Characterization of *Mycobacterium tuberculosis* isolates with discordant rifampicin susceptibility test results

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DECLARATIONS

I, *Yonas Tesfagabir Ghebrekristos,* hereby declare that the work on which this dissertation is based on my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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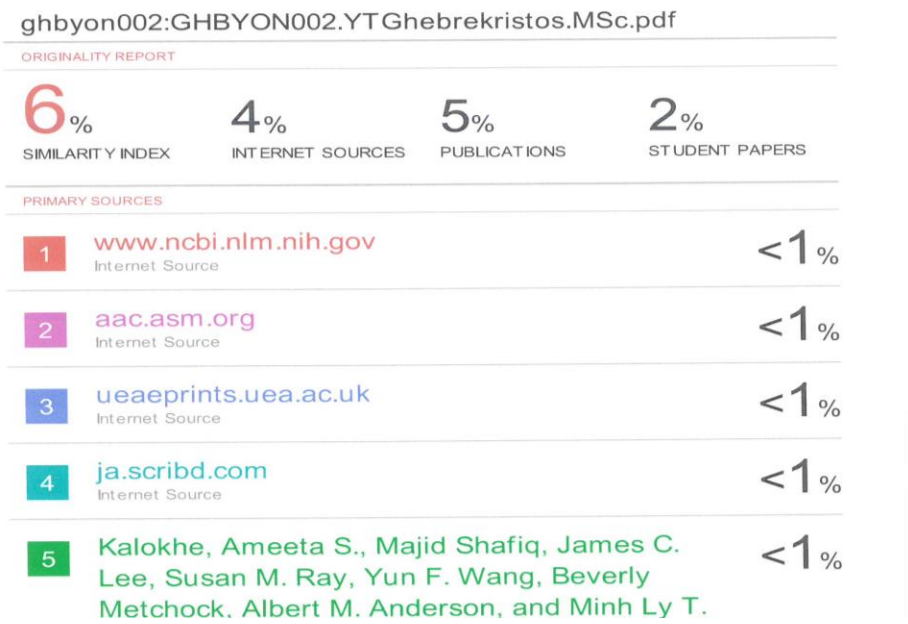

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PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

1. Ghebrekristos Y, Simpson J, Wojno J, Allen V, da Silva M, Nicol M, Beylis N. Characterization of Mycobacterium tuberculosis complex isolates with discordant rifampicin susceptibility results detected in a routine TB laboratory in Cape Town. Presented as a poster at the 46th Union World Conference on Lung Health and Tuberculosis held in Cape Town, South Africa on 04 December 2015

2. Ghebrekristos Y. Presented at Clinical Laboratory Science Postgraduate Research Day at University of Cape Town on 02 September 2015

3. Beylis N, Nicol M. Resolving discordant susceptibility results. Presented at the European Society of Clinical Microbiology and Infectious Diseases postgraduate technical workshop: Diagnosis and Management of drug-resistant tuberculosis held in Cape Town, South Africa held on 18-19 January 2016

4. Laboratory based national guideline that is now used within National Health Laboratory Service TB laboratories (South Africa) detailing steps on how to detect possible false RIF^{R} results by Xpert MTB/RIF and on how to troubleshoot discordant Xpert RIF^{R} and LPA RIF^{S} results.

5. Beylis N, Ghebrekristos Y. Resolving discordant susceptibility results. Presented at the TB forum Tygerberg Hospital, Cape Town September 2016

6. Moodley, C. Presented at the National Health Laboratory Service Research Day in University of Cape Town, Cape Town on 08 September 2016

7. Ghebrekristos, Y, Beylis N. MSc work to be presented at the International Pathology Day in University of Cape Town, Cape Town on 15 November 2017

PREFACE

This dissertation is submitted for the degree of Master of Science in Medicine (MSc Med) in Medical Microbiology at the Division of Medical Microbiology, Department of Clinical Sciences, University of Cape Town, South Africa. The study was approved by the Human Research Ethics Committee (HREC) of the University of Cape Town, South Africa (HREC REF: 737/2014). The study was supported by National Health Laboratory Service (NHLS) Grant (South Africa) and Med Microbiology. The work reported in this dissertation resulted from a collaborative effort between TB laboratories Groote Schuur Hospital and Green Point Complex TB laboratories, NHLS, Cape Town, South Africa.

The aim of this dissertation was to describe the two most commonly encountered discordant rifampicin (RIF) susceptibility results encountered in the routine National Tuberculosis Control Programme (NTBCP) testing algorithm of South Africa. Part 1 of the study focuses on discordant RIF resistant (RIF^R) result by Xpert MTBB/RIF (Xpert) and RIF susceptible (RIF^S) result by line probe assay (LPA) and determines which result is the true result as determined by sequencing. Part 2 of the study focuses on RIF^R isolates with miscellaneous *rpo*B mutations detected by LPA and to determine the minimum inhibitory concentration (MIC) level of isolates with these specific mutations when routine liquid drug susceptibility testing is expected to miss resistance.

The MSc candidate performed DNA extractions for purposes of the study for LPA and *rpo*B PCR. The MSc candidate performed and interpreted the following assays; repeat LPA, *rpo*B PCR and MIC. PCR products of *rpo*B PCR were sent to Inqaba Biotechnical Industries (Pretoria, South Africa) for Sanger sequencing and sequencing results were sent back to the MSc candidate and the MSc candidate interpreted sequencing results.

The submitted material is the work of the MSc candidate, unless stated otherwise by acknowledgments.

ABSTRACT

Background: The Xpert MTB/RIF assay was adopted as the initial diagnostic test for patients with presumptive tuberculosis (TB) by the South African National TB Control programme in December 2010. Rifampicin (RIF) resistance detected by the Xpert MTB/RIF (Xpert) is confirmed by a line probe assay (LPA) (GenoType MTBDR*plus*) and/or phenotypic (culture-based) drug susceptibility testing (DST) by MGIT (Mycobacterial Growth Indicator Tube) on the culture isolate from a 2nd specimen.

Although both the Xpert and LPA target the rifampicin resistance determining region (RRDR) of the *rpo*B gene, discordant RIF results (Xpert RIF resistant (RIF^R), LPA RIF susceptible (RIF^S)) have been reported. In addition, in cases where genotypic tests detect an *rpo*B mutation, inferring RIF resistance, routine phenotypic DST may report a RIF susceptible result. This is usually due to disputed *rpo*B mutations.

Aim: The aims of this study are to determine 1) whether the discordance between Xpert and LPA is due to false RIF^R by Xpert or false RIF^S by LPA and to elucidate the causes of false results and 2) the frequency and types of *rpo*B mutations expected to test susceptible on routine phenotypic DST and their corresponding RIF MIC (minimum inhibitory concentration).

Methods*:* Consecutive isolates with discordant Xpert RIF^R and LPA RIF^S results were selected during routine review. For the Xpert, parameters including bacterial DNA load and cycle threshold (Ct) of the probes were evaluated. In addition, isolates with a pattern of any absent *rpo*B WT band and absent MUT band on the LPA strip ("miscellaneous *rpo*B mutations") were selected for MIC testing using the MGIT 960 system and EpiCenter TB eXiST software. Sanger sequencing of the *rpo*B gene from codon 462 to 591 was performed on all selected isolates.

Results and discussion: ***Discordant Xpert/LPA results***: From the total of 1542 patients with RIF^R results by Xpert, 106 (6.9%) had a discordant LPA RIF^S result. Sequencing results were available for 101 isolates of which 78 (77.2%) had no *rpo*B mutation detected and these were categorized as false RIF^R by Xpert. Mutations were detected by sequencing in the remaining 23 (22.8%); these were categorized as false RIF^S by LPA. Probe delay occurred in 56/76 (73.7%) cases compared with 104/1436 (7.2%) controls (p <0.0001). Probe delay with ΔCt max value between 4.1 and 4.9 and is also a significant predictor of false RIF^R by Xpert (p value <0.001). Double probe delay was observed only in the false RIF^R by Xpert group and occurred in 12/76 (15.8 %) with p value <0.001. "Very Low" bacterial load occurred in 47/76 (61.8%) cases compared with 192/1436 (13.4%) controls (p <0.0001) and is a significant predictor of false RIF^R by Xpert. A RIF^R result by Xpert that is determined by probe delay where the ΔCt max is >4 and there is a Very Low bacterial load has a positive predictive value (PPV) of 64.2 % of being false and where the ΔCt max is between 4.1 and 4.9 with Very Low bacterial load, the PPV of a false result increases to 85.7%. For the false RIF^S results by LPA, the majority 11/23 (47.8%) were due to technical errors. In 6/23 (26.1%) it was due to mixed infection and in 2/23 (8.7%) there was laboratory mix up. In the remaining 4/23 (17.4%) the cause could not be determined and mixed infection or a laboratory mix up could not be excluded.

Discordant genotypic/phenotypic results: RIF resistance was detected in 1502 patients by LPA, of which 169 (11.3%) had a miscellaneous mutation. In addition, a further 21 isolates were selected from "Part 1" of the study, where sequencing confirmed that the *rpo*B mutation was not one of the high level / high confidence *rpo*B mutations. A total of 178 isolates had both MIC and *rpo*B sequencing results. In our study 140/178 (78.7%) isolates with miscellaneous *rpo*B mutations (n=158) or previously described disputed *rpo*B mutations (n=20) had MIC values ranging from ≤0.0625 µg/ml to 1.0 µg/ml. An MIC >1.0 µg/m was determined for 38/178 (21.3%) that would have tested RIF^R by MGIT DST.

Conclusion: Arising from this study is a laboratory based guideline that is now used within NHLS TB laboratories detailing steps on how to detect possible false RIF^R results by Xpert MTB/RIF and on how to troubleshoot discordant Xpert RIF^R and LPA RIF^S results. A database has been created from the results obtained in this study that lists specific *rpo*B mutations and their corresponding MIC value and has the potential to assist clinicians in individualizing the patient TB treatment regimen.

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ABBREVIATIONS

%	Percentage
ΔCt max	Delta cycle threshold maximum
ΔCt min	Delta cycle threshold minimum
AC	Amplification control
AFB	Acid fast bacilli
BD	Becton Dickinson
BSC	Biosafety cabinet
CC	Conjugate control
CM	Common Mycobacteria
CI	Confidence interval
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
DST	Drug susceptibility testing
E. coli	*Escherichia coli*
EMB	Ethambutol
EQA	external quality assurance programme
GPT	Green Point
GSH	Groote Schuur Hospital
GU	Growth unit
HIV	Human immunodeficiency virus
INH	Isoniazid
IRR	Incident rate ratio
kDa	kiloDalton

LJ	Löwenstein–Jensen
LIS	Laboratory Information System
LPA	Line probe assay (MTBDR*plus*)
MDR	Multidrug-resistant
MGIT	Mycobacterium Growth Indicator Tube
MIC	Minimum inhibitory concentration
Ml	Milliliter
MTB	*Mycobacterium tuberculosis*
MS	Microsoft
MIRU	Mycobacterial Interspersed Repetitive Units
MUT	Mutation
NALC	N-acetyl-L-cysteine
NaOH	Sodium hydroxide
NGS	Next generation sequencing
NHLS	National Health Laboratory Service
NPV	Negative predicative value
NTBCP	National Tuberculosis Control Program
NTM	Non-tuberculous mycobacteria
PCR	Polymerase chain reaction
PD	Pharmacodynamic
PK	Pharmacokinetic
PPV	Positive predicative value
PZA	Pyrazinamide
QC	Quality control

RIF	Rifampicin
RIF^R	Rifampicin resistant
RIF^S	Rifampicin susceptible
RR	Rifampicin resistant
RRDR	Rifampicin Resistance Determining Region
SA	South Africa
Sens	Sensitivity
SIRE	Streptomycin, isoniazid, rifampicin, ethambutol
SNP	Single nucleotide polymorphism
Spec	Specificity
STR	Streptomycin
TB	Tuberculosis
µg	microgram
V2	Version 2
VL	Very low
VNTR	Variable Number of Tandem Repeats
WGS	Whole genome sequencing
WHO	World Health Organization
WT	Wild type
Xpert	Xpert MTB/RIF
ZN	Ziehl Neelsen

GLOSSARY OF TERMS

Borderline RIF resistance is defined as a discrepancy between liquid and solid culture based DST methods, with the liquid-based phenotypic DST method reporting RIF^S but the solid method phenotypic DST reporting RIF^R and genotypic testing (either or both of Xpert and LPA and / or *rpo*B sequencing) detects an *rpo*B mutation and the MIC is >0.5 and ≤1.0 µg/ml (Van Deun et al., 2013).

Clonal heterogeneity is clonal diversity with different MIRU-VNTR patterns due to one clone of MTB that has evolved over a shorter period of time from susceptible to resistant (Cohen et al., 2012).

Delta Ct max (ΔCt max) is defined as the difference between the Ct values of the latest and earliest probes of Xpert MTB/RIF (Lawn and Nicol, 2011).

Delta Ct min (ΔCt min) is the smallest Ct difference between any pair of probes (Xpert MTB/RIF) (Lawn and Nicol, 2011).

Disputed *rpo*B mutation is an *rpo*B mutation that is phenotypically RIF^S with routinely used liquid based methods and thus give discordant genotypic / phenotypic rifampicin results (Van Deun et al., 2013).

a. There is a RIF susceptible MGIT result OR
b. The MIC result is either susceptible (<0.0625ug/mml) or low-level RIF resistant (> 0.0625 and <=1g/ml)

False RIF^S result by LPA: Rifampicin resistance was reported by Xpert, but LPA reported a RIF susceptible result. There was a mutation detected in the RRDR of the *rpo*B gene by sequencing.

False RIF^R result by Xpert: Rifampicin resistance was reported by Xpert, but LPA reported RIF susceptible. There was no mutation detected in the RRDR of *rpo*B gene by *rpo*B sequencing.

Heteroresistance is defined as the presence of both susceptible and resistant bacteria in a specimen or MTB culture (Hofmann-Thiel et al., 2009)

A high level / high confidence *rpo*B mutation is one of the following mutations: S531L / H526Y / H526D / D516V

a. It is clearly identified by the LPA as the following *rpo*B band patterns: [[WT8 absent and MUT3 present]; [WT7 absent and MUT 2A present]; [WT7 absent and MUT2B present] and [WT3&4 absent and MUT 1 present] respectively
b. The expected MIC would be high level (>32ug/ml) (Van Deun et al., 2013)
c. The MGIT RIF result is always resistant (concordant LPA and MGIT results = R)

Low level RIF resistance occurs when both liquid and solid media phenotypic DST results show susceptibility to RIF at the routine critical concentration recommended for each method (no discrepancy between the two phenotypic methods) but the genotypic test (either or both of Xpert and LPA and / or *rpo*B sequencing) detects an *rpo*B mutation and the MIC is >0.0625 and ≤0.5 µg/ml and the MGIT DST result would be expected to be RIF susceptible (Van Deun et al., 2013).

Miscellaneous *rpo*B mutations are all *rpo*B mutation in the RIF resistance determining region (RRDR) that are detected by the LPA except the high level / high confidence *rpo*B mutations. It is identified by the LPA pattern of an (any) [absent WT band and absent MUT bands]. At the time of selection of isolates for this study, the MIC, MGIT result and actual *rpo*B mutation (that would be determined by sequencing), are not known.

Mixed infection is the presence of different clones of MTB with different MIRU-VNTR patterns at two or more loci in the same specimen occurring as a result of multiple infections (Cohen et al., 2012).

Probe delay is an Xpert analysis where MTB is detected and one or more probes have delayed onset of fluorescence with delta Ct max value of >4 (Lawn and Nicol, 2011).

Probe dropout is an Xpert analysis where MTB is detected and one or more probes have a Ct value of zero (Lawn and Nicol, 2011).

Susceptible *rpo*B mutation

a. A miscellaneous / disputed *rpo*B mutation where there is an MIC result available for it and the MIC is ≤0.0625ug/ml (Gumbo, 2010)
b. MGIT would test RIF susceptible

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Figure 7: MIC value in isolates with mutations in codon 526. The majority (84%) of miscellaneous *rpo*B mutations in codon 526 translate to low level RIF resistance or RIF^S by phenotypic testing.

Figure 8: LPA strip of an isolate from patient D6 showing absent *rpo*B WT8 and absent MUT bands which is reported as RIF^R. DNA sequencing detected L533P. The MIC was 0.25µg/ml which is lower than the critical concentration (1µg/ml) and would be expected to test RIF susceptible by MGIT DST

Figure 9: LPA strip of an isolate from patient F4 showing absent *rpo*B WT3&4 probes and absent MUT bands as well as absent *kat*G WT probe and *kat*G MUT1 probe present and therefore reported as MDR. DNA sequencing showed D516Y mutation and MIC value was ≤0.5µg/ml which is lower than the critical concentration (1 µg/ml); would be expected to test RIF^S by MGIT DST.

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CHAPTER 1

1. Literature review

1.1 Introduction

Tuberculosis, caused by *Mycobacterium tuberculosis* (MTB), is the greatest killer due to a single infectious agent worldwide (WHO, 2016). In 2015, MTB was responsible for approximately 1.8 million deaths (WHO, 2016). Over 95% of tuberculosis (TB) deaths occur in low- and middle-income countries. Multidrug-resistant TB (MDR-TB) is defined as MTB that is resistant to at least both of the first-line anti-TB drugs isoniazid (INH) and rifampicin (RIF). It poses a serious challenge for global TB control and makes successful treatment difficult. In 2015, there were an estimated 480 000 people who developed MDR-TB worldwide (WHO, 2016).

In South Africa, the same is true that TB is the greatest infectious killer and South Africa was among the six countries that had the largest number of incident cases in 2015 (WHO, 2016). There were 450,000 new cases of active TB reported in South Africa in 2015, equating to 1% of the total South African population and South Africa had the second highest absolute number of notified RIF-resistant (RR)/MDR cases globally with 18 734 (WHO, 2016).

Globally, in 2015, approximately 3.9% of new TB cases were MDR-TB and 21% of previously treated cases were MDR-TB (WHO, 2016). In South Africa, 2.1% of new cases and 4.3% of retreatment cases were MDR-TB (WHO, 2016). In 2014 South Africa ranked tenth (amounting to 2.1% of the total global burden) among the 27 high burden MDR-TB countries (WHO, 2015).

Lack of access to early diagnosis of drug resistant TB is one of the major obstacles toward the correct management and control of drug resistant TB. The development of commercially available rapid molecular tests that are applicable in high TB prevalence low-income settings, show great promise in meeting this need. Two such examples are the GenoType® MTBDR*plus* line probe assay

(LPA) (Hain Lifescience, Nehren, Germany) and the Xpert MTB/RIF (Xpert) (Cepheid, Sunnyvale, USA). The LPA and Xpert assays are genotypic assays that detect mutations in the MTB genome conferring RIF (and isoniazid (INH) in the case of the LPA) resistance. Xpert detects MTB in specimens in less than one day (usually 3-4 hours) and the LPA (if performed directly on smear positive specimens or on a cultured isolate of MTB) also has a short turnaround time of 24-48 hours. In addition, they both have a high sensitivity for the detection of RIF resistance (Xpert [pooled estimate] 95% (Steingart et al., 2014) and LPA [pooled estimate] 98%) (Morgan et al., 2005)). In 2008, the World Health Organization (WHO) endorsed the use of the LPA for the detection of drug resistant TB in areas with a high prevalence of HIV and MDR-TB (WHO, 2010b). In 2010, the WHO recommended Xpert for both the diagnosis of TB and the rapid detection of RIF resistance (WHO, 2010c, Boehme et al., 2010). Both LPA and Xpert were evaluated in comparison with culture based (phenotypic) drug susceptibility testing methods.

Rifampicin, first introduced in 1972, is a key drug in the four-drug treatment regimen of tuberculosis. RIF has early bactericidal effects on metabolically active MTB. It also has excellent sterilizing activity against semi-dormant (i.e. slow or non-growing) MTB (Somoskovi et al., 2001, Almeida Da Silva and Palomino, 2011). The introduction of RIF to combined therapy reduced the duration of TB treatment from 18 months to a short course of six months (Grosset, 1978, Mitchison, 1992, Somoskovi et al., 2001). RIF-resistance is a key determinant for treatment failure (Lew et al., 2008, Ahmad and Mokaddas, 2014). Detection of RIF-resistance is regarded as a surrogate for the detection of MDR-TB, since the prevalence of RIF mono-resistance is generally considered to be low (Lawn and Nicol, 2011, Somoskovi et al., 2001).

RIF inhibits mycobacterial transcription by binding to the β-subunit of bacterial deoxyribonucleic acid (DNA) dependent ribonucleic acid (RNA) polymerase (Telenti et al., 1993, Ramaswamy and Musser, 1998).The important catalytic region of RNA polymerase of bacteria has a molecular mass of 400kDa and is evolutionarily conserved among all cellular organisms (Campbell et al., 2001,

Archambault and Friesen, 1993). Mutations that confer RIF resistance almost entirely occur in the RIF Resistance Determining Region (RRDR) of the *rpo*B gene of organisms such as *Escherichia coli* (Ezekiel and Hutchins, 1968) and MTB (Ramaswamy and Musser, 1998). The RRDR is 81 base pairs long comprising 27 codons (from codon 507 to 533 using the *Escherichia coli* numbering scheme). More than 95% of RIF resistant (RIF^R) MTB strains contain a mutation, either a single nucleotide polymorphism (SNP) or a deletion, of a nucleotide in the RRDR (Somoskovi et al., 2001, Telenti et al., 1993, Ramaswamy and Musser, 1998). A SNP is a change in a single nucleotide from the wild-type nucleotide at that position.

1.2 Drug resistance in *Mycobacterium tuberculosis*

Members of the MTB complex (including *Mycobacterium tuberculosis*, *Mycobacterium africanum* and *Mycobacterium bovis* among others*)* as well as the non-tuberculous mycobacteria (NTM) are naturally resistant to many commonly used antibiotics due to the slow uptake of drugs across the hydrophobic lipid-rich cell wall (Viveiros et al., 2003). *Mycobacterium bovis* and *Mycobacterium canettii* are naturally resistant to pyrazinamide (PZA) (Ramaswamy and Musser, 1998).

Acquired TB-drug resistance in previously susceptible MTB occurs wherever anti-TB drugs are used for the treatment of TB. Factors that contribute to the emergence of MTB drug resistance include diagnostic delay (leading to high bacterial burden), ineffective treatment (due to drug shortages, inappropriate regimens and treatment interruption), pharmacokinetic variability and possibly patients with underlying immune suppression (Barnes, 1987, Gordin et al., 1996, Reichman, 1997).

With increasing anti-TB drug use and misuse, drug resistant MTB isolates have emerged through the selection of pre-existing drug resistant mutants. TB drug resistance is an amplification of a natural phenomenon (Palomino and Martin, 2014). MTB drug resistance is not acquired due to horizontal gene transfer

(Zainuddin and Dale, 1990) on plasmids or transposons, but through chromosomal mutations that occur spontaneously during bacterial multiplication.

The drug resistant MTB mutants that arise spontaneously are subsequently selected during inappropriate anti-TB treatment (e.g. monotherapy) resulting in a predominant mutant MTB population. Thus, most MTB drug resistance is a result of specific mutations in target genes. Gene alterations that change the structure of the target protein but do not compromise the protein's function allow the mutant strain to be selected in the presence of the drug. The most common mechanism of MTB resistance in general is due to SNP; other mechanisms include drug efflux (Louw et al., 2009).

1.3 Rates of spontaneous mutations giving rise to INH & RIF resistance in *Mycobacterium tuberculosis*

Mutations in the *rpo*B gene alter the amino acid sequence of RNA polymerase in such a way that RIF cannot bind to the enzyme and thus will not inhibit its function. Spontaneous mutations in the *rpo*B gene that cause resistance to RIF occur very rarely, at an average of 1 in 100 million cells. This means that the chance of 1 mutation occurring in the RRDR of the *rpo*B gene is approximately once every time 100 million bacilli divide in a drug free environment (Grange, 1990). Genetic mutations resulting in resistance to INH occur more frequently or at a rate of approximately 10^{-7} per cell division (Grange, 1990). More than 10^{7} bacilli may be present per lesion in lung cavities (Canetti, 1965). Therefore, if INH is used as a mono-therapy for a patient with cavitary TB, there will be a strong likelihood of there being at least one bacillus with INH resistance within the cavity that may be selected out during INH mono-therapy (Almeida Da Silva and Palomino, 2011).

The occurrence of MDR TB due to spontaneous mutations occurring for both RIF and INH simultaneously is extremely rare; no single gene mutation associated with MDR TB exists and mutations resulting in resistance to different drugs arise independently. By using INH and RIF the probability that any single bacillus in a population will carry resistance against both drugs simultaneously is about 10^{-15}

(10^{-7}x10^{-8}) (Iseman and Madsen, 1989, Grange, 1990). This is the main reason that a multidrug regimen is used in anti-TB treatment (Cohn et al., 1959, Gillespie, 2007, Almeida Da Silva and Palomino, 2011).

1.4 Molecular mechanism of rifampicin resistance

Within the RRDR, the most common *rpo*B mutations causing RIF resistance in MTB worldwide are S531L, H526D, H526Y and D516V. These mutations account for 75-85% of *rpo*B mutations causing RIF resistance (Somoskovi et al., 2001, Huitric et al., 2006, Rigouts et al., 2007). These four *rpo*B mutations almost always give rise to high-level RIF resistance (minimum inhibitory concentration (MIC) above 16 µg/ml) (Ohno et al., 1996, Somoskovi et al., 2001, Huitric et al., 2006, Cavusoglu et al., 2006). When performing conventional phenotypic drug susceptibility testing (DST) with liquid culture-based methods such as the Mycobacterial Growth Indicator Tube (MGIT; Becton Dickinson, MD), these particular "high level" *rpo*B mutations would typically give concordant RIF^R results (both MGIT and the genotypic method would give a RIF^R result). These high level *rpo*B mutations have also been termed "non-disputed" or "high confidence" *rpo*B mutations.

The remaining 15-25% "less common" *rpo*B mutations are associated with variable RIF MICs (Huitric et al., 2006, Ohno et al., 1996) and may not be detected by conventional liquid-based phenotypic DST methods such as MGIT, which uses the critical concentration of 1µg/ml RIF. The *rpo*B mutations have been termed "disputed *rpo*B mutations" if they test phenotypically susceptible with routinely used liquid based methods and they thus lead to discordant genotypic / phenotypic rifampicin results. Some are termed "low level" RIF resistance mutations when MIC testing reveals MICs <1 µg/ml but >0.0625 µg/ml. The clinical significance of the disputed *rpo*B mutations is not entirely clear but there is some information that indicates that the majority of patients infected with MTB strains that harbour these disputed *rpo*B mutations have clinical failure with

standard (drug susceptible) TB regimens (Ohno et al., 1996, Williamson et al., 2012, Van Deun et al., 2013).

Less commonly, RIF resistance may arise from mutations in the *rpo*B gene that occur either upstream or downstream of the RRDR (Somoskovi et al., 2001). These too may lead to either high or low-level RIF resistance (Siu et al., 2011).

1.5 Diagnostic test methods commonly used for detecting rifampicin resistance in *Mycobacterium tuberculosis* and their limitations

Principles of and methods for drug susceptibility testing

Drug susceptibility testing (DST) is used to determine whether a particular organism is susceptible or resistant to the drugs used in therapy. A patient infected with a susceptible organism has a greater probability of treatment success if that drug is used as treatment (Isenberg, 1988, Leekha et al., 2011). A patient infected with a resistant strain has a high probability of failing treatment if that drug is used (Isenberg, 1988). Standardised and reliable methods to ensure accurate and reproducible results are required to provide guidance for the treatment of each patient. A false resistant or false susceptible result may lead to a clinician replacing an effective regimen with a less effective one. This in turn will have an impact not only on the patient's health, but also result in an increased expenditure for the healthcare system and may also result in the spread of drug-resistant strains in the community (Pinto and Menzies, 2011).

Drug susceptibility of MTB may be determined phenotypically by observing growth or metabolic inhibition of the MTB organisms in a medium with an anti-TB drug. It may also be performed genotypically by detecting known mutations in genes that confer resistance to anti-TB drugs.

