therapy, the HIV epidemic has seriously hampered TB management and severely affected treatment outcomes (8) while the TB-HIV co-epidemic has fuelled the escalation of both MDR-TB and XDR-TB (9). The introduction of new rapid molecular diagnostic tests in South Africa, notably the Xpert® MTB/ RIF assay (Cepheid, USA) and line probe assays (LPAs) for the detection of drug-resistant TB has markedly improved patient management with decreased result turnaround times (TATs) for testing. Culture-based phenotypic drug susceptibility testing (DST), considered to be the gold standard for drug resistance determination, is important for MDR-TB confirmation and the assessment of drug resistance to second-line and new drugs in the management of MDR-TB and XDR-TB (10). However, conventional DST is labour intensive, time consuming and generally takes between 2 - 3 weeks' incubation to provide meaningful treatment directing results. In addition, second-line DST is fraught with challenges due to variability of methodology, reproducibility in performance and reliability of results. Currently, there are no rapid genotypic diagnostic tests in the South African TB diagnostic algorithm for the determination of resistance to second-line anti-TB drugs (10). Molecular based assays designed to detect specific drug resistance-encoding mutations in *M. tuberculosis* have the advantage of achieving faster TATs (within 48 hours) for resistance reporting when compared to conventional DST, and in the process alerting clinicians to the emergence of drug resistance in *M. tuberculosis* strains from individual patients. Early detection

of drug resistance is crucial to prevent the transmission of drug-resistant TB and averting mortality as previously described (9, 11).

A current limitation of molecular assays is that they do not accommodate all mutations conferring resistance to anti-TB agents. A WHO Expert Group determined in 2013 that the Genotype® MTBDR*sl* VER 1.0 (Hain Lifescience, Germany) cannot replace phenotypic DST but it may be used as a rule-in test for XDR-TB (12). In a meta-analysis published in 2014, Theron and colleagues (5) reported respective pooled sensitivity and specificity indices of 83.1% (95% CI, 78.7 - 86.7%) and 97.7% (95% CI, 94.3% - 99.1%) for FLQs and 76.9% (95% CI, 61.1 - 87.6%) and 99.5% (95% CI, 97.1 - 99.9%) for SLID for culture isolates using the Genotype® MTBDR*sl* VER 1.0 assay. They concluded that since the assay only targets selected mutations involving *gyr*A (FLQ) and *rrs* (SLID) gene loci, mutations encoding resistance to FLQ and SLID that occur outside these regions would be missed by the assay (5).

Genotype® MTBDR*sl* VER 2.0 is redesigned based on MTBDR*sl* VER 1.0 and accommodates additional mutations for the molecular detection of resistance to FLQ involving *gyr*A and *gyr*B and SLID resistance covering both *rrs* and *eis* genes (13). The probes target commonly occurring mutations that encode resistance to these agents. The *gyr*A probes target codons 85 to 97 of the gene and *rrs* probes target nucleic acid positions 1401 to 1484. The inclusion of additional targets for selected mutations in *gyr*B region (codons 536 to 541) and *eis* promoter region (-10 to -14) for low-level KAN resistance are reported to improve the performance of the assay for the detection of FLQ and SLID resistance (13).

In order to prioritise and facilitate the identification of pre-XDR-TB (resistance to INH, RIF and either resistance to any FLQ or SLID, but not both) (8) and XDR-TB in South Africa's high-burden setting, the implementation of Genotype® MTBDR*sl* VER 2.0 would be a fundamental improvement to case detection and management. An evaluation of this assay was undertaken with the objective of assessing diagnostic performance as well as to determine inter-operator and inter-laboratory performance of Genotype® MTBDRsl VER 2.0 in the detection of second line drug resistance mutations in *M. tuberculosis* complex culture isolates compared to phenotypic DST.

MATERIALS AND METHODS

Setting and study design. The evaluation of the Genotype® MTBDR*sl* VER 2.0 was conducted at the Centre for Tuberculosis, National Institute for Communicable Diseases (CTB, NICD), Johannesburg, South Africa. The laboratory is a designated WHO Supranational Laboratory (SRL) accredited to ISO 15189:2012 (14). The Human Research Ethics Committee of the University of Witwatersrand, Johannesburg, South Africa approved the study (M150752). The study was structured with two interrelated components involving the validation of characterised *M. tuberculosis* isolates and a reproducibility assessment using a subset of these isolates tested by selected National Health Laboratory Service (NHLS) laboratories in South Africa, performing diagnostic tests involving culture of *M. tuberculosis*.

Mycobacterial isolates. A total of 268 repository isolates collected between 2012 and 2014 were tested. These comprised of 92 phenotypically well-characterized *M. tuberculosis* complex isolates using whole genome sequencing (WGS) and 176 anonymized *M. tuberculosis* clinical isolates exhibiting rifampicin mono-resistance (RIF-R) or MDR based on phenotypic DST, tested at CTB or NHLS TB Referral Laboratory, Braamfontein, Johannesburg. Phenotypic testing for higher generations of FLQ and SLID was introduced in 2015 (2). These isolates originated from Gauteng, Limpopo, Northern Cape, North West, KwaZulu-Natal and Mpumalanga provinces, collected during surveillance activities for rifampicin resistance in South Africa. The fully susceptible ATCC® *M. tuberculosis,* H37Rv 27294 reference strain (15) was used as the DST and LPA positive quality control culture.

Phenotypic DST. Bactec™ MGIT 960 DST using EpiCenter software (Becton, Dickinson and Company Diagnostic Systems, Sparks, MD, USA) for interpretation of results was performed according to manufacturer's recommendations (16) and considered as the gold standard for resistance determination. The following critical concentrations of drugs recommended by WHO for testing of drug-resistant TB using Bactec™ MGIT 960 DST were used: Ofloxacin (OFX) 2.0 µg/ml, AMK 1.0 µg/ml, KAN 2.5 µg/ml and CAP 2.5 µg/ml (17).

Genotype® MTBDR*sl* VER 2.0. The Genotype® MTBDR*sl* VER 2.0 assay was performed according to manufacturer's instructions (13). Each strip contains 27 reaction zones with probes for all specific targeted regions. Seven probes for *gyr*A (A90V, S91P, D94A, D94N/Y, D94G, and D94H) and 2 probes for *gyr*B (N538D, E540V) are used to detect FLQ resistance. SLID resistance is detected by selected *rrs* (A1401G, C1402T and G1484T) and *eis* (C-14T and C-12T)

probes. The presence of all wild type bands and absence of mutation bands indicated susceptibility. The development of specific mutation bands (defined mutation) or the absence of wild-type bands (undefined mutation) (18) related to a specific gene on the hybridization strip was interpreted as resistance to the respective drug (13). Heteroresistance was demonstrated when both wild type and mutation band(s) were present and was recorded as resistant for interpretation purposes. SLID resistance referred to resistance to at least one of the 3 injectable drugs (AMK, KAN and CAP) (5).

Discordance resolution by whole genome sequencing. WGS was used to resolve discordance between phenotypic and genotypic methods. WGS was performed using the MiSeq® platform (Illumina®, San Diego, USA). Library preparation was performed using the Illumina Nextera XT® library preparation kit and sequencing reaction using the MiSeq® version 3 cartridge (2 x 300bp). Variant detection was performed using the resequencing module with the reference strain *M. tuberculosis* H37Rv (NC_000962) on CLC Genomics Workbench (v7.5.1).

Reproducibility. In order to evaluate the inter-laboratory performance of the assay, a panel of 10 *M. tuberculosis* complex EQA isolates constituting a subset of the isolates evaluated in the present study, comprising 10 isolates present in triplicate was distributed to 5 NHLS laboratories performing diagnostic culture of *M. tuberculosis* as well as LPA (19). An overall agreement of ≥ 90% was deemed an acceptable score. Inter-operator reproducibility was performed by comparing findings on 45 EQA isolates tested by two operators at CTB, working independently.

Statistical analysis. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), based on agreement between the DST gold standard and the index assay and 95% confidence intervals (95% CIs) were calculated for both the antibiotic class and individual drugs. Agreement between the gold standard and index assay was calculated using the McNemar test. Statistical analyses were performed using Stata: Release 13 (20).

RESULTS

A total of 268 *M. tuberculosis* complex isolates were included in the study and the frequency of resistance to OFX, KAN, AMK and CAP among these isolates as detected by the two methods feature in Table 1. Of these, 42 isolates from the NHLS Braamfontein Laboratory had phenotypic DST performed for OFX and KAN. As the DNA for the 42 isolates was not available, WGS could not be performed. Resistance to one or more of the drugs under evaluation was observed in 120/268 (44.8%) of the *M. tuberculosis* isolates included in the study. All 27 probe bands of Genotype® MTBDR*sl* VER 2.0 strips were interpretable in all samples tested with successful positive and negative quality controls (14). Phenotypically susceptible isolates were correctly classified as susceptible by the assay.

Detection of FLQ Resistance. Sensitivity, specificity and accuracy indices for FLQ (OFX) resistance using the Genotype® MTBDR*sl* VER 2.0 were determined as 100%, 98.9% and 99.3% respectively, when compared to the gold standard (Table 2). There were 85/120 (70.8%) isolates resistant to FLQ as tested by DST. Genotype® MTBDR*sl* VER 2.0 detected 92 mutations in the *gyr*A and *gyr*B genes among the 85 FLQ (OFX) resistant isolates. The distribution of mutations is summarised in Table 3. The majority of mutations, 52/92 (56.5%) was observed at codon 94. Other *gyr*A mutations detected by the assay were at codon 90 (29/92; 31.5%) and at codon 91 (9/92; 9.8%).

The diversity of single defined mutations at *gyr*A codon 94 included the following (see Table 3): *gyr*A MUT3C (D94G) 23/52 (44.2 %), *gyr*A MUT3A (D94A) 7/52 (13.5%), *gyr*A MUT3D (D94H) 6/52 (11.5%) and *gyr*A MUT3B (D94N / D94Y) 5/52 (9.6%). In 3/52 (5.8%) isolates, both *gyr*A mutations MUT3B and MUT3D (D94N / D94Y & D94H) were detected. Other *gyr*A defined mutations detected at codons 90 and 91 were: *gyr*A MUT1 (A90V) 25/29 (86.2%) and *gyr*A MUT2 (S91P) 7/9 (77.8%) (See Table 3).

Heteroresistance in *gyr*A was observed in 14/85 (16.5%) OFX resistant isolates. Eight isolates amongst these exhibited two mutation bands involving codons 94, 91 and 90. These were as follows: 4/85 (4.7%) WT/*gyr*A MUT1 and MUT3B (A90V and D94N/D94Y); 2/85 (2.4%) WT/*gyr*A MUT2 and MUT3B (S91P and D94N/D94Y); and 2/85 (2.4%) WT/*gyr*A MUT3B and MUT3C respectively (See Table 3).

There were 8/85 (9.4%) isolates with undefined mutations that were interpreted as resistant. The following wild-type bands were not detected amongst these isolates; *gyr*A WT2 4/85 (4.7%), *gyr*A WT3 1/85 (1.1%) and *gyr*B WT1 3/85 (3.3%). WGS performed on 6/8 available isolates confirmed defined mutations at *gyr*A (A90V, S91P and D94Y) missed by the assay, and *gyr*B mutations at codons 274 and 499 that are not covered by the assay (Table 4). All 8 of these isolates were phenotypically resistant to OFX. An isolate that harboured *gyr*A MUT3C (D94G) mutation confirmed by WGS with an A94G mutation was phenotypically susceptible to OFX. In the selection of isolates tested, no defined mutations were detected by the assay in the *gyr*B region (i.e. mutations N538D and E540V).

Detection of SLID Resistance. The sensitivity, specificity and accuracy of Genotype® MTBDR*sl* VER 2.0 for SLID resistance was 89.2%, 98.5% and 96.3 % respectively, when compared with phenotypic DST. Individual SLID sensitivity and specificity assessments were respectively: 93.8% and 98.5% for AMK, 89.2% and 98.5% for KAN and 86.2% and 95.9% for CAP (Table 2).