1.5.1 Phenotypic DST methods

Phenotypic ("culture-based") TB drug susceptibility testing is considered to be highly accurate and reliable and is currently regarded as the gold standard for

diagnosing drug-resistant TB. Phenotypic DST will detect most mechanisms of resistance including those for which no mutation causes is known to cause the resistance. Phenotypic susceptibility testing methods are the most widely used methods and classify MTB isolates into drug-resistant or drug-susceptible, based on their ability to grow in the presence of a “critical concentration” of the test drug. This critical concentration is defined as the lowest concentration of a drug that will inhibit ≥95% of wild type (WT) strains of MTB that have never been exposed to the drug and that does not inhibit resistant strains isolated from patients not responding to the drug (Canetti et al., 1963). The critical concentration is an epidemiological cut-off used to differentiate between WT (susceptible) and non-WT (resistant) strains (Canetti et al., 1963, Bottger, 2011) rather than a clinical breakpoint used to predict clinical efficacy (Kim, 2005). The MIC is defined as the minimum concentration of drug required to inhibit a visible growth of more than 99% of MTB population (Angeby et al., 2012).

It was observed that WT strains of MTB that have never been exposed to RIF do not exhibit great variation in MIC to RIF (Canetti et al., 1963, Angeby et al., 2012). Since drug resistance occurs due to the selection of pre-existing mutants, both drug resistant mutants and drug susceptible wild types are likely to be present in any large population of MTB bacilli in a culture medium. Resistance was deemed clinically significant when at least 1% of the population of bacilli grew at the critical concentration. A smaller proportion of mutant organisms was not thought to have clinical significance (Canetti et al., 1963).

A comparison of laboratory findings and clinical outcomes revealed that an increase in mutant cells in a population and growth of >1% of the population at the critical concentration of drug could predict therapeutic failure. Resistant cells below 1% were typically successfully controlled with standard anti-TB treatment (Canetti et al., 1963).

Recent studies have criticized the use of a single critical concentration for MTB DST, suggesting that determining the specific MIC of each isolate, as is widely used in general bacteriology, should be considered for MTB (Bottger, 2011).

Phenotypic DST may be performed directly from specimens or indirectly from cultured isolates.

Direct phenotypic DST is performed from a decontaminated specimen or untreated specimen (the latter only if the specimen is from a normally sterile site) that is TB microscopy-smear-positive with acid alcohol fast bacilli (AFB). The specimen is inoculated directly onto drug-containing media and drug-free control media. The advantages of direct DST are that it has a reduced turnaround time compared with indirect DST, and the DST is more representative of the MTB complex population in the original sample. The disadvantages are that the method can only be performed on a smear-positive specimen and it could result in increased expenditure where there is high incidence of smear-positive specimens containing NTM (O'Grady et al., 2011, WHO, 2010a).

Indirect phenotypic DST involves isolation of pure growth of MTB from clinical specimens in culture media followed by inoculation of the isolate into a drug containing medium.

Phenotypic DST methods for MTB include the absolute concentration method, the resistance ratio method and the 1% proportion method (Canetti et al., 1963). The former two are used less often as they are labour intensive. The WHO recommends that laboratories performing phenotypic DST perform the proportion method, which can be performed either directly or indirectly Worldwide, the proportion method is the preferred method among the conventional methods and is considered the gold standard (Kim, 2005). Drug susceptibility results for the above three methods do not differ significantly for RIF and INH (WHO, 2010a).

Indirect DST methods depend on the primary culture becoming positive for MTB. Once performed, DST results may take from two to six weeks; the total process could therefore take one to three months. This delay has a great impact in patients who may suffer clinical deterioration during inadequate therapy and patients who may continue to transmit drug-resistant TB into the community. Therefore, there is a need for more rapid methods that can provide accurate and reliable DST results.

1.5.1.1 Proportion drug susceptibility testing methods

Conventional phenotypic DST by the proportion method can be performed using either solid media such as agar or egg-based media (e.g., Löwenstein–Jensen (LJ) media enriched with glycerol and asparagine) or liquid media that contains serum or bovine albumin (e.g. MGIT) (Kim, 2005).

1.5.1.1.1 Solid media based proportion method

The solid agar proportion method depends on the critical concentration of the drug and the critical proportion of resistant bacilli in a population. It involves comparing the number of colony forming units that grow in the drug-containing media to that in the drug-free media (Canetti et al., 1963).

The number of colonies obtained from the control drug-free media indicates the number of viable bacilli contained in the corresponding dilution. The number of colonies obtained from the drug-containing media indicates the number of resistant bacilli in the inoculum dilution. The ratio between the number of colonies from the drug-containing media and the number of colonies from the control media is used to show whether the strain is susceptible or resistant. If the ratio is below the critical proportion (1%) then the isolate is reported susceptible and if the result is above the critical proportion then the isolate is reported resistant. The control media should have at least 20 countable colonies for the DST to be interpreted unless the isolate is resistant (Canetti et al., 1963, Canetti et al., 1969). The RIF critical concentration using LJ media is 40 µg/ml (Canetti et al., 1963, Canetti et al., 1969).

Technical errors could cause false susceptibility results that may lead to discordance with a genotypic test that detects resistance. Therefore, it is important that the inoculum is prepared correctly; if the inoculum is too small, the sample size will not be enough to detect the resistant mutant's critical proportion leading to false susceptible results. If the inoculum is too large the spontaneously occurring mutants may be present in a large enough amount to give a false resistant result. Contamination with NTM can also lead to a false resistant result (Canetti et al., 1963, Canetti et al., 1969).

1.5.1.1.2 Liquid media based proportion method

Various liquid media based DST methods have been used to provide susceptibility results as they have a more rapid turn-around time than DST on solid media (WHO, 2007). Early liquid media based DST methods include the BACTEC 460 system (Becton Dickinson Diagnostic Systems, Sparks, MD) which provides faster susceptibility results (Siddiqi et al., 1981, Roberts et al., 1983). However, the requirement for radioactive waste disposal makes it unsafe, difficult and expensive (Siddiqi et al., 2012). The BACTEC 460 system has since been replaced by the non-radiometric BACTEC MGIT 960 system (Becton Dickinson Diagnostic Systems, Sparks, MD) which was endorsed by the WHO in 2007 (WHO, 2007). Other liquid DST methods include non-commercial microscopic observation of drug susceptibility (MODS), nitrate reductase assay in liquid medium (NRA) and commercially available systems like the Trek Sensititre MYCOTB MIC plate (Treck Diagnostic Systems, Cleveland, OH).

Mycobacterial Growth Indicator Tube (MGIT)

The BACTEC MGIT (Mycobacterial Growth Indicator Tube) manual and automated systems are commercial test methods based on liquid media originally introduced for the rapid detection of mycobacterial growth (primary culture of MTB). The BACTEC MGIT 960 SIRE kit (Becton Dickinson, Franklin Lakes, New Jersey, USA) is an indirect qualitative DST method for the detection of “first-line” drug resistance to anti-TB drugs, namely streptomycin (STR), INH, RIF, and ethambutol (EMB). It takes 4 to 13 days to obtain a DST result after the MGIT culture is positive for MTB. This test was developed with critical concentrations for STR (1 µg/ml), INH (0.1 µg/ml), RIF (1 µg/ml) and EMB (5 µg/ml) (Rusch-Gerdes et al., 1999, Siddiqi SH, 2006).

There is a fluorescent compound quenched by oxygen at the bottom of the culture medium MGIT tube; once oxygen is consumed by actively growing microorganisms, the compound fluoresces and is detected by the BACTEC MGIT 960 instrument. The DST is based on the method in which the growth of MTB isolates in a drug-containing tube is compared to growth in a drug-free control

tube. The BACTEC MGIT 960 instrument continuously analyses fluorescence of the drug-containing tube compared to that of the drug-free control tube.

Routine RIF susceptibility testing in MGIT is performed at a critical concentration of 1 µg/ml and MIC testing at various drug concentrations can be performed with the aid of EpiCenter TB eXiST software (Becton Dickinson, Heidelberg, Germany). The MGIT 960 only allows a maximum of 13 days incubation period for the growth control tube to reach the required growth unit (GU>400) for interpretation. If the required growth unit is not reached within the 13 days of incubation, an invalid result is obtained. The EpiCenter TB eXiST software is useful as it allows the MGIT system to incubate slower growing MTB strains for longer, thus susceptibility results can be obtained after a longer incubation period (i.e. up to 42 days). The Epicenter System has been used to determine the RIF MICs of MTB isolates with various *rpo*B mutations for RIF (Springer et al., 2009, Sirgel et al., 2013).

Limitations of traditional (non-EpiCenter) MGIT DST include failing to obtain a valid RIF susceptibility result for a slow growing MTB isolate. The limited incubation time when using routine MGIT DST may also fail to detect some slow growing MTB isolates that harbour particular *rpo*B mutations that have been associated with possible loss of fitness (Rigouts et al., 2013). Errors or discordant RIF susceptibility results may also arise for liquid based DST if the inoculum is not prepared correctly.

The critical concentration of 1 µg/ml used in routine MGIT DST for RIF has been criticised as being too high. For certain *rpo*B mutations, the MGIT result is reported as susceptible, causing a discordant overall result between the genotypic and the liquid based phenotypic tests. Solid media (LJ)-based phenotypic testing correctly identifies these MTB isolates as RIF^R (Van Deun et al., 2009, Van Deun et al., 2011).

1.5.2 Genotypic drug susceptibility testing

Molecular or genotypic DST depends on the detection of mutations that confer drug resistance. If a mutation is detected and known to be associated with resistance, the MTB is reported to be drug resistant whereas if no mutation is detected the MTB is reported as susceptible (i.e. wild type). Mutations associated with resistance to many of the anti-TB drugs have been described (Zhang and Young, 1994, Telenti, 1998, Johnson et al., 2006, Almeida Da Silva and Palomino, 2011). Genotypic methods can be categorized into two groups: probe based methods and sequence based methods.

1.5.2.1 Line probe assays (LPA)

Two LPA assays; the GenoType MTBDR*plus* (Hain Lifescience, Nehren, Germany) and the INNO LiPARifTB (Innogenetics, Ghent, Belgium), are available to determine MTB susceptibility to RIF. Both assays are reverse hybridization assays with a sensitivity for detection of RIF resistance of 98% (Morgan et al., 2005, Barnard et al., 2008) and 92% (Tortoli and Marcelli, 2007), respectively.

In South Africa, GenoType MTBDR*plus* is the line probe assay routinely used in the public sector and this thesis will focus on this assay. In the remaining thesis, the term “LPA” refers exclusively to the GenoType MTBDR*plus* assay.

GenoType MTBDR*plus* line probe assay

The GenoType MTBDR*plus* LPA was incorporated into the routine TB diagnostic testing algorithm within the state sector in South Africa in 2008. It replaced routine phenotypic DST (i.e. MGIT proportion method) as the first screen for MTB drug resistance. At the time, DST by LPA was performed only on patients failing first line TB regimens, retreatment patients or on patients suspected clinically to have MDR-TB (e.g. having an MDR-TB contact). It was performed on direct sputum specimens that are smear positive for AFB, as well as on cultures positive for AFB.

In the most recent TB testing algorithm within the South African TB Control Programme, the LPA is now used to confirm RIF resistance that has previously

been detected by Xpert - confirmation of RIF resistance was recommended in the WHO endorsement of the Xpert MTB/RIF assay in 2010. The LPA is also performed on a subsequent culture if the initial screening Xpert does not detect MTB (Figure 6).

Principle

The LPA is a qualitative test for the extraction and identification of MTB and its resistance to INH and RIF based on DNA-STRIP technology. For RIF, the presence of *rpo*B mutations is inferred by the absence of hybridization to one (or less commonly more) wild type (WT) probe with or without presence of hybridization to mutation probe (MUT) in the RRDR of the *rpo*B gene, spanning from codon 505 to codon 533. For INH, the *kat*G and *inh*A gene are examined.

The procedure includes DNA extraction from a smear positive respiratory specimen or from the culture isolate, multiplex amplification with biotintylated primers and a reverse hybridization to membrane strips coated with target-specific oligonucleotides. Highly specific binding of complementary DNA strands is ensured by stringent conditions (e.g. buffer composition and 45^0C temperature). The reverse hybridization binding is followed by a biotin-streptavidin mediated detection of hybridized amplified products (Hain-Lifescience, July 2013). The staining that results from the biotin-streptavidin hybridization step results in a band at that particular reaction zone (“probe”) (Figure 1).

Figure 1: Facsimile of "RIF resistant region of the *rpo*B gene" from LPA MTBDR*plus* package insert version 2. The Rifampicin Resistance Determining Region (RRDR) of the *rpo*B gene that is included in the LPA spans from codon 505 to codon 533 and is divided into 8 WT probes (*rpo*B WT1-8). *rpo*B MUT1-3 represent corresponding mutation probes for the most commonly encountered *rpo*B mutations in MTB.

*RIF = rifampicin, LPA = line probe assay, WT = wild type probe, MUT = mutation probe.

Interpretation

If interpretation is manual, the strip is "read" by the naked eye, using a template that is provided in each kit. Interpretation may be performed by an automated scanner (Genoscan, Hain Lifesciences) which interfaces with the laboratory information system (LIS); this is used in high throughput laboratories such as Green Point NHLS (National Health Laboratory Service) in Cape Town.

Each LPA strip has 27 reaction zones including internal controls. For RIF susceptibility, the LPA strip is divided into 12 probes; eight probes represent the WT (susceptible) and four MUT probes represent the (4 most common) mutant (resistant) sequences of nucleotides within the RRDR of *rpo*B gene. The LPA includes internal quality control bands on the DNA strip which are the conjugate control (CC) which verifies the efficiency of conjugate binding and substrate reaction for the hybridization of probe to amplicon, the amplification control (AC) which verifies that amplification occurred and that there was no inhibition of the PCR, and the locus control bands for the *rpo*B, *kat*G and *inh*A genes which detect a genetic region specific for the respective locus.

The package insert states that a probe band must be interpreted as being present if its intensity is equal to or stronger than that of the AC band for that strip. The probe band is interpreted as absent if its intensity is less than that of the AC band for the same strip (Hain-Lifescience, July 2013) (Figure 2).

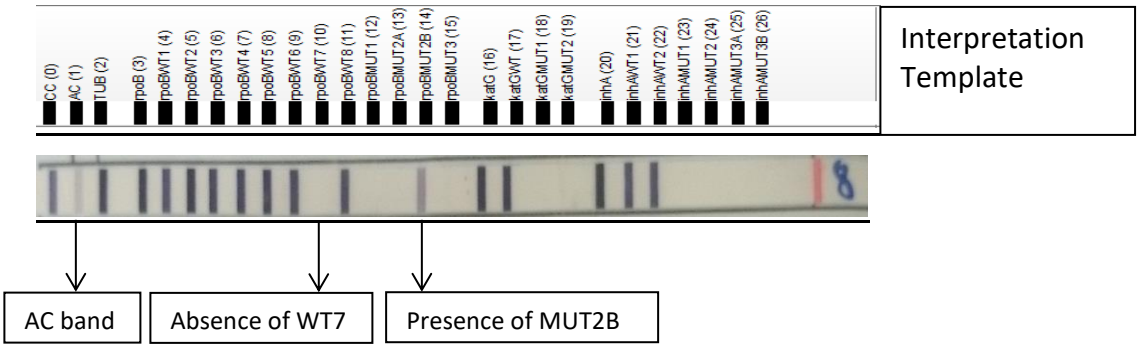


Figure 2: An LPA strip with interpretation template showing all the 27 reaction zones aligned to MTB isolate number 8 showing absence of *rpo*B WT7 and presence of *rpo*B MUT2B. This is interpreted as RIF^R. (The isolate strip also shows the presence of all WT *kat*G and *inh*A bands and absence of *kat*G and *inh*A MUT bands; this is interpreted as INH susceptible).

* AC = amplification control, LPA = line probe assay, MTB = *Mycobacterium tuberculosis*, WT = wild type, MUT = mutation, RIF^R = rifampicin resistant, INH = isoniazid

TUB band

If present, the TUB band indicates the presence of MTB complex. If the TUB probe is absent the specimen/culture is considered negative for MTB complex. The TUB band must be present in order to proceed to interpret the *rpo*B, *kat*G and *inh*A band patterns.

Wild type (WT) probes

Table 1: Interpretation of the LPA. Both A and B must be determined in order to interpret the antibiotic result.

(A) Wild type (WT) probes present/absent	**(B) Mutation (MUT) probes present/absent**	**(A+B) Interpretation of drug susceptibility**
Present (all)	Absent	Susceptible
Absent (one or two)	Present (usually one) or absent*	Resistant
Present (all)	Present (usually one)	Resistant (heteroresistance)

*Present: the intensity of the particular band is as strong as or stronger in intensity than the AC band.

*Absent: the intensity is weaker than the AC band. *AC = amplification control

Mutation (MUT) probes

The presence of a mutation (MUT) band indicates resistance to the drug. The LPA incorporates four mutation probes for *rpo*B. These are the sequences of the most commonly encountered *rpo*B mutations that account for the majority of RIF resistance (Telenti et al., 1993, Van Deun et al., 2013). The corresponding amino acid change that occurs for each of these *rpo*B mutations is provided in the package insert (Figure 3) (Hain-Lifescience, July 2013). For example, if WT3 and WT4 are absent, and MUT1 band is present, it corresponds to *rpo*B mutation in codon 516 called mutation D516V where aspartic acid (D) is replaced by valine (V). The remaining three most common *rpo*B mutations encountered clinically are H526Y, H526D where histidine (H) is replaced by tyrosine (Y) and aspartic acid (D), respectively, and S531L where serine (S) is replaced by leucine (L). These are identified by the following patterns on the LPA: absent WT7 plus present MUT2A (H526Y); absent WT7 plus present MUT2B (H526D) and absent WT8 plus present MUT3 (S531L).

For the remaining less commonly encountered *rpo*B mutations, either in the same codons 516, 526 or 531 (where aspartic acid, histidine, and serine are replaced by amino acids other than valine, tyrosine and leucine respectively) or in other

codons (between 505 to 533), there is no corresponding MUT band incorporated in the LPA strip; the replacing amino acid is not encoded for by its known sequence in a probe; however, a list of possible amino acid changes is provided in the package insert (Figure 3), based in the most commonly encountered sequences worldwide (Telenti et al., 1993). These less common *rpo*B mutations are still interpreted and reported as conferring RIF resistance since there is a nucleotide substitution/deletion in the WT sequence. The resulting pattern on the LPA is absent WT probe band, and absent MUT band. The clinical significance of some of these mutations are unknown (Van Deun et al., 2009) and those with an asterisk (in package insert; Figure 3) have only been detected “in silico” (not from clinical specimens/isolates). Since the corresponding phenotypic RIF result when testing at the critical concentration of 1µg/ml with MGIT or with other liquid based proportion method DST may be susceptible (thus giving a discordant phenotypic result), these *rpo*B mutations have been termed by some authors as “disputed *rpo*B mutations” (Van Deun et al., 2013, Rigouts et al., 2013).

In addition to differentiating high level RIF mutations from possible low level / susceptible RIF mutations, the LPA is able to also detect heteroresistance for a particular drug, as long as the proportion of the mutant population is greater than 5% in the specimen/culture isolate (Zetola et al., 2014). RIF heteroresistance is inferred if all *rpo*B wild type (*rpo*B WT) probes are present and (usually) one of the *rpo*B mutation (*rpo*B MUT) bands is also present. Heteroresistance may be due to the presence of more than one MTB population (i.e. “mixed infection”) in the specimen/culture.

Failing wild type band(s)	Codons analyzed	Developing mutation band	Mutation
rpoB WT1	505-509		F505L
			T508A
			S509T
rpoB WT2	510-513		E510H
			L511P*
rpoB WT2/WT3	510-517		Q513L*
			Q513P
			del514-516
rpoB WT3/WT4	513-519	*rpoB* MUT1	D516V
			D516Y
			del515
rpoB WT4/WT5	516-522		del518*
			N518I
rpoB WT5/WT6	518-525		S522L
			S522Q
rpoB WT7	526-529	*rpoB* MUT2A	H526Y
		rpoB MUT2B	H526D
			H526R
			H526P*
			H526Q*
			H526N
			H526L
			H526S
			H526C
rpoB WT8	530-533	*rpoB* MUT3	S531L
			S531Q*
			S531W
			L533P

Figure 3: Facsimile of "Table of mutations in the *rpo*B gene and the corresponding wild type and mutation bands" from LPA MTBDR*plus* package insert version 2. LPA has 8 *rpo*B WT bands (WT1-WT8) and four *rpo*B MUT bands (MUT1, MUT2A, MUT2B and MUT3) and spans from codon 505 to 533. Mutations in the *rpo*B gene are due to SNPs resulting in a change of amino acid e.g. L533P indicates that amino acid leucine (L) in codon 533 was replaced by amino acid proline (P) due to a SNP where thymine (T) was substituted by cytosine (codon CTG to CCG).

*WT=wild type, MUT=mutation, SNP = single nucleotide polymorphism

*Failing wild type band (s) = absent wild type band (s)

Limitations of the LPA that could lead to a false RIF susceptibility testing result and thus discordance between LPA and phenotypic DST

1. The LPA assay screens for SNPs; therefore, there is a possibility that an *rpo*B mutation that does not cause an amino acid change (i.e. a silent mutation) will be detected as an absence of wild type probe, and interpreted as RIF resistant.
2. The assay only detects RIF resistance that originates from the mutations within the RRDR of the *rpo*B gene. Resistance due to *rpo*B mutations originating from outside of the RRDR of *rpo*B gene or due to non *rpo*B genes (Siu et al., 2011) or other mechanisms of resistance (Louw et al., 2009) are not detected by the LPA. A resulting RIF susceptible (RIF^S) result by LPA could give rise to a discordant result with phenotypic RIF susceptibility testing.
3. In cases of heteroresistance where both a susceptible and a resistant pattern is expected, but resistance is due to a disputed *rpo*B mutation (thus there will be an absent WT probe and absent MUT probe, the LPA will be interpreted as RIF^S since only the wild type pattern will be visible on the strip. The heteroresistance will be missed by the LPA.
4. In 2013, the LPA was updated to a 2nd version ("V2"), in order to improve the intensity of the bands that developed on hybridization, specifically, *rpo*B WT6, WT7 and WT8. These bands had often looked faint in version 1, resulting in uninterpretable results for RIF. In addition to improving the intensity of the *rpo*B bands in V2, the L533P mutation (corresponding to a pattern of [WT8 absent and no mutation present]) was excluded from the WT8 probe. The reason for this exclusion was that isolates with the L533P mutation were found to be phenotypically susceptible to RIF at the critical concentration of 1 µg/ml that is used in liquid based DST methods (Van Deun et al., 2013). However, this contradicted findings of later studies where patients infected with MTB that harboured the L533P mutation were failing treatment. Thus, the L533P mutation was considered to be clinically

significant (Williamson et al., 2012). In a later version of V2, Hain reincorporated the L533P into the WT8 probe.

1.5.2.2 Xpert MTB/RIF

Principle

The Xpert MTB/RIF (Cepheid, Sunnyvale, USA) assay is based on hemi-nested real-time PCR and molecular beacon technology in a closed system for the detection of RIF^R MTB (Lawn and Nicol, 2011). Mutations in the 81-bp RRDR of the *rpo*B gene (codons 507 to 533) are detected by five overlapping molecular beacons. Molecular beacons are single stranded oligonucleotide hybridization probes that form a hairpin (i.e. stem-loop) structure. The probe loop is a sequence complementary to WT sequence. On either side of the probe loop, there are two sequences that are complementary to each other and together form the stem. The fluorophore (fluorescent dye) is covalently attached to the one end of the stem and quencher (non-fluorescent dye) is covalently attached to the other end (Figure 4). When a beacon binds to a matching sequence of DNA from the specimen, it fluoresces indicating the presence of one of the WT gene sequences that is characteristic of RIF^S MTB. If a beacon fails to bind at all or there is delayed binding to the matching sequence, it indicates potential RIF-resistance (i.e. absence of wild type) (Lawn and Nicol, 2011, Steingart et al., 2014). Cycle threshold (Ct) is defined as the number of cycles of PCR that is required for a fluorescent signal to occur. This fluorescent signal must exceed the background level or cross the threshold, the point at which the fluorescence significantly rises above the background level. Delta Ct max (ΔCt max) is defined as the difference between the Ct values of the latest and earliest probes (Lawn and Nicol, 2011).

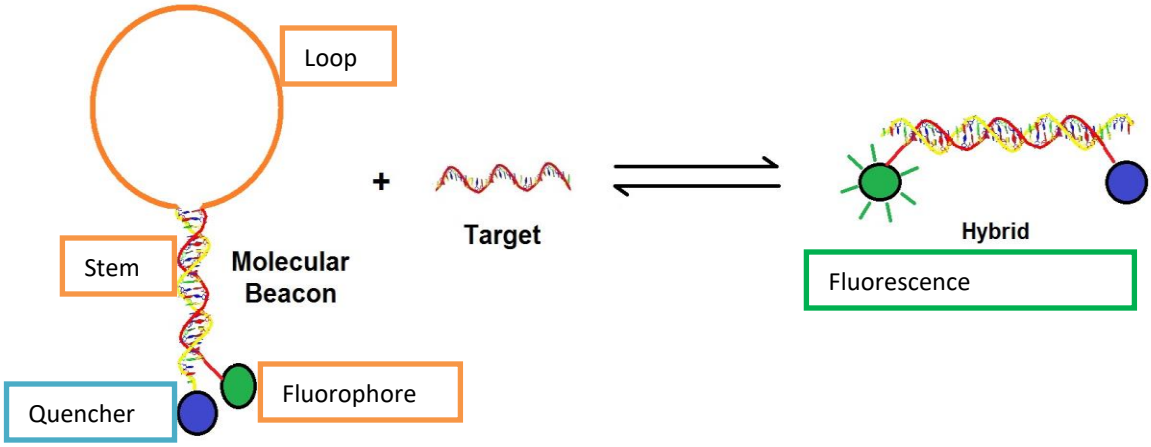


Figure 4: Molecular beacon operation. The molecular beacon contains a loop region that is complementary to the target sequence. The stem region contains complimentary nucleotides with a fluorophore and quencher dye attached to either side of the stem arm. When the target sequence is present, the stem opens due to the more stable probe-target duplex where the loop region of the beacon binds to the complementary sequence, causing the loop to extend resulting in the quencher releasing from the fluorophore with an increased fluorescent signal that is read by the instrument. In the absence of the target sequence the complementary region in the stem remains hybridized keeping the quencher in close contact with the fluorophore and no fluorescence occurs.

The Xpert assay detects MTB DNA and RIF resistance at the same time by amplifying the RRDR which is probed with five molecular beacons (probes A – E) for *rpo*B mutations (Figure 5). Each beacon is labeled with a different fluorophore. The molecular beacons were designed to hybridize the WT sequence of the RRDR. If there is a mutation within the RRDR then it interferes with hybridization in such a way that the probe is retained in a non-fluorescing state. A mutation(s) in the RRDR results in one or more of the corresponding probes to either fail to fluoresce completely (termed probe "***drop out***" and indicated by one or more probes with a Ct value of zero) or have a delayed onset of fluorescence (termed ***"probe delay"*** and calculated by the instrument by subtracting the lowest probe Ct value from the highest probe Ct value) (Blakemore et al., 2010, Lawn and Nicol, 2011).

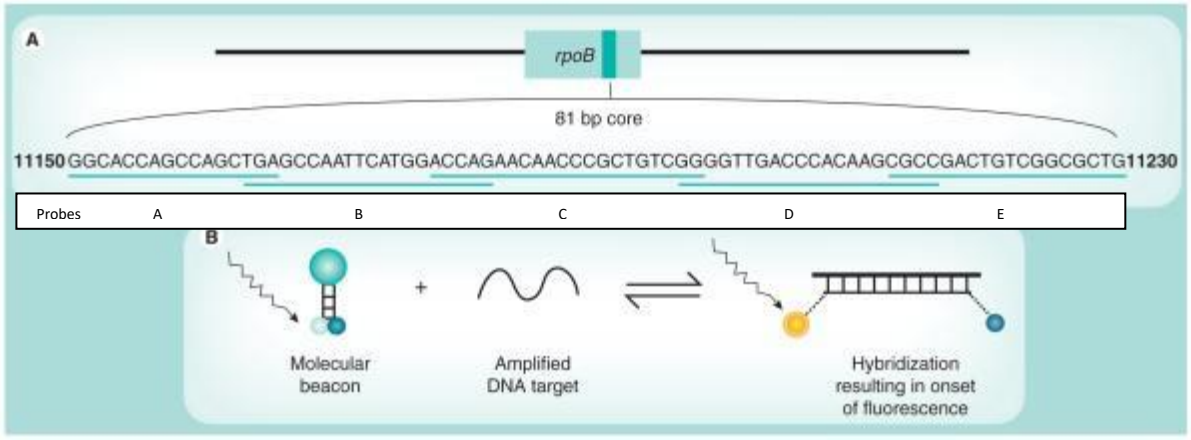


Figure 5: *rpo*B gene rifampicin resistance determining region and molecular beacon technology. A) The *rpo*B gene, the nucleotide sequence of 81bp RRDR and the five (A-E) overlapping molecular beacon probes covering the core region. B) A schematic of the molecular beacon in stem-loop hairpin conformation. The stem brings the dye and quencher to quench the fluorescence. The loop region contains a complementary sequence to the target sequence. Hybridization induces a conformational change that leads to separation of the fluorophore and quencher resulting in an increased fluorescence signal. (Figure adopted from Lawn, Nicol 2011)
*RRDR = rifampicin resistant determining region

Automated interpretation by the GeneXpert system is done by using the fluorescent signals and embedded calculation algorithms. MTB is reported as detected when at least two probes fluoresce within the valid range (Ct value ≤38) and the delta Ct min (ΔCt min is the smallest Ct difference between any pair of probes) is less than 2. If MTB DNA is detected, the result is reported as High, Medium, Low or Very Low depending on the Ct value (Table 2) (Blakemore et al., 2011, Lawn and Nicol, 2011). The Ct value is indirectly proportional to the baseline load of MTB bacteria in the specimen such that a lower Ct value is obtained when there is a higher concentration of MTB DNA in the sample and a higher Ct value means there is a lower starting concentration of MTB DNA in the sample (Blakemore et al., 2011, Lawn and Nicol, 2011).

Table 2: Relationship between semi quantitative report results and the actual Ct value

MTB detected (load)	**Ct value range of earliest probe**
High	≤16
Medium	16 < Ct ≤ 22
Low	22 < Ct ≤ 28
Very Low	28 <Ct ≤38

*MTB = Mycobacterium tuberculosis, Ct =cycle threshold

The RIF result by Xpert is reported as "susceptible" by the instrument if MTB is detected and the ΔCt max is ≤ 4.0. RIF resistance is reported if MTB is detected and the delta ΔCt max is >4.0 (delay) or MTB is detected and one or more of the probes fail to fluoresce at all (dropout: Ct=0) (Cepheid, May 2012).

<u>Limitation of Xpert assay that may lead to discordant results between Xpert and phenotypic DST</u>

1. The Xpert assay is dependent on the number of organisms present in a sample. When the bacterial load is Very Low, it has been reported to lead to discordant or/and a false RIF^R result (Ocheretina Oksana, 2016).
2. Reliable results are dependent on proper adherence to recommendations supplied by the manufacturer from sample collection, transport and storage to correct sample processing.
3. The assay only detects RIF resistance that originates from mutations in the RRDR of the *rpo*B gene. Resistance due to other mutations originating outside the gene regions or other genes or other mechanism of resistance will not be detected.
4. Previous versions of Xpert MTB/RIF:
    a. In 2010-11, false RIF resistance was reported by Xpert MTB/RIF (version G3 cartridge); this was primarily due to a delay in hybridization and it was determined that the delta Ct max cutoff was too high. In light of this, the criteria for determining RIF resistance by Xpert when resistance was detected due to a delay in probe hybridization was changed in a subsequent version of the Xpert

MTB/RIF cartridge (G4) from ΔCt max >3.5 to >5 then from ΔCt max >5 to ΔCt max >4 (Lawn and Nicol, 2011).The improved Xpert MTB/RIF cartridge G4 has been in use in the South African public sector since 2012.

b. False RIF^S results also occurred with the G3 assay as this version did not detect the L533P mutation. This was shown in a study in Switzerland (Somoskovi et al., 2013) where the presence of the mutation L533P was confirmed by sequencing on the corresponding culture isolates. Initially two sputum specimens were submitted for Xpert testing and MTB was detected on both specimens and RIF-indeterminate on first sample and RIF^R on second sample using G4 cartridges. Because the patient was from Switzerland (a low MDR-incident country) the assay was repeated on 4 subsequent sputum specimens using G3 cartridges and all four tested RIF^S. In addition, on repeat testing of the first specimen, using the version G4 cartridge, RIF^R was detected with delayed hybridization (ΔCt max >5) of probe E (that corresponds to L533P). Further analysis using MGIT 960 showed RIF^S at the critical concentration of 1 µg/ml (Somoskovi et al., 2013) which correlates with other findings of mutation L533P conferring low level resistance (Van Deun et al., 2009, Rigouts et al., 2013).

c. False RIF^R results also occurred with the G3 cartridge due to problems with probe B. An improvement in the G4 cartridge include modification of probe B beacon sequence to reduce false RIF^R by improving the stability of the wild type hybrid target at an elevated annealing temperature. (Other improvements include modification of the assay setting and PCR cycling to reduce error rates and false RIF^S results (FIND, 2011, Osman et al., 2014)).

1.5.2.3 DNA Sequencing

Sequencing of known resistance loci

DNA sequencing remains the gold-standard for genotypic detection of mutations conferring TB drug resistance. Currently, Sanger sequencing is the most accessible form of DNA sequencing for MTB. The advantages of Sanger sequencing include high accuracy (Sanger et al., 1977) and it can be utilized as a reference when validating new sequencing tests. DNA sequencing can be used to detect any alterations that confer RIF-resistance in the RRDR of *rpo*B gene and beyond (e.g. upstream or downstream of the RRDR) (Telenti et al., 1997, Musser, 1995). However, Sanger DNA sequencing for detection of mutations associated with MTB drug resistance is not routinely available in most diagnostic laboratories.

Sanger DNA sequencing is based on the chain termination method to sequence a DNA fragment. Once a specific primer binds to the denatured single strand of DNA, DNA polymerase initiation occurs and DNA replication begins at the primer site. The elongation is terminated when a dye-labeled dideoxynucleotide is inserted. This happens because the dye-labeled dideoxynucleotide lacks the 3'-OH group required for the formation of a phosphodiesterase bond between two subsequent nucleotides. The content of the reaction produces DNA fragments of various lengths that can be separated via electrophoresis by size and nucleotide type and subsequently sequenced (Sanger et al., 1992).

The major limitation of DNA sequencing testing is that it is costly and not available in routine TB laboratories; as such is not routinely used for TB diagnosis within the National TB Program. Its use is restricted to troubleshooting discordant results between routinely used test methods (such as Xpert and LPA) or for diagnosis when routine methods such as Xpert and LPA provide no conclusive results.

An additional advantage of DNA sequencing is that it is able to detect heteroresistance by showing dual peaks chromatogram pattern in the *rpo*B gene (Kumar et al., 2014).

Next generation sequencing (NGS) has more recently become more widely available and is available in reference / research laboratories in some countries. NGS involves shorter read lengths and higher error rates but requires higher sequence depth to determine the consensus sequence (Tucker et al., 2009). It involves sequencing of the entire genome of MTB, with comparison to a reference sequence. NGS will likely soon replace Sanger sequencing as it is high throughput and has great potential for TB diagnosis (Pankhurst et al., 2016, Ocheretina et al., 2015).

DNA sequencing may be used to resolve discordance between LPA or Xpert and phenotypic RIF susceptibility results.

1.6 South African National TB Control Program for patients with presumptive TB

The Xpert assay has been adopted as the initial screening test for patients with presumptive TB by the South African National TB Control programme (NTBCP), Figure 6. RIF resistance detected by Xpert is confirmed by LPA and/or phenotypic DST using MGIT on a culture isolate from a second sputum specimen.

In the Western Cape Province, two spot sputum specimens are collected one hour apart from a patient with presumptive TB and Xpert is performed on one sample. The second specimen is stored at 4-8^{0}C in the laboratory until further testing which is based on the Xpert result (Figure6).

Where Xpert detects MTB complex that is susceptible to RIF, the patient is started on the WHO standard TB drug susceptible regimen and the second specimen is processed for TB microscopy only. The microscopy result is used to monitor response to treatment and for contact tracing. If Xpert detects RIF^{R}-MTB complex, the patient is started on an MDR-TB regimen and the 2nd specimen undergoes processing for mycobacterial culture and DST, which is in most cases the LPA.

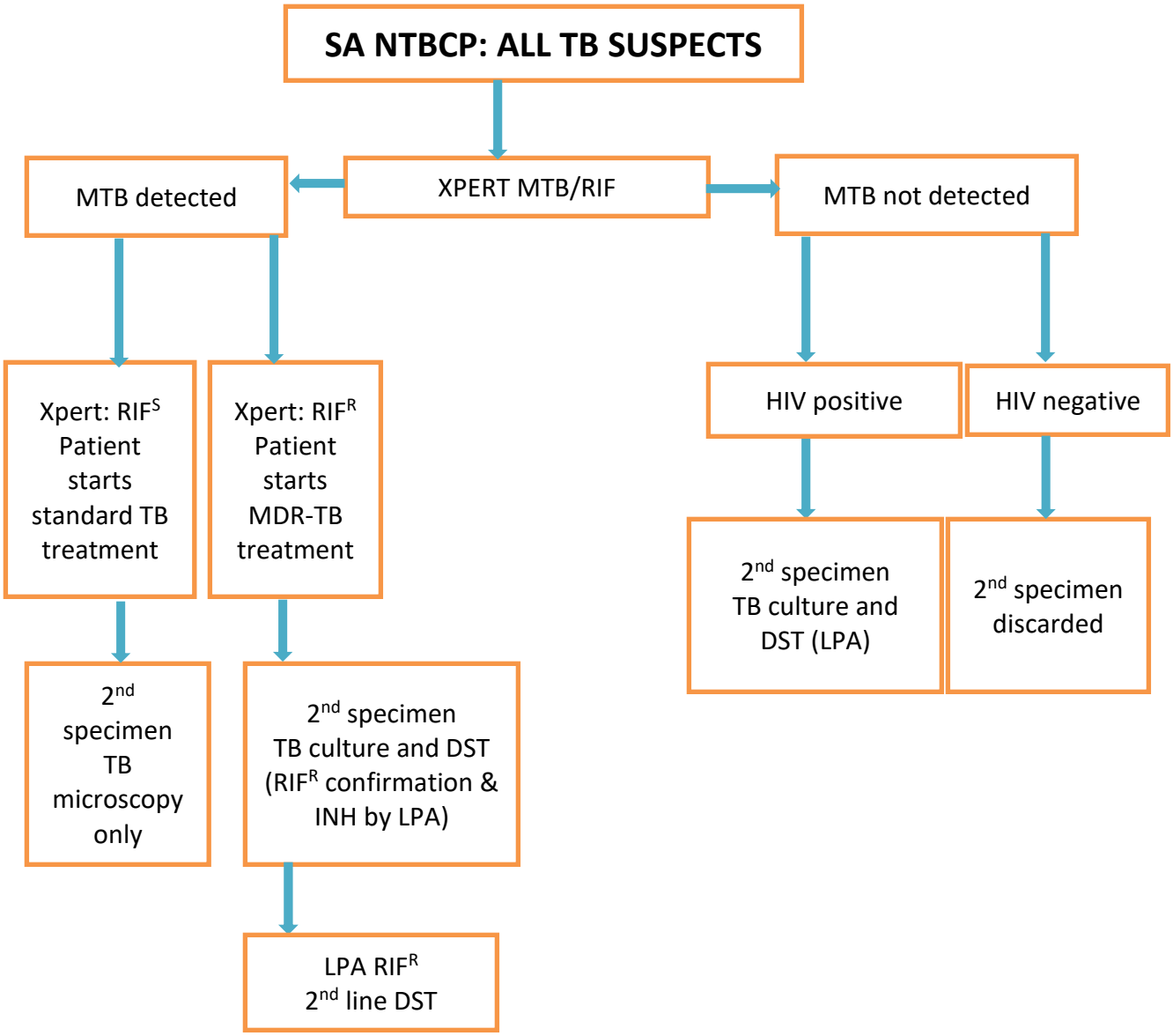


Figure 6: South African tuberculosis program diagnostic algorithm incorporating Xpert MTB/RIF as the initial screening test. SA NTBCP= South African National TB Control Program, TB= tuberculosis, MTB= *Mycobacterium tuberculosis* RIF= rifampicin, ^{S}= susceptible, ^{R}= resistant, MDR= multi-drug resistant, HIV= human immunodeficiency virus, DST= drug susceptibility testing, INH= isoniazid, LPA= line probe assay (GenoType MTBDR*plus*).

Key limitations of the NTBCP algorithm in relation to screening for resistance are:

1. Rifampicin resistance that (rarely) occurs due to mechanisms other than mutations in the RRDR of the *rpo*B gene will be missed and falsely reported as Xpert RIF^S (whether tested by Xpert or LPA). The NTBCP laboratory testing algorithm guide informs clinicians to submit a specimen requesting phenotypic DST if RIF^R is suspected despite a RIF^S result by Xpert or LPA, so that the approximately 2% of RIF resistance that is missed by Xpert/LPA can be detected by phenotypic DST.
2. Xpert is performed directly on a clinical specimen (this specimen cannot be used for further testing e.g. culture once it has been processed for Xpert); the subsequent confirmatory assay (LPA or MGIT) is performed on a culture isolate from a second submitted specimen. Thus, it is possible for discordant results in general to arise because the two submitted specimens may contain different amounts of TB bacilli, and / or different strains of MTB. In addition, specimen mix up / mislabelling may occur before specimens reach the laboratory.
3. INH is not tested

1.7 Discordant rifampicin susceptibility results

In general, utilizing different test methods to test for the same parameter may result in discordance since one method may be superior in sensitivity or specificity to the other. It is important to understand the limitation of each assay in determining RIF susceptibility. Access to the gold standard method may be limited, making it challenging to determine the true result. If only one test method is being utilized discordance may arise if testing is repeated for some reason.

Discordant RIF susceptibility results may arise between two different genotypic methods, between a genotypic method and a phenotypic method, or between two different phenotypic methods.

1.7.1 Discordance between genotypic RIF results (Xpert and LPA)

Although both the Xpert and LPA target the same *rpo*B gene region (the RRDR), discordant RIF susceptibility results between the two assays have been reported (Berhanu R, 2015, Ocheretina Oksana, 2016).

1.7.1.1 Xpert RIF^R: LPA RIF^S

Particular Xpert assay parameters (e.g. bacterial load, delayed probe hybridization) have been found to be associated with discordant Xpert and LPA RIF results (Berhanu R, 2015) and also with false Xpert RIF^R results (Ocheretina Oksana, 2016).

In a study that was performed in Haiti, 2024/9890 (20.5%) respiratory samples were Xpert (G4) positive for MTB. Of the 2024, 410 (20.3%) had Very Low MTB loads detected by Xpert. From samples with non-Very Low MTB loads, all 87 (5.4%) which were RIF^R on Xpert, were classified true Xpert RIF^R because all isolates from these patients harboured an *rpo*B mutation by Sanger sequencing. Of the 410 samples with Very Low MTB loads, 35 (8.5%) samples tested Xpert RIF^R of which only 22/35 had positive cultures. Only 10/22 (45.5%) had an *rpo*B mutation detected by Sanger sequencing of which 8/10 tested RIF^R by phenotypic DST and 2/10 had silent mutation T508T and were RIF^S by phenotypic DST. However, 12/22 (54.5%) had no *rpo*B mutation detected by Sanger sequencing and all tested RIF^S by phenotypic DST. In addition, LPA was performed directly on 11/12 specimens and 10/11 specimens were LPA RIF^S (considered a true LPA result) and one failed the LPA test. The false Xpert RIF resistance was due to probe delay in 91% (10/11) and in 1/11 there was probe drop out of three probes. From the 11/12 specimens which had probe delay, there were double probe delays of D+E (n=6), probe D (n=4) and probe E (n=1). This study concluded that all Xpert RIF^R results with a Very Low level of MTB should be confirmed by culture based DST prior to reporting and should consider bacterial load of samples to determine specificity for the detection of RIF resistance (Ocheretina Oksana, 2016).

A study conducted in India reported 25 discordant RIF results between Xpert (G4) and LPA from a total of 145 smear positive sputum samples; 21/25 were Xpert RIF^S and LPA RIF^R, 4/25 were Xpert RIF^R and LPA RIF^S. *rpo*B sequencing and MGIT DST were performed on all discordant isolates from the same specimen. There were 18/21 cases that were shown to be false Xpert RIF^S where mutations L533P (n=14) and S531L (n=4) were detected on isolates by *rpo*B sequencing. There were 2/21 isolates that were resistant by MGIT DST but no mutation was detected by *rpo*B sequencing and the study could not rule out mixed infection. Three of the four cases with Xpert RIF^R were categorized as false Xpert RIF^R because there were no mutations detected by *rpo*B sequencing and all three were RIF^S by MGIT-DST. Two (one of each category) could not be confirmed by *rpo*B sequencing or MGIT DST due to contamination (Rufai et al., 2014). Mutations detected by four Xpert probes (A,B,C and D) were all concordant with mutations detected in similar codon regions by LPA but probe E did not detect a mutation in 52% of samples that had mutations detected by LPA in similar codon regions (wild type 8 absent). All 18 false Xpert RIF^S results had LPA RIF^R reported due to an absent WT8 and absent MUT band in the 14 samples (L533P) and an absent WT8 band plus MUT3 present in four samples (S531L). In all 18 cases, the LPA pattern corresponded with the *rpo*B mutation detected by sequencing. The study suggested that each country carry out an evaluation that represents the population with each version of the assays because some probes may have different utility, geographically (Rufai et al., 2014).

1.7.1.2 Xpert RIF^S: LPA RIF^R

Strydom et al compared three genotypic assays including LPA version 2.0, Xpert G3 and the Anyplex MTB/NTM (Seegene, South Korea) on 115 MTB culture isolates collected from Tswane Academic Division, NHLS, South Africa. LPA version 1.0 was used to select isolates retrospectively. Consecutive isolates were selected to make up a calculated sample size of 50 susceptible to both INH and RIF, 20 monoresitant (10 INH monoresistant and 10 RIF monoresistant), 20 isolates with unidentified mutations (absent WT and absent MUT bands) and 30 MDR isolates. Of the total selected 5 isolates were excluded; 4 were duplicates

and one had poor banding pattern using LPA version 2.0. There were six discordant RIF results among the three assays as determined by sequencing. Only one isolate had discordant RIF result between LPA and Xpert assays in which LPA version 2.0 detected RIF resistance whereas Xpert did not detect RIF resistance. Sanger sequencing of the *rpo*B gene did not detect any mutation. The LPA assay showed a heteroresistant pattern for RIF displaying *rpo*BMUT3 band and the isolate tested RIF^R by MGIT DST at 1.0 µg/ml (Strydom et al., 2015). Xpert was reported to miss RIF resistance in a mixed population due to wild type masking effect which is one of the limitations of the Xpert assay (Lawn and Nicol, 2011). This discordance phenomenon would not be detected by the routine diagnostic algorithm of the NTBCP and will not be the focus of this dissertation.

The focus of this dissertation will be the phenomenon of discordance Xpert RIF^R: LPA RIF^S, where the Xpert reports MTB resistant to RIF and the LPA reports MTB that is susceptible to RIF. This is the most common discordant scenario for the genotypic tests that is encountered in SA, since screening for MTB according to the routine diagnostic algorithm of the SANTBCP starts with Xpert (Figure 6).

1.7.2 Discordance between genotypic and phenotypic RIF results

Not all *rpo*B mutations confer the same level of RIF resistance; some mutations confer high level resistance (a high RIF MIC) which typically results in a concordant phenotypic RIF^R result with liquid based (MGIT) DST. Some mutations confer low level or “borderline” RIF resistance which would be missed by liquid based (e.g. MGIT) DST and thus give a discordant RIF result.

1.7.2.1 Discordance between genotypic and phenotypic RIF results, specifically RIF^R (genotypic) and RIF^S (phenotypic)

Low level rifampicin resistance occurs when both liquid and solid media phenotypic DST results show susceptibility to RIF at the routine critical concentration recommended for each method (no discrepancy between the two phenotypic methods) but the genotypic test (either or both of Xpert and LPA and

/ or *rpo*B sequencing) detects an *rpo*B mutation (Van Deun et al., 2009, Van Deun et al., 2013). For low level RIF resistance, the MIC lies between 0.5 and 0.0625 µg/ml.

Borderline rifampicin resistance is defined as a discrepancy between liquid and solid culture based DST methods, with the liquid-based phenotypic DST method reporting RIF^S but solid method phenotypic DST reporting RIF^R (Van Deun et al., 2013, Van Deun et al., 2009) and genotypic testing detecting an *rpo*B mutation and the MIC is >0.5 and ≤1.0 µg/ml.

The *rpo*B mutations that confer to either low level or borderline rifampicin resistance have been termed disputed *rpo*B mutations (Van Deun et al., 2009, Van Deun et al., 2013, Rigouts et al., 2013).

Over a six-month period in 2009, in the NHLS Braamfontein TB laboratory, Johannesburg, the LPA detected *rpo*B alterations in single isolates from 422 patients, of which 396 (93.8%) had concordant RIF^R results using the MGIT DST method at the critical concentration of 1 µg/ml. Of the isolates with concordant RIF^R results; 371/422 (88%) isolates had one of the four most commonly encountered *rpo*B mutations (S531L, H526D, H526Y or D516V). The remaining 51/422 (12%) of *rpo*B mutations were those less commonly encountered, identified by the LPA as having “an absent *rpo*B WT band and no corresponding MUT band”; in 26/51 (51%) the MGIT result was susceptible to RIF (Beylis N, 2012).

Van Deun *et al.* studied MTB isolates from 19 TB-confirmed patients who were failing the standard susceptible TB regimen, and detected a discrepancy between genotypic and liquid-based phenotypic DST methods for RIF whereby isolates which had *rpo*B mutations conferring RIF resistance were susceptible to RIF by MGIT DST (Van Deun et al., 2009). The study recommended that a prolonged incubation time and a larger inoculum size may be necessary to detect the resistance of poorly growing low-level RIF-resistant strains and that the RIF critical concentration used with liquid phenotypic methods might be too high.

Rigouts *et al.* showed similar results in that MGIT DST failed to identify RIF resistance in isolates harbouring certain, less common, RIF resistance-conferring mutations associated with low level resistance – these mutations were associated with poor clinical outcome and therefore appear to be clinically relevant (Rigouts et al., 2013). The mutations were L511P, D516Y, H526N, H526L, L533P, I572F and these mutations have been reported to confer low level RIF resistance in other studies (Ohno et al., 1996, Van Deun et al., 2011, Van Deun et al., 2013).

The critical concentration of RIF for liquid DST methods such as MGIT is 1.0 µg/ml; however, the relevance of this concentration to microbiologic and clinical outcomes is unclear. Gumbo used antimicrobial pharmacokinetic / pharmacodynamic (PK/PD) parameters and population pharmacokinetic variability using Monte Carlo simulation to conclude that the critical concentration for RIF should be lowered to 0.0625 µg/ml (Gumbo, 2010).

Similarly, a study by Bottger *et al*. has criticized the DST method used for MTB where the critical concentration is an epidemiological cutoff and is not based on clinical outcome information or PK/PD parameters, as is done for routine bacteriology testing (Bottger, 2011). The study further emphasizes the importance of MIC testing for MTB in assisting the clinician in developing individualized regimens for drug-resistant TB. The level of RIF resistance seems to correlate specifically with the particular codon in the *rpo*B RRDR region, and in addition with the actual SNP/amino acid change that occurs within that codon (Ohno et al., 1996).

In the study performed in Haiti, 153 consecutive clinical isolates resistant to RIF by both Xpert and LPA were selected and tested phenotypically by MGIT and solid agar for RIF susceptibility. There were 16 isolates which were susceptible to RIF by the MGIT DST method and 11 on solid media at the respective critical concentration. The RIF MIC was determined using the microplate assay for all of the discordant isolates. Five isolates were classified as borderline RIF^R, with *rpo*B mutations H526L (n=4) and H526C (n=1). Nine isolates were classified as low level RIF^R with *rpo*B mutations L511P (n=5) with MIC values between 0.125 and

0.25 µg/ml and with double mutations L511P and M515T (n=2) with MIC values between 0.25 and 0.5 µg/ml. The remaining two isolates from two patients harboured an identical silent mutation, a SNP inT508 (AAC-ACT); both were susceptible at the lowest MIC (0.031 µg/ml) tested. It was concluded that it is necessary not only to detect the presence of an *rpo*B mutation, but also to identify the mutation in order to accurately diagnosis RIF resistance. The study also suggested creating a user-friendly database that determines links the specific *rpo*B mutation to its level of RIF resistance (MIC) (Ocheretina et al., 2014).

In a study conducted in Australia, *rpo*B sequencing was performed on 214 consecutive isolates that were phenotypically (MGIT) RIF^S but resistant to other first line drugs (202 were INH mono-resistant and 12 had other resistance patterns). Of the 214 isolates, 207 had *rpo*B sequencing results of which five had *rpo*B mutations; L511P (n=3), D516G (n=1) and L533P (n=1) and 202 had no *rpo*B mutation detected. RIF MIC testing was performed using the MGIT method at concentrations between 0.12 and 1 µg/ml. Isolates with the mutations L511P (n=1) and L533P (n=1) had an MIC value of 1.0 µg/ml and isolates with L511P (n=1) and D516G (n=1) had an MIC value of 0.5 µg/ml. The remaining isolate with mutation L511P had an MIC value of 0.25 µg/ml. On average, the MIC values of the five isolates with an *rpo*B mutation were 7.3 times higher than the control group of 12 isolates (that were all fully susceptible with no *rpo*B mutation). This difference was statistically significant $p<0.0001$ (95% CI 3.9 -13.7) (Ho et al., 2013).

A study from New Zealand described 94 MTB isolates that were resistant to INH and susceptible to RIF by MGIT DST. Xpert as well as *rpo*B sequencing was performed retrospectively on the stored isolates. *rpo*B mutations were detected in four cases and all four *rpo*B mutations were so-called disputed (low level resistance) mutations, single or combined (L511P and M515I, H526N and A532V, D516Y and H526L) (Williamson et al., 2012).

A study conducted in Germany selected 143 stored isolates that were INH-resistant and RIF-susceptible by MGIT DST. These were further tested for *rpo*B

mutations by sequencing. Four (2.8%) isolates had at least one *rpo*B mutation within the RRDR, two isolates had L533P with a MGIT MIC value of 0.5 µg/ml, one had double mutations D516Y and N518D with an MIC value of ≤0.25 µg/ml and the remaining one had double mutations D516Y and E510H with an MIC of 1.0 µg/ml (Andres et al., 2014).

Table 3: *rpo*B mutations described to confer low level RIF resistance and their MIC values

Mutations (n)	MIC value (µg/ml)	Reference
L511P (5)	0.125 to 0.25	(Ocheretina et al., 2014)
L511P (3)	0.25, 0.5 and 1.0	(Ho et al., 2013)
D516G (1)	0.5	(Ho et al., 2013)
D516Y (1)	0.25	(Williamson et al., 2012)
H526L (4)	0.5 to 4.0	(Ocheretina et al., 2014)
H526L (1)	0.5	(Williamson et al., 2012)
L533P (1)	1.0	(Ho et al., 2013)
L533P (2)	0.5	(Andres et al., 2014)
L511P and M55T (2)	0.25 to 0.5	(Ocheretina et al., 2014)
L511P and M55I (1)	0.5	(Williamson et al., 2012)
D516Y and N518D (1)	≤0.25	(Andres et al., 2014)
D516Y and E510H (1)	1.0	(Andres et al., 2014)
H526N and A532V (1)	0.5	(Williamson et al., 2012)

A silent mutation in the *rpo*B gene is a mutation that does not result in an amino acid change of the protein and does not alter the phenotype of *Mycobacterium tuberculosis* against RIF (i.e. would be true RIF^S). Both Xpert and LPA would detect a silent mutation situated within the RRDR that would result in a false genotypic RIF^R result, and a discordant phenotypically susceptible result. Silent mutations have been described to cause discordance between genotypic and phenotypic test methods and if reported as RIF^R have the potential to lead to improper management of patients (Mathys et al., 2014, Alonso et al., 2011, Ocheretina et al., 2014, Mokaddas et al., 2015).