Genotype® MTBDR*sl* VER 2.0 detected 56 defined mutations and 6 undefined mutations in either the *rrs* or *eis* genes amongst the 65/120 (54.2%) isolates phenotypically resistant to SLID (Table 3). The most frequently observed mutation 48/56 (85.7%), for SLID resistance was the *rrs* MUT1 (A1401G) with four of these isolates displaying heteroresistance. All 48 isolates were confirmed as phenotypically resistant to a SLID (AMK, KAN or CAP). Two isolates had *rrs* WT1 bands absent with no corresponding mutation band observed, 1/2 tested wild type by WGS, both being phenotypically susceptible to SLID. Similarly, there were 4/65 (6.2%) isolates that were interpreted as resistant (absence of *eis* WT2 and no mutation band observed), 3/4 were phenotypically resistant to KAN while 1/4 was phenotypically susceptible to KAN. WGS tested 2/4 as wild type with a C-10T/G-10A mutation observed in 1/4 isolates. The *eis* MUT1 (C-14T) mutation was observed in 8/65 (12.3%) of the SLID resistant isolates and confirmed phenotypically as KAN resistant. In 6/65 (9.2%) isolates phenotypically resistant to KAN, no *rrs* or *eis* mutations were observed. These were interpreted as susceptible to SLID by the assay. DNA available for 3/6 of these isolates tested wild type by WGS (Table 4). Amongst five isolates (5/65; 7,7%) that were phenotypically susceptible to CAP, 2/5 had the *rrs* MUT1 (A1401G) mutation and 3/5 with *eis* missing WT2, and interpreted as resistant to SLID by the assay. WGS performed on these isolates revealed no resistance conferring mutations for *gid*B and *tly*A regions. The mutation, *rrs* MUT2 (G1484T) was not observed amongst any of the isolates tested.

Detection of XDR-TB. Amongst the isolates tested 31/120 (25.8%) were XDR by phenotypic DST (Table 5). Agreement between Genotype® MTBDR*sl* VER 2.0 and phenotypic DST was calculated as 97.0% for the detection of XDR-TB. Genotype®MTBDR*sl* VER 2.0 correctly identified 27/31 (87.1%), missing 4/31 (12.9%) isolates, all of which were phenotypically SLID (KAN) resistant but sensitive by the assay. WGS performed for 2/4 missed by the assay, were wild type for *rrs* and *eis;* however, both isolates were phenotypically resistant to KAN (SLID). All isolates with a defined mutation showed phenotypic resistance.

Reproducibility. Amongst the 5 laboratories that tested the panel of 30 isolates, the overall performance ranged between 93% – 96 % (Table 6). The inter-operator assessment showed 100% agreement.

DISCUSSION

To our knowledge, the evaluation of Genotype® MTBDR*sl* VER 2.0 in conjunction with a reproducibility assessment is the first such study conducted in a high TB-HIV setting in Africa. Genotype® MTBDR*sl* VER 2.0 has shown an improvement in the sensitivity and specificity for the determination of molecular resistance to both FLQ 100% (95% CI, 95.8 - 100%); 98.9% (95% CI, 96.1 - 99.9%) and SLID 89.2% (95% CI, 79.1 - 95.6%); 98.5% (95% CI, 95.7 - 99.7%), in comparison with the pooled sensitivity and specificity of the Genotype® MTBDR*sl* VER 1.0 reported by WHO Expert Group (12) and a meta-analysis by Theron and colleagues (5). Agreement between phenotypic gold standard, and Genotype® MTBDR*sl* VER 2.0 for FLQ (OFX) and SLID was 99.3% and 96.3% respectively across a wide distribution of mutations. The assay was found to be highly reproducible in terms of inter-laboratory (93 - 96%) and inter-operator (100%) assessment.

FLQ resistance in *M. tuberculosis* is ascribed mainly to *gyr*A mutations, with 57.5% of mutations detected at codon 94 and 31.5% at codon 90 (21). Consistent with published data, the highest frequency of mutations conferring FLQ resistance was observed in *gyr*A codons 94 and followed by codon 90 (Table 3). Furthermore, *gyr*A mutations D94G and D94N were observed in majority of the isolates tested. Previous studies have indicated that isolates harbouring these mutations exhibit high levels of resistance to FLQ (21, 22). The *gyr*A MUT3D/ D94H mutation, reported as a rare *in silico* mutant (13, 23), was observed in 9/85 (10.6%) of mutations identified from these clinical isolates. Heteroresistance involving either FLQ or SLID was detected in 18/85 (21.2%) with multiple mutation bands observed in some isolates (Table 3) and were confirmed as phenotypically resistant. Cohen et. al., 2011, reported on mixed-strain infections in a hospital setting in KwaZulu-Natal, South Africa (24). Further investigation is required to elucidate the frequency and clinical relevance of mixed infections across a more widespread South African setting. Based on our findings interpretation of these strains as resistant was consistent with the phenotypic DST results.

Undefined mutations with the absence of *gyr*A wild-type bands were confirmed as phenotypically resistant. WGS results available for four isolates confirmed the presence of *gyr*A mutations that the assay missed. The Genotype® MTBDR*sl* VER 2.0 guideline for interpretation of results states that, “only bands with intensities as strong as or stronger than the amplification control zone (AC) are to be considered” (13). Three out of four of these discordant isolates had *gyr*A MUT2/S91P

bands with intensities less than the AC zone, therefore interpreted as negative and reported as resistant due to the absence of *gyr*A wild-type bands. No weak intensity mutation bands were detected in the fourth isolate. An isolate phenotypically susceptible to OFX with *gyr*A MUT 3C/D94G bands and a confirmed Asp94Gly mutation on WGS had *gyr*A WT bands with less intensity than AC control band. The discrepant OFX phenotypic DST is possibly due to a mixed population. The inclusion of selected *gyr*B probes offered limited enhancement to the identification of FLQ mutations as only the absence of *gyr*B WT was observed in 3/268 (1.1%) of isolates. Genotype® MTBDR*sl* VER 2.0 is limited to detecting mutations in selected areas of the QRDR regions of *gyr*A and *gyr*B only; therefore, FLQ resistance mechanisms outside these regions are likely to be missed but does not appear to be a major concern in our setting presently.

Consistent with published data, (23), *rrs* MUT1 A1401G (translating to high level SLID resistance) was the most frequently observed mutation (77.5%) amongst tested isolates. The overall SLID sensitivity has improved in the new assay to 89.2% compared with previously published data (5, 12). The inclusion of *eis* promoter region probes improved the detection of SLID resistance as observed in 8/62 (12.9%) low-level KAN resistant isolates, all phenotypically KAN resistant and not associated with *rrs* mutations. In 2 isolates with *rrs* WT1 bands absent with no corresponding mutation band observed and wild type by WGS, interpreted as resistant to SLID by the assay, was phenotypically susceptible to SLID. The omission of WT-bands in line probe assay is not a reliable indication of phenotypic resistance and would require confirmatory phenotypic DST (25). In 6 phenotypically KAN-resistant isolates that were AMK-susceptible, no mutations were detected by the assay or WGS. Although other factors related to phenotypic resistance cannot be excluded, the discrepant results may be due to the inherent challenges of in-house (non-standardised) second-line drug preparations available for testing (25). Five isolates that were phenotypically susceptible to CAP, interpreted as SLID resistant by the assay and wild type by WGS, displayed resistance only to KAN and AMK phenotypically. As indicated by Georghiou et al., (26) *rrs* MUT1 A1401G mutation is a moderate predictor of CAP resistance. Therefore, CAP resistance should be confirmed phenotypically prior to exclusion of the drug from a treatment regimen (27).

There were 31 isolates identified as phenotypically XDR and the Genotype® MTBDR*sl* VER 2.0 achieved 97.0% agreement between the index and gold standard tests. Of the four XDR cases misclassified by the assay, all showed FLQ resistance but missed SLID resistance. All were phenotypically KAN resistant. One isolate was classified as XDR by LPA with *gyr*A MUT1

(A90V) and a missing *eis* WT2 only (C-12T/G-10A) but was phenotypically susceptible to KAN. We used strict criteria and resistance to any one SLID was accepted as a criterion to classify a strain as XDR. Due to limited availability of standardised drug preparations for second-line DST, in routine practice, laboratories would normally only test one drug out of this class for resistance determination. This is a limitation of the data available for this sample set, as mutations in the assay are known to correlate well with OFX resistance and is evident in our observations. As reported, correlation of these mutations with higher FLQs is uncertain and may still show phenotypic susceptibility despite the presence of these mutations, thus phenotypic testing remains important for these drugs (17, 28).

The reproducibility assessment of selected routine laboratories showed excellent performance consistent with other data on molecular diagnostics (Table 6) (29). Genotype® MTBDR*sl* VER 2.0 LPA performed well with excellent reproducibility and high sensitivity and specificity for FLQ and SLID resistance determination. The average reporting turn-around-times for Genotype® MTBDR*sl* VER 2.0 LPA varied from 2 to 4 days subsequent to the identification of a positive TB culture, whereas, second-line phenotypic DST results were only available in 14 to 21 days. The longer TAT for DST results is largely dependent on the viability/fitness of the isolate, especially for XDR isolates. It also highlights the importance of direct testing in providing clinically relevant and reliable results.

Furthermore, adoption of the assay in laboratories already performing the Genotype assays was relatively easy and quick to implement. Genotype® MTBDR*sl* VER 2.0 LPA is therefore suitable for testing clinical isolates as a rapid screening tool for detection of second-line drug resistance in countries with a high burden of MDR-TB.

A limitation of the study was that only culture isolates were used and not clinical specimens, as this study was performed to compare directly the Genotype® MTBDR*sl* VER 2.0 with the gold standard, phenotypic DST. However, there is a need for further studies evaluating the Genotype® MTBDR*sl* VER 2.0 on clinical specimens from patients with MDR-TB, thereby enhancing the turn-around time for resistance reporting and pre-XDR/XDR case detection in high-risk settings. Another limitation was that of the FLQs, only OFX was tested and not any of the other later generation FLQs (isolates collected between 2012 and 2014). This probably explains the higher sensitivity found in our study compared with other studies (30). Phenotypic DST for higher

generation FLQs (moxifloxacin) was introduced in 2015 as per WHO recommendations (17, 28). A general limitation of second-line drug testing is the limited availability of standardized drug preparations (25). A further limitation was that samples from the Braamfontein TB Referral Laboratory could not be included for WGS resolution testing, as the DNA was not available.

In a press release by the WHO in May 2016 the use of the Genotype® MTBDR*sl* VER 2.0 assay as "an initial test, instead of phenotypic culture-based DST" to detect FLQ and SLID resistance in confirmed RIF-R and MDR patients is recommended (31, 32). Appropriately trained laboratory staff, quality assurance and availability of laboratory infrastructure are requisite recommendations of the WHO to implement use of this assay (31, 32). Our study provides support and evidence for these recommendations and the implementation of the assay in South Africa.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

Table 1: Summary of MTBDRsl VER 2.0 LPA and DST Results

Genotype® MTBDRsl VER 2.0 Assay and Phenotypic DST Results				
DRUG TYPE	**MGIT SL-DST**		**MTBDRsl VER 2.0**	
(n=number of isolates)	**RESISTANT**	**SUSCEPTIBLE**	**RESISTANT**	**SUSCEPTIBLE**
FLQ (OFX) (n=267)	85(31.8%)	182 (68.2%)	87 (32.6%)	180 (67.4%)
AG (KAN) (n=268)	65 (24.3%)	203 (75.7%)	60 (22.4%)	208(77.6%)
AG (AMK) (n=226)*	32(14.2%)	194(85.8%)	33 (14.6%)	193 (85.4%)
CAP (n=226)*	29 (12.8%)	197 (87.2%)	33(14.6%)	193 (85.4%)

* Excludes isolates from NHLS Braamfontein TB Referral Laboratory

Table 2: Performance of Genotype® MTBDRsl V2 Assay

Pooled Result (n=268)	% SENS	95% CI	% SPEC	95% CI	PPV (%)	95% CI	NPV (%)	95% CI	Diagnostic Efficacy (%)
FQ (OFX) (n=267)	100.0	95.8-100	98.9	96.1–99.9	97.7	91.9–99.7	100.0	98.0-100.0	99.3
SLID (AG/CP)	89.2	79.1-95.6	98.5	95.7–99.7	95.1	86.3–99.0	96.6	93.2-98.6	96.3
AMI (n=226)*	93.8	79.2–99.2	98.5	95.5–99.7	90.9	75.7–98.1	99.0	96.3-99.9	97.8
KANA (n=268)	89.2	79.1–95.6	98.5	95.7–99.7	95.1	86.3–99.0	96.6	93.2-98.6	96.3
CAP (n=226)*	86.2	68.3–96.1	95.9	92.2–98.2	75.8	57.7–88.9	97.9	94.8-99.4	94.7

* Excludes isolates from NHLS Braamfontein TB Referral Laboratory

Table 3: Genotype® MTBDRsl VER 2.0 Assay - Hybridisation patterns observed in LPA resistant strains (FLQ n = 85; SLID n = 65)

Genotype® MTBDRsl V2 Assay Results				
PHENOTYPIC RESISTANCE DETECTED	**HYBRIDIZATION BAND(S) OBSERVED**	**MUTATION(S) DETECTED**	**No. of mutations n***	**%**
FLQ	*gyr*A MUT1	A90V	25	27.2
	WT/*gyr*A MUT1 & MUT3B	A90V & D94N/D94Y	4	4.3
	*gyr*A MUT2	S91P	6	6.5
	WT/*gyr*A MUT2	S91P	1	1.1
	WT/*gyr*A MUT2 & MUT3B	S91P & D94N/D94Y	2	2.2
	*gyr*A WT2 Absent	None	4	4.3
	*gyr*A WT3 Absent	None	1	1.1
	*gyr*A MUT3A	D94A	6	6.5
	WT/*gyr*A MUT3A	D94A	1	1.1
	*gyr*A MUT3B	D94N / D94Y	3	3.3
	WT/*gyr*A MUT3B	D94N / D94Y	2	2.2
	WT/*gyr*A MUT3B & MUT3C	D94N / D94Y & D94G	2	2.2
	*gyr*A MUT3B & MUT3D	D94N / D94Y & D94H	3	3.3
	*gyr*A MUT3C	D94G	21	22.8
	WT/*gyr*A MUT3C	D94G	2	2.2
	*gyr*A MUT3D	D94H	6	6.5
	*gyr*B MUT1	N538D	0	0.0
	*gyr*B MUT2	E540V	0	0.0
	*gyr*B WT1 Absent	None	3	3.3
CAP AMK KAN	*rrs* MUT1	A1401G	44	71.0
	WT/*rrs* MUT1	A1401G	4	6.5
	rrs WT1 Absent	None	2	3.2
CAP VIO AMK KAN	*rrs* MUT2	G1484T	0	0.0
Low-level KAN	*eis* MUT1	C-14T	8	12.9
	eis WT2 Absent	None	4	6.5

*FLQ mutations (n=92)

*SLID mutations (n=62)

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Table 4. WGS* results for MTBDRsl VER 2.0 Isolates with Undefined Mutations and Discordant DST

MGIT DST (OFX)	MGIT DST (KAN)	MGIT DST (AMK)	MGIT DST (CAP)	MTBDRsl VER 2.0 *gyrA*	WGS *gyrA*	MTBDRsl VER 2.0 *gyrB*	WGS *gyrB*	MTBDRsl VER 2.0 *rrs*	WGS *rrs* (nt)**	MTBDRsl VER 2.0 *eis*	WGS *eis* (nt)**
R	S	S	S	Missing WT 3	Asp94Tyr Ala90Val	wt	wt	wt	wt	wt	wt
R	S	S	S	Missing WT 2	Ser91Pro Ala90Val	wt	wt	wt	wt	wt	wt
R	S	S	S	Missing WT 2	Ser91Pro Ala90Val	wt	wt	wt	wt	wt	wt
R	S	S	S	Missing WT 2	Ser91Pro	wt	wt	wt	wt	wt	wt
R	S	S	S	wt	wt	Missing WT 1	Ser274Arg Asn499Ser	wt	wt	wt	wt
R	S	S	S	wt	wt	Missing WT 1	Ser274Arg Asn499Ser	wt	wt	wt	wt
S	R	R	S	wt	Gly247Ser	wt	wt	wt	wt	Missing WT 2	wt
S	R	S	S	wt	Gly247Ser	wt	wt	wt	T517C	Missing WT 2	wt
S	R	R	S	wt	wt	wt	wt	wt	wt	Missing WT 2	C-10T/ G-10A
S	S	S	S	wt	wt	wt	wt	Missing WT 1	wt	wt	wt
S	R	R	S	wt	wt	wt	wt	*rrs* MUT1 / A1401G	A1401G	wt	wt
S	R	R	S	wt	wt	wt	wt	*rrs* MUT1 / A1401G	A1401G	wt	wt
S	R	S	S	*gyrA* - MUT 3C / D94G	Asp94Gly	wt	wt	wt	wt	wt	wt
R	R	S	S	*gyrA* - MUT 3C / D94G	Asp94Gly	wt	Val457Leu	wt	wt	wt	wt
R	R	R	S	*gyrA* - MUT2 / S91P	Ser91Pro	wt	Val457Leu	wt	wt	wt	wt

*DNA available for 10/14 isolates with Undefined mutations & 9/20 Discordant isolates **Nucleotide

Table 5: MGIT DST and Genotype® MTBDRsl VER 2.0 analysis in XDR Isolates identified (n=31)

MGIT DST (FLQ)	MGIT DST (SLID)	MTBDRsl VER 2.0 (FLQ-*gyrA*/*gyrB*)	MTBDRsl VER 2.0 (SLID-*rrs*)	MTBDRsl VER 2.0 (SLID-*eis*)	Frequency n (%)
R	R	*gyr*A MUT1/A90V	*rrs* MUT1/A1401G	wt	5 (16.1)
R	R	*gyr*A MUT1/A90V	*rrs* MUT1/A1401G	*eis* MUT1/ C-14T	1 (3.2)
R	R	*gyr*A MUT1/A90V	wt	*eis* MUT1/ C-14T	5 (16.1)
R	R	*gyr*A MUT1/A90V	wt[1]	wt[1]	1 (3.2)
R	R	*gyr*A MUT2/S91P	wt[2]	wt[2]	1 (3.2)
R	R	*gyr*A MUT3A/D94A	*rrs* MUT1/A1401G	wt	2 (6.5)
R	R	*gyr*A MUT3B/ D94N/D94Y	wt[2]	wt[2]	1 (3.2)
R	R	*gyr*A MUT3B/ D94N/D94Y	wt	*eis* MUT1/ C-14T	1 (3.2)
R	R	*gyr*A MUT 3C/D94G	*rrs* MUT1/A1401G	wt	8 (25.8)
R	R	*gyr*A MUT 3C/D94G	wt[1]	wt[1]	1 (3.2)
R	R	*gyr*A MUT3D/D94H	*rrs* MUT1/A1401G	wt	4 (12.9)
R	R	*gyr*A MUT1/A90V *gyr*B WT absent	wt	*eis* MUT1/ C-14T	1 (3.2)

[1] WGS not available

[2] WGS results: wt

Table 6: GenoType® MTBDRsl VER 2.0 - REPRODUCIBILITY ASSESSMENT OF PARTICIPANT LABORATORIES

STRAIN NUMBER (3 Isolates each)	Laboratory A	Laboratory B	Laboratory C	Laboratory D	Laboratory E
Strain 1	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
Strain 2	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
Strain 3	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
Strain 4	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
Strain 5	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
Strain 6	No consensus Excluded	No consensus Excluded	No consensus Excluded	No consensus Excluded	No consensus Excluded
Strain 7	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
Strain 8	2 /3 (67%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
Strain 9	3/3 (100%)	2 /3 (67%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
Strain 10	3/3 (100%)	2 /3 (67%)	1/3 (33%)	2 /3 (67%)	2 /3 (67%)
Overall score	26/27 (96%)	25/27 (93%)	25/27 (93%)	26/27 (96%)	26/27 (96%)

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CHAPTER 4

4 RESULTS OF PHASE 3 OF STUDY

4.1 Prospective validation of the GenoType® MTBDR*sl* VER 2.0 Line Probe Assay on clinical samples (direct sputum testing)[2]

A prospective validation of the GenoType® MTBDR*sl* VER 2.0 was conducted in phase 3 of the study to evaluate the performance of the assay on clinical samples and addresses the objective of the study as described in chapter 1.16.2. All LPA testing, data analysis and co-ordination of sample collection from the selected NHLS TB laboratories was conducted by the student. Genotyping data was sourced from the routine testing performed on surveillance samples at CTB.

4.1.1 ABSTRACT

Introduction

South Africa has one of the highest burdens of drug-resistant tuberculosis (DR-TB) globally with 490 000 cases of multi-drug resistant (MDR)-TB cases reported by WHO in 2016. Rapidity in diagnosis of DR-TB would enable early and potentially appropriate and effective treatment. Molecular-based assays designed to detect specific drug resistance mutations for *M. tuberculosis*, offer the advantage of faster TAT and accurate results. The WHO recommends the use of GenoType® MTBDRplus VER 2.0 line probe assay (LPA) as an initial direct DST for the detection of resistance to RIF and INH and GenoType® MTBDRsl VER 2.0 LPA to detect resistance to second-line anti-TB drugs.

Methods

GenoType® MTBDRsl VER 2.0 LPA is designed for molecular detection of resistance-conferring mutations in genes encoding resistance to fluoroquinolones

[2] Editorial format for Journal of Clinical Microbiology was applied to this chapter

(FLQ) (*gyr*A and *gyr*B*)* and second-line injectable drugs (SLID) (*rrs* and *eis)*. A total of 178 smear positive clinical samples collected between April 2016 and November 2016 were tested. Sensitivity, specificity and diagnostic accuracy of the MTBDRsl VER 2.0 were determined with the BACTEC MGIT™ 960 system DST as a reference method, using WHO 2013 recommended critical concentrations for FLQ (OFX 2.0 µg/ml, MFX 0.5 µg/ml) and SLID (KAN 2.5 µg/ml, AMK 1.0 µg/ml and CAP 2.5 µg/ml).

Results

Directly comparable results between LPA and DST were available for 134 clinical samples. The specificity of the GenoType® MTBDRsl VER 2.0 against conventional DST was relatively high for FLQ and SLID and varied from 89.2% for OFX to 90.8% for CAP. The sensitivity values ranged from 74.5% for OFX to 84.7% for CAP. PPVs ranged from 71.7% for MFX to 89.3% for both KAN and CAP.

Conclusion

MTBDRsl VER 2.0 LPA performed well on clinical samples with good sensitivity and specificity for FLQ and SLID. The results are comparable with data published in the WHO 2016 Policy Guidance Document. The inclusion of this assay as an initial direct test in the South African National TB Program will enable faster diagnosis and laboratory-based treatment options for DR-TB patients.

Key Words

Genotype® MTBDR*sl* VERSION 2.0; Drug Resistant (DR)-TB; MDR-TB; XDR-TB, line probe assay, LPA, fluoroquinolones, second-line injectable drugs, molecular diagnostic testing, phenotypic drug susceptibility testing

4.2 INTRODUCTION [3]

The primary aim of the WHO-endorsed End TB Strategy (2016 – 2035) is *"to end the global TB epidemic"* (WHO Global TB Report 2016, 2017). This includes the reduction of TB deaths by 95% and TB incidence by 90% as compared with these rates in 2015 (WHO Global TB Report, 2016, 2017). In order to achieve these targets, implementation of molecular diagnostic testing methods which provide speedier diagnosis with standardised quality-assured results are required to improve National TB Management Programs globally. South Africa (SA) has one of the highest burdens of drug-resistant tuberculosis (DR-TB) globally, with 18 734 laboratory confirmed rifampicin-resistant (RR-) or multi-drug resistant TB (MDR-TB), defined as resistance to isoniazid (INH) and rifampicin (RIF), cases reported in 2014 (WHO SA, SA NDOH, and USAID 2016). Moreover, the introduction of shorter regimens for the treatment of RR/MDR TB to ensure improved patient treatment outcomes requires prompt identification and treatment of pre-XDR-TB (resistance to INH, RIF and resistance to either any FLQ or any SLID, but not both) (Niehaus et al., 2015) and XDR-TB (MDR-TB with additional resistance to any fluoroquinolone (FLQ) and at least one second-line injectable drug (SLID) (WHO Global TB Report 2014). The challenges of detection and treatment of DR-TB continue. Early detection and appropriate management of both susceptible and DR-TB cases are necessary to improve patient outcomes and curb transmission within communities.

Phenotypic drug susceptibility testing (DST) is considered as the gold standard for individualised patient treatment management but has long turn-around times for results to become available and presents challenges in reproducibility. DST for second-line TB drugs requires extended turnaround times for results, is expensive and not standardised. The implementation of rapid molecular assays for the detection of resistance to second-line drugs is therefore of major interest.

In May 2016, the WHO recommended the use of the line-probe assay (LPA) GenoType® MTBDR*sl* VER 2.0 LPA as an initial rapid screening test for the detection of resistance to second-line anti-TB drugs in patients with confirmed rifampicin resistance (RR) or MDR-TB, as an alternative to culture-based phenotypic DST

[3] A summarised introduction of the literature and methods is described. A comprehensive literature review and methods are discussed in chapters 1 and 2. The format for headings and tables follows the numbering in accordance with other chapters.

(WHO Rapid diagnostic test, 2016; WHO Policy Guidance - molecular line probe assays, 2016). The recommendations pertain to direct testing of clinical samples, regardless of smear status and indirect testing of culture isolates from these patients (WHO Rapid diagnostic test, 2016).