1.7.2.2 Discordance between genotypic and phenotypic RIF results, specifically RIF^S (genotypic) and RIF^R (phenotypic)

Since more than 95% RIF^R MTB strains contain a mutation in the RRDR of the *rpo*B gene (Telenti et al., 1993) and Xpert and LPA utilize the RRDR to detect mutations, both assays may miss a small proportion of MTB RIF resistance due to other mutations or mechanisms. Several mutations outside the RRDR have been reported as being associated with RIF resistance. *rpo*B mutation V176F was shown to be associated with high level of RIF resistance with MIC value 256 µg/ml (Heep et al., 2001). V146, I572F were also shown to be associated with high level RIF resistance (McCammon et al., 2005, Siu et al., 2011). These mutations would not be detected by any genotypic assay targeting the RRDR.

Heteroresistance in MTB may be a cause of discordant laboratory test results. Heteroresistance is defined as the presence of both susceptible and resistant bacteria in a specimen or MTB culture. It may be due to a mixed infection (the presence of different clones of MTB in the same patient) or due to one clone of MTB that has evolved over a shorter period of time from susceptible to resistant, so that both are detected by the laboratory test(s). The detection of heteroresistance is important for patient management (Richardson et al., 2002, Cohen et al., 2012); however, detection is challenging and very dependent on the method that is used (Shamputa et al., 2006, Hingley-Wilson et al., 2013, Folkvardsen et al., 2013, Zetola et al., 2014).

The LPA is able to detect RIF resistance in mixed infection if the resistance is due to a high confidence mutation (one that confers high level RIF resistance (i.e. pattern of all WT bands are present plus a MUT band is present) and if more than 5% of the population is resistant (Tolani et al., 2012, Folkvardsen et al., 2013). If resistance is due to a disputed *rpo*B mutation, it will not be detected by the LPA due to lack of a corresponding MUT band. In one study, the phenotypic assay (MGIT 960) was found to be more sensitive in detecting resistance within a mixed culture than genotypic assays (LPA and sequencing) (Folkvardsen et al., 2013).

Discordance manifest as genotypic RIF^S: phenotypic RIF^R will not be the focus of Part 2 of this dissertation.

1.7.3 Discordance of rifampicin results between different phenotypic assays MGIT RIF^S: LJ RIF^R

Both LJ (solid) and MGIT 960 (liquid) DST are based on the agar proportion method but discordant RIF susceptibility results have been reported between the two assays (Van Deun et al., 2009, Van Deun et al., 2013). This may be related to the different critical concentrations used and to the particular *rpo*B mutation that is responsible for the resistance. Discordance between different phenotypic assays is not the subject of this dissertation.

1.8 Clinical relevance of discordant susceptibility testing results

1.8.1 Discordant genotypic RIF results: Xpert RIF^R versus LPA RIF^S

The studies that described discordance between Xpert and LPA did not include any information about patients' clinical outcomes (Ocheretina Oksana, 2016, Berhanu R, 2015). Discordant results would be expected to cause confusion for the clinicians and may lead to mismanagement and possible poor clinical outcome as well as possibly to continued transmission, e.g. if a patient with false resistance is treated for MDR when the patient has susceptible TB and vice versa. Labeling a patient as having MDR-TB may also lead to psychological stress for the patient as well as have implications for their employment.

1.8.2 Discordant genotypic versus phenotypic results

In an observational cohort study performed in China, outcomes of patients with discordant RIF results (genotypic versus phenotypic) were better in the group of

patients who were treated with a drug-resistant regimen, than in those treated with a susceptible-TB regimen (Pang et al., 2014).

The clinical importance of discordant RIF results of MTB strains with “disputed *rpo*B mutations” remains unclear despite some studies reporting poor clinical outcome of patients infected with these strains. However, the studies are mostly observational case series with very small sample sizes. When RIF resistance is highly likely despite a susceptible phenotypic result, an alternative genotypic assay (such as sequencing) will assist in determining whether the MTB strain harbours a mutation conferring low level resistance. MIC testing will determine the level of RIF resistance. The frequency of strains with low level RIF resistance might be underestimated especially where liquid-based phenotypic susceptibility methods are utilized as the primary DST screening test.

The frequency as well as the nature of discordant RIF susceptibility results between Xpert and LPA should be examined. In addition, the clinical implications of discordant RIF results between Xpert and LPA should be described.

Since the current NTBCP laboratory diagnostic algorithm employs two genotypic tests used consecutively, discordant results may arise between them and also between the two and a subsequent phenotypic DST, if performed. Phenotypic DST (MGIT in the case of NTBCP algorithm) is often used to determine the true RIF result in a case of discordant Xpert and LPA. Phenotypic DST for RIF is limited, however, as *rpo*B mutations giving rise to low level RIF resistance, will test susceptible on MGIT testing at a concentration of 1 µg/ml. DNA sequencing may therefore be a useful adjunctive test to determine which the true susceptibility result is.

In cases of discordant Xpert/LPA RIF susceptibility results there are no clear guidelines available in South Africa with regards to managing the patient and there is no clear laboratory trouble-shooting algorithm on how to further investigate the discordant RIF results. There is no standard method of reporting the discordant results by the laboratories performing the routine tests, and sequencing is not

readily available. In addition, the prevalence of discordant Xpert and LPA RIF results in South Africa is not known.

The prevalence of potential disputed *rpo*B mutations prevalence in South Africa is also not known. In addition, the clinical significance and optimal regimen for treatment of patients infected with MTB strains harbouring these *rpo*B mutations is not known.

1.9 Aims

The overall aim is to describe the two most commonly encountered discordant RIF results encountered in the routine NTBCP testing algorithm of South Africa.

1. Discordant Xpert:LPA results
    a. To determine the frequency of discordant Xpert RIF^R and LPA RIF^S results in Cape Town
    b. To determine whether the discordance between an Xpert RIF^R and LPA RIF^S is due to a
        i. false Xpert RIF^R result, or
        ii. false LPA RIF^S result;
    c. To identify the underlying reason for false Xpert and false LPA results
    d. To develop a laboratory trouble-shooting algorithm for laboratories to assist in decreasing the frequency of discordant Xpert:LPA results
        i. To determine, from our data, whether there are any risk factors in the Xpert/LPA test parameters that are associated with false RIF results, and
        ii. To develop a guide for laboratories to use to determine when a possible false result occurs so that this is not reported
2. Discordant genotypic RIF^R (Xpert or LPA) and phenotypic RIF^S (MGIT) results for RIF
    a. To determine the frequency and types of miscellaneous *rpo*B mutations as identified by the LPA in Cape Town, South Africa. Miscellaneous *rpo*B mutations we define as any *rpo*B mutation detected by the LPA that is not a high confidence (non-disputed) *rpo*B mutation. The specific pattern on the LPA that determines a miscellaneous *rpo*B mutation is any absent *rpo*B wild type band and absent *rpo*B mutation bands
    b. To determine the MICs that occur with specific miscellaneous *rpo*B mutations

c. To determine which of the miscellaneous *rpo*B mutations correlate with high level RIF resistance and which lead to low level / no RIF resistance (and thus would be disputed *rpo*B mutations)
d. To develop a database for all miscellaneous *rpo*B mutations detected in this study that will potentially assist clinicians with individualized management of drug resistant TB patients

CHAPTER 2

2. METHODOLOGY

2.1 Summary of study design

The study was conducted in two National Health Laboratory Service (NHLS) TB laboratories in Cape Town, South Africa: Green Point Complex TB laboratory (GPT) and the microbiology laboratory at Groote Schuur Hospital (GSH).

As per the South African NTBCP TB diagnostic algorithm, all Xpert RIF^R results are confirmed by LPA performed directly on second specimen or on a culture isolate obtained from a second specimen.

Part 1: Discordant Xpert RIF^R and LPA RIF^S

Part 1 of the study includes MTB isolates from patients with discordant Xpert RIF^R and LPA RIF^S results in the study period. Sanger sequencing of the *rpo*B gene from codon 462 to 591 was performed to determine which of the Xpert or LPA was the false test result. The sequencing was performed on the same culture isolates on which LPA had been routinely performed. (Isolates that had disputed *rpo*B mutations by Sanger sequencing underwent MIC testing by the MGIT 960 system and EpiCenter TB eXiST software with RIF concentrations 0.0625, 0.125, 0.25, 0.5 and 1 µg/ml as they were also included in Part 2 of this study).

A comparator group of isolates for Part 1 was included. These were isolates from specimens that had been submitted during the same study period that had concordant Xpert RIF^R and LPA RIF^R results. These specimens were routinely processed in the same way as the discordant isolates' specimens. The comparator group isolates did not undergo *rpo*B sequencing because funding was limited; however, we considered them true RIF^R since the Xpert and LPA RIF results were concordant (both resistant) and the probes indicating the location of the mutation corresponded with each other e.g. if there was a mutation in probe D of Xpert, there was a mutation in WT7 of the LPA (Table 1).

The same Xpert parameter results that were analyzed for the discordant group were available for the comparator group.

Part 2: Discordant genotypic (LPA) RIF^R and phenotypic (MGIT) RIF^S results

Part 2 of the study includes MTB isolates that were interpreted and reported according to the LPA package insert as RIF^R, specifically with an LPA pattern of an absent WT band with no MUT bands detected (irrespective of whether an Xpert result was available). We termed these "miscellaneous *rpo*B mutations"; they were all *rpo*B mutations detected by the LPA except the 4 high confidence *rpo*B mutations S531L, H526Y, H526D and D516V. At this point of selection, we did not know what the corresponding RIF MIC would be (whether susceptible (≤0.0625 µg/ml), "low level" (between 0.125 µg/ml and 1µg/ml) or high level (>1 µg/ml)).

A comparator group of isolates for Part 2 was included. This comprised isolates from routinely processed LPA batches during the study period that were LPA RIF^R (by virtue of an LPA pattern of an absent WT band plus a present MUT band i.e. a high level *rpo*B mutation); some of these were also used as high level RIF^R controls. Isolates that were LPA RIF^S (where sequencing detected no mutation) were used as RIF^S controls (expected MIC ≤0.0625 µg/ml).

All isolates selected for Part 2 of the study and the comparator group underwent *rpo*B sequencing and MIC testing with RIF concentrations of 0.0625, 0.125, 0.25, 0.5 and 1 µg/ml.

2.2 Selection of isolates to be tested

Specimens from patients being routinely tested for TB were submitted to various NHLS laboratories for initial Xpert testing according to the NTBCP algorithm (Figure 6). With the roll-out of Xpert in the NHLS, many peripheral (non-TB-culture) laboratories perform Xpert on the first specimen and then refer the second specimen to a central TB culture laboratory, either the GPT or GSH laboratory. Peripheral NHLS laboratories that performed Xpert on specimens from patients that are included in this study are Tygerberg hospital, Paarl, Khayelitsha, George and Vredendaal laboratories. Thus, patients

included in the study were seen by clinics and hospitals serviced by these peripheral and central NHLS laboratories.

2.2.1 Inclusion criteria for Part 1 isolates (Xpert RIF^R and LPA RIF^S)

1. An MTB isolate with an LPA RIF^S result, for which the patient had a recent (within 7 days) Xpert RIF^R result. In most cases, the specimen for Xpert would have been submitted on the same day as the specimen for which culture and LPA was performed. Specimens collected more than one week apart were excluded from the study.
2. The isolates selected were from specimens that had been submitted to the NHLS laboratories between 1 January 2014 and 31 October 2015.
3. The comparator isolates:
    i. Were selected retrospectively using the laboratory information system (LIS).
    ii. Were from specimens that were submitted to both GPT and GSH laboratories during the study period.
    iii. Included consecutive isolates with a LPA RIF^R result that was concordant with a recent (i.e. within seven days) Xpert RIF^R result for the same patient
    iv. Underwent no further tests to confirm RIF resistance; however, the probes involved in resistance for both the Xpert and the LPA corresponded

Discordant isolates that fit the inclusion criteria for Part 1 of the study were identified in one of two ways:

1. Staff in the laboratory performing the LPA noticed the discordant Xpert RIF result

During the routine review of a batch of LPA results, isolates with LPA RIF^S results are checked against the patient's corresponding Xpert result using the LIS. If the Xpert RIF result was discordant, i.e., RIF^R, the isolate was included in the study. For the study, a

comment was added onto the laboratory LPA report acknowledging the discordant results. A statement was added that further testing was to be performed and to call the laboratory for further information. Isolates from GPT laboratory were sent to GSH laboratory for further testing that included phenotypic DST to RIF at a critical concentration of 1µg/ml (using MGIT method) and *rpo*B sequencing.

2. The clinician / nurse managing the patient noticed the discordant result and informed the laboratory

This usually occurred when the Xpert test was performed at a peripheral laboratory. A comment acknowledging the discordant result and informing the clinician of further testing to be performed was added to the lab report. If the LPA had been performed at GPT laboratory, the isolate was referred to GSH for further testing.

2.2.2 Inclusion criteria for Part 2 isolates (LPA RIF^R and MGIT RIF^S)

1. MTB isolates with the specific LPA pattern of [absent WT band and absent MUT band]. Thus this group includes all *rpo*B mutations detected by the LPA except the more commonly encountered "high confidence" *rpo*B mutations (specifically S531L, H526D, H526Y and D516V) that confer high level RIF resistance (which are identified by an LPA pattern of [absent WT band plus present MUT band]). Consecutive isolates meeting this criterion were selected from routine LPA batches of GPT and GSH TB laboratories.
2. The corresponding specimens for these cultures were submitted between 01 November 2014 and 31 May 2015.
3. Comparator isolates
    a. Isolates with concordant results between LPA and MGIT (i.e. both LPA RIF^R and MGIT RIF^R, and LPA RIF^S and MGIT RIF^S) were included from the same period of study
    b. Were randomly selected and included as overall RIF^R and RIF^S comparison groups, respectively

i. The isolates were selected from the most recent batch of LPA that was performed and one isolate was selected for each batch of sequencing and there were a total of 15 isolates

1. RIF^R isolates had MGIT RIF resistant result and on LPA had one *rpo*B WT probe absent and one *rpo*B MUT probe present (high confidence / high level *rpo*B mutations)
2. RIF^S isolates had a MGIT RIF susceptible result and on LPA had all *rpo*B WT probes present and absent *rpo*B MUT probes

c. Were from specimens that were submitted to both GPT and GSH laboratories

d. Underwent sequencing and MIC testing the same way as the case isolates

e. Underwent reproducibility testing by repeating *rpo*B PCR and *rpo*B sequencing

Table 1: Comparison of codons analyzed by both Xpert and LPA; MTB isolate exhibiting *rpo*B mutation L511P would have an Xpert mutation in probe B (drop out or delay) and an LPA pattern of absent WT2

Xpert			LPA		
	Codons analyzed			Codons analyzed	
Probe A	507	512	WT1	505	509
Probe B	511	517	WT2	510	513
			WT3	513	517
Probe C	516	526	WT4	516	519
			WT5	518	522
Probe D	522	529	WT6	522	525
			WT7	526	530
Probe E	528	533	WT8	530	533

*MTB = mycobacterium tuberculosis, WT = wild type, Xpert = Xpert MTB/RIF, LPA = line probe assay

2.3 Laboratory Test Methods

2.3.1 Xpert MTB/RIF

Procedure

Routine Processing

The peripheral and central NHLS TB laboratories that routinely perform Xpert testing make use of a NHLS standard operating procedure (SOP) that all NHLS laboratories are expected to follow (NHLS GPL3695, 2014); this SOP is in keeping with the manufacturer's instructions. In addition, NHLS laboratories performing Xpert are expected to adhere to an external quality assurance programme (EQA)(NHLS GPL3964, 2016). Participation in the EQA programme by the laboratories involved in this study was 100% and all received 100% in both EQA rounds during the study periods in 2014 and 2015.

Xpert interpretation

The interpretation was performed by the instrument, according to set algorithms developed by the manufacturer. All parameter results including MTB detection, RIF susceptibility, Ct values and bacterial loads were automatically determined and interpreted by the GeneXpert system and downloaded onto the LIS. The results were archived according to the manufacturer's instructions by qualified and competent technologists.

Quality control

Internal and external controls were included by each laboratory according to the prescribed SOP (NHLS GPL3695, 2014, NHLS GPL3964, 2016).

Study processing / analysis of Xpert

For each included isolate, the Xpert probe parameters were recorded and it was determined whether resistance was due to probe drop-out or probe delay. In the case of probe delay, the investigators determined the ΔCt max value by subtracting the earliest probe Ct value from the latest probe Ct value. The probe(s) involved in delayed hybridization or drop-out were also recorded. The graphs generated by the Xpert instrument were analyzed for any unusual sigmoid curves.

2.3.2 GenoType® MTBDR*plus* Line Probe Assay (LPA)

Procedure

Routine sample processing

Specimens were decontaminated using N-acety-L-cysteine- sodium hydroxide (NaOH-NALC) to a final concentration of 1% or 1.5% at GPT and GSH laboratories, respectively. This is in accordance with the MGIT manufacturer's instructions (Siddiqi SH, 2006).

After refrigerated centrifugation at 3000×g, the sediment was re-suspended in approximately 0.5 ml of phosphate buffer. Then, 0.5 ml was inoculated into a MGIT tube and incubated according to manufacturer's instructions. Once the MGIT culture became positive and the culture was determined to be Ziehl Neelsen (ZN) positive for AFB (Ruiz et al.), LPA was performed on the culture.

The extraction and amplification procedures of the LPA were performed according to the manufacturer's instructions (Hain-Lifescience, July 2013). This assay was performed as a routine diagnostic procedure in both GPT and GSH laboratories.

Routine interpretation and reporting

The results were interpreted as per manufacturer's instructions (Hain-Lifescience, July 2013).

The high throughput GPT laboratory uses the GenoScan (Hain Lifesciences) to interpret the LPA strips. The interpretation read-out as well as the image of the strips is available on the computer for reading, archiving and printing. Individual strips were stuck onto the worksheet for manual interpretation, if this was deemed necessary. The smaller GSH laboratory does not use GenoScan since it processes smaller numbers of LPA; routine interpretation was performed manually. The LPA strips were stuck onto the LPA worksheet that is provided by the manufacturer with adhesive tape. Interpretation on the worksheet was performed by the technologist using the template card provided in the kit.

Routine result entry and review

At GPT laboratory, entry of the LPA results into the LIS was through the interface. A technologist checked the results, and then a pathologist reviewed the results on the LIS

using the printout of the strips from the scanner. At GSH laboratory, a technologist interpreted the results which were subsequently checked (not-blinded) by another technologist to ensure quality and minimize any interpretation errors. After agreement, one of the technologists would enter the result manually onto the LIS, and the result was then reviewed by a pathologist using the LPA worksheet with adhered strips.

During routine review of each LPA result, the pathologist would check all other TB results for the patient on the LIS; this included the corresponding Xpert as well as other LPA results, if any, on other specimens. At the GSH laboratory, if on review of an LPA RIF^S result it was found that the patient had a recent Xpert RIF^R result; the LPA was repeated from the culture to exclude any laboratory error / mix up. If the repeat LPA RIF result remained RIF^S, the isolate was selected for inclusion in the study and further testing. If repeat LPA was RIF^R (concordant with Xpert), the isolate was not included in the study. This repeat testing did not routinely occur at the GPT laboratory; the culture was instead referred to GSH laboratory for further testing (routine (reported) MGIT DST and sequencing (for study purposes only). Xpert testing of the culture isolate was not performed due to budgetary constraints, and repeat Xpert testing of the first specimen was not possible since the specimen would have been discarded (and would have been too old for repeat testing at the time discordant results were recognized).

Quality control

Internal and external controls were included in every batch of LPA processed by each laboratory according to the prescribed SOP (NHLS MIC1645, 2014).

Study processing / analysis of LPA for the study

1. Repeating the LPA

The LPA was repeated retrospectively for all discordant isolates from GPT that were included in Part 1 of the study to determine whether the error (in the case of false LPA RIF^S) was with the procedure, or with interpretation. This repetition was performed between two and six weeks after routine results had been obtained and the repeat was performed on the same MGIT culture (aliquot) isolate referred from GPT to GSH for further testing. It was performed according to manufacturer's instructions and laboratory

SOP (NHLS MIC1645, 2014). The LPA was not repeated for the comparator group because all the probes involved in resistance for the Xpert corresponded to the probes on the LPA.

2. Repeating the interpretation

Repeat interpretation of the routinely performed LPA, interpretation of repeat LPA and comparison of routinely processed and repeat LPAs were performed as follows:

a. Photos were taken from the computer of the routinely processed LPA strips that had been archived by the GenoScan scanner at the GPT laboratory. The routinely processed LPA strips from GSH laboratory were still available on the original worksheet. This step was a blinded exercise where each LPA was interpreted according to manufacturer's instructions without referring to the original interpretation and without referring to other (e.g. Xpert, phenotypic DST or sequencing) available results. Two investigators independently performed the "new" interpretation and their results were compared.

b. Isolate mix up as a cause for discordant results was looked for by examining strips within a particular LPA batch that included a case patient (patient with discordant Xpert/LPA results)'s LPA. For example, if the case patient's Xpert RIF^R result indicated a mutation in probe D, LPAs in the same batch that had a WT absent in WT probe 7 were examined and the corresponding patient's own Xpert and other results were checked to make sure that that patient's own Xpert/LPA/other TB results were all concordant with the patient's LPA result.

c. The false LPA RIF^S isolates were stratified into four categories after analyzing all available results (i.e. repeat interpretation of the original LPA, repeat LPA, sequencing, Xpert and phenotypic susceptibility tests). The 4 categories are Technical error, mixed infection, Laboratory mix up, and Indeterminate (Table 2).

    i. Technical error:

        1. "Questionable interpretation": The routine and repeated LPA patterns remain the same; however, the interpretation of

routine and repeat LPA (by study investigator) differs from what was reported for the routine LPA

2. "Procedure error": The pattern of *rpo*B WT bands on the repeat LPA is different to the pattern on the strip of the routine LPA.

ii. Mixed infection: Mixed infection was determined either by LPA or by sequencing results. If the LPA showed heteroresistance where all WT and MUT probes were present for an antibiotic the case was deemed to have mixed infection. Mixed infection was also deemed likely when sequencing was performed on 2 or more isolates from the same patient and contradicting results (one WT and the other with mutation) were found.

iii. Laboratory mix-up: The repeat LPA shows a RIF^R pattern. The routine LPA shows a susceptible pattern, the interpretation of which (by study investigator) does not change (susceptible). Laboratory mix-up was determined to have definitely occurred when the LPA batch containing the false RIF^S LPA strip (apparently belonging to the case patient) was examined and another LPA strip (apparently belonging to an unrelated patient) was found in the batch that has a pattern for RIF resistance, specifically matching the involved Xpert probe for the case patient (e.g. LPA for unrelated patient has absent WT8 and case patient's MTB mutation is in the corresponding Xpert probe E). In addition, on searching the LIS database, another TB result (from a specimen taken within one week of the LPA specimen receipt date) for the unrelated patient is found which is RIF-susceptible. Thus, the unrelated patient has true RIF-susceptible TB.

iv. "Indeterminate": The cause of the discordance is uncertain. The repeat LPA pattern is the same as that of the original; both have presence of all 8 *rpo*B WT bands that are as intense or more intense than the AC band and are also as intense as each other (no *rpo*B WT band is lighter than the other WT *rpo*B bands in the same strip)

and there is no MUT band present. The interpretation of repeat LPA is not different to interpretation that was reported for the routine LPA or the interpretation by study investigator of the routine LPA. Thus, the interpretation is not questionable (all *rpo*B WT bands are clear and as intense as the AC as well as all other *rpo*B WT bands), and procedure error has not occurred as the repeat LPA shows the same pattern as the original LPA pattern. However, the following cannot be ruled out:

1. Specimen (pre-laboratory) or isolate (laboratory) mix-up (on examining LPA batches, no obvious mix-ups were found);
2. Mixed infection since multiple specimens were not submitted from the case patient

Table 2: Classification of false RIF^S by LPA into four categories

Routine LPA: Reported RIF result (by routine laboratory)	**Routine LPA: Interpretation of RIF result (by study investigators)**	**Repeat LPA: Interpretation of RIF result (by study investigators)**	***rpo*B sequencing result**	**Error category**
Susceptible	Inconclusive	Inconclusive	Mutation detected	Technical (Questionable interpretation)
Susceptible	Susceptible	Resistant	Mutation detected	Technical (Procedure error) (cannot rule out specimen / isolate mix up)
Susceptible	Susceptible (heteroresistant pattern on LPA strip)	(hetero) Resistant (heteroresistant pattern on LPA strip)	Mutation detected (only one isolate underwent sequencing)	Mixed infection (determined by LPA)
Susceptible	Susceptible	Susceptible	Mutation detected in one isolate and no mutation detected in another isolate (both isolates are from the same patient)	Mixed infection (determined by sequencing) (cannot rule out specimen / isolate mix up)
Susceptible	Susceptible	Resistant	Mutation detected	Laboratory mix-up
Susceptible	Susceptible	Susceptible	Mutation detected	Indeterminate Cannot rule out mixed infection, laboratory error or specimen/isolate mix-up

*****LPA = line probe assay, RIF = rifampicin

2.3.3 DNA sequencing of the *rpo*B gene of *Mycobacterium tuberculosis*

Isolate retrieval

For isolates that were selected at GSH laboratory, the primary MGIT cultures were available and used for sequencing. These had been stored at room temperature for periods ranging from one to 4 weeks. For isolates that were selected at GPT laboratory, an aliquot of the primary culture was transported at room temperature to the GSH laboratory for testing.

Procedure

2.3.3.1 *rpo*B PCR

Preparation of template

MGIT cultures were mixed by vortexing and an aliquot of 200 µl was transferred into a labeled clean Eppendorf tube (2ml screw cap). The aliquot was boiled at 95-100°C for 30 minutes. The mixture was centrifuged at 12000×g for 5 minutes and 100 µl of the supernatant was transferred to a clean tube and used as template DNA. The extracted DN/A was stored at 4°C until required. Two µl of the extracted DNA was added to 18 µl of PCR master-mix for the PCR reaction. The remaining extracted DNA was stored at -20°C (NHLS MIC1645, 2014).

PCR amplification

A segment of the *rpo*B gene starting at codon 462 and ending at codon 591, containing the RRDR (507-533), was amplified by PCR using the following primers: (Ho et al., 2013).

*rpo*B-F: 5'-GACGACATCGACCACTTCGGCAAC-3′

*rpo*B-R: 5'-GAACGGGTTGACCCGCGCGTACA-3′

Upon receipt of primers, a working stock solution was made up to a final concentration of 10 pmol/µl (NHLS VIR0241, 2016). The primers were synthesized by the University of Cape Town, South Africa (NHLS VIR0241, 2016).

The PCR reaction mixture (master mix) was made up as follows: each 20µl PCR mixture contained 10µl KapaTaq 2x Ready Mix with Mg^{2+} (Biosystems, Boston, USA), 1µl of the forward and reverse 10µM primers, 6µl distilled water, and 2µl of genomic DNA. The

KapaTaq Ready Mix contained everything required for PCR except the primers and the template (0.05U/µl KapaTaq DNA polymerase, reaction buffer with Mg^{2+} and 0.4mM each dNTP) (Schleper et al., 1997).

Addition of DNA

Adding the template DNA was performed in a separate room. Two µl of template DNA was added to the 18µl PCR mixture and mixed with a pipette and placed into a thermocycler for amplification in a dedicated amplification room. The amplification parameters included an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 58°C for 45 seconds, and elongation at 72°C for 45 seconds, with a final elongation step at 72°C for 5 minutes.