The rationale for this phase of the study includes early detection of second-line drug resistance to assist the current national program for management of drug resistant tuberculosis. The aim was to evaluate the performance of GenoType MTBDRsl VER 2.0 LPA, including reproducibility of the findings for decontaminated smear-positive clinical (direct) samples with known RR or MDR results coupled with the identification of *M. tuberculosis* complex and the molecular detection of resistance to second-line TB drugs.

4.3 METHODS

4.3.1 Ethical consideration

Residual DNA from clinical samples was used with no patient identifiers without compromising routine testing turn-around-times. The Human Research Ethics Committee of the University of Witwatersrand, Johannesburg, South Africa, approved the study (M150752).

4.3.2 Setting and Sample Selection

The study was a prospective evaluation of the performance of the GenoType MTBDRsl VER 2.0 LPA conducted at the Centre for Tuberculosis, National Institute for Communicable Diseases (CTB, NICD) and selected National Health Laboratory Service (NHLS) TB laboratories in SA. Residual DNA from concentrated sediment of clinical samples and MGIT culture isolates was collected from the CTB, NICD (the designated Reference Laboratory for the study) and selected NHLS TB laboratories (the designated Testing Laboratories for the study) performing TB culture and LPA testing.

Smear-positive sputum samples sent to the selected laboratories for routine TB diagnostic testing, i.e. smear microscopy, TB culture and MTBRplus VER 2.0 LPA (assay for RIF and INH) were selected. Only samples with confirmed RR or MDR-TB by the Xpert MTB/Rif or MTBRplus VER 2.0 LPA were tested with MTBDRsl VER 2.0 LPA. Routine DST continued in parallel without interference. An overview of the selection process is detailed in Chapter 2, figure 2.1b

The samples were used to evaluate the accuracy and reproducibility of the assay. To accomplish these objectives, samples comprising of the following were obtained:

- 161 decontaminated smear-positive sediments with a resistance profile of RR-TB on Xpert MTB/Rif testing or MDR-TB on testing by MTBRplus VER 2.0 LPA and 141 corresponding MGIT culture isolates were tested at CTB.
- 80 smear-negative clinical samples randomly selected from samples with a resistance profile of RR-TB on Xpert MTB/Rif testing submitted to CTB between 22 June 2016 and 06 July 2016.

- 410 residual DNA from smear positive clinical samples identified as RR-TB or MDR-TB by MTBRplus VER 2.0 LPA testing were collected between September 2016 and January 2017 from selected NHLS laboratories. There were 344 corresponding MGIT isolates received between November 2016 and March 2017 available for LPA second-line testing.
- The clinical samples and isolates tested were from patients' in Gauteng, Free State, Northern Cape, North West Province, Western Cape and Eastern Cape provinces in SA.
- The ATCC® *M. tuberculosis,* H37Rv 27294 (ATCC, 2006) was used as the reference strain for phenotypic DST and LPA testing.

4.3.3 GenoType® MTBDRsl VER 2.0 Line Probe Assay Testing

The GenoType® MTBDR*sl* VER 2.0 assay was performed according to manufacturer's instructions (HAIN LifeScience, 2015). DNA extraction, PCR amplification and reverse hybridisation of amplicons are integral processes performed in LPA methodology and are detailed in chapter 2.12.

The presence of specific mutation bands (defined mutations) or the absence of wild type and corresponding mutation bands (undefined mutations) were interpreted as resistant to the related drug group (Daum et al., 2012; Gardee et al., 2017). Heteroresistance, defined as the presence of wild type and mutation bands, was interpreted as resistant (HAIN LifeScience, 2015). Samples with indeterminate banding or discordant with phenotypic DST results were retested from stored DNA.

4.3.4 Phenotypic Drug Susceptibility Testing

Phenotypic DST for second-line anti-TB drugs, fluoroquinolones (FLQ), moxifloxacin (MXF) and ofloxacin (OFX), and the second-line injectable drugs (SLID), aminoglycosides, amikacin (AMK) and kanamycin (KAN) together with the cyclic peptide, capreomycin (CAP) was performed using BACTEC MGIT™ 960 system (Becton Dickinson, USA). The WHO 2013 guidelines for critical concentrations (CC) for FLQ (OFX 2.0 µg/ml, MFX 0.5 µg/ml) and SLID (KAN 2.5 µg/ml, AMK 1.0 µg/ml

and CAP 2.5 µg/ml), as shown in Chapter 2, Table 2.2, (WHO Companion Handbook, 2014) were used.

4.3.5 Genotyping

Genotyping was performed to ascertain strain relatedness of XDR isolates using two methods: spacer oligonucleotide typing (spoligotyping) and 24-loci mycobacterial interspersed repetitive units-variable number tandem repeats (MIRU-VNTR) typing. Spoligotyping was performed using a commercial kit as per manufacturer's protocol (Ocimum Biosolution, Hyderabad, India). The resulting spoligotypes were entered in an excel sheet as a binary code representing either a positive or negative hybridization result (n and o, respectively) and were entered in the SpolDB4 database (http://www.pasteur-guadeloupe.fr/tb/bd_myco.html Pasteur Institute of Guadeloupe). In this database, SIT (Spoligotype International Type) designates spoligotyping shared by two or more patient isolates, as opposed to "unique" which designates patterns reported for a single isolate.

The 24-loci MIRU-VNTR was performed using quadruplex version of the GenoScreen MIRU typing kit (GenoScreen, Lille, France). Sizing of PCR fragments was determined using the ABI 3500 XL genetic analyser (Applied Biosystems, CA, USA) with LizMarker (Bioventures, TN, USA) as a size standard. MIRU-VNTR alleles were determined by using Gene Mapper software version 5.0 (Applied Biosystem, USA).

4.3.6 Reproducibility and Agreement

Reproducibility, defined as the ability of a method to achieve the same results on repeat testing of the same samples at different sites or different operators (Banoo et al., 2008; Ebentier, et.al., 2013). Repeat testing of samples for the assessment of inter-laboratory and inter-operator reproducibility and agreement of the assay was performed at CTB, NICD. An overall agreement of ≥90% was deemed as acceptable (Fattorini et al.,2012; Migliori et al., 2000).

4.3.7 Statistical analysis

Estimates for sensitivity, specificity, positive and negative predictive values, diagnostic accuracy and 95% confidence intervals (95% CIs) were calculated for both the antibiotic class and individual drugs were calculated as described (Banoo et al., 2008; Sauerbrei and Blettner, 2009). Evaluation of agreement (concordance) between different sites and sample types was determined using Cohen's Kappa (**κ**). The **κ** statistic measures the degree of interrater agreement, a measure of test reliability, and was interpreted using the following scale: <0.20 =poor; 0.21 – 0.40 = fair; 0.41 – 0.60 = moderate; 0.61 -0.80 = good; 0.81 -1.0 = very good (Kwiecien et al., 2011). Statistical analyses were performed using Stata: Release 13 (Stata Press 2013).

4.4 RESULTS

4.4.1 Detection of second-line resistance from clinical samples - GenoType® MTBDRsl VER 2.0 versus phenotypic DST

A total of 161 decontaminated sputum sediments and 134 of their corresponding MGIT isolates from CTB were tested between April 2016 to November 2016, using GenoType® MTBDRsl VER 2.0 LPA to assess the diagnostic performance of the assay for clinical samples. MTB Complex DNA (positive TUB band) was detected in all 161(100%) of the sediments tested. The sediment DNA yielded interpretable LPA results for 153/161 (95.0%) of the samples tested. There were 8/161 (5.0%) indeterminate LPA results from smear-positive sediment DNA. MTBC DNA was detected in all 134 (100%) MGIT isolates and yielded interpretable LPA results for all isolates tested. Phenotypic DST results were available for 125/134 (93.3%) MGIT isolates and resistance to one or more drugs under evaluation was observed in 79/125 (63.2%) of the MGIT isolates tested.

Between September 2016 and January 2017, 410 residual smear-positive sediment DNA and 344 corresponding MGIT isolates were received between November 2016 and March 2017, from NHLS TB laboratories (testing laboratories) were included for evaluation. The samples were retested at the CTB, NICD reference laboratory to establish inter-operator and inter-laboratory reproducibility of the GenoType® MTBDRsl VER 2.0 LPA. Of the samples retested 406/410 (99.0%) sediment DNA and 334/344 (97.1%) MGIT isolates detected MTBC DNA and yielded interpretable LPA results.

4.4.2 Performance of GenoType® MTBDRsl VER 2.0 on clinical samples

Based on the gold standard, phenotypic DST, the performance indices for clinical samples for the GenoType® MTBDRsl VER 2.0 were determined and are detailed in Table 4.1.

Table 4.1: Performance of Genotype® MTBDRsl VER 2.0 Assay on clinical samples (MGIT DST Gold Standard)

Results (n=125)	SENS (%)	95% CI	SPEC (%)	95% CI	PPV (%)	95% CI	NPV (%)	95% CI	Diagnostic Accuracy (%)	k
FLQ (n=125)	77.2	67.2 - 85.3	85.4	79.0 - 90.5	75.5	65..6 - 83.8	86.5	80.2 - 91.5	82.4	0.623
OFX	74.5	60.4 - 85.7	89.2	79.8 - 95.2	82.6	68.6 - 92.2	83.5	73.5 - 90.9	83.2	0.647
MFX	76.7	61.4 - 88.2	84.1	74.4 - 91.3	71.7	56.5 - 84.0	87.3	78.0 - 93.8	81.6	0.599
SLID (n=124)*	81.9	75.5 - 87.2	91.5	86.6 - 95.1	90.3	84.7 - 94.4	84.0	78.2 - 88.7	86.0	0.735
KAN	76.9	64.8 - 86.5	89.8	79.2 - 96.2	89.3	78.1 - 96.0	77.9	66.2 - 87.1	83.1	0.663
AMI	84.5	72.6 - 92.7	89.2	79.1 - 95.6	87.5	75.9 - 94.8	86.6	76.0 - 93.8	87.1	0.739
CAP	84.7	73.0 - 92.8	90.8	81.0 - 96.5	89.3	78.1 - 96.0	86.8	76.4 - 93.8	87.9	0.757

*** Failed DST**

Table 4.2: Performance of Genotype® MTBDRsl VER 2.0 Assay on clinical samples (LPA on clinical isolates as Gold Standard) (n=129)

Drug Class	SENS (%)	95% CI	SPEC (%)	95% CI	PPV (%)	95% CI	NPV (%)	95% CI	Diagnostic Accuracy (%)	k
FLQ	88.1	77.1 - 95.1	88.6	78.7 - 94.9	86.7	75.4 - 94.1	89.9	80.2 - 95.8	88.4	0.766
SLID	91.3	82.0 - 96.7	90.0	79.5 - 96.2	91.3	82.0 - 96.7	90.0	79.5 - 96.2	90.7	0.813

The specificity of the GenoType® MTBDRsl VER 2.0 against conventional DST was relatively high for FLQs as well as SLIDs and varied from 89.2% for OFX to 90.8% for CAP while the corresponding sensitivity values ranged from 74.5% for OFX to 84.7% for CAP. PPVs ranged from 71.7% for MFX to 89.3% for both KAN and CAP (Table 4.1).

The GenoType® MTBDRsl VER 2.0 performance indices for 129 clinical isolates compared with their corresponding clinical samples are shown in table 4.2. Overall agreement and κ- values for clinical isolates (cultures) compared with clinical samples were determined for FLQ at 88.4% and 0.77 and for SLID at 90.7% and 0.81, respectively.

4.4.3 Detection of FLQ Resistance

The phenotypic DST results for FLQ showed 48/125 (38.4%) OFX-resistant and 42/125 (33.6%) MFX-resistant isolates. The assay detected defined and undefined mutations in *gyr*A and *gyr*B genes in 46/125 (36.8%) corresponding clinical samples tested.

Amongst the clinical samples the majority of mutations were detected at *gyr*A codon 94, with 29/46 (63.0%) confirmed as phenotypically resistant to OFX and MFX. The diversity of single defined mutations observed at *gyr*A codon 94 were, *gyr*A MUT3C (D94G) 19/46 (41.3%), *gyr*A MUT3B (D94N / D94Y) 7/46 (15.2%), *gyr*A MUT3A (D94A) 2/46 (4.3%) and *gyr*A MUT3D 1/46 (2.1%). In addition, other *gyr*A mutations observed were *gyr*A MUT1 (A90V) 12/46 (26.1%), 4/12 (33.3%) were phenotypically resistant to OFX and MFX, 7/12 (58.3%) were phenotypically OFX-resistant and MFX-susceptible and 1/12 (8.3%) was OFX-susceptible and MFX-resistant. Heteroresistance in *gyr*A was observed in 2/46 (4.3%) OFX and MFX resistant isolates. No defined mutations were detected by the assay at *gyr*A MUT2 (S91P) and in the *gyr*B region (i.e. mutations N538D and E540V).

Six samples (6/46, 13.0%) with undefined mutations, i.e. absence of wild type bands, were interpreted as resistant by LPA. Three samples, 3/46 (6.5%) showed the wild type band absent for *gyr*A WT3, 2/3 were phenotypically susceptible to OFX and

MFX. One sample, 1/46 (2.1%) had a *gyr*A WT1 absent and was phenotypically susceptible to OFX and MFX. Two isolates (2/46, 4.3%) that were phenotypically OFX-susceptible and MFX-resistant had the *gyr*B WT absent in the clinical samples and corresponding isolates when tested by LPA.

4.4.4 Detection of SLID Resistance

There were 63/124 (50.8%) phenotypically resistant SLID isolates. The individual SLID resistant isolates were as follows; 63/124 (50.8%) KAN, 56/124 (45.2%) AMK and 57/124 (46.0%) CAP. The majority of defined mutations detected by GenoType® MTBDRsl VER 2.0 assay for SLID resistance amongst the clinical samples tested were 50/63 (79.4%) *rrs* MUT1 (A1401G). Both *rrs* MUT1 (A1401G) and *rrs* MUT2 (G1484T) mutations were detected in one clinical sample (1/63,1.6%) and showed phenotypic resistance to all SLID tested (KAN, AMK, CAP). Amongst the corresponding isolates tested *rrs* MUT1 (A1401G) was detected in 54/63 (85.7%) by the assay. There were 2/124 (1.6%) *eis* MUT1 (C-14T) mutations observed in the SLID resistant isolates and these were confirmed as phenotypically KAN-resistant.

4.4.5 Detection of Pre-XDR and XDR-TB

Amongst the isolates tested, 38/125 (30.4%) were phenotypically pre-XDR TB. There were 14/38 (36.8%) FLQ-susceptible and SLID-resistant samples and 8/38 (21.1%) FLQ-resistant and SLID-susceptible samples confirmed by phenotypic DST and correctly identified as pre-XDR by the assay. Thirteen of the pre-XDR isolates (13/38, 34.2%) were missed by the assay. These tested as susceptible to FLQ and SLID by LPA on both clinical samples and the corresponding isolates.

Phenotypic DST results showed 38/125 (30.4%) XDR-TB isolates. Amongst the clinical samples tested, the assay correctly identified 31/38 (81.6%) as XDR but failed to detect 7/38 (18.4%) when compared with phenotypic DST results (Table 4.3). The assay missed 1 isolate with phenotypic SLID resistance and 2 isolates with phenotypic FLQ resistance when tested on clinical samples as well as the corresponding isolates. One clinical sample had weak band development and was

assessed as indeterminate for LPA. The corresponding isolate was confirmed as XDR when tested using phenotypic DST and LPA.

Amongst the XDR isolates, *gyr*A MUT3C (D94G) was detected in 15/38 (39.5%) and *rrs* MUT1 (A1401G) mutation in 33/38 (86.8%). The majority of the samples, 13/38 (34.2%) had mutations at both *gyr*A MUT3C (D94G) and *rrs* MUT1 (A1401G) and all were confirmed as phenotypically XDR. The assay detected heteroresistance in *gyr*A MUT3C (D94G) and *rrs* MUT1 (A1401G) in one clinical sample and the corresponding MGIT isolate.

Table 4.3: Analysis of XDR Isolates detected by MTBDRsl VER 2.0 - confirmed by phenotypic DST (n = 38/125)

*FLQ (OFX 2.0 µg/ml; MFX) 0.5 µg/ml); SLI (KAN 2.5 µg/ml; AMK 1.0 µg/ml; CAP 2.5 µg/ml)

Drug Resistance Phenotype					MTBDRsl VER 2.0 Clinical Samples				MTBDRsl VER 2.0 Clinical Isolates				No of Isolates
OFX	MFX	KAN	AMK	CAP	FLQ (*gyr*A)	FLQ (*gyr*B)	SLID (*rrs*)	SLID (*eis*)	FLQ (*gyr*A)	FLQ (*gyr*B)	SLID (*rrs*)	SLID (*eis*)	(n)
R	R	R	R	R	*gyr*AMUT 3C / D94G	WT	*rrs*MUT1 / A1401G	WT	*gyr*AMUT 3C / D94G	WT	*rrs*MUT1 / A1401G	WT	13
R	R	R	R	R	WT + *gyr*AMUT 3C / D94G	WT	WT + *rrs*MUT1 / A1401G	WT	WT + *gyr*AMUT 3C / D94G	WT	WT + *rrs*MUT1 / A1401G	WT	1
R	R	R	R	R	*gyr*AMUT3B / D94N/D94Y	WT	*rrs*MUT1 / A1401G	WT	*gyr*AMUT3B / D94N/D94Y	WT	*rrs*MUT1 / A1401G	WT	5
R	S	R	R	R	*gyr*AMUT1/ A90V	WT	*rrs*MUT1 / A1401G	WT	*gyr*A MUT1 / A90V	WT	*rrs*MUT1 / A1401G	WT	4
R	S	R	R	R	*gyr*AMUT1/ A90V	WT	*rrs*MUT1 / A1401G	WT	*gyr*A WT3 Absent	WT	*rrs*MUT1 / A1401G	WT	1
R	R	R	R	R	*gyr*AMUT3A / D94A	WT	WT	*eis*MUT1 / C -14T	*gyr*AMUT3A / D94A	WT	WT	*eis*MUT1 / C -14T	1
R	R	R	R	R	*gyr*AMUT1 / A90V	WT	WT	WT	*gyr*AMUT1 / A90V	WT	WT	WT	1
S	R	R	R	R	*gyr*AMUT1 / A90V	WT	WT	*eis*MUT1 / C -14T	WT	*gyr*B WT Absent	*rrs*MUT1 / A1401G	WT	1
R	R	R	R	R	WT	WT	WT	WT	WT	WT	WT	WT	1
R	R	R	R	R	WT	WT	*rrs*MUT1 / A1401G	WT	WT	WT	*rrs*MUT1 / A1401G	WT	1
R	R	R	R	R	*gyr*A WT3 Absent	WT	*rrs*MUT1 / A1401G	WT	*gyr*A WT3 Absent	WT	*rrs*MUT1 / A1401G	WT	1
R	S	R	R	R	WT	WT	WT	WT	WT	WT	WT	WT	2
S	R	R	R	R	WT	WT	WT	WT	WT	*gyr*B WT Absent	*rrs*MUT1 / A1401G	WT	2
R	R	S	R	R	WT	WT	*rrs*MUT1 / A1401G	WT	WT	WT	*rrs*MUT1 / A1401G	WT	1
R	R	R	S	R	*gyr*AMUT3C / D94G	WT	*rrs*MUT1 / A1401G	WT	*gyr*AMUT3C / D94G	WT	*rrs*MUT1 / A1401G	WT	1
S	R	R	R	R	WT	*gyr*B WT Absent	*rrs*MUT1 / A1401G	WT	WT	*gyr*B WT Absent	*rrs*MUT1 / A1401G	WT	2

4.4.6 Genotyping of XDR isolates

Spoligotyping and 24-loci MIRU-VNTR analyses were available for all 38 XDR-TB isolates. There were 6 strain families identified by spoligotyping namely, Beijing, LAM4, LAM3, 1, X1 and EAI1_SOM originating from 4 provinces (Eastern Cape, KZN, North West and Mpumalanga). Majority were Beijing strain 30/38 (78.9%) with the other strain families represented by T1 3/38 (7.9%), LAM4 2/38 (5.3%), and one each (2.6%) for LAM3, EAI1_SOM and X1. The 30 strains belonging to the Beijing family were divided into two clusters by 24-loci MIRU-VNTR typing. There were 17 and 5 distinct isolates in the two respective clusters. All of these strains originated from the Eastern Cape province in SA.

4.4.7 Discordant Samples Detected by GenoType® MTBDRsl VER 2.0

Discordance was observed in 32/125 (25.6%) clinical samples when compared with the corresponding isolate phenotypic DST results for the specific drugs tested. Amongst these 8 clinical samples were discordant for both FLQ and SLID, 16 showed FLQ-discordance only whilst 7 were SLID-discordant only. There were 6/16 clinical samples that harboured the *gyr*A MUT1 (A90V) mutation. These were phenotypically OFX-resistant and MFX-susceptible. Clinical samples detected as wild type for *gyr*A and *gyr*B by the assay, were phenotypically OFX-resistant and MFX-susceptible in 5/16 isolates and OFX and MFX-resistant in an additional 5/16 isolates. The LPA results for the corresponding 16 MGIT isolates were concordant with the clinical samples. Clinical samples and isolates with undefined mutations detected for *gyr*B on LPA were phenotypically OFX-susceptible and MFX-resistant (Table 4.4).

GenoType® MTBDRsl VER 2.0 assay missed 8 clinical samples which tested as wild type for *rrs* and *eis* genes but were phenotypically resistant to all SLID tested (KAN, AMK and CAP). The LPA on 5/8 corresponding isolates tested as wild type for *rrs* and *eis* genes and *rrs* MUT1/ A1401G mutation was detected in a 3/8 corresponding isolates. Five phenotypically KAN-resistant and AMK and CAP susceptible isolates tested as wild type on LPA, i.e. no *eis* MUT1/C -14T was detected.

Table 4.4: Analysis of Discordant results between phenotypic DST and MTBDRsl VER 2.0 (n = 32/125)

FLQ* Drug Resistance Phenotype			MTBDRsl VER 2.0 Clinical Samples		MTBDRsl VER 2.0 MGIT		No. of Isolates
OFX	**MFX**		***gyr*A**	***gyr*B**	***gyr*A**	***gyr*B**	**(n)**
R	R		WT	WT	WT	WT	6
R	R		WT	WT	*gyr*A MUT3C/ D94G	WT	1
S	R		WT	*gyr*B WT Absent	WT	*gyr*B WT Absent	2
S	R		WT	WT	WT	*gyr*B WT Absent	2
S	R		WT	WT	WT	WT	1
R	S		WT	WT	WT	WT	5
R	S		*gyr*A MUT1/ A90V	WT	*gyr*A MUT1/ A90V	WT	8
R	S		WT	WT	*gyr*A MUT1/ A90V	WT	1
S	S		*gyr*A MUT3C /D94G	WT	WT	WT	1
SLID* Resistance Phenotype			**MTBDRsl VER 2.0 Clinical Samples**		**MTBDRsl VER 2.0 MGIT**		
KAN	**AMK**	**CAP**	***rrs***	***eis***	***rrs***	***eis***	**(n)**
S	R	R	*rrs* MUT1/ A1401G	WT	*rrs* MUT1/ A1401G	WT	1
S	S	S	*rrs* MUT1/ A1401G	WT	WT	WT	1
R	R	R	WT	WT	WT	WT	8
R	R	R	WT	WT	*rrs* MUT1/ A1401G	WT	3
R	S	S	WT	WT	WT	WT	6

*FLQ (OFX 2.0 µg/ml; MFX) 0.5 µg/ml); SLID (KAN 2.5 µg/ml; AMK 1.0 µg/ml; CAP 2.5 µg/ml)

4.4.8 Performance in Smear Negative Clinical Samples

Eighty randomly selected smear-negative clinical samples were tested using GenoType® MTBDRsl VER 2.0 assay, and of these 32/80 (40%) tested positive for MTBC- specific DNA. There were 19/80 (23.8%) smear-negative clinical samples with interpretable second-line LPA results (Table 4.5). The corresponding MGIT culture results were as follows: 10/32 (31.3%) negative, 6/32 (18.8%) contaminated and 16/32 (50.0%) positive. Amongst these positive cultures 7/16 (43.8%) had phenotypic results available. Of the 13/32 (40.6%) failed second-line LPA samples, 5 MGIT cultures were positive with 3 phenotypic DST results available. Two of these were susceptible to both FLQ (OFX and MFX) and SLID (KAN, AMK and CAP) and one was KAN-resistant and susceptible to the rest of the drugs tested.

Table 4.5: Performance of GenoType® MTBDRsl VER 2.0 Assay on Negative Smear Clinical Samples (n = 80)

No. of Clinical Samples tested	MTBC DNA detected	MGIT Culture result		
	(n)	Positive (n)	Negative (n)	Contaminated (n)
80	32	16	10	6
Interpretable LPA Results (n = 19/80)	**(n)**	**MGIT DST**		
FLQ =R low-KAN=R	1	N/A		
FLQ =S SLID=Hetero-R	1	N/A		
FLQ =R SLID=Hetero-R	3	N/A		
FLQ =R SLID=R	1	N/A		
FLQ =S SLID=S	13	FLQ, SLID = S (n = 5)		

*N/A = Not Available

4.4.9 Reproducibility and Agreement

The inter-laboratory and inter-operator reproducibility and agreement evaluation of the GenoType® MTBDRsl VER 2.0 assay was performed on 410 residual DNA samples from smear-positive clinical samples.