PCR cycling conditions:

95°C for 5 minutes
95°C for 1 min
58°C for 45 sec } 35 cycles
72°C for 45 sec
72°C for 5 min
15°C for hold

Detection of PCR product

Agarose (2%) gel electrophoresis was used to determine the presence or absence of PCR product and determine the size of the PCR product.

Interpretation of PCR product

Interpretation of the gel

1. Presence of a single band size of 400 base pairs (bp) - positive for PCR product
2. Absence of a band of 400bp - negative for PCR product

Figure 1: *rpo*B PCR of nine isolates (V17 to D18), a negative control (E18) and a positive control H37Rv (F18). All isolates had PCR product of 400bp thus MTB is detected and sent for sequencing. *MW VI = molecular weight marker VI (was used as a ladder), PCR = polymerase chain reaction,

MTB = *Mycobacterium tuberculosis*, bp = base pairs

2.3.3.2 DNA sequencing

The *rpo*B sequencing of all amplified study isolate products was Sanger sequencing and was performed by Inqaba Biotechnical Industries (Pretoria, South Africa). PCR products underwent the chain termination method to sequence the segment that had been amplified (*rpo*B gene from codon 462 to 591).

Procedure

Once a single band of 400 bp PCR product was obtained, the PCR product as well as both forward and reverse primers were sent to Inqaba Biotechnical Industries for Sanger sequencing. At Inqaba, the PCR products were cleaned using Exonuclease I (New England Biolabs, Beverly, MA) and Shrimp Alkaline Phosphatase (New England Biolabs, Beverly, MA) mixture and incubated first at 37°C for 30 minutes then at 95°C for 5 minutes. Sanger sequencing was performed with the ABI BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions using amplification primers (Industries, 2015). Labeled

products were cleaned with Zymo Research DNA sequencing clean-up kit (Zymo Research, Orange, CA) according to the manufacturer's instructions. The cleaned products were analyzed on an ABI 3500XL Genetic Analyser (Applied Biosystems, Foster City, CA, USA) (Industries, 2015).

Interpretation of DNA sequencing

The DNA sequence data were analyzed and aligned manually against the reference sequence of H37Rv using DNAman software (LynnonBioSoft, Quebec, Canada); MFinder software was used to analyze any mismatches. MFinder software starts by scanning a user specified directory for matching forward and reverse complement AB1 files. Base calling was done for both forward and reverse complement files using Phred with a cutoff parameter of 0.05. The forward and reverse complement files were then merged using a Biojava gapped aligner. Blastn was then used to find an alignment with H37Rv reference sequence. Mismatches in the alignment were recorded. A report for each of the sequence pairs was then generated indicating the position of the mismatches.

All sequence results were analyzed for any sequence mismatch; if there was no nucleotide sequence mismatch then it was reported as "*no mutation detected*". If there were any nucleotide mismatches the result was reported as "*mutation detected*" and further analyzed if the mismatch caused a codon change that led to an amino acid change. In the cases where mismatches were detected, the actual codon and amino acid change were reported. It was reported as a change from the original amino acid followed by codon number (according to the *E. coli* numbering scheme) and resultant amino acid and the nucleotide change (Table 3).

Table 3: DNA sequences of two isolates (A14 and B14) aligned against the reference sequence H37Rv.

H37v - 514 TTCATGGACCAGAACAACCCGCTGTCGGGGTTGACCCACAAGCGCCGACTGTCGGCGCTG 533
A14 - 514 TTCATGGACCAGAACAACCCGCTGTCGGGGTTGACCCACAAGCGCCGACTG**CA**GGCGCTG 533
B14 - 514 TTCATGGACCAGAACAACCCGCTGTCGGGGTTGACCCACAAGCGCCGACTGTCGGCGC**C**G 533

Isolate	Codon involved	Original nucleotide	Nucleotide change	Original amino acid	Amino acid change	Mutation abbreviation
A14	531	TCG	CAG	Serine (Ser) S	Glutamine (Gln) Q	S531Q
B14	533	CTG	CCG	Leucine (Leu) L	Proline (Pro) P	L533P

The sequence shown here is a segment of the RRDR (from codon 514 to 533). Isolate A14 underwent a double nucleotide polymorphism in codon 531; TCG was substituted with CAG causing an amino acid change from serine (S) to glutamine (Q) and the mutation is coded as S531Q. Isolate B14 underwent a single nucleotide polymorphism in codon 533; where CTG was substituted with CCG causing an amino acid change from leucine (L) to proline (P) and the mutation is coded as L533P.

For Part1 of the study *rpo*B sequencing results were used as a gold standard test to determine whether the Xpert RIF results were false resistant or the LPA RIF results were false susceptible.

Quality control

PCR products were sequenced using bidirectional (i.e. forward and reverse) primers for maximum coverage and reproducibility of results.

Controls were performed with every *rpo*B PCR and *rpo*B sequencing batch run as follows:

1. H37Rv ATCC 27294 was used as the positive control for *rpo*B PCR and as a wild type control for sequencing
2. Only master mix reagents were used as the blank control for *rpo*B PCR
3. Ultra-pure water was used as the negative control for *rpo*B PCR
4. Isolates that were LPA RIF^R with MUT band present (high confidence *rpo*B mutations) were randomly selected and sequenced and were used as mutant controls for sequencing

2.3.4 Rifampicin minimum inhibitory concentration testing

All MTB isolates that were selected for Part 2 of the study underwent MIC testing. Because the isolates were obtained during the same study period, MTB isolates that originated from Part 1 of the study that were determined to have a disputed *rpo*B mutation by sequencing were included in Part 2 of the study, and underwent MIC testing.

MIC testing was performed using the MGIT 960 system and EpiCenter TB eXiST software with RIF concentrations of 0.0625, 0.125, 0.25, 0.5 and 1 µg/ml according to manufacturer's instructions (Siddiqi SH, 2006, Springer et al., 2009).

Procedure

Preparation of Drug

Rifampicin concentrations (Becton Dickinson Diagnostic Systems, Sparks, MD) were prepared according to manufacturer's instructions.

For each isolate, six MGIT tubes were labeled as follows with their corresponding RIF concentrations: tube 1 (growth control tube), tube 2 (0.0625 µg/ml), tube 3 (0.125 µg/ml), tube 4 (0.25 µg/ml), tube 5 (0.5 µg/ml) and tube 6 (1.0 µg/ml). 100µl of the reconstituted RIF was added to the appropriately labeled corresponding MGIT tubes. No drug was added to the growth control tube. All MGIT tubes with antibiotic added are called test MGIT tubes.

Preparation of inocula

The primary MGIT culture was sub-cultured by transferring 0.5 ml of culture to a new MGIT tube (with supplement MGIT added) and incubated at 37°C in the automated MGIT instrument. Once the MGIT tube flagged positive for growth, "test" and "control" inocula were prepared from the tube when the growth unit (GU) was approximately 500 (day 1 or day 2 after flagging positive). A purity check was performed by streaking the MGIT culture onto a blood agar plate which was incubated for 48 hours at 37°C.

Sub-culture MGIT tubes that were processed one or two days after flagging positive, were mixed well by vortexing and allowed to stand for one to two minutes to ensure that the larger clumps had settled. The upper layer of approximately 3.5 ml was aspirated into a clean sterile tube and this was termed the standard inoculum. Sub-culture MGIT tubes

that were processed between three and five days after flagging positive were mixed by vortexing and a 1:5 dilution was made in saline; this was used as the standard inoculum.

Control inoculum was prepared by diluting the standard inoculum 1:100 in saline (100 µl standard inoculum + 9.9 ml saline). Then 0.5ml of the “control” inoculum was transferred to the “control MGIT tube” containing 0.8 ml of MGIT supplement.

Addition of MTB and drug into MGIT tubes and incubation

For the “test MGIT tubes”, 0.5 ml of the standard inoculum was transferred to each test tube containing 0.8 ml of MGIT supplement and respective RIF concentration with final RIF concentrations of 1 µg/ml, 0.5 µg/ml, 0.25 µg/ml, 0.125 µg/ml and 0.0625 µg/ml. All MGIT tubes (control and test tubes) were inoculated at the same time and incubated at 37°C in the BACTEC MGIT 960 instrument.

Interpretation

The interpretation was performed according to the manufacturer’s instructions. Briefly, an isolate was considered ***resistant*** if the time to positivity of the RIF containing tube was faster than that of the growth control tube or if the growth unit (GU) of the RIF containing tube was ≥ 100 when the growth control tube reached a GU of ≥ 400. Conversely, an isolate was considered ***susceptible*** when the growth unit of the RIF containing tube remained < 100 after the growth control tube reached a GU of ≥ 400. The MIC value was reported as the lowest concentration of the drug at which the isolate was susceptible (Figure 2). If tube 1.0 µg/ml has a GU of <100 and the remaining tubes (0.5, 0.25, 0.125 and 0.0625 µg/ml) have GU >100 when a GU of ≥400 is reached in the growth control tube then the MIC is 1.0 µg/ml. If the GU is >100 in all test tubes (1.0, 0.5, 0.125, and 0.0625 µg/ml) when the growth control tube reached a GU of ≥ 400; the MIC value is >1.0 µg/ml.

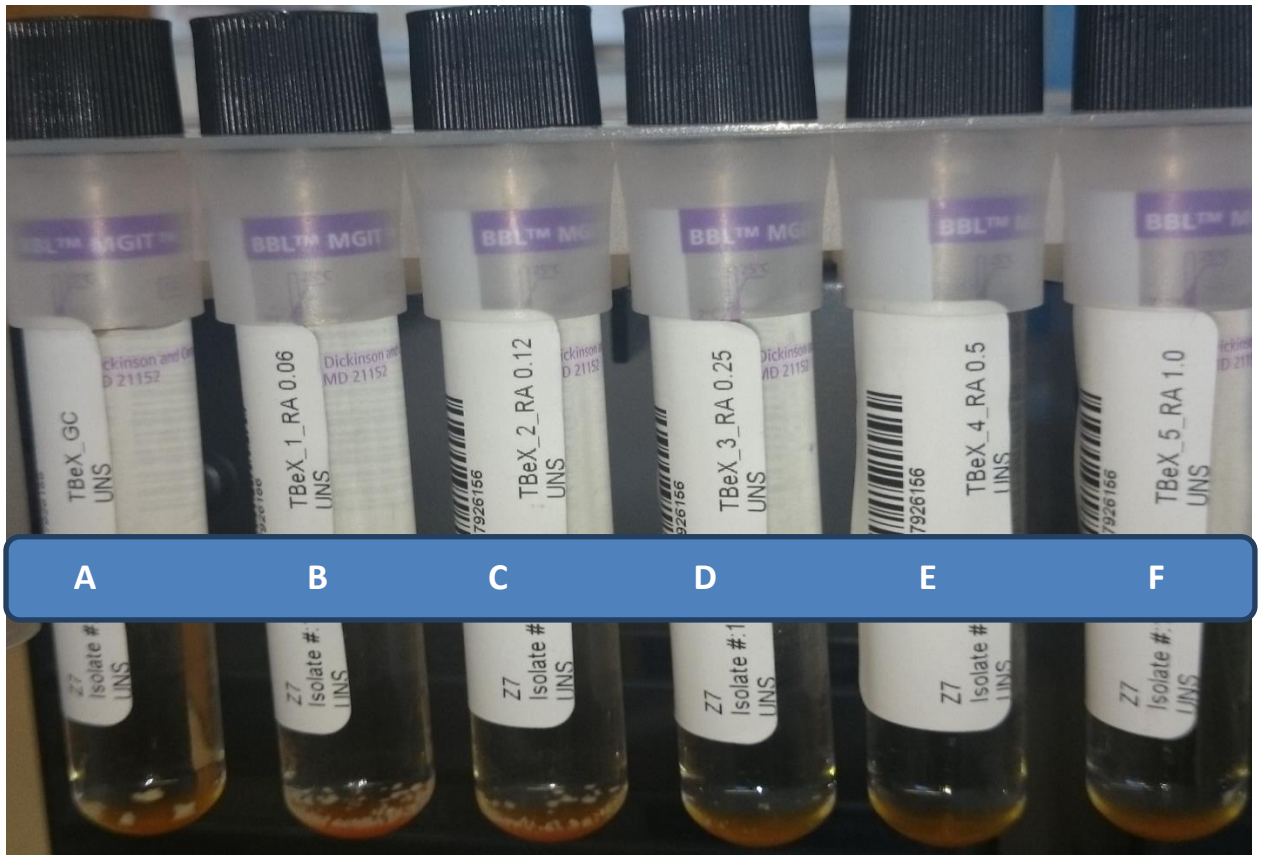


Figure 2: MGIT MIC test for isolate Z7 showing six MGIT tubes; there is visible growth in tubes A) Growth control tube, B) TBeX 1 (0.0625µg/ml) and C) TBeX 2 (0.125µg/ml). There is no visible growth in tubes D) TBeX 3 (0.25 µg/ml), E) TBeX 4 (0.5 µg/ml) and F) TBeX 5 (1 µg/ml). Tubes A, B, and C had growth units of >400 reading from the MGIT instrument and tubes D, E, and F had growth units of zero. The lowest concentration at which the growth unit was <100 when tube A (growth control) had growth unit just exceeding 400 is considered the MIC value. The MIC value of isolate Z7 is reported as 0.25 µg/ml, i.e. tube D.

*MIC = minimum inhibitory concentration, MGIT= Mycobacterium Growth Indicator Control Tube

Quality control

MGIT tube control

Quality control was performed on new (lot numbers or shipment) MGIT tubes as per manufacturer's instructions where H37Rv ATCC 27294 was used as the positive control.

Rifampicin drug control

Quality control was performed on new (lot numbers or shipment) antibiotic reagents as follows:

1. H37Rv was used as a susceptible control with an expected MIC value of ≤0.0625µg/ml.
2. Randomly selected isolates with known mutations (D516V, H526Y and S531L) that confer high level RIF resistance were used as resistant controls.
3. Randomly selected isolates whose sequencing revealed no *rpo*B mutations were included as RIF susceptible controls (expected MIC $\leq$ 0.0625ug/ml).

Details of reagent, lot number and expiry date were recorded and controlled before being used. All equipment used in the analysis was calibrated and serviced according to manufacturer's instructions.

2.4 Ethics

This study received ethical approval from the Human Research Ethics Committee of the Faculty of Health Sciences at the University of Cape Town (HREC REF: 737/2014).

2.5 Data recording and analysis

Part 1 of the Study

Xpert parameter results of cases and comparator group were recorded on an MS Excel spreadsheet. Xpert parameters were used to calculate sensitivity, specificity, positive predicative value (PPV) and negative predictive values (NPV) and predict a false Xpert RIF^R (Table 4).

Table 4: Calculations of sensitivity, specificity, positive predictive value and negative predicative values

False RIF^R by Xpert		**Not false RIF^R (True RIF^R) by Xpert**	
Positive Parameter	**A**	Positive parameter	**B**
Negative parameter	**C**	Negative parameter	**D**

Sensitivity = A/(A+C) Specificity = D/(D+B)

PPV = A/(A+B) NPV = D/(D+C)

*RIF^R = rifampicin resistant, PPV = positive predictive value, NPV = negative predictive value

The LPA probe pattern for RIF was recorded for both cases and comparator group. The original interpretation and repeat interpretation of the routine LPA by investigators was also recorded and compared. The interpretation and probe pattern of the repeat LPA was also recorded and compared to the routine and repeat interpretation of the original LPA.

Stata version 13.1 was used to obtain continuous Poisson regression to determine the probability of false RIF^R by Xpert with regards to Xpert parameters and this was indicated in incidence rate ratio (IRR) with 95 % confidence intervals (CI), among isolates with discordant RIF^R results.

The sequencing result was used to determine whether the discordance was due to false RIF^R by Xpert or false RIF^S by LPA. If sequencing detected no mutation, the overall result was categorized as “false RIF^R t by Xpert”. If sequencing detected an *rpo*B mutation within the RRDR, the overall result for the patient was “false RIF^S by LPA”.

For Part 1 and Part 2 of the Study

Sequencing results were recorded as follows;

- “No mutation detected” was recorded if there was no mutation detected within the sequenced *rpo*B gene which covered the RRDR
- If a mutation was detected in the sequenced *rpo*B gene, the following were recorded;
    - The nucleotide polymorphism and/or deletion (from wild type nucleotide/s to mutant nucleotide/s),
    - Codon position number where the mutation occurred,
    - Original amino acid and substitute amino acid

- A mutation was abbreviated with original amino acid abbreviation followed by codon position number followed by substitute amino acid abbreviation
- The mutation detected was compared to the Xpert probes and LPA probe bands pattern

MIC results of both cases and comparator group were recorded. MIC value of ≤0.0625 µg/ml was considered RIF^S, MIC value >0.0625 and ≤1.0 µg/ml was considered low level RIF^R and MIC of >1.0 µg/ml was considered high level RIF^R.

Excel was used to graphically determine MIC distribution among the miscellaneous *rpo*B mutations determined by the LPA.

CHAPTER 3

3. RESULTS

3.1 Part 1. Discordant Xpert RIF^{R} and LPA RIF^{S}

From January 2014 to November 2015 there were 210,378 samples processed for Xpert at both GPT and GSH laboratories. Xpert detected MTB complex in 27,940 (13.3%) specimens and RIF resistance in 1542 (5.5%); this excludes duplicate specimens, i.e., more than one specimen per patient.

For those patients with a RIF^{R} by Xpert, the LPA result on the subsequent 2nd specimen for each patient was reported as RIF^{R} in 1436 (93.1%) and as RIF^{S} (i.e. discordant) for 106 (6.9%) (Figure 1). *rpo*B DNA sequencing was performed on the discordant 106 isolates of which 101 (95.3%) had sequencing results available. Sequencing results were not obtained for five (4.7%) isolates due to the cultures being contaminated with other bacteria (n=3) or with NTM (n=2). The bacterial contamination was confirmed by the presence of bacterial growth on 2% blood agar. The presence of NTM was confirmed by the GenoType CM (common mycobacteria) LPA (HAIN, Lifescience, Nehren, Germany), which detects MTB complex as well as various non-tuberculous mycobacteria in addition to MTB.

Of the 101 MTB isolates with DNA sequencing results available, a total of 78 (77.2%) had no mutation detected and these were categorized as “false RIF^{R} by Xpert”. DNA sequencing detected a mutation in the RRDR of the *rpo*B gene in 23 (22.8%) isolates and these were categorized as “false RIF^{S} by LPA”. For the latter group, the probe involved in the resistance detected by Xpert corresponded with the *rpo*B mutation detected by sequencing in all 23 cases.

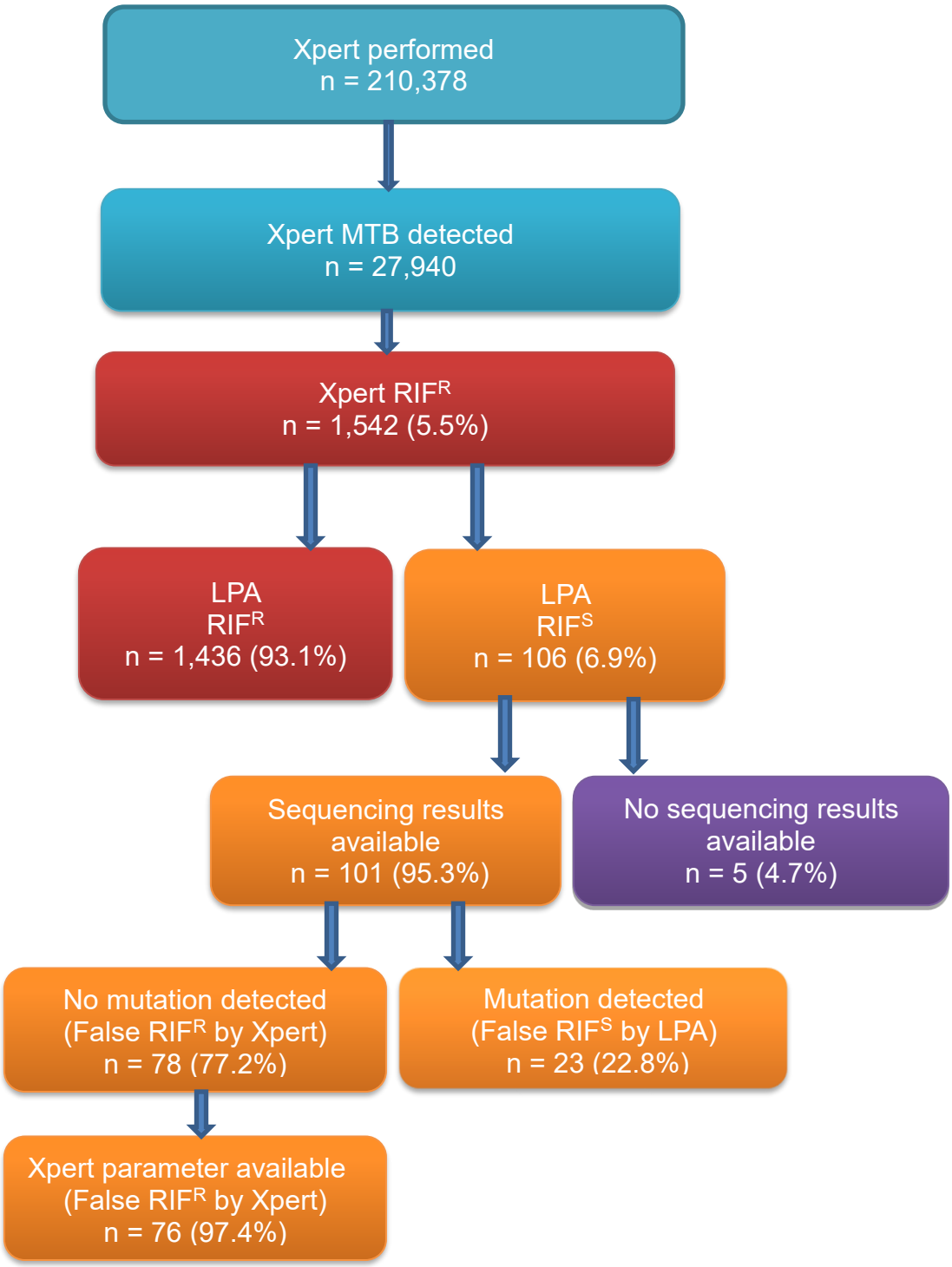


Figure 1: The number of patients with discordant Xpert (RIF^{R}) and LPA (RIF^{S}) results and the proportions with mutations not detected (false Xpert) and mutations detected (false LPA) by sequencing.

*MTB = *Mycobacterium tuberculosis*, RIF^{R} = Rifampicin resistant, RIF^{S} = Rifampicin susceptible, LPA = Line probe assay

3.1.1 False RIF^R by Xpert (n=78)

Of the 78 cases with false RIF^R by Xpert (no mutation detected by sequencing), 76 (97.4%) had Xpert parameter values available. The parameter values of the remaining two could not be obtained because the parameter values were not available on the LIS and readouts had not been archived. Xpert parameters were available for the entire control group of 1436 patients that had concordant RIF^R results for Xpert and LPA.

The total number of samples with RIF^R results by Xpert during the study period for which Xpert parameters were available was 1512; this comprised 1436 true RIF^R (the comparator group of patients with concordant Xpert and LPA results (controls)) plus 76 false RIF^R cases by Xpert (cases).

The following Xpert parameters were compared:

1. Probe delay versus probe dropout

We assessed the value of probe delay and probe dropout in identifying false RIF^R results.

Probe delay (ΔCt max >4) occurred in 56/76 (73.7%) cases compared with 104/1436 (7.2%) controls (*p* <0.0001). Probe dropout occurred in 20/76 (26.3%) cases compared with 1332/1436 (92.8%) in the control group (*p* <0.0001) (Table 1).

Table 1: Accuracy of probe delay (ΔCt max > 4) vs. probe dropout for detecting a false RIF^R result

Xpert RIF^R parameter	True RIF^R by Xpert n (%)	False RIF^R by Xpert n (%)	Total n (%)
Probe delay (ΔCt max > 4)	104 (7.2)	56 (73.7)	160 (10.6)
Probe dropout	1332 (92.8)	20 (26.3)	1352 (89.4)
Total	1436 (100)	76 (100)	1512 (100)

Xpert RIF^R parameter	IRR	95% CI	Sensitivity	Specificity	PPV	NPV
Probe delay ΔCt max > 4	23.66	14.59 - 38.38	73.7	92.8	35	98.5

RIF^R = Rifampicin resistant, ΔCt = Delta cycle threshold maximum, IRR = Incident rate ratio, CI = Confidence interval, PPV = Positive predictive value, NPV = Negative predictive value

Probe delay was a significant predictor of false RIF^R by Xpert; an Xpert RIF^R result that is due to probe delay was 23.66 more likely to be false RIF^R than an Xpert RIF^R result that is due to a probe dropout (95%CI: 14.59 - 38.78) (Table 1).

2. A probe delay value (delta Ct max) of between 4.1 and 4.9

We assessed the value of ΔCt max between 4.1 and 4.9 for identifying false RIF^R. Of the 1512 cases with RIF^R by Xpert, 62 (4.1%) had resistance determined by probe delay with a ΔCt max value between 4.1 and 4.9. The remaining 1450 (95.9%) Xpert RIF^R cases had probe dropout or probe delay with ΔCt max above 5 (Table 2).

Of the 1436 controls, 20 (1.4%) had a ΔCt max value between 4.1 and 4.9 compared with the cases where 42/76 (55.3%) had a ΔCt max of 4.1-4.9 ($p < 0.0001$).

Table 2: Accuracy of probe delay of ΔCt max 4.1 - 4.9 vs. probe delay ΔCt max ≥5 or probe dropout for detecting a false RIF^R result

Xpert RIF^R parameter	True RIF^R by Xpert n (%)	False RIF^R by Xpert n (%)	Total n (%)
Probe delay ΔCt max 4.1 - 4.9	20 (1.4)	42 (55.3)	62 (4.1)
Probe delay ΔCt max ≥ 5 or probe dropout	1416 (98.6)	34 (44.7)	1450 (95.9)
Total	1436 (100)	76 (100)	1512 (100)

Xpert RIF^R parameters	IRR	95% CI	Sensitivity	Specificity	PPV	NPV
Probe delay ΔCt 4.1 - 4.9	28.89	19.87 - 41.99	55.3	98.6	67.7	97.7

RIF^R = Rifampicin resistant, ΔCt = Delta cycle threshold maximum, IRR = Incident rate ratio, CI = Confidence interval, PPV = Positive predictive value, NPV = Negative predictive value

Probe delay with ΔCt max value between 4.1 and 4.9 was a significant predictor of false RIF^R by Xpert. A result of RIF^R by Xpert where probe delay with a ΔCt max value of

between 4.1 and 4.9 was 28.89 times more likely to be false RIF^R than an Xpert result of RIF^R that where there was probe dropout or a probe delay with a ΔCt max value ≥ 5 (Table 2).

3. Bacterial load (quantitative)

We assessed the value of MTB bacterial load in identifying false RIF^R results. A total of 239 (15.8%) patients had RIF^R results by Xpert with readouts of 'Very Low' bacterial load of which 47 were false RIF^R making up 61.8% of the total cases that had false RIF^R by Xpert (Table 3).

Table 3: Comparison of proportion of patients with true vs. false Xpert RIF^R stratified by bacterial load (as determined by Xpert); and the accuracy of a Very Low bacterial load result for detecting false RIF^R

Xpert Parameter	True RIF^R by Xpert n (%)	False RIF^R by Xpert n (%)	Total n (%)
Very Low	192 (13.4)	47 (61.8)	239 (15.8)
Low	331 (23.0)	10 (13.2)	341 (22.5)
Medium	479 (33.4)	9 (11.8)	488 (32.3)
High	434 (30.2)	10 (13.2)	444 (29.4)
Total	1436 (100)	76 (100)	1512 (100)

RIF^R = Rifampicin resistant, IRR = Incident rate ratio, CI = Confidence interval, PPV = Positive predictive value, NPV = Negative predictive value

Xpert RIF^R parameters	IRR	95% CI	Sensitivity	Specificity	PPV	NPV
Very Low	8.58	5.57 - 13.21	61.8	86.6	19.7	97.7

MTB bacterial load of Very Low occurred in 47//76 (61.8%) cases compared with 192/1436 (13.4%) controls (p <0.0001) and VL MTB bacterial load is a significant predictor of false RIF^R by Xpert.