The agreement between the NHLS testing laboratories and the Reference laboratory (CTB) sites was high, with overall agreement and κ- values of 97.7% and 0.936 for FLQ and 98.7% and 0.936 for SLID respectively (Table 4.6).

Table 4.6: Reproducibility and Agreement Analysis of GenoType MTBDRsl VER 2.0 for Clinical Samples (n= 409)

	Result Interpretation MTBDRsl VER 2.0							
	Testing Lab (NHLS)			Reference Lab (CTB)				
Drug Class	S	R*	IND	S	R*	IND	Agreement (%)	κ
FLQ	304	100	5	298	97	14	97,7	0,936
SLI	321	80	8	313	80	16	98,7	0,936
Low-level KAN**		2			4		99,7	0,798

* Hetero-Resistance interpreted as Resistant
S=susceptible; R = resistant; IND = Indeterminate

The agreement between clinical samples and isolates was determined for 425 clinical samples and corresponding MGIT isolates collected from all testing sites. An overall agreement of 81.3% for FLQ and 82.7% for SLID with moderate-rated κ-values of 0.564 and 0.601 were observed respectively.

4.5 Discussion

In order to reduce the transmission of TB in a high burden DR-TB setting such as South Africa (SA), the implementation of a rapid diagnostic test for the detection of pre-XDR and XDR-TB is imperative. To the extent of our knowledge this is the first study conducted in SA to evaluate the performance of the GenoType® MTBDRsl VER 2.0 assay on clinical samples.

In this study the performance indices (sensitivity and specificity estimates) of the GenoType® MTBDRsl VER 2.0 were as follows respectively; FLQ 77.2% (CI: 67.2 – 85.3%); 85.4% CI: 79.0 – 90.5%) and SLID 81.9% (CI: 75.5 - 87.2%), 91.5% (CI: 86.6– 95.5%), when measured against phenotypic DST, as the gold standard (Table 4.1). The results obtained are comparable with data published in the WHO 2016 Policy Guidance - molecular line probe assays (2016). The performance of GenoType® MTBDRsl VER 2.0 on clinical samples improved when applying LPA for culture isolates as a reference method (Table 4.2). Overall there was a higher yield of interpretable GenoType® MTBDRsl VER 2.0 LPA results from MGIT isolates and smear-positive sediments than from smear-negative sediments. Majority of the smear-negative sediments showing uninterpretable LPA results had weak banding or "no target probe detection". This suggests that the number of AFB in these clinical samples tested was most likely below the limit of detection for the assay (150 bacteria/ml) (HAIN LifeScience, 2015).

Fluoroquinolone cross-resistance for OFX and MFX was confirmed in 78.3% of clinical samples with mutations in *gyr*A region at codons 94 (63.0%) and 90 (26.1%). These findings confirm results previously reported, that the highest frequency of FLQ resistance are observed at *gyr*A codons 94 (57.5%) and 90 (22.5%) (Kambli et al., 2014). There were eleven isolates phenotypically OFX-resistant and MFX-susceptible, 6/11 were detected with a *gyr*A MUT1 (A90V) mutation and 5/11 clinical samples were detected as wild type for *gyr*A and *gyr*B by the assay. Since MFX differs structurally from OFX, and other FLQs, it may not be affected by resistance mechanisms of the organism applicable to other FLQs, such as efflux, thereby preventing the emergence of drug resistance (Pestova et al., 2000). As reported previously (Sirgel et al., 2012), isolates harbouring a *gyr*A MUT1 (A90V) mutation may require phenotypic confirmation before excluding MFX from a treatment

regimen, especially in the South African setting where MDR-TB patients were treated with OXF before the introduction of MFX (NDOH SA 2014).

The *rrs* MUT1/ A1401G mutation is reported to confer high level resistance in KAN and AMK with low level resistance in CAP (Ajbani et al., 2012; Kambli et al., 2015). In this study the *rrs* MUT1/ A1401G mutation was detected by the GenoType® MTBDRsl VER 2.0 LPA in majority of the clinical samples and corresponding MGIT isolates 54/63 (85.7%). The assay did not detect any mutations in 8 clinical samples in either *rrs* or *eis* regions for which the isolates were phenotypically resistant to KAN, AMK and CAP. Phenotypic resistance in these isolates may be due to mutations outside the regions tested by the assay or may be mediated by other resistance mechanisms.

The GenoType® MTBDRsl VER 2.0 detected *eis* MUT1 (C-14T) in 2 phenotypically KAN-resistant samples. These were interpreted as low-level KAN resistant by the assay. Isolates with *eis* promoter region mutations may be phenotypically susceptible to AMK and CAP suggesting the potential use of these drugs for treating TB caused by strains that exhibit low-level KAN resistance.

The GenoType® MTBDRsl VER 2.0 correctly identified 81.6% of the XDR-TB, consistent with that reported by Tagliani et al.(2015) and, 65.8% pre-XDR-TB amongst the clinical samples tested. The predominant phylogenetic linage amongst the XDR-TB isolates was the Beijing strain originating from Eastern Cape province in SA, also reported previously by Chihota et al. (2012). It is well documented that Beijing strains are associated with the spread of drug resistance (Merker et al., 2015; Chihota et al., 2012; Hove et al., 2011; Roetzer et al., 2011). As recommended by the WHO (WHO Rapid diagnostic test, 2016; WHO Policy Guidance - molecular line probe assays, 2016) testing clinical samples with GenoType® MTBDRsl VER 2.0 for the early identification of pre-XDR-TB and XDR-TB cases will assist in curbing the transmission of these resistant strains.

There were 19/80 (23.8%) interpretable results detected by the assay for smear-negative clinical samples from RR-TB cases. This is an important finding in our high burden TB-HIV setting. Smear-negative patients, especially TB-HIV co-infected patients, infected with DR-TB are likely to be identified earlier for appropriate

treatment when tested using the assay (Gandhi et al., 2006; Migliori and Sotgiu, 2010).

The assay showed excellent reproducibility between the NHLS testing laboratories and the Reference laboratory (CTB) sites with agreement and κ- values of 97.7% and 0.936 for FLQ and 98.7% and 0.936 for SLID respectively (Table 4.6), interpreted as a very good measure of reliability (Kwiecien et al., 2011). On average, GenoType® MTBDRsl VER 2.0 LPA results were available within 2 – 4 days after the receipt of sputum samples. An advantage of LPA is the inclusion of specific probes for the molecular detection of resistance; it can therefore be performed on isolates not suitable for phenotypic based tests. The challenge with TB culture and phenotypic DST in particular, is the extended TAT of these conventional methods. Phenotypic DST results were only available 14 – 21 days after confirmation of a pure TB culture. A further limitation of phenotypic DST is the lack of robustness affecting viability of the culture strains that often results in the delay of diagnosis of TB infection, especially in pre-XDR and XDR patients.

The limitations experienced during the present study was the high percentage (8/161, 5.0%) of indeterminate LPA results from smear-positive sputum DNA, loss of viability of culture isolates required for retesting phenotypic DST of discordant isolates, and the unavailability of WGS data for resolution of discordance.

The data presented by this study accurately evaluated the GenoType® MTBDRsl VER 2.0 LPA in a high burden DR-TB setting, in SA, compared with published data (WHO Policy Guidance - molecular line probe assays, 2016). We recommend that the implementation of this assay in the National TB Management Program as an initial screening test would be extremely invaluable for the rapid identification of pre-XDR-TB and XDR-TB patients for inclusion in appropriate MDR-treatment regimens. We reiterate the assertions by the WHO (WHO Policy Guidance - molecular line probe assays, 2016;WHO 2016a WHO Rapid diagnostic test, 2016) that this application of rapid molecular technology does not exclude the need to perform phenotypic DST as a confirmatory test to monitor the development of further resistance in patients with DR-TB.

CHAPTER 5

5 Evaluation of standardised Becton Dickinson® (BD) second-line DST lyophilised drug kit in comparison with the Genotype® MTBDRsl VER 2.0 LPA and in-house drug preparations for the detection of second-line drug resistance among *M. tuberculosis* clinical isolates [4]

This chapter addresses the fourth objective of the study as described in chapter 1.16.2.

5.1 ABSTRACT

The increase in the incidence of multidrug-resistant tuberculosis (MDR-TB) globally makes the availability of reliable drug susceptibility testing (DST) for second-line anti-TB drugs a priority for proper patient management and favourable treatment outcomes. We evaluated the Becton Dickinson® (BD) second-line DST lyophilised drug kit for BACTEC MGIT™ 960 system using a combined reference standard (CRS); in-house drug phenotypic DST and Genotype® MTBDR*sl* VER 2.0 line probe assay (LPA). The WHO 2013 recommended critical concentrations for fluoroquinolones (FLQ), ofloxacin 2.0 µg/ml, moxifloxacin 0.5 µg/ml and second-line injectable drugs (SLID), kanamycin 2.5 µg/ml, amikacin 1.0 µg/ml and capreomycin 2.5 µg/ml were used. The assay showed very good overall agreement (FLQ 99.2 % and SLID 97.4%) between the in-house drugs and BD second-line lyophilised drug kit. The sensitivity and specificity indices using the CRS were estimated for FLQ (98.3%, 100%) and SLID (95.2%, 98.5%) respectively. The second-line lyophilised drug kit is standardized thus providing an alternative to the challenges and complexities of drug preparation and quality control of phenotypic DST for diagnostic TB laboratories globally.

[4] Editorial format for the International Journal of Tuberculosis and Lung Disease was applied to this chapter.

Key Words

Becton Dickinson® (BD) second-line lyophilised drug kit, Genotype® MTBDR*sl* VERSION 2.0; MDR-TB; XDR-TB, line probe assay, LPA, fluoroquinolones, second-line injectable drugs, phenotypic drug susceptibility testing, DST

5.2 INTRODUCTION [5]

The global emergence of drug-resistant tuberculosis (DR-TB), which continues to be a threat, has hampered the efforts of National TB Management Programs to control the disease. In 2016, the World Health Organization (WHO) reported 600 000 new cases of rifampicin-resistant (RR) TB of which an estimated 490 000 were multidrug-resistant (MDR) TB (WHO Global TB Report 2017). As one of the goals of the WHO-endorsed End TB Strategy (2016 – 2035), the WHO has recommended global access to drug susceptibility testing (DST), which includes both phenotypic and genotypic methods (WHO Global TB Report, 2016 & 2017). South Africa is listed amongst the 30 high TB/HIV burden countries globally, with one of the largest numbers of incident cases in the world (WHO Global TB Report 2016 & 2017). The challenges of identification and treatment of DR-TB cases continue. Timely detection and appropriate treatment of all TB cases is essential to achieve acceptable patient outcomes and control TB transmission within communities.

Conventional phenotypic DST is required to perform first- and second-line TB DST for management and diagnosis of TB disease (NDOH SA 2014; WHO Companion Handbook, 2014). The success of phenotypic DST, considered as the "Gold Standard" methodology, is dependent on the propagation of *Mycobacterium tuberculosis* isolates in pure culture. Phenotypic DST for second-line TB drugs is expensive, time consuming and requires highly competent and skilled staff. Standardisation of drug preparation and critical concentrations for anti-TB agents used are often a challenge. These methodological differences are reflected in the variability of results obtained from different laboratories. Furthermore, repeat testing due to contamination or growth failure of *M. tuberculosis* isolates inevitably results in extended testing periods.

Standardisation and quality assurance systems for second-line DST are crucial for assuring reliable results. An essential priority is the development and implementation of tests, based on the media used for phenotypic DST, at well-established critical concentrations of anti-TB agents recommended by the WHO, which are the most widely used criteria for management of DR-TB. For certain antibiotics (e.g. RIF, INH)

[5] A summarised introduction of the literature and methods is described. A comprehensive literature review and methods are discussed in chapters 1 and 2. The format for headings and tables follows the numbering in accordance with other chapters.

the association between drug resistance mechanisms and the genes responsible for conferring resistance are known and well documented, however, in the case of other antibiotics e.g. bedaquiline (BDQ), these mechanisms are not completely understood (Domínguez et al., 2016). The use of standardised reagents such as Becton Dickinson® (BD) second-line DST lyophilised drug kit to enable the reporting of reliable results is aimed at directing early effective treatment to reduce the extent of person-to-person transmission of MDR-TB and XDR-TB (Siddiqi, 2014; WHO Global TB Report 2016 & 2017). Studies have indicated that rapidity in diagnosis of TB and DR-TB in particular, would lead to early appropriate and effective treatment (Theron et al., 2014; Gandhi et al., 2006; Niehaus et al., 2015).

In May 2016, the WHO recommended the use of Genotype® MTBDR*sl* VER 2.0 line probe assay (LPA) as an initial rapid screening test for the detection of resistance to second-line anti-TB drugs in patients with confirmed RR or MDR-TB. Not all resistance-determining mechanisms for TB are known and genotypic DST assays used may not detect resistance-related mutations such as those that confer resistance but are outside the gene target region of the assay(s) (Drobniewski, 2013; WHO Expert Group, 2013). As reported previously (Gardee et al. 2017), the presence of defined or undefined mutations observed by Genotype® MTBDR*sl* VER 2.0 LPA may not show phenotypic resistance for a particular drug, therefore phenotypic DST remains important for confirmation of susceptibility / resistance. The WHO recommendations assert that LPA does not replace the use of second-line phenotypic DST as a confirmatory test (WHO Rapid diagnostic test, 2016; WHO Policy Guidance - molecular line probe assays, 2016).

The aim of this study was to evaluate the performance and reproducibility of standardised Becton Dickinson® (BD) second-line DST lyophilised drug kit in comparison with the Genotype® MTBDR*sl* VER 2.0 LPA and in-house drug preparations for the detection of second-line drug resistance among *M. tuberculosis* clinical isolates.

5.3 METHODS

5.3.1 Ethical consideration

Characterised repository isolates and clinical isolates from routine testing, with no patient identifiers, collected between 2012 and 2014, were selected for the present study. The Human Research Ethics Committee of the University of Witwatersrand, Johannesburg, South Africa, approved the study (M150752).

5.3.2 Setting and Sample Selection

This evaluation was conducted at the Centre for Tuberculosis, National Institute for Communicable Diseases, National Health Laboratory Service (CTB, NICD/NHLS) and selected NHLS TB laboratories in South Africa. The study was structured with two inter-related components involving the resistance profile validation of characterised *M. tuberculosis* isolates and a proficiency assessment and verification of the performance of laboratories, using a subset of these isolates by selected NHLS TB laboratories.

A representative panel of 142 RR / MDR *M. tuberculosis* isolates; comprising 50 reference isolates and 92 clinical isolates collected between 2012 and 2014 from 6 provinces in South Africa were used. The ATCC® *M. tuberculosis,* H37Rv 27294 (ATCC, 2006) was used as the reference strain for phenotypic DST and LPA testing.

5.3.3 Phenotypic Drug Susceptibility Testing

Phenotypic DST for second-line anti-TB drugs included the FLQ drugs, moxifloxacin (MXF) and ofloxacin (OFX) and SLIDs comprising the aminoglycosides, amikacin (AMK) and kanamycin (KAN), as well as the cyclic peptide capreomycin (CAP). DST was performed using the Becton Dickinson (BD) second-line DST lyophilised drug kit and EpiCenter software on the BACTEC MGIT™ 960 system (Becton Dickinson, USA). The WHO 2013 recommended critical concentrations (CC) for FLQ (OFX 2.0 µg/ml, MFX 0.5 µg/ml) and SLID (KAN 2.5 µg/ml, AMK 1.0 µg/ml and CAP 2.5 µg/ml), as shown in Table 2.2, (WHO Companion Handbook, 2014) were used.

A lower prevalence of MXF 2.0 µg/ml resistance compared to MFX 0.5 µg/ml was anticipated (Zignol et al., 2016), thus testing for the clinical breakpoint (CB) of MXF was performed on a subset of 58 isolates.

5.3.4 Genotype® MTBDR*sl* VER 2.0 Line Probe Assay Testing

The Genotype® MTBDR*sl* VER 2.0 assay was performed according to manufacturer's instructions (Hain Lifescience, 2015) as described in chapter 2.11.

The presence of specific mutation bands (defined mutations) or the absence of wild type without corresponding mutation bands (undefined mutations) were interpreted as resistant to the related drug group (Gardee et al., 2017; Daum et al., 2013). Heteroresistance, defined as the presence of wild type and mutation bands in the same isolate, was interpreted as resistant (Hain Lifescience, 2015).

5.3.5 Whole genome sequencing

Whole genome sequencing (WGS) was performed using the MiSeq® platform using the MiSeq® version 3 cartridge (2 x 300bp) (Illumina®, San Diego, USA) and used to resolve discordance between phenotypic DST and LPA findings. Library preparation was performed using the Nextera XT® kit. Variant detection was performed using the resequencing module with the reference strain *M. tuberculosis* H37Rv (NC_000962) on CLC Genomics Workbench (v7.5.1).

5.3.6 Reproducibility and Agreement

Reproducibility was defined as the ability of a method to achieve the same results on repeat testing of the same samples at different sites or different operators (Banoo et al., 2008; Ebentier, et.al., 2013). For the assessment of inter-laboratory reproducibility and agreement of the assay, a panel comprising 10 selected *M. tuberculosis* isolates present in triplicate was distributed to each of six NHLS TB

laboratories performing phenotypic DST. An overall agreement of ≥90% was deemed as acceptable (Fattorini et al., 2012; Migliori et al., 2000).

5.3.7 Analysis of results

Evaluation of agreement between different assays and sites was determined using Cohen's Kappa (**κ**) and interpreted as described previously Kwiecien et al., 2011). The **κ** statistic measures the degree of interrater agreement, a measure of test reliability and was interpreted using the following scale: <0.20 =poor; 0.21 – 0.40 = fair; 0.41 – 0.60 = moderate; 0.61 -0.80 = good; 0.81 -1.0 = very good Kwiecien et al., 2011). To determine agreement under comparative target conditions, two procedures were used together as a combined reference standard (CRS), i.e. in-house drug phenotypic DST and Genotype® MTBDR*sl* VER 2.0 LPA, on the same isolate. If an isolate tested susceptible / resistant by in-house DST and had the corresponding wild types or mutations in the *gyr*A and *gyr*B (FLQ) or *rrs* and *eis* (SLID) genes present, the isolate was considered as having the target condition (susceptibility or resistance). Discordant results were resolved using WGS data for the isolate. Sensitivity, specificity, positive and negative predictive values (PPV and NPV), 95% confidence intervals (95% CI) and diagnostic accuracy were calculated using CRS. Statistical analyses were performed using Stata: Release 13 (Stata Press, 2013).

5.4 Results

5.4.1 Performance of phenotypic DST using BD second-line lyophilised drug kit

In order to accurately determine the performance of the BD second-line DST lyophilised drugs in comparison with in-house drug preparations on Bactec MGIT™ 960 DST, 17/142 (12.0%) isolates were excluded from the analysis due to Epicentre AST errors, loss of viability or contamination. There were 125/142 (88.0%) isolates in the final analysis that yielded interpretable results for all three assays tested.

The overall agreement and κ- values of 99.2% and 0.985 for FLQ and 97.4% and 0.943 for SLID, respectively. The inter-laboratory performance for reproducibility amongst the seven sites assessed for the BD second-line lyophilised drug kit compared with Genotype® MTBDR*sl* VER 2.0 LPA ranged between 90 - 100%, with all sites meeting the acceptance criteria.

The sensitivity, specificity and accuracy indices using the CRS were estimated for FLQ (98.3%, 100% and 99.2%) and SLID (95.2%, 98.5% and 97.3%) respectively, and the individual drug performance is shown in Table 5.1. FLQ resistance was observed in 57/125 (45.6%) and SLID resistance in 42/125 (33.6%) of the isolates tested. There were 31/125 (24.8%) isolates classified as XDR-TB. All probe bands of the Genotype® MTBDR*sl* VER 2.0 LPA were interpretable in all samples tested (Hain Lifescience, 2015)

Table 5.1: Performance of *Combined Reference Standard vs BD Second-Line Lyophilised Drug Kit

Drug (n=129)	SENS (%)	95% CI	SPEC (%)	95% CI	PPV (%)	95% CI	NPV (%)	95% CI	Diagnostic Accuracy (%)
OFX 2.0 µg/ml	98.3	91.1 - 100.0	100.0	94.5 - 100.0	100.0	93.9 - 100.0	98.5	91.8 - 100.0	99.2
MFX 0.5 µg/ml	86.2	74.6 - 93.9	100.0	94.6 -100.0	100.0	92.9 - 100.0	89.3	80.1 - 95.3	93.6
KAN 2.5 µg/ml	93.5	82.1 - 98.6	96.9	89.2 - 99.6	95.6	84.9 - 99.5	95.4	87.1 - 99.0	95.5
AMK 1.0 µg/ml	88.6	75.4 - 96.2	100.0	94.6 - 100.0	100.0	91.0 - 100.0	93.0	84.3 - 97.7	95.5
CAP 2.5 µg/ml	95.2	83.8 - 99.4	98.5	92.1 - 100.0	97.6	87.1 - 99.9	97.1	89.9 - 99.6	97.3

*Combined Reference Standard = In-house drugs phenotypic DST & Genotype® MTBDRsl VER 2.0 LPA

5.4.2 Detection of FLQ Resistance

Amongst the 57/125 (45.6%) phenotypically FLQ-resistant isolates, the Genotype® MTBDR*sl* VER 2.0 LPA detected the majority of defined and undefined mutations at *gyr*A codons 94 and 90 respectively. The mutations observed were; 16/57 (28.1%) *gyr*AMUT3C / D94G, 5/57 (8.8%) *gyr*AMUT3B / D94N/D94Y, 4/57 (7.0%) *gyr*AMUT3A / D94A, 3/57 (5.3%) *gyr*AMUT3D / D94H and 11/57 (19.3%) *gyr*AMUT1 / A90V. Multiple mutations were observed in three FLQ-resistant isolates with *gyr*AMUT3B / D94N/D94Y and *gyr*AMUT3D / D94H in two isolates (2/57, 3.5%) and one isolate showing *gyr*AMUT1 / A90V and *gyr*AMUT3C / D94G. Undefined mutations were observed at *gyr*A WT3 (11/57, 19.3%) and one at *gyr*A WT2.

In the subset of 58 isolates tested for MXF 2.0 µg/ml the performance indices were respectively, sensitivity (87.5%, 95% CI, 47.3 - 99.7%), specificity (100.0%, 95% CI, 92.9 - 100.0%), and diagnostic accuracy 98.3%. There were 7/58 (12.1%) phenotypically MXF-resistant isolates. Among the strains tested at both MXF critical concentrations, the majority of mutations were detected at codon 94, with *gyr*AMUT3C / D94G detected in 18/58 (31.0%) at MFX 0.5 µg/ml and 5/58 (8.6%) at MFX 2.0 µg/ml. In contrast, *gyr*AMUT1 / A90V showed resistance in 9/58 (15.5%) at MFX 0.5 µg/ml and 1/58 (1.7%) at MFX 2.0 µg/ml. These results were consistent with both phenotypic DST assays performed and were confirmed by WGS data.

5.4.3 Detection of SLID Resistance

SLID resistance was observed in 41/125 (32.8%) of isolates tested. The predominant defined mutation observed using Genotype® MTBDR*sl* VER 2.0 LPA was *rrs*MUT1/A1401G and was present in 38/41 (92.7%) isolates. Phenotypic resistance to KAN, AMK and CAP using in-house drugs and BD second-line lyophilised drugs was found in 36/38 (94.7%) of isolates with the *rrs* mutation. SLID with *eis* undefined mutation was observed in 3/41 (7.3 %) isolates. All three isolates were phenotypically resistant to KAN and AMK and susceptible to CAP with *eis* WT only observed by WGS.

5.4.4 Discordant Samples

When using the combined reference standard, discordance was observed in 15/125 (12.0%) FLQ and 6/125 (4.8%) SLID isolates as shown in Table 5.2. There was one isolate resistant to OFX and MFX 0.5 µg/ml by in-house drugs and only OFX-resistant by BD drugs where no mutations were detected in *gyr*A and *gyr*B regions by LPA and WGS. The remaining FLQ discordant results occurred at MFX 0.5 µg/ml critical concentration rather than with OFX. There were 2/41 (4.8%) SLID-phenotypically resistant isolates with wild type only observed in *rrs* and *eis* regions on both LPA and WGS testing.