Xpert Very Low bacterial load parameter was a significant predictor of false RIF^R compared to combined non-Very Low quantitative loads (Low, Medium or High). A RIF^R result by Xpert with a Very Low bacterial load was 8.58 times more likely to be false RIF^R

than a RIF^R result with Non-Very Low bacterial load (IRR: 8.58, [95%CI: 5.57 – 13.21]) (Table 3).

4. Xpert probes

We assessed the value of delay of specific probes, as well as 'double probe delay' for identifying False RIF^R. When compared to probe B; probes A, C, D and D+E were more likely to be involved in a false RIF^R result while probe E was not a statistically significant predictor of false RIF^R by Xpert.

Double probe delay is defined as probe delay when two probes each have a ΔCt max value > 4 but the ΔCt max value of each of the remaining probes is ≤ 4. There were 12 cases in the study period where RIF^R was due to a double probe delay (all involved probes D & E) and all of these were false RIF^R by Xpert. The control group had no RIF^R results due to delay of more than one probe (all 3 Xpert results in the control group where RIF resistance involved more than one probe ([A+B] and [A+B+D]) were determined by drop-out) (Table 4).

Table 4: Comparison of probes involved in Xpert RIF^R and the probability of a result with these values being false RIF^R

Xpert probe	True RIF^R by Xpert n (%)	False RIF^R by Xpert n (%)	Total n (%)
A	71 (4.9)	7 (9.2)	78 (5.2)
B	130 (9.1)	1 (1.3)	131 (8.6)
C	9 (0.6)	1 (1.3)	10 (0.7)
D	170 (11.8)	12 (15.8)	182 (12)
D+E	0 (0)	12 (15.8)	12 (0.8)
E	1053 (73.3)	43 (56.6)	1096 (72.5)
A+B	2 (0.2)	0 (0)	2 (0.1)
A+B+D	1 (0.1)	0 (0)	1 (0.1)
Total	1436 (100)	76 (100)	1512 (100)

False RIF^R by Xpert; Xpert probe	IRR	Robust Std. Err.	z	P >|z|	95% CI	
A	11.75	12.46	2.33	0.020	1.47	93.83
C	13.09	18.02	1.87	0.062	.88	194.39
D	8.63	8.94	2.08	0.037	1.14	65.65
D+E	131.00	130.55	4.89	0.000	18.56	923.73
E	5.14	5.18	1.62	0.104	.71	37.04
A+B	.00	.00	-7.82	0.000	6.44e-06	.00
A+B+D	.00	.00	-6.77	0.000	4.44e-06	.00
B (reference)	1	.001	1	0.000	.001	.054

RIF^R = Rifampicin resistant IRR: Incident rate ratio CI = Confidence interval

5. Combination of parameters: Very Low bacterial load plus probe delay

A total of 67 RIF^R results by Xpert had both a probe delay (ΔCt max > 4) and a Very Low bacterial load. Of these, 43 (64.2%) results were false RIF^R. Furthermore, in 42/67 (62.7%) results with Very Low bacterial load the ΔCt max value was between 4.1 and 4.9 and 36 (85.7%) were false RIF^R by Xpert (Table 5). This is in contrast with only 6 controls (14.3%) that had a VL bacterial load and ΔCt max value between 4.1 and 4.9.

Table 5: Combinations of bacterial load and probe delay for assessing the accuracy of a RIF^R result by Xpert

Xpert parameter	True RIF^R by Xpert n (%)	False RIF^R by Xpert n (%)	Total n (%)	Sens	Spec	PPV	NPV
VL + ΔCt > 4	24 (35.8)	43 (64.2)	67 (100)	56.6	98.3	64.2	97.7
Non (VL + ΔCt > 4)	1412 (97.7)	33 (2.3)	1445 (100)				
VL + ΔCt 4.1 - 4.9	6 (14.3)	36 (85.7)	42 (100)	47.4	99.6	85.7	97.3
Non (VL + ΔCt 4.1 - 4.9)	1430 (97.3)	40 (2.7)	1470 (100)				
VL + ΔCt ≥ 5	24 (77.4)	7 (22.6)	31 (100)	29.2	98.3	22.6	95.3
Non (VL + ΔCt ≥ 5)	1412 (95.3)	69 (4.7)	1481 (100)				

RIF^R = Rifampicin resistant, Sens = Sensitivity, Spec = Specificity, PPV = Positive predictive value, NPV = Negative predictive value, VL = Very low bacterial load, ΔCt = Delta cycle threshold maximum

The PPV of VL and ΔCt max >4 is 64.2% and PPV of VL and ΔCt max between 4.1 and 4.9 is 85.7 with NPV of 97.7% and 97.3% respectively.

6. ΔCt max 4.1 - 4.9 versus Very Low bacterial load

When comparing the parameters Very Low bacterial load and ΔCt max between 4.1 and 4.9 to each other and to other parameters, ΔCt max 4.1 - 4.9 (IRR: 15.7 [95% CI: 9.73 – 25.34]) was a stronger predictor of false RIF^R than Very Low bacterial load (IRR: 2.97 [95% CI: 1.78 – 4.93]) (Table 6).

Table 6: Comparison of Xpert RIF^R parameters Very Low bacterial load and ΔCt max 4.1 - 4.9 in predicting false RIF^R

Xpert parameter	IRR	Robust Std. Err.	Z	P >|z|	95% CI	
VL	2.97	0.77	4.19	0.000	1.78	4.93
ΔCt 4.1 - 4.9	15.70	3.83	11.28	0.000	9.73	25.34
_cons	.019	.003	-21.18	0.000	.012	.027

RIF^R = Rifampicin resistant, VL = Very low bacterial load, ΔCt = Delta cycle threshold maximum

IRR = Incident rate ratio, CI = Confidence interval

3.1.2 False RIF^S results by LPA (n=23)

Of the discordant isolates (Xpert RIF^R and LPA RIF^S) that had *rpo*B sequencing results available (n=101), 23 (22.8%) had a mutation detected by sequencing and were thus classified as false RIF^S by LPA. All the mutations detected by sequencing lie within the RRDR of the *rpo*B gene and thus would be expected to be detected by the LPA. In all 23 cases, the Xpert probe that failed to hybridize covered the region where the identified mutation lies (true RIF^R by Xpert) (Table 7).

Table 7: List of 23 isolates that had false RIF^S by LPA and true RIF^R by Xpert showing Xpert probe involved in resistance corresponding with the *rpo*B mutation detected by sequencing

Study number	Xpert RIF result	Xpert probe involved	LPA RIF result	Sequencing result
U16	R	A	S	L511P
L	R	A	S	L511P
H14	R	D	S	H526L
J	R	E	S	L533P
P5	R	E	S	L533P
Q10	R	E	S	L533P
A17	R	A	S	L511P
Q2	R	A	S	L511P
E	R	E	S	L533P
W	R	E	S	L533P
O2	R	A	S	L511P
F4	R	B	S	D516Y
N	R	D	S	H526L
F6	R	D	S	H526N
B	R	D	S	H526N
S5	R	E	S	H531L
L2	R	E	S	S531L
Q15	R	E	S	L533P
O5	R	D	S	H526N
D6	R	E	S	L533P
Z3	R	A	S	L511P
A	R	A	S	L511P
M15	R	D	S	H526N

RIF^S = Rifampicin susceptible, LPA = Line probe assay, RIF^R = Rifampicin resistant

The causes of false RIF^S results by LPA were grouped as follows (Table 8):

i. Technical error

There were 11 cases where on further examination by study investigators, the routine LPA result was doubtful and a technical error causing the discordance for RIF between Xpert and LPA was deemed likely. These were further divided into questionable interpretation (n=6) or procedure error (n=5).

a. Questionable interpretation (Figure 2)

Six tests (U16, H14, P5, Q10, A17 and Q2) were interpreted as RIF^S during routine reporting of the LPA. However, on examination by the study investigators, it was found that the patterns on both routine and repeat LPA strips was not clearly a wild type pattern and interpretation of this LPA result for RIF was deemed inconclusive. The original LPA strip was obtained and re-examined by the study investigators, who were blinded to the repeat LPA result at this point. It was noted that for all six cases, all wild type (WT) bands for *rpo*B were present (darker than the AC band) and there was no mutation band (MUT) present, in keeping with an interpretation of "susceptible" (as was done routinely). However, it was also noted that one WT band was lighter than adjacent WT bands for *rpo*B in the strip, even though it was the same intensity as the AC band in the strip. This lighter *rpo*B WT band corresponded to the mutation detected by sequencing. The LPA was repeated by the study investigator and the same pattern was observed, where the involved WT band was equal in intensity or more intense than the corresponding AC band; thus, interpretation would also be as susceptible, according to the package insert. Overall, the investigators assessed the LPA pattern for RIF as inconclusive and would recommend repeat testing (Table 8).

b. Procedure error

Five study isolates (L, J, E, D6 and M15) showed a susceptible pattern on the original LPA (presence of all WT bands and no MUT bands). The study investigators agreed with the interpretation of this. However, on repeat LPA testing, the *rpo*B pattern changed. The repeat LPA pattern had one WT band for *rpo*B clearly absent (no MUT bands were present), implying a RIF^R result. The location

of the absent WT band corresponded with the mutation that was detected by sequencing. Thus, an error in procedure or isolate mix-up had presumably occurred causing the routine LPA's false RIF^{S} result and discordance between Xpert and LPA (Table 8).

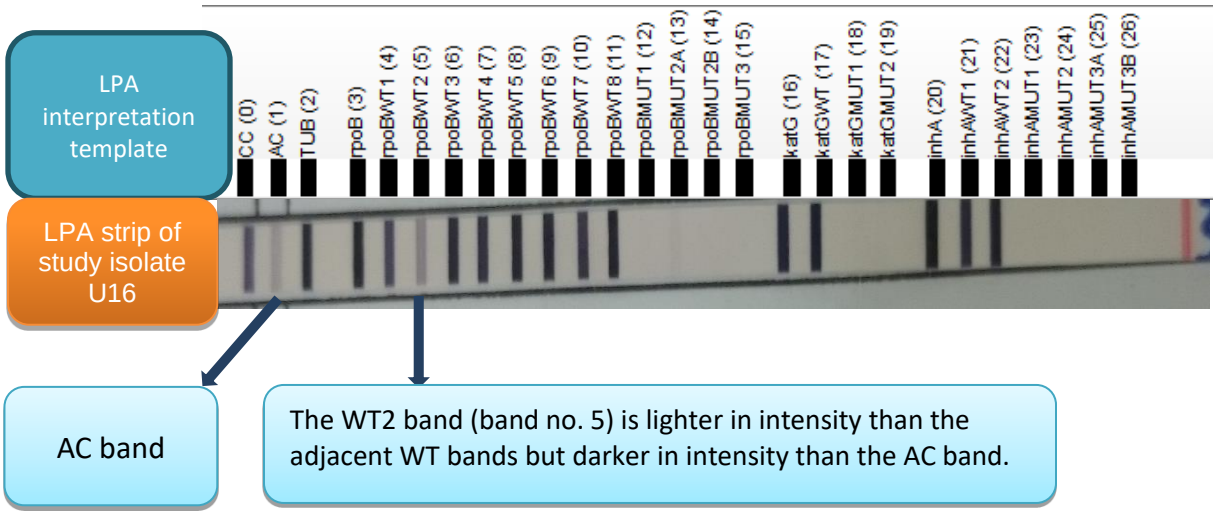


Figure 2: "DNA strip" of the LPA showing the formation of bands where hybridization had occurred between amplicons from the isolate and the probe. Since the WT2 band is darker in intensity than the AC band, it is interpreted according to package insert as present, and the overall result as susceptible to rifampicin since all *rpo*B WT bands are present and there are no MUT bands. However, DNA sequencing detected L511P in this case which corresponds to absent *rpo*B WT2. The intensity of the WT2 band is not equal to that of the other *rpo*B WT bands, resulting in what we term "questionable interpretation".

*WT = wild type, MUT = mutation, LPA = Line probe assay, AC = Amplification control

Table 8: False RIF^S results by LPA that were due to technical error

Study number	Routine LPA: *rpo*B pattern	Routine LPA: Reported RIF result (by routine laboratory)	Routine LPA: Interpretation of RIF result (by study investigators)	Repeat LPA: *rpo*B pattern	Repeat LPA: Interpretation of RIF result (by study investigators)	*rpo*B sequencing result
Questionable interpretation (change in interpretation)						
U16, A17, Q2	All WT bands present, no MUT bands. WT2 band is as strong as AC band	Susceptible	Inconclusive *rpo*B WT2 band is lighter than other *rpo*B WT bands on same LPA strip	All WT bands present, no MUT bands. WT2 band is as strong as AC band but is lighter than other WT bands on strip	No change in repeat LPA pattern. Would change interpretation to inconclusive.	L511P
H14	All WT bands present, no MUT bands. WT7 band is as strong as AC band	Susceptible	Inconclusive. *rpo*B WT7 band is lighter than other *rpo*B WT bands on same LPA strip	All WT bands present, no MUT bands. WT7 band is as strong as AC band but is lighter than other WT bands on strip	No change in repeat LPA pattern. Would change interpretation to inconclusive.	H526L
P5 and Q10	All WT bands present, no MUT bands. WT8 band is as strong as AC band	Susceptible	Inconclusive. *rpo*B WT8 band is lighter than other *rpo*B WT bands on same LPA strip	All WT bands present, no MUT bands. WT8 band is as strong as AC band but is lighter than other WT bands in LPA strip	No change in repeat LPA pattern. Would change interpretation to inconclusive-.	L533P
Procedure error (change in *rpo*B pattern on repeat testing)						
L	All WT bands present, no MUT bands. WT2 band is as	Susceptible	Inconclusive. *rpo*B WT2 is lighter than other *rpo*B	The LPA pattern changes: WT2 band is now	Resistant	L511P

	strong as AC band		bands on same LPA strip	clearly absent; no MUT bands		
M15	All WT bands present, no MUT bands. WT7 band is as strong as AC band	Susceptible	Susceptible	WT7 is clearly absent; no MUT bands	Resistant	H526N
J, E and D6	All WT present, no MUT bands. WT8 as strong as AC band	Susceptible	Susceptible	WT8 band is clearly absent; no MUT bands	Resistant	L533P

RIF = Rifampicin, RIF^S = Rifampicin susceptible, LPA = Line probe assay, WT = Wild type, AC = Amplification control, MUT = mutation

ii. Mixed infection

There were six cases that fit the study definition of mixed infection.

Three of these cases were deemed to have mixed infection based on the LPA indicating heteroresistance. The remaining three cases were deemed to have mixed infection on the basis of results of sequencing that was performed on more than one isolate from the same patient (Table 9). (Specimen/isolate mix-up could also be the cause of discordance for these three isolates).

The mutations detected by *rpo*B sequencing were L511P, D516Y, H526L, H526N and L533P.

Table 9: False RIF^S results by LPA as a consequence of mixed infection

Study number	**Routine LPA: *rpo*B pattern**	**Routine LPA: Reported RIF result (by routine laboratory)**	**Routine LPA: Interpretation of RIF result (by study investigators)**	**Repeat LPA: *rpo*B pattern**	**Repeat LPA: Interpretation of RIF result (by study investigators)**	***rpo*B sequencing result**
W	All WT bands present; no MUT bands. (All WT bands plus a MUT band for katG & inhA present)	Susceptible to RIF. heteroresistant to INH	Susceptible to RIF. heteroresistant to INH	WT8 band absent; no MUT bands. (All WT bands plus MUT band for katG & inhA present)	Resistant (to both RIF and INH)	L533P
F4	All WT bands present; no MUT bands (All WT bands plus a MUT band for katG present)	Susceptible to RIF; heteroresistant to INH	Susceptible to RIF; heteroresistant to INH	WT3,4 absent, no MUT bands (All WT bands plus a MUT band for katG present)	Resistant to both RIF and INH	D516Y
N	All WT bands present; no MUT bands (All WT bands plus a MUT band for katG present)	Susceptible to RIF; heteroresistant to INH	Susceptible to RIF; heteroresistant to INH	WT7 absent, no MUT bands (All WT bands plus a MUT band for katG present)	Resistant to both RIF and INH	H526L
O2	All WT bands present; no MUT bands	Susceptible	Susceptible	All WT bands present, no MUT bands	Susceptible	L511P

F6	All WT bands present; no MUT bands	Susceptible	Inconclusive. *rpo*B WT7 band is lighter than other *rpo*B WT bands on same LPA strip	WT7 absent, no MUT bands	Resistant	H526N
B	All WT bands present; no MUT bands	Susceptible	Susceptible	All WT bands present, no MUT bands	Susceptible	H526N

RIF = Rifampicin, RIF^S = Rifampicin susceptible, LPA = Line probe assay, WT = Wild type, AC = Amplification control, MUT = mutation

iii. Laboratory mix up

There were two isolates implicated in a laboratory mix up. Both isolates involved had *rpo*B mutation L531L detected by *rpo*B sequencing. In both cases, the routine LPA had all *rpo*B WT bands present and no *rpo*B MUT probe bands and was correctly interpreted as RIF^S by routine testing. However, after repeating the LPA on each isolate the *rpo*B WT8 band was clearly absent plus there was a clear MUT3 band present. On further examination of each of the LPA batches that included the "case patients''' LPA strips that had been processed in the routine laboratory, it was noted that for each case, there was an LPA pattern from an isolate that belonged to a different patient ("non-case patient") with the same *rpo*B (and INH) banding pattern as the repeated LPAs (absent *rpo*B WT8 plus MUT 3 band). In each case, the LPA strip of the non-case patient's isolate was located immediately adjacent to the LPA strip of the study case. It was determined that a mix up (swap) during the routine processing of the LPA batch in the routine laboratory had occurred; most likely during DNA extraction. When the LPA was repeated, the aliquot for DNA extraction was taken from the MGIT tube of the correct patient (name and laboratory number were checked). On searching the Laboratory Information System, results of RIF-susceptible by either Xpert or LPA on other specimens submitted within 7 days of the implicated isolate's specimen were found for the non-case patients. The lab reports were

amended and the clinicians involved were informed of the correct results. The relevant laboratory was informed about the laboratory mix up and corrective actions were taken.

iv. Indeterminate

There were four isolates for which all LPA *rpo*B WT band intensities appeared stronger than that of the AC band on the routine LPA. These were thus routinely interpreted and reported as RIF^S. On repeating of the LPA, the pattern remained unchanged. Sequencing of the isolates on which the LPA was performed revealed a mutation (which matches the probe implicated in resistance by Xpert). Mixed infection could not be excluded for these patients as no repeat samples were submitted for culture and DST (which would include routine Xpert and LPA). In addition, mix up of specimens / isolates could not be ruled out as a cause for the discordant results (Table 10).

Table 10: False RIF^S results with no determined cause

Study number	**Routine LPA: *rpo*B pattern**	**Routine LPA: Reported RIF result (by routine laboratory)**	**Routine LPA interpretation of RIF result (by study investigators)**	**Repeat LPA: *rpo*B pattern**	**Repeat LPA: Interpretation of RIF result (by study investigators)**	***rpo*B sequencing result**
A, Z3	All WT bands present; no MUT bands	Susceptible	Susceptible	All WT bands present; no MUT bands	Susceptible	L511P
O5	All WT bands present; no MUT bands	Susceptible	Susceptible	All WT bands present; no MUT bands	Susceptible	H526N
Q15	All WT bands present; no MUT bands	Susceptible	Susceptible	All WT bands present; no MUT bands	Susceptible	L533P

RIF = Rifampicin, RIF^S = Rifampicin susceptible, LPA = Line probe assay, WT = Wild type, MUT = mutation

3.2 Part 2. Discordant LPA RIF^{R} and MGIT RIF^{S}

During the six-month study period the LPA detected RIF resistance in 1502 patients, of which 169 (11.3%) had a “miscellaneous *rpo*B mutation”. In addition, a further 21 isolates were selected from “Part 1” of the study, where sequencing confirmed that the *rpo*B mutation was not one of the high level / high confidence *rpo*B mutations (not one of S531L / H526Y / H526D / D516V). All of the isolates underwent RIF MIC testing.

A total of 178 isolates had both MIC and *rpo*B sequencing results. There were 12 excluded isolates overall (one from Part 1 and 11 from Part 2 of the study) (Figure 3). Sequencing results could not be obtained for seven isolates because the cultures were contaminated with other bacteria. MIC could not be performed on five isolates due to loss of viability (n=3 isolates with mutations H526P, D516Y, L533P) and contamination (n=2 isolates with mutations H526P, H526L).

Isolates that were sequenced and their MIC distribution appear in Table 11. Of the 178 isolates with MIC results, 140 (78.7%) had an MIC ≤1 µg/ml (low-level RIF^{R} or RIF^{S}). Fifteen isolates were used as reproducibility controls for *rpo*B sequencing and as quality control strains for MIC testing.

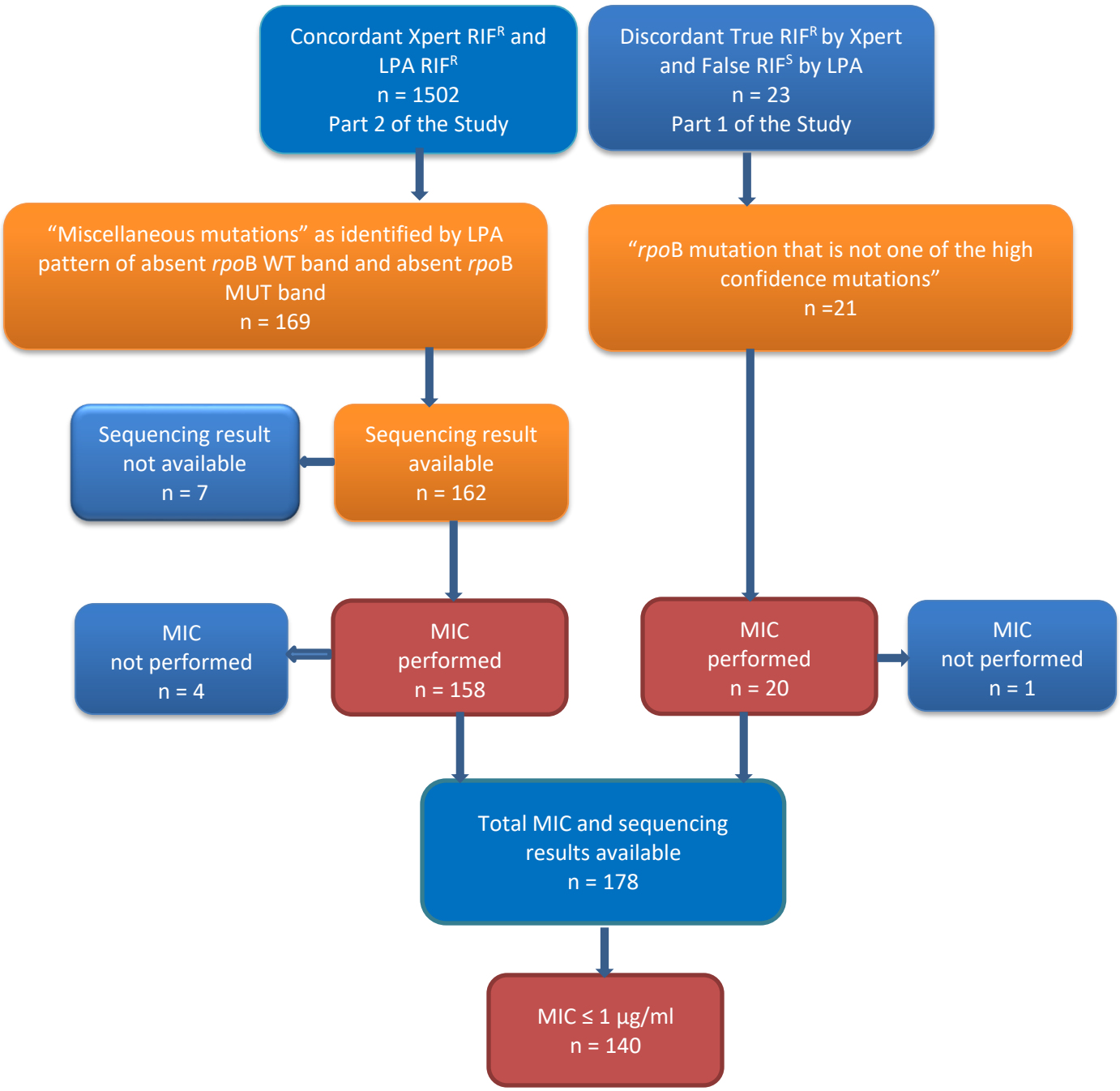


Figure 3: Origin of isolates analyzed in Part 2 of the study. All isolates were proven to have *rpo*B mutations other than the 4 most commonly encountered (high confidence) *rpo*B mutations by *rpo*B sequencing. A total of 178 were included in the analysis where both sequencing and MIC results were available.

*MIC = minimum inhibitory concentration, LPA = line probe assay, RIF^R = rifampicin resistant

Table 11: Relationship between *rpo*B mutations and MIC values (MGIT 960 EpiCenter)

	MIC (µg/ml)	MGIT 960 with EpiCenter					no. of isolates
	RIF Susceptible	Low level RIF^R				High level RIF^R	
*rpo*B mutations detected	≤0.0625	0.125	0.25	0.5	1	>1.0	Total
L511P	23	27	3	0	0	0	53
H526N	6	10	3	0	0	0	19
L533P	0	2	10	12	3	0	27
D516Y	0	0	10	15	4	1	30
H526L	0	0	0	1	6	4	11
S531W	0	0	0	0	0	4	4
D516F	0	0	0	0	0	5	5
D516G	1	0	0	0	0	0	1
Q513P	0	0	0	0	0	3	3
Q513K	0	0	0	0	0	2	2
S522L	0	0	0	0	0	2	2
H526R	0	0	0	0	0	1	1
S531C	1	0	0	0	0	0	1
S531F	0	0	0	0	0	1	1
S531Q	0	0	0	0	0	1	1
L511P/D549E	0	1	0	0	0	0	1
D516G/I572M	0	0	0	0	0	1	1
H526N/S531W	0	0	0	0	0	1	1
Q510L/D516Y	0	0	0	0	0	1	1
D516Y/I572M	0	0	0	0	0	1	1
D516G/L533P	0	0	0	0	0	1	1
L511P/M515V/V581A	0	0	0	0	1	0	1
del 518	0	0	0	0	0	1	1
del517/518	0	0	0	1	0	0	1
del 514/515	0	0	0	0	0	1	1
del 514/515/516	0	0	0	0	0	3	3
del 515/516/517	0	0	0	0	0	1	1
del 513/514/515	0	0	0	0	0	1	1
del 515/516/517/518	0	0	0	0	0	1	1
del 511/512/513/514	0	0	0	0	0	1	1
Total	31	40	26	29	14	38	178

MIC = minimum inhibitory concentration, MGIT = mycobacterial growth indicator tube, RIF^S = Rifampicin susceptible, RIF^R = Rifampicin resistant

L511P, H526N, L533P and D516Y accounted for 72.5% (n=129) of the total isolates with miscellaneous *rpo*B mutations that had sequence and MIC results (Figure 4). Of these 129 isolates; only one (D516Y) was high level RIF resistance with an MIC>1.0 µg/ml. The remaining 128 (99.2%) had an MIC of ≤1 µg/ml; 99 (76.8%) had an MIC between 0.125 and 1 µg/ml (low level RIF resistant) and 29 (22.5%) were RIF susceptible with an MIC ≤0.0625 µg/ml (Figure 5).