Table 5.2: Analysis of Discordant results between *Combined Reference Standard vs BD second-line phenotypic DST (n = 21/125)

FLQ Drug Resistance Phenotype									
In-House SL MGIT DST			**MTBDRsl VER 2.0 LPA**		**BD SL MGIT DST**			**WGS**	
OFX 2.0 µg/ml	**MFX 0.5 µg/ml**	**MFX 2.0 µg/ml**	***gyr*A**	***gyr*B**	**OFX 2.0 µg/ml**	**MFX 0.5 µg/ml**	**MFX 2.0 µg/ml**	**Amino Acid Change**	**n**
R	R	S	*gyr*AMUT1 / A90V	WT	R	S	S	Ala90Val	4
R	R	S	*gyr*AMUT3A / D94A	WT	R	S	S	Asp94Ala	3
R	R	Not Done	*gyr*AMUT3B D94N/D94Y	WT	S	S	S	Asp94Asn	1
R	R	S	*gyr*AMUT3B/ D94N/D94Y + *gyr*AMUT3D/ D94H	WT	R	R	R	Asp94His	2
R	R	S	*gyr*AMUT3C /D94G	WT	R	R	R	Asp94His	1
R	R	S	*gyr*AWT1 missing	WT	R	S	S	Gly88Ala	1
S	S	S	*gyr*AWT3 missing	WT	S	S	S	WT	2
R	R	S	WT	WT	R	S	S	WT	1
SLID Resistance Phenotype									
In-House SL MGIT DST			**MTBDRsl VER 2.0 LPA**		**BD SL MGIT DST**			**WGS**	
KAN 2.5 µg/ml	**AMK 1.0 µg/ml**	**CAP 2.5 µg/ml**	***rrs***	***eis***	**KAN 2.5 µg/ml**	**AMK 1.0 µg/ml**	**CAP 2.5 µg/ml**	**Amino Acid Change**	**n**
R	R	R	*rrs* MUT1/ A1401G	WT	R	S	R	A1401G	1
R	R	R	WT	WT	R	R	R	WT	2
R	S	S	WT	*WT*	S	S	S	WT	2
R	R	R	WT	WT	S	S	S	WT	1

5.5 Discussion

The performance of standardised BD second-line lyophilised drugs on phenotypic DST using the Bactec MGIT™ 960 TB System was excellent. It addresses an important gap to improve standardisation across laboratories and thereby enhance the quality and reliability of results for patient care.

The assay showed very good overall agreement (FLQ 99.2 % and SLID 97.4%) between the in-house drugs and BD second-line lyophilised drug kit. Consistent with published data (Migliori et al., 2000; Fattorini et al., 2012), the overall reproducibility was also good (90% -100%), considering the inherent challenges of phenotypic DST assays such as contamination, strain fitness and viability.

Our study used a combined reference standard to address variability in testing, providing a robust comparator. The diagnostic accuracy was above 95% for all drugs except MFX 0.5 µg/ml. This is understandable, as the critical concentration for moxifloxacin has been disputed (Zignol et al., 2016) and a recent WHO Expert Group meeting has considered reducing the MFX-CC for 0.5 µg/ml to 0.25 µg/ml and the MFX-CB from 2.0 µg/ml to 1.0 µg/ml (WHO Expert Group, 2017; WHO/HTM/TB/2017.22). As strains may have MIC's close to the CC, this revision is likely to resolve these issues. This was also apparent from the discordance testing where many of the *gyr*A mutations tested susceptible at MFX 0.5 µg/ml with the BD second-line lyophilised drug kit.

The data shows that the majority of mutations encoding FLQ resistance were observed in *gyr*A among codons 94 and 90 and for SLID at the *rrs*1401 codon. Amongst the FLQ-resistant isolates, 79.3% cross-resistance was observed between OFX at 2.0 µg/ml and MXF at 0.5 µg/ml with SLID (KAN/AMK/CAP) cross-resistance observed in 94.7% of isolates harbouring *rrs* MUT1/A1401G mutation. These results reflect data previously reported for FLQ and SLID cross-resistance (Kambli et al., 2014 & 2015). Phenotypically resistant FLQ and SLID isolates where no mutations were detected by LPA and WGS possibly harbour mutations which are located outside the *gyr*A and *gyr*B or *rrs* and *eis* regions or exhibit resistance caused by other mechanisms. The two (2) SLID discordant isolates, which most likely reflect technical errors in DST testing, highlights the complexity of phenotypic DST testing.

Since culture-based DST methods remain the WHO recommended reference standard (WHO Global TB Report 2017) the implementation of standardised DST methods provides reliability in testing to routine laboratories globally. The BD second-line lyophilised drug kit offers routine laboratories a standardised ready-to-use option for phenotypic DST. The inherent challenges and complexities of drug preparation and quality control of these preparations are not required when using the kit.

A limitation of this study was the paucity of data available for phenotypic DST comparison for MFX 2.0 µg/ml as this critical concentration was only implemented in the latter part of 2014 within the South African TB diagnostic algorithm.

5.6 Conclusion

The BD second-line lyophilised drug kit on Bactec MGIT™ 960 phenotypic DST showed good correlation between the in-house drugs and genotypic methods allowing for standardised and simplified phenotypic DST for routine laboratories.

This study provides evidence for the implementation of this kit which addresses an essential gap for TB laboratories globally to enable the reliable diagnostic assessment of DR-TB patients.

CHAPTER 6

6 SUMMARY OF MAIN FINDINGS AND CONCLUDING REMARKS

High rates of DR-TB are indicative of the failure of a country's national TB management program to function effectively, including the failure to control and treat drug susceptible TB successfully, and prevent DR-TB transmission. Currently, South Africa is listed amongst the 14 countries that appear in all three WHO 2016 -2020 revised "high burden country" (HBC) lists (WHO Global TB Report 2015 & 2016).

The South African National Department of Health implemented new rapid diagnostic testing methods, including the Genotype® MTBDR*plus* VER 2.0 LPA (2008) and Xpert® MTB/Rif (2011) to improve the turn-around-time from specimen collection to laboratory-based diagnosis of TB disease in patients (WHO SA, SA NDOH, and USAID 2016). These strategic improvements have assisted in offering, high quality laboratory testing that is faster and standardised. However, there are crucial questions to be addressed:

(a) How should the gaps that still exist in the rapid diagnosis of pre-XDR and XDR-TB be closed?
(b) What change(s) can be implemented to alleviate the challenges related to non-standardised second-line phenotypic DST?

6.1 Rapid diagnostic testing for the detection of resistance to second-line anti-TB drugs for pre-XDR and XDR-TB

The present study was conducted to evaluate the performance and reproducibility of the newly designed Genotype® MTBDRsl VER 2.0 LPA to detect second-line drug resistance in *M. tuberculosis* clinical isolates and in decontaminated sputum samples with known status of RR-TB detected by the Xpert MTB/Rif or MDR-TB detected by the MTBRplus VER 2.0 LPA. The findings of the study showed an overall improvement in the performance of the Genotype® MTBDRsl VER 2.0 LPA on clinical isolates (Tagliani et al.,2015) . The respective sensitivity and specificity indices for FLQ resistance detection for Genotype® MTBDRsl VER 2.0 LPA were 100% (95%

CI, 95.8 - 100%) and 98.9% (95% CI, 96.1 - 99.9%) while for SLID the indices were 89.2% (95% CI, 79.1 - 95.6%) and 98.5% (95% CI, 95.7 - 99.7%) respectively (Gardee et al., 2017). The WHO recommended the use of Genotype® MTBDR*sl* VER 2.0 LPA as an initial rapid screening test for the detection of FLQ and SLID resistance in confirmed RR-TB and MDR-TB patients in May 2016 (WHO Policy Guidance - molecular line probe assays, 2016).

The assessment of the performance of the assay on smear-positive decontaminated sputum samples by microscopy, showed good sensitivity, specificity and diagnostic accuracy for the detection of FLQ resistance 76.8% (95% CI, 63.6 - 87.0%), 89.7% (95% CI, 80.8 - 95.5%) and 84.2%, and for SLID resistance status, 86.2% (95% CI, 75.3 - 93.5%), 91.2% (95% CI, 81.8 - 96.7%) and 88.7%, respectively. The results are comparable with data published in the WHO 2016 Policy Guidance Document (WHO Policy Guidance - molecular line probe assays, 2016).

6.2 Standardised phenotypic DST for second-line anti-TB drugs

The lack of standardised phenotypic DST methods for second-line anti-TB drugs (liquid or solid culture media) and drug preparations used may, at least in part, explain the variability of results obtained by laboratories. Adequate training of laboratory staff is essential to ensure reliability and reproducibility of results (WHO SL-DST POLICY, 2008). Clear guidelines and recommendations on specific DST methodology implemented within the National TB Diagnostic Algorithm will help to ensure consistency and reliability of results obtained by laboratories within the country.

As part of the present study, the performance and reproducibility of phenotypic DST using standardised Becton Dickinson® (BD) second-line DST lyophilised drug kit was evaluated. Training was provided to key laboratory staff in the new methods in all participating laboratories. A proficiency-testing panel of *M. tuberculosis* isolates was distributed to laboratories to assess participant performance and inter-laboratory reproducibility of the drug kit. Results were analysed using a combined reference standard (CRS), i.e. in-house drug phenotypic DST and Genotype® MTBDR*sl* VER

2.0 LPA on the same isolate to assess the performance of the new standardised assay.

The results obtained showed excellent agreement (FLQ 99.2% and SLID 97.4%) between the in-house prepared drug concentrations and the standardised BD second-line DST lyophilised drug kit. Phenotypic resistance of the isolates tested correlated well with the results of the resistance mutations detected by Genotype® MTBDR*sl* VER 2.0 LPA for *gyr*A, *gyr*B, *rrs* and *eis* regions and were confirmed by WGS testing. Inter-laboratory reproducibility for the standardised BD second-line lyophilised drug kit was >90% for all participants (Migliori et al., 2000; Fattorini et al., 2012).

6.3 Concluding Remarks

Prompt diagnosis of pre-XDR and XDR-TB patients is critical to ensure the correct choice of treatment regimen for improved patient outcomes. The incorporation of a validated rapid diagnostic test and standardised phenotypic DST assay into the TB diagnostic algorithm of the country is likely to lead to significant improvement of South Africa's TB Control Program. The results obtained on the performance and diagnostic accuracy of the Genotype® MTBDR*sl* VER 2.0 LPA for clinical isolates of *M. tuberculosis* complex were submitted to WHO for assessment of the assay (WHO Policy Guidance - molecular line probe assays, 2016). In the process, the present study has provided further evidence to support the suitability of the Genotype® MTBDR*sl* VER 2.0 LPA (Gardee et al., 2017) and the standardised BD second-line DST drug kit for implementation into South Africa's TB diagnostic algorithm.

In January 2017, the NDOH and NHLS implemented the Genotype® MTBDR*sl* VER 2.0 LPA as per WHO recommendations into South Africa's TB diagnostic algorithm (Appendix E) (WHO Rapid diagnostic test, 2016; WHO Policy Guidance - molecular line probe assays, 2016). The successful evaluation of the standardised BD second-line DST lyophilised drug kit has prompted the implementation of the assay for use in NHLS TB laboratories, with support from the CTB –NTBRL at NICD.

6.4 Limitations of the study

One of the limitations of the study was that the collection of samples and isolates used may not have been completely representative of the DR-TB burden in all provinces of South Africa. Another limitation was the limited number of smear-negative sputum samples, that were available for testing the performance of Genotype® MTBDR*sl* VER 2.0 LPA, especially in the face of the HIV/AIDS epidemic where smear negativity in HIV-positive TB patients is common. Furthermore, regarding proficiency and reproducibility assessments, no historical data was available for comparison with the results of this study, and no formal or inter-laboratory proficiency testing schemes (PTS) or external quality assessment (EQA) programs are currently implemented at NHLS TB laboratories for phenotypic and genotypic SL-DST testing of *M. tuberculosis* isolates for susceptibility to second-line anti-TB drugs. A pilot SL-DST phenotypic and genotypic PTS managed by the CTB, NICD was conducted in 2017 to address this gap.

6.5 Future work

The assessment of the performance and cost effectiveness of testing smear-negative sputum on direct Genotype® MTBDR*sl* VER 2.0 LPA and the implementation of decentralised SL-DST in the South African National TB Management Program are high priorities for the near future. Also, as recommended by WHO, implementation of laboratory quality assurance programs is an essential requirement for ensuring good quality, reliable and reproducible results (WHO 2015). Thus, early implementation of an on-going PTS program for phenotypic and genotypic SL-DST in NHLS laboratories is paramount.

The use of drug resistance data gathered from surveillance and surveys will assist in measuring the achievement of goals set by the WHO-endorsed End TB Strategy (2016 – 2035), that is, *"to end the global TB epidemic"* (WHO Global TB Report, 2016).

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8 APPENDICES