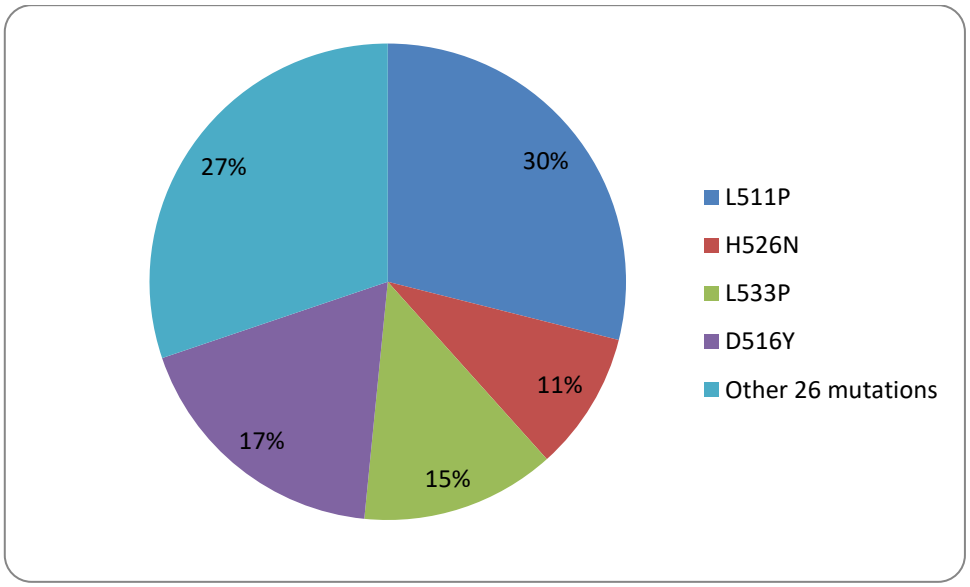


Figure 4: Proportion of *rpo*B mutations detected from LPA RIF^R with absent *rpo*B WT and MUT bands pattern. Four mutations made up 72.5% of the isolates with miscellaneous mutations as detected by LPA. The remaining 27.5% isolates had 26 other different mutations.

*LPA = Line probe assay, RIF = Rifampicin, RIF^R = Rifampicin resistant, WT = Wild type, MUT = Mutation probe

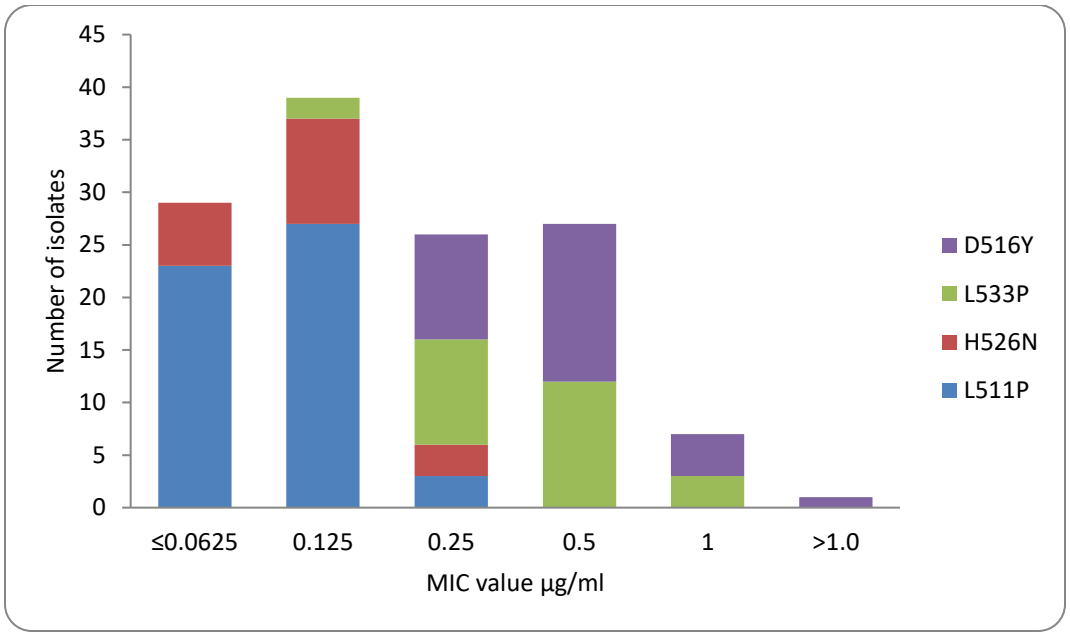


Figure 5: MIC distribution of isolates with the four most common miscellaneous *rpo*B mutations L511P, D516Y, H526N and L533P. *MIC = minimum inhibitory concentration

The remaining 26 less common mutations occurred in 49 isolates of which 37 (75.5%) had an MIC > 1.0 µg/ml; seven (14.3%) had an MIC of 1.0 µg/ml and five (10.2%) had an MIC ≤ 0.5 µg/ml (Figure 6). Seventeen of the 26 less common miscellaneous mutations were either double mutations (7) or double deletions (10). Fourteen (82.4%) of 17 isolates with a double mutation / deletion had an MIC >1ug/ml.

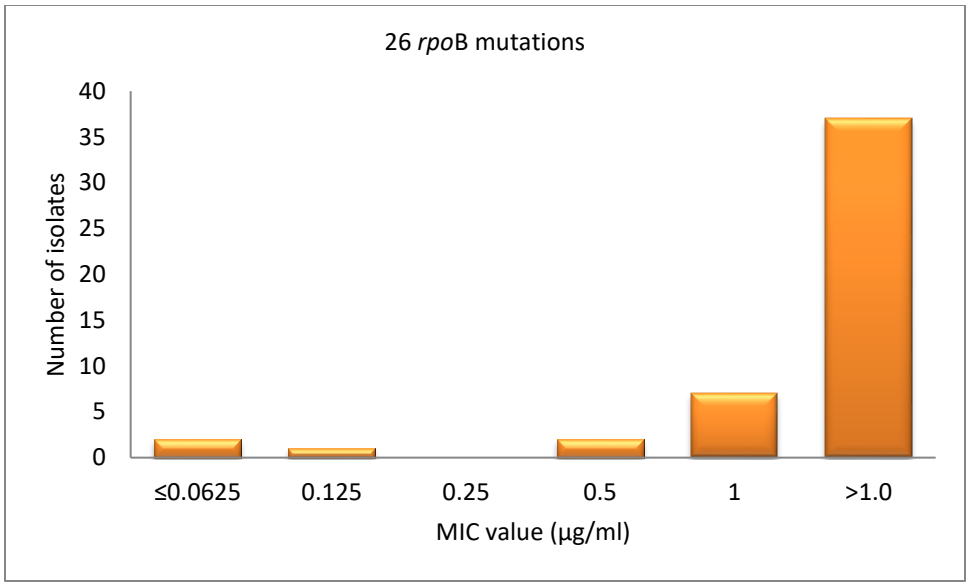


Figure 6: MIC distribution of the 26 less common miscellaneous *rpo*B mutations identified by LPA and DNA sequencing *MIC = minimum inhibitory concentration

Controls: The MIC value of all isolates from the control group were within the expected MIC range; there were ten isolates with *rpo*B mutations that are known to confer high level RIF resistance which all had an MIC >1 µg/ml and five MTB (H37Rv) isolates with no *rpo*B mutation had an MIC ≤ 0.0625 µg/ml. These isolates were also used as reproducibility controls for *rpo*B PCR and *rpo*B sequencing; *rpo*B PCR and sequencing results were 100% reproducible (Table 12).

Table 12: *rpo*B reproducibility and MIC values of control isolates

Isolate	*rpo*B sequencing results	Repeat *rpo*B sequencing	MIC results (µg/ml)
1	D516V	D516V	>1.0
2	D516V	D516V	>1.0
3	H526D	H526D	>1.0
4	H526	H526D	>1.0
5	H526Y	H526Y	>1.0
6	H526Y	H526Y	>1.0
7	S531L	S531L	>1.0
8	S531L	S531L	>1.0
9	S531L	S531L	>1.0
10	S531L	S531L	>1.0
11	No mutation detected (H37Rv)	No mutation detected (H37Rv)	≤0.0625
12	No mutation detected (H37Rv)	No mutation detected (H37Rv)	≤0.0625
13	No mutation detected (H37Rv)	No mutation detected (H37Rv)	≤0.0625
14	No mutation detected (H37Rv)	No mutation detected (H37Rv)	≤0.0625
15	No mutation detected (H37Rv)	No mutation detected (H37Rv)	≤0.0625

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 DISCUSSION

4.1.1 Part 1. Discordant Xpert RIF^R and LPA RIF^S

In South Africa, the most common discordant scenario involving TB tests that are used in the routine National testing algorithm is when the Xpert MTB/RIF reports RIF^R and the confirmatory LPA performed on the patient's second sample reports RIF^S.

In Cape Town, we found that 6.9% (106) of the 1542 patients with RIF^R results reported by Xpert had a discordant RIF^S result by LPA which was lower than the 16.3% discordance reported in Johannesburg (Berhanu R, 2015).

In our study the true RIF result was determined by sequencing the same isolate on which the (routine) LPA was performed. The study that was conducted in Johannesburg did not perform sequencing (Berhanu R, 2015).

We showed that Xpert MTB/RIF parameters can be utilized to predict false RIF^R results by Xpert. Xpert parameters have similarly been used to determine the specificity of RIF^R results by Xpert in the study by Ocheretina et al (Ocheretina Oksana, 2016).

4.1.1.1 False RIF^R by Xpert (n=78)

Seventy eight of the total discordant cases that had sequencing results (101) were deemed to be false RIF^R by Xpert as sequencing did not detect an *rpo*B mutation in the RRDR. Xpert parameters such as the Ct value not only provide a semi quantitative measure of the bacterial load in a specimen (Hanrahan et al., 2014, Blakemore et al., 2011, Helb et al., 2010) but can be utilized to predict RIF susceptibility discordance (Berhanu R, 2015), to determine a false RIF^R result by Xpert in discordant cases (Ocheretina Oksana, 2016) and potentially may be used to remove a likely false RIF^R result before it is reported to clinicians (Appendix A).

We found that probe delay was a significant predictor of false RIF^R by Xpert; an Xpert RIF^R result that has been determined due to probe delay was 23.66 times more likely to be false RIF^R than an Xpert RIF^R result that is due to a probe dropout (95%CI: 14.59 - 38.78). This finding is similar to the findings of Ocheretina *et al,* where 91% of false RIF^R (10/11) had probe delay (Ocheretina Oksana, 2016), and Berhanu *et al,* where discordance was 12.74 times more likely to occur with Xpert RIF^R results due to probe delay than due to dropout (Berhanu R, 2015). We further classified the probe delay into two groups: probe delay where the ΔCt max value was between 4.1 and 4.9 and probe delay with ΔCt max ≥5. Probe delay with ΔCt max value between 4.1 and 4.9 was a significant predictor of false RIF^R by Xpert. An Xpert MTB/RIF RIF^R result due to probe delay where probe delay has a ΔCt max value of between 4.1 and 4.9 is 28.89 times more likely to be false RIF^R than an Xpert MTB/RIF RIF^R result that is due to probe delay with a ΔCt max value ≥ 5 or where RIF^R is due to probe dropout. In the context of a RIF^R result by Xpert, we recommend that the Xpert MTB/RIF RIF^R result be changed to inconclusive and Xpert be repeated on a repeat specimen.

An MTB bacterial load of “Very Low” is also a significant predictor of an Xpert false RIF^R result. In our study 61.8% (47/76) of the false RIF^R results by Xpert had a Very Low bacterial load. This is similar to the proportion of 54.5% (12/22) determined by Ocheretina *et al* where they recommend that all RIF^R results by Xpert with a Very Low bacterial load must be confirmed by culture based DST prior to reporting the RIF result as resistant (Ocheretina Oksana, 2016). A RIF^R result by Xpert with a Very Low bacterial load is 8.58 times more likely to be false RIF^R than a RIF^R result with a Non-Very Low bacterial load.

In the context of a discordant RIF result between Xpert and LPA, where there is drop-out or delay with a ΔCt max value ≥5, a Very Low bacterial load on the Xpert read-out could be used to consider that the Xpert RIF^R result is likely the false result and that the LPA is likely the true (RIF^S) result. We would recommend that a provisional comment is added to the LPA report noting the discordance, suggesting that the LPA is the true RIF result, and requesting a repeat specimen for repeat Xpert testing.

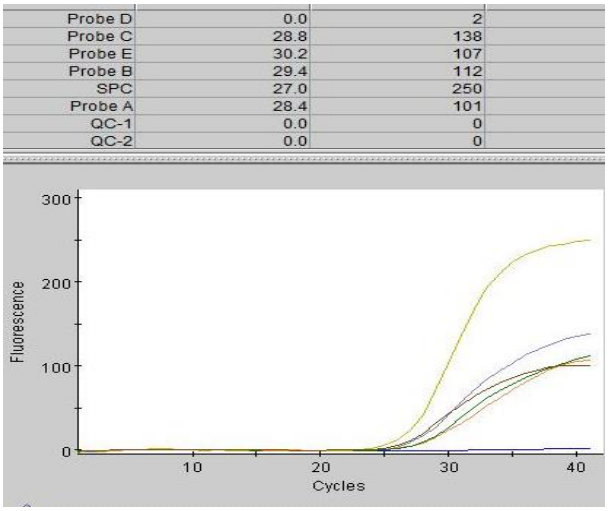
It might be prudent, in a low MDR prevalence setting, that when a RIF^R result by Xpert MTB/RIF is obtained that has a Very Low bacterial load to investigate further (e.g., repeat

the Xpert test on a second specimen) before excluding RIF from the treatment regimen. However, in a setting of relatively high RIF resistance prevalence such as South Africa, it would be costly to repeat Xpert testing for all RIF^R results with a Very Low bacterial load. In our study, 15.8% of the total RIF^R results had a Very Low bacterial load and 80.3% of these had concordant RIF^R results by LPA. In addition, withholding all RIF^R results with a Very Low bacterial load until RIF resistance is confirmed by the LPA would delay commencement of the patient on an appropriate TB regimen and negate the benefit of the Xpert.

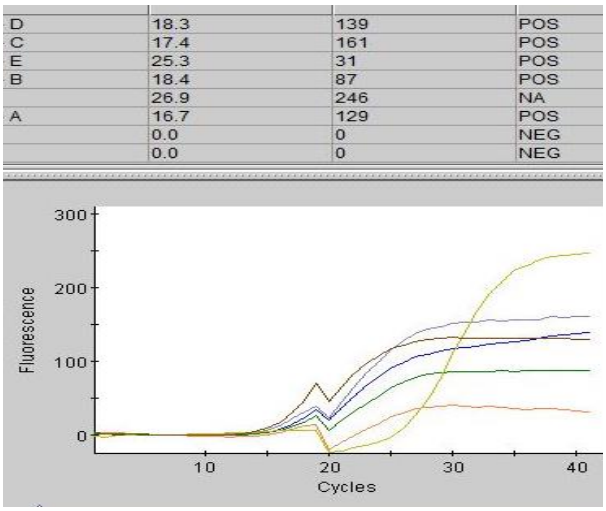
The number of probes involved in determining the RIF^R result by Xpert can also be used to predict the true RIF susceptibility result. If RIF^R result by Xpert is detected due to double probe delay (not drop-out) (specifically probes D and E), we strongly recommend the Xpert test is repeated on another sample prior to releasing the resistant result. In our study there were 12 RIF^R results by Xpert due to double probe delay (probes D and E) and all were found to be false RIF^R by Xpert. This finding is similar to that found in the study by Ocheretina *et al* (Ocheretina Oksana, 2016) where 6/12 false RIF^R were due to double probe delay (also involving probes D and E).

A combination of the above predictive Xpert MTB/RIF parameters can be used to further strengthen the predictive value of false RIF^R by Xpert. A RIF^R result by Xpert that is determined by probe delay where the ΔCt max is >4 and there is a Very Low bacterial load has a positive predictive value (PPV) of 64.2 % of being false. If RIF^R is due to probe delay where the ΔCt max is between 4.1 and 4.9 and in addition there is a Very Low bacterial load, the PPV of a false result increases to 85.7%.

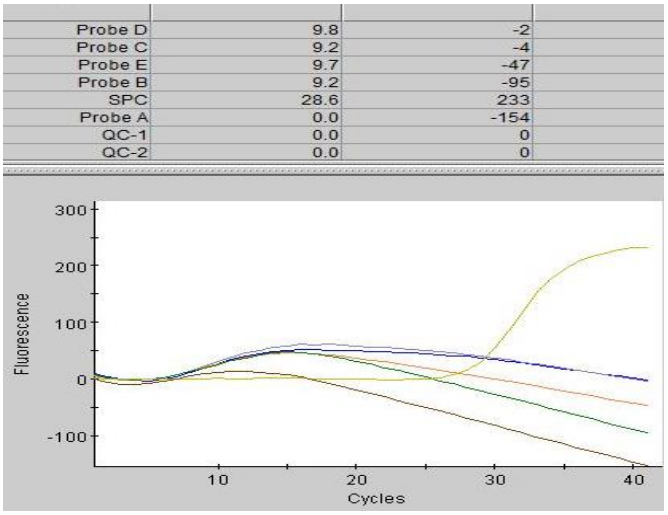
The Xpert graphs (fluorescence of probes) were analyzed for any abnormal shape of the curve that may result from disrupted amplification. This could arise from an air bubble that may be introduced in the cartridge during sample processing or by a high level of fluorescent background (e.g. a very high load of AFB's in an aspirate sample). When the Xpert graph is irregular or abnormal; the result should not be reported and a repeat test is recommended (Figure 1).



A



B



C

Figure 1: A) Sample O5 graph showing the sigmoid shape that is expected for all probes that bind to wild type sequence, and the lack of binding and fluorescence that occurs with true RIF resistance (in this case due to a Ct value dropout in probe D); the RIF^R result should be reported. B) Sample graph with abrupt and distorted fluorescence of probes due to an air bubble in the cartridge; the RIF^R result should not be reported and a repeat test is recommended. C) Sample T2 Xpert MTB/RIF graph showing a high level of fluorescent background due to high bacterial load. The normal sigmoid curve is absent. This RIF^R result should not be reported and a repeat test is recommended. *RIF^R = rifampicin resistant

We recommend that laboratories performing Xpert MTB/RIF should have a dedicated person who reviews all Xpert RIF^R results and checks the Xpert probe readouts for any Xpert parameters that may predict false RIF^R prior to releasing results to clinician.

In our practice, at the point of review of the Xpert MTB/RIF RIF^R result, we change the automatically generated RIF^R result to RIF inconclusive in all cases where the ΔCt max value lies between 4.1 and 4.9, no matter the bacterial load value. We also change the resistant result to RIF inconclusive when RIF resistance is determined by the presence of a double probe delay. We add a comment stating that the RIF resistant result has been changed to RIF inconclusive as it is suspicious for false RIF resistance, and we request that a repeat specimen be submitted for repeat Xpert MTB/RIF testing. This occurs before the LPA has been processed and resulted.

In a case of Xpert / LPA discordance (at the point of review of the LPA result), the Xpert parameters are again examined (to make sure that there are no parameters that would lead us to change the Xpert result to inconclusive); if there is a very low bacterial load, but the ΔCt max value is ≥5 (or there is probe drop-out), we report the RIF^S result by LPA and add a comment to the LPA report suggesting that the likely explanation of the discordance is possible false RIF^R resistant result by Xpert, we suggest that the likely true result is the RIF^S by LPA and we request a repeat specimen for repeat Xpert testing.

4.1.1.2 False RIF^S by LPA (23)

Twenty three percent of isolates were deemed false RIF^S by LPA as an *rpo*B mutation in the RRDR was detected.

Out of 23, 11 LPA strips were false RIF^S due to technical errors. Seven of the 11 had questionable interpretation where the routine LPA strip should have been interpreted as RIF inconclusive rather than RIF^S. The routine interpretation of RIF^S was made according to the package insert provided by the manufacturer where it states that “a probe band must be interpreted as being present if its intensity is equal to or stronger than that of the AC band for that strip. The probe band is interpreted as absent if its intensity is less than that of the AC band for the same strip” (Hain-Lifescience, July 2013). The intensity

comparison is subjective and sometimes it is difficult to determine if a band is absent or present when the intensity of the AC band is very light. Upon re-reading the routine LPA it was found that the intensity of one *rpo*B WT band was as strong as the AC but was clearly lighter than the other *rpo*B WT bands and this WT with light intensity corresponds to the mutation detected by sequencing. The same reading error was made by the automated Genoscan scanner that is used routinely in the GPT laboratory (Figure 2). We suggest that the LPA manufacturer to amend the interpretation criteria and comparing intensity of an *rpo*B WT band should not only be against the intensity of AC band but also against all other *rpo*B WT bands in the strip.

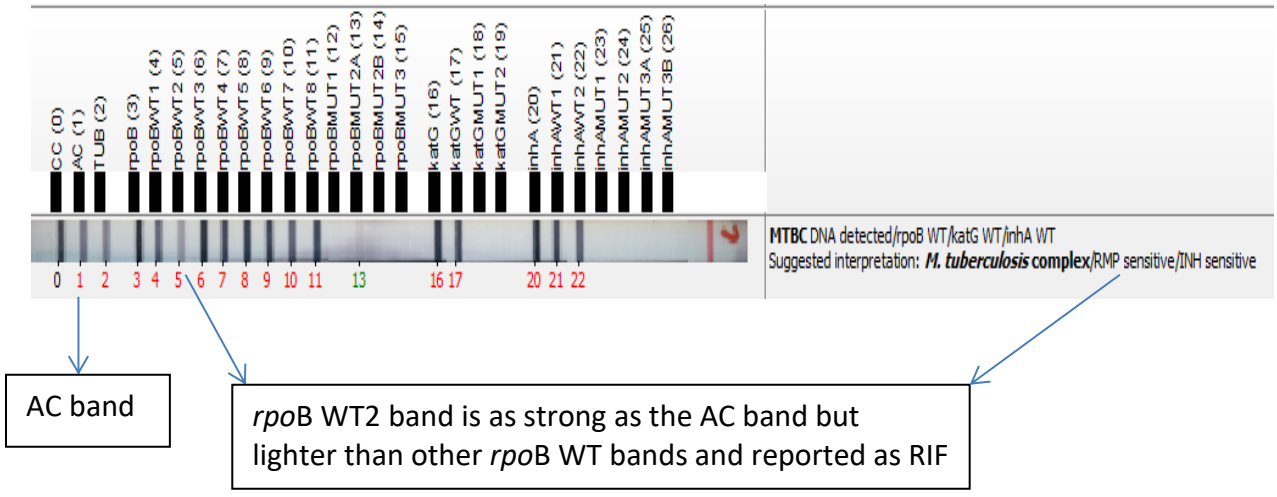


Figure 2: "DNA strip" of the LPA read by the automated Genoscan as susceptible to both RIF and INH. Since the WT2 band is darker in intensity than the AC band, it is interpreted according to package insert as present, and the overall result as susceptible to rifampicin since all *rpo*B WT bands are present and there are no MUT bands. However, DNA sequencing detected L511P in this case which corresponds to absent *rpo*B WT2. The intensity of the WT2 band is not equal to that of the other *rpo*B WT bands, resulting in what we term "questionable interpretation" (Figure 3).

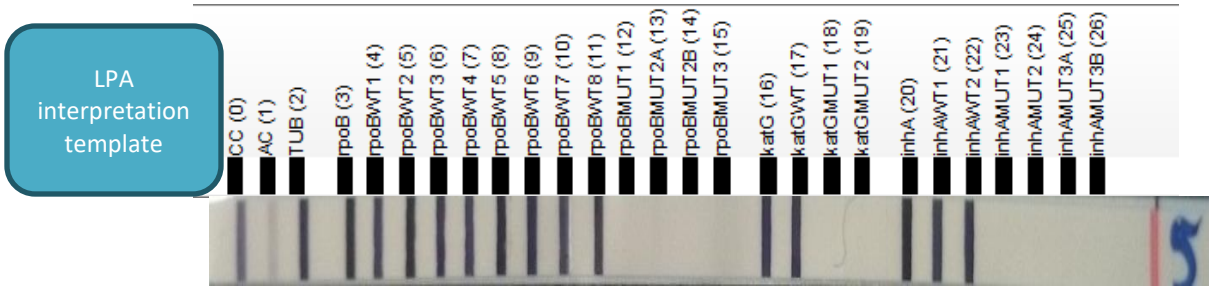


Figure 3: "DNA strip 5" of a wild type MTB strain (where DNA sequencing detected no mutation in the *rpo*B gene. All (*rpo*B, *kat*G and *inh*A) WT bands are darker in intensity than the AC. In addition, all *rpo*B WT bands are of equal intensity to each other. The correct interpretation of this LPA pattern is susceptible to both rifampicin and isoniazid.

*WT = wild type, MUT = mutation, LPA = Line probe assay, AC = Amplification control

The remaining four of 11 LPAs were incorrectly reported as RIF^S as there was an error that occurred during the routine procedure. The routinely processed LPA strips had clearly RIF^S patterns (all *rpo*B WT bands were stronger in intensity than the AC band of the same strip and the *rpo*B WT bands were as strong as each other). However, on repeat testing of the same isolate, the LPA pattern was different: it was clearly RIF^R with one *rpo*B WT band clearly absent or lighter than the AC band. The particular absent *rpo*B WT band corresponded to the mutation detected by sequencing for each case. Thus, we concluded that the change in the result could have been from a laboratory mix-up, or an error occurring during the procedure, which would include an error within the LPA kit itself. In 2013 the GenoType MTBDRplus version 1 LPA was updated to a newer version (2) in order to improve the intensity of some *rpo*B WT probes that were often light and difficult to interpret leading to unnecessary and costly repeat testing. In addition, the MUT probe for L533P was removed from the version 2 as it had been found to lead to discordant genotypic / phenotypic RIF results and was thought to be a less clinically significant *rpo*B mutation at the time (Van Deun et al., 2013). The four isolates were routinely processed using the earlier version 2; hence on three isolates the L533P mutation was not detected. Version 2 GenoType MTBDR*plus* was subsequently updated to incorporate the L533P mutation. The remaining one isolate had the H526N mutation, with the LPA pattern of

absent *rpo*B WT7 and absent MUT bands on repeat of the LPA which is the expected pattern for this particular *rpo*B mutation; the absence of this pattern on the routinely performed LPA could be explained by the improvement of intensity of bands in the earlier version 2 resulted in a WT7 band appearing as strong as AC. Laboratory mix up or heteroresistance however, cannot be excluded. The repeat LPA performed on the four isolates by the study investigator was performed with the newest version 2 LPA.

There were six cases with mixed infection. Detection of mixed infection is challenging and it also depends on the laboratory methods used. DNA sequencing can detect heteroresistance by showing a dual peaks chromatogram pattern (Kumar et al., 2014), however phenotypic DST and even LPA have been regarded as superior to Sanger sequencing for the detection of heteroresistance (Folkvardsen et al., 2013). We did not detect dual peak chromatogram patterns in any of the sequencing results.

Despite the limitations of determining whether mixed infection in TB cases is present, we deemed 3 of the cases of false RIF^S by LPA as having probable mixed TB infection as we had results of Sanger sequencing for 2 sequential isolates for each case (sequencing was inadvertently performed on an isolate from a second specimen that had been submitted from the patient within a week of the index specimen being submitted). Whereas one sequencing result detected the presence of a mutation (thus classifying the case as false RIF^S by LPA), the sequencing performed on the second isolate detected no *rpo*B mutation in the RRDR. Another possible cause of discordance of this group could be specimen mix-up.

The remaining three cases with mixed infection were categorized as such as there was a heteroresistant pattern for INH on the routinely performed as well as the repeat LPA strips. One can report heteroresistance by the LPA for a drug when a MUT band as well as all WT bands are present. For RIF, each *rpoB* MUT band that is included in the LPA corresponds to a high level *rpo*B mutation (S531L, H526Y, H526D or D516V). Thus, when heteroresistance is visible for RIF on the LPA, it is due to presence of a high-level RIF mutation, in addition to presence of a susceptible strain. In the case of heteroresistance involving the presence of RIF^S and RIF^R due to a miscellaneous *rpo*B mutation, the LPA pattern will appear susceptible as only wild type (WT) *rpo*B bands will be present with no

MUT band present. The current version of the LPA does not include MUT bands for sequences belonging to the disputed *rpo*B mutations.

Two cases of false RIF^S by LPA were erroneously reported as RIF^S as they were involved in a laboratory isolate mix up that most likely occurred during DNA extraction, the first step of 3 in the LPA procedure. A definite laboratory mix up occurred but we were unable to determine the exact error. Possible errors that may have occurred are that the DNA strips may have been mislabeled or placed in the incorrect LPA tray wells or PCR amplicon tubes or MGIT tubes may have been switched around.

It is important that all laboratory procedures are streamlined and quality controlled to limit any laboratory mix-ups of specimens / isolates. At the time of reporting the LPA result it is important to ensure that the RIF^S result by LPA is not discordant with any RIF result by Xpert on the patient. In the case of discordance, one of the easiest causes to rule out is laboratory mix up. Where a RIF^R result by LPA is expected (to match resistance result of Xpert), the entire LPA batch should be checked to see if there are any other strips (usually but not always adjacent to the susceptible strip in question) that have a RIF^R pattern and particularly if that pattern corresponds with the probe detecting a mutation in the patient's Xpert result.

There were four cases that could not be definitively categorized as either technical error, specimen / isolate mix-up or mixed infection. The repeat LPA patterns remained the same as the routine LPA patterns and the interpretation of RIF^S remained. The *rpo*B WT bands were all present and their intensities were stronger than the AC band (and no WT band was lighter in intensity than the other WT bands). Specimen mix up could not be ruled out and since there was only one specimen submitted from each patient mixed infection could not be ruled out either.

Some possible causes of discordant Xpert / LPA results are pre-laboratory and laboratory specimen mix-up or laboratory contamination. Adhering to good laboratory practice by working aseptically and cleaning of working environment, following procedure according to the manufacturer's instruction and processing negative and positive controls with each batch is important. If the control result is not the expected result (e.g., LPA negative control shows any WT bands), it must be investigated further and the assay must be

repeated prior to releasing any result from that batch. False RIF^S by LPA may occur if an isolate exhibiting a miscellaneous *rpo*B mutation is contaminated with a true RIF^S isolate resulting in the LPA strip will show all *rpo*B WT bands and reported false RIF^S. Laboratory mix-up or contamination can be minimized by ensuring there are continuous training and competency checks of technical staff and implementing internal control checks of each process regarding the particular assay.

The findings of this study have assisted our laboratory in developing a guide that laboratorians in this lab as well as in other TB laboratories in the organization can use to deal with discordant RIF results. The guide is divided into sections starting with how to identify a possible false RIF^R result by Xpert. It includes how to exclude false RIF^S result by LPA and lastly how to deal with a discordant RIF result. The guide has been circulated to all NHLS TB laboratories (Appendix A)

Table 1: Guideline for TB laboratories to investigate possible false RIF^R by Xpert and discordance with a subsequent RIF^S by LPA **Full guideline available as Appendix A*

1. All RIF^R results by Xpert should be checked prior to releasing the result. During Xpert review check the Xpert parameters for a possible false RIF^R result. a. Probe delay where the ΔCt max value is between 4.1 and 4.9 • Report RIF inconclusive, repeat Xpert on second specimen b. Double probe delay (specifically delayed hybridization of probes D and E) • Report RIF inconclusive, repeat Xpert on second specimen c. An abnormal graph fluorescence curve • Report RIF inconclusive, repeat Xpert on second specimen
2. If a RIF^S result by LPA is reported, but this is discordant with an already-reported Xpert result of RIF^R
a. Check the Xpert readout and graph (as point 1 above) b. If Very Low MTB bacterial load was detected by Xpert, then it is likely false RIF^R by Xpert c. Note which probe is involved (Ct=0 or delay >4) in the Xpert RIF^R result • If for example the RIF^R result by Xpert was due to probe dropout or probe delay of probe D, it is expected that the LPA has an absent *rpo*B WT7 band. The LPA WT7 band may be equal or darker in intensity than the AC band (especially if the AC bands of most strips in the batch are very light) but intensity of WT7 should be compared to intensity of all the other *rpo*B WT bands. If it is lighter than the others *rpo*B WT bands it is possible that it should be interpreted as absent rather than present (and hence resistant rather than susceptible for RIF) d. Check LPA result again and repeat if indicated • Obtain the LPA strip if possible. If not possible, obtain a photo of the strip (in a case where Genoscan was in use). Look again at the intensity of all the *rpo*B WT bands. Although the LPA package insert states that comparison of intensity of the band is made only with the AC band, in some cases of false RIF^S results by LPA, the AC band is particularly light, and the WT2 / WT7 band in particular is more intense than the AC band; however, the WT2/7 band may be lighter than the adjacent *rpo*B WT

bands in a case where Xpert has detected resistance due to probe delay / dropout in probes A / D respectively

e. If there is no obvious questionable *rpo*B WT bands, consider repeating the LPA
f. Batches of LPA should be checked for any indication of contamination; specimen / culture mix up i.e. laboratory contamination# I should be ruled out prior to reporting of any results

3. All previous and current results of a patient should be checked prior to reporting results to clinicians;
    a. Actively look for all previous molecular (Xpert and LPA) results on the patient, as well as any phenotypic DST results
    b. Examine the probe patterns and graphs of each Xpert and LPA result to make sure no technical errors occurred
    c. Multiple specimens / isolates with repeatedly discordant results may be an indication of true mixed infection

4. Repeat testing
    a. If interpretation of all performed tests seems correct according to the probe patterns expected, and the Xpert graphs look normal, repeat testing is recommended:
    b. LPA should be repeated as not always possible to repeat Xpert on the same specimen at the time of discordance and repeating the Xpert on culture may be considered
    c. If the Xpert parameters indicate likely false RIF resistance, the Xpert should be repeated on a second sample that should be submitted (a comment must be added to original Xpert report)
    d. If repeat testing gives the same result, further tests may be done, if available
        - Phenotypic DST (MGIT method most commonly available); the limitations of particularly liquid based phenotypic should be considered i.e. MGIT DST may miss disputed *rpo*B mutations that confer low level RIF resistance
    e. If the Xpert and LPA results are not altered after all results are checked, the isolate (on which LPA was performed) may be referred for *rpo*B sequencing

4.1.2 Part 2. Discordant genotypic / phenotypic results: Xpert /LPA RIF^{R} and MGIT RIF^{S}

In selecting isolates to include in this part of the study, we termed all *rpo*B mutations other than the non-disputed (high confidence) *rpo*B mutations (D516V, H526Y, H526D and S531L) (Figure 4) as “miscellaneous *rpo*B mutations”. The isolates with miscellaneous *rpo*B mutations were selected based on the LPA pattern of absence of any *rpo*B WT band and absence of an *rpo*B MUT band.

In this study we showed that the less common, “miscellaneous” mutations detected by the LPA accounted for 11.3% of RIF resistance in our setting. A similar proportion was found in strains from Bangladesh (13.1%) and Kinshasa (10.6%) (Van Deun et al., 2013, Van Deun et al., 2015). Our study was performed in two laboratories that render service to most of the Western Cape public health sectors and is therefore representative of the Western Cape strain population. Similar studies should be performed in other regions of South Africa to determine the proportion of disputed *rpo*B mutations.

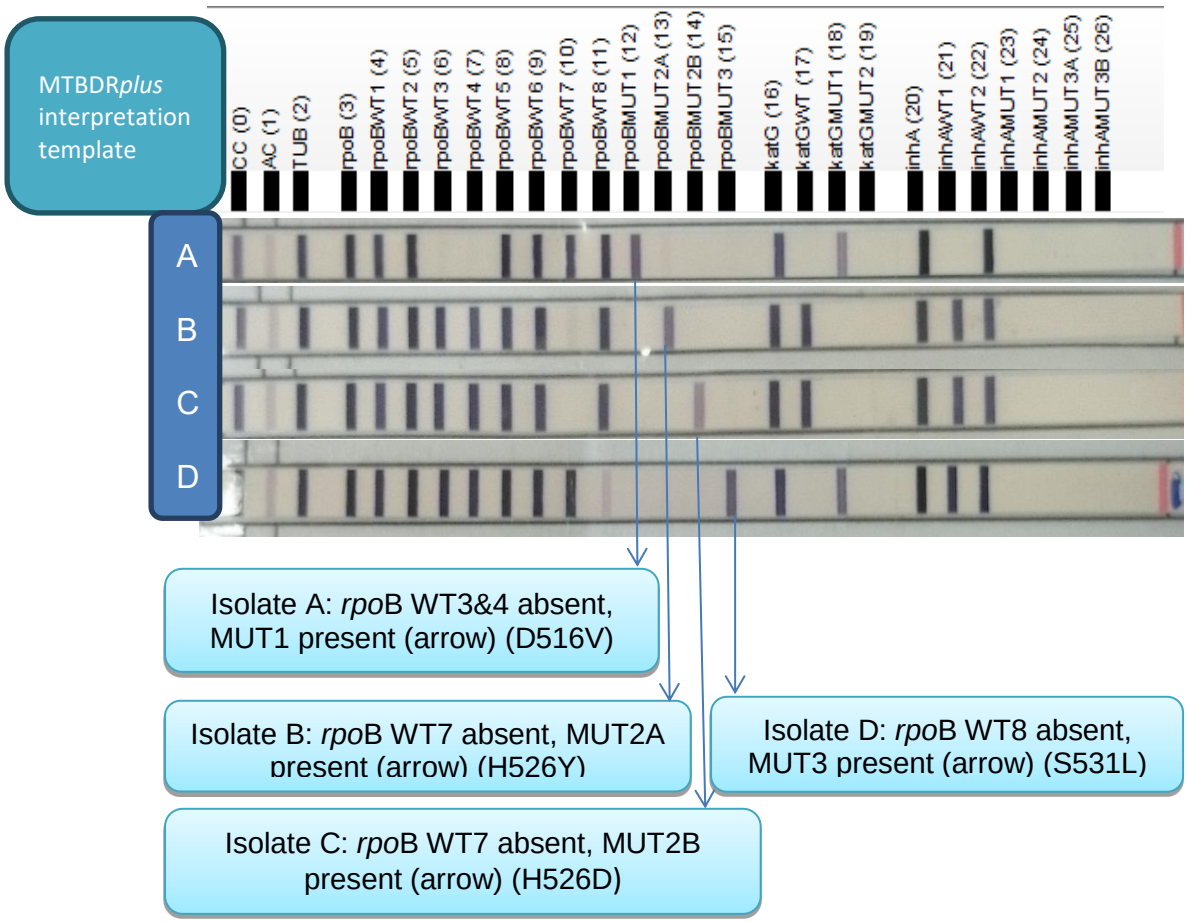


Figure 4: LPA strip patterns from isolates of MTB that harbour the 4 most common non-disputed (high confidence) *rpo*B mutations that confer high level RIF resistance. A) Isolate A harbours the D516V mutation (absent *rpo*B WT3&4 bands and presence of MUT1 band). B) Isolate B harbours the H526Y mutation (absent WT7 band and presence of MUT2A band). C) Isolate C harbours the H526D mutation (absent *rpo*B WT7 band and presence of MUT2B band) D) Isolate D harbours the S531L mutation (absent *rpo*B WT8 band and presence of MUT3 band). Isolates harbouring these 4 mutations were not the subject of Part 2 of this study

*LPA = line probe assay, MTB = *Mycobacterium tuberculosis*, RIF = rifampicin WT = wild type probe, MUT = mutation probe

In our study 140/178 (78.7%) isolates with miscellaneous *rpo*B mutations (n=158) or confirmed (previously described) disputed *rpo*B mutations (n=20) had MIC values ranging from ≤0.0625 µg/ml to 1.0 µg/ml. These would be reported as RIF^S by MGIT DST. Only 38/178 (21.3%) had an MIC value of >1.0 µg/m that would have tested RIF^R by MGIT

DST. A laboratory based study in Johannesburg found that, of 51 isolates with miscellaneous *rpo*B mutations as determined by LPA pattern, 25 (49%) tested RIF^R by MGIT DST, and the other 26 (51%) tested RIF^S by MGIT DST. The latter would be expected to have low level rifampicin MICs or be susceptible to rifampicin (Beylis N, 2012).

L511P (miscellaneous mutations in codons spanning 510-511)

Isolates that harbour the L511P mutation are identified on the LPA as an absent *rpo*B WT2 probe with no corresponding MUT band (Figure 5). According to the GenoType MTBDR*plus* package insert, the *rpo*B WT2 region spans *rpo*B codons 510-513 and the most commonly encountered mutation in this regon is L511P (Hain-Lifescience, July 2013). Twenty three (42.6%) had an MIC ≤0.0625 µg/ml (susceptible to RIF) and the remaining 31 (57.4%) had MICs ranging between 0.125 and 0.25 µg/ml (low level RIF resistance). None of the isolates had an MIC above 1.0 µg/ml thus none would test RIF^R by MGIT DST. Thus if the LPA determines RIF^R by virtue of [WT2 absent, no MUT band] it can be assumed that in most cases it is due to L511P, and that in 42.6% of cases it translates to true RIF^S (begging the question of whether RIF^R should be routinely reported by the LPA (or perhaps whether this particular mutation should be reported at the point of reporting RIF^R by LPA) and whether RIF should be included in the TB regimen) while in the remaining 57.4% it translates to low level RIF resistance where RIF at high doses may be added to the regimen (van Ingen et al., 2011). Thus it must be determined whether this mutation should be reported on the final LPA laboratory report, along with implications for phenotypic DST and level of RIF resistance. RIF is the most effective TB drug, and these patients would be treated by MDR therapy as per national guidelines, and that this is less effective and more toxic, with current regimens.

There is limited data on the clinical relevance of the L511P mutation but some observational studies indicate that patients infected with MTB that harbours this *rpo*B mutation fail standard TB regimens and require extended / drug resistant TB regimens (Williamson et al., 2012) or regimens that contain high dose RIF. This may imply that despite a low level RIF resistance or even a susceptible MIC level, standard regimens are not effective. More clinical studies that include such patients are needed to determine

the clinical significance of such mutations and whether they should be reported to clinicians when they are detected by molecular tests.

In addition to L511P, the LPA package insert lists E510H as a mutation that could be detected in this codon region; we did not detect this mutation in the 54 isolates tested though one isolate had double mutation of L511P and D549E (which is outside the RRDR). E510H has very rarely been described in clinical isolates. The E510H mutation has been reported in combination with D516Y in an isolate with MIC of 1.0 µg/ml (Andres et al., 2014).

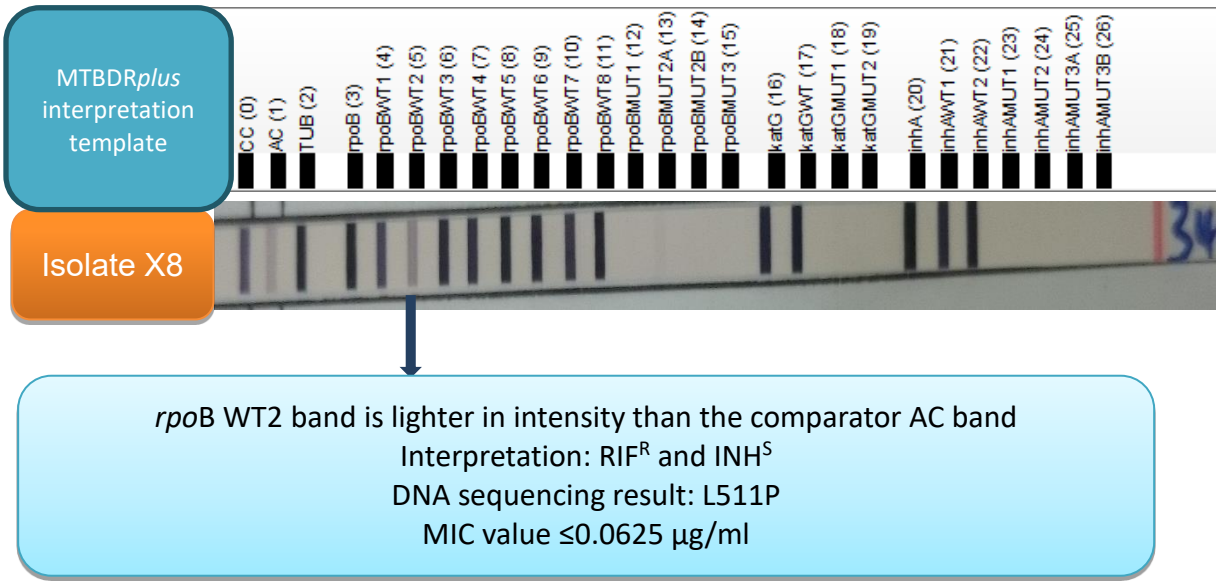


Figure 5: An MTB isolate that harbours L511P from patient X8. The LPA pattern shows that all the *rpo*B WT bands except WT2 are stronger in intensity than the AC band. WT2 is lighter in intensity than AC and the LPA is thus interpreted as RIF^R. The MIC was ≤0.0625 µg/ml which is lower than the critical concentration (1 µg/ml) used for MGIT and would thus test RIF^S by MGIT DST; leading to a discordant genotypic / phenotypic RIF result.

*WT = wild type probe, MUT = mutation probe, AC = Amplification control, RIF^R = rifampicin resistant, RIF^S = rifampicin susceptible, INH^S = isoniazid susceptible, LPA = line probe assay MIC = minimum inhibitory concentration, DST = drug susceptibility testing

H526N, H526L and H526R (miscellaneousmutations detected in codons spanning 526-529)

There were 31 isolates with miscellaneous mutations as determined by the LPA in the region spanning codons 526-529 [LPA pattern of absent *rpo*B WT7 and absent MUT bands] (Figure 6). All isolates harboured a mutation in codon 526 of which 19 were H526N; 11 were H526L and 1 was H526R.

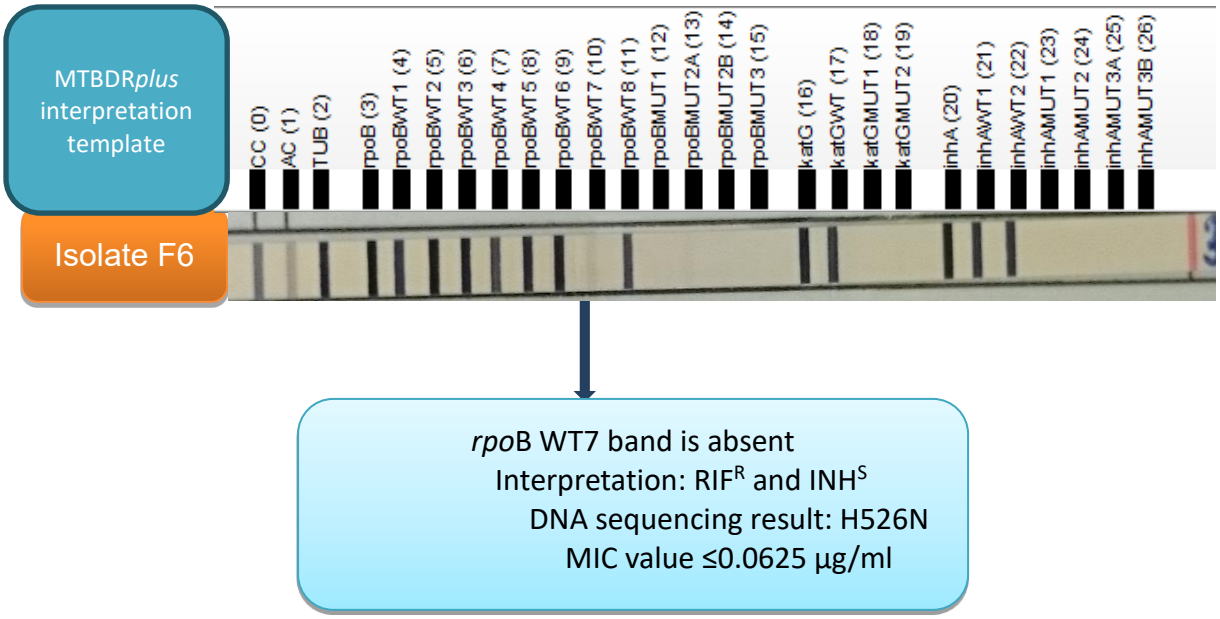


Figure 6: An LPA of an isolate from patient F6 showing absent *rpo*B WT7 probe and absent MUT bands, reported as RIF^R. DNA sequencing showed H526N mutation and the MIC in this case was ≤0.0625 µg/ml which is lower than the critical concentration (1 µg/ml) thus would be expected to test RIF^S by MGIT DST (and lead to a discordant genotypic / phenotpic RIF result).

*WT = wild type probe, MUT = mutation probe, RIF^R = rifampicin resistant, RIF^S = rifampicin susceptible, INH^S = isoniazid susceptible LPA= line probe assay, MIC= minimum inhibitory concentration, DST = drug susceptibility testing

All isolates with H526N had an MIC ≤0.25 µg/ml: 6 had an MIC ≤0.0625 µg/ml ; 10 had an MIC of 0.125 µg/m and 3 had an MIC of 0.25 µg/ml. Our findings correlate with those of van Deun *et al* where 4/5 isolates with H526N were reported to confer low level RIF resistance and contrary to our finding one isolate was RIF^R by MGIT DST at 1.0 µg/ml (Rigouts et al., 2013). For isolates with the H526L mutation the MIC value ranged from

0.5 µg/ml (n=1) through 1.0 µg/ml (n=6) to >1.0 µg/ml (n=4). The MIC values were similar to other reports on isolates with H526L confering borderline RIF resistance (the term borderline is used for MTB isolates which are RIF^S at 0.5 to 1.0 µg/ml using MGIT DST but RIF^R at the same concentration by proportion method on solid agar) (Ocheretina et al., 2014, Van Deun et al., 2009). One isolate harbouring H526R had an MIC of >1.0 µg/ml (Figure 5).

In our study, all isolates with *rpo*B mutation H526N had an MIC value ≤0.25 µg/ml conferring low level rifampicn resistance. An MIC value of ≤1.0 µg/ml should be expected for all isolates harbouring the H526N mutation and if the laboratory could detect the actual amino acid / nucleotide change this result could be communicated to the clinician with a comment suggesting low level RIF resistance for treatment regimen consideration. Sequencing is not available routinely and the LPA that is more readily available does not differentiate between different amino acid changes; hence all mutations in codon 526 (besides H526Y and H526D) will appear with the LPA pattern of absent WT7 and absent MUT band . If future clinical studies deem it important to report low level RIF^R mutations, it may be useful for future versions of the LPA to include sequences for the more common amino acid changes in codon 526, such as 526N , 526L and 526R.

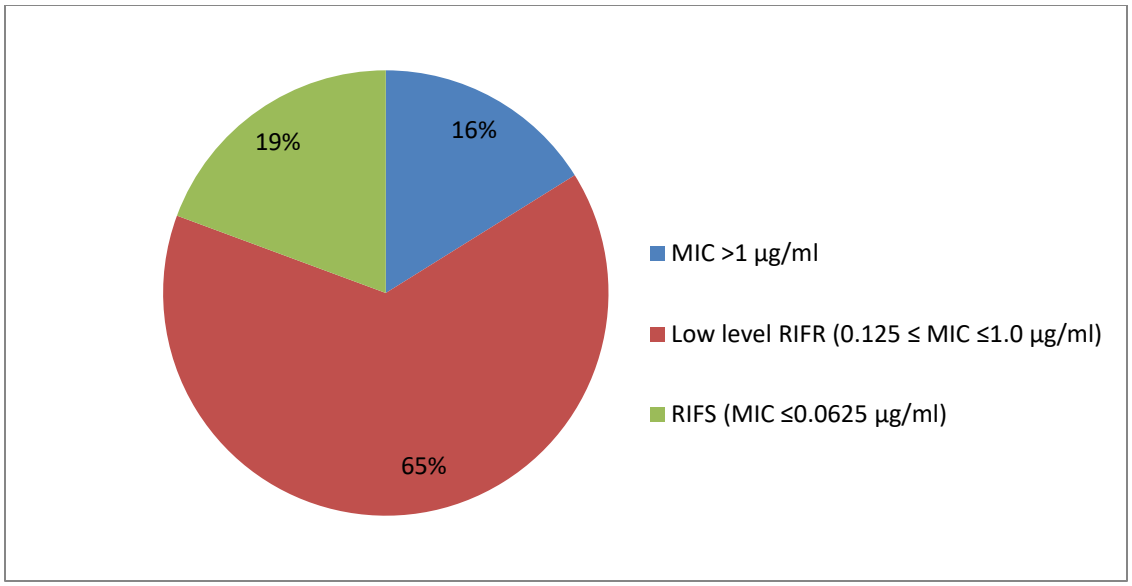


Figure 7: MIC value in isolates with mutations in codon 526. The majority (84%) of miscellaneous *rpo*B mutations in codon 526 translate to low level RIF resistance or RIF^S by phenotypic testing.

*MIC = minimum inhibitory concentration, RIF^R = rifampicin resistant, RIF^S = rifampicin susceptible

In comparison with the LPA pattern of [WT2 absent, no MUT] where 98.1% of mutations in this study were due to L511P, LPA pattern of [WT7 absent, no MUT] can be due to one of 3 mutations which have variable MICs (Figure 7). However, 84% of them translate to a MGIT RIF^S result. It is not possible for the LPA to differentiate between the disputed *rpo*B mutations that occur in codon 526, thus it is more difficult for the laboratory to comment on the level of MIC expected for mutations detected in this *rpo*B region. Further studies with more isolates would add value here. A mutation detected in this codon region by LPA should lead to confirmation by phenotypic DST, ideally by MIC testing. Again the clinical relevance of these mutations is unclear. Orchereina *et al* described four isolates with the H526L mutation with MICs ranging from 0.5 to 4 µg/ml (Ocheretina et al., 2014) and one isolate with 0.5 µg/ml (Williamson et al., 2012); all patients failed the standard TB regimen. Williamson *et al* described one isolate with mutation H526N with a low level MIC of 0.5 µg/ml from a patient who had failed the standard TB regimen (Williamson et al., 2012).

S533P, S531W, S531C, S531F and S531Q (miscellaneous mutations in codons spanning 530-533)

There were 34 isolates with miscellaneous *rpo*B mutations as identified by the LPA with absent *rpo*B WT8 and absent MUT bands (Figure 8). According to the LPA package insert the mutations in this region span from codon 530 to 533. There were five different mutations detected by sequencing namely L533P (n=27 (79.4%)), S531W (n=4), S531C (n=1), S531F (n=1) and S531Q (n=1). The MIC values for all isolates with L533P mutation were ≤1 µg/ml (but >0.0625 µg/ml). These low level MIC values are similar to those determined in previous studies (Rigouts et al., 2013, Ho et al., 2013, Andres et al., 2014) which reported MIC values ranging from 0.5 µg/ml (Andres et al., 2014) to 1.0 µg/ml (Ho et al., 2013). Rigouts *et al* showed that all tested isolates (n=14) with L533P were RIF^S at 0.5 µg/ml and 1.0 µg/ml using MGIT DST but all tested RIF^R at the same concentration using solid (LJ) DST (Rigouts et al., 2013). This finding is similar to ours where all the isolates with L533P tested RIF^S at 1.0 µg/ml but we had 24 (88.9%) that were RIF^S at 0.5 µg/ml. The one isolate with S531C had a RIF^S MIC of ≤0.0625 µg/ml similar to that previously reported with MIC value ≤0.125 µg/ml (Berrada et al., 2016). The remaining six isolates with S531F, S531Q, S531W mutations all had an MIC >1.0 µg/ml (high level RIF resistance and would be expected to test RIF^R by the MGIT DST method); these were previously reported to have an MIC of >8 µg/ml (Berrada et al., 2016).

In summary, of the 34 isolates that had the LPA pattern of [absent *rpo*B WT8 and absent MUT bands], 27 (79.4%) had low level RIF resistance by MIC testing, one was RIF^S (MIC≤0.0625 µg/ml) and the remaining 6 (17.6%) had RIF MIC >1.0 µg/ml. All isolates with *rpo*B mutation L533P had an MIC value between 0.0625 µg/ml and ≤1 µg/ml, conferring low level RIF resistance. Since this mutation accounted for the majority (79.4 %) of mutations detected by this LPA pattern, the laboratory reporting RIF resistance when this LPA pattern is detected could add a comment that in about 80% of cases, it translates to low level rifampicin resistance. Future versions of the LPA may consider including a MUT band for this particular mutation, should it be deemed clinically significant and should it be determined that it should be reported.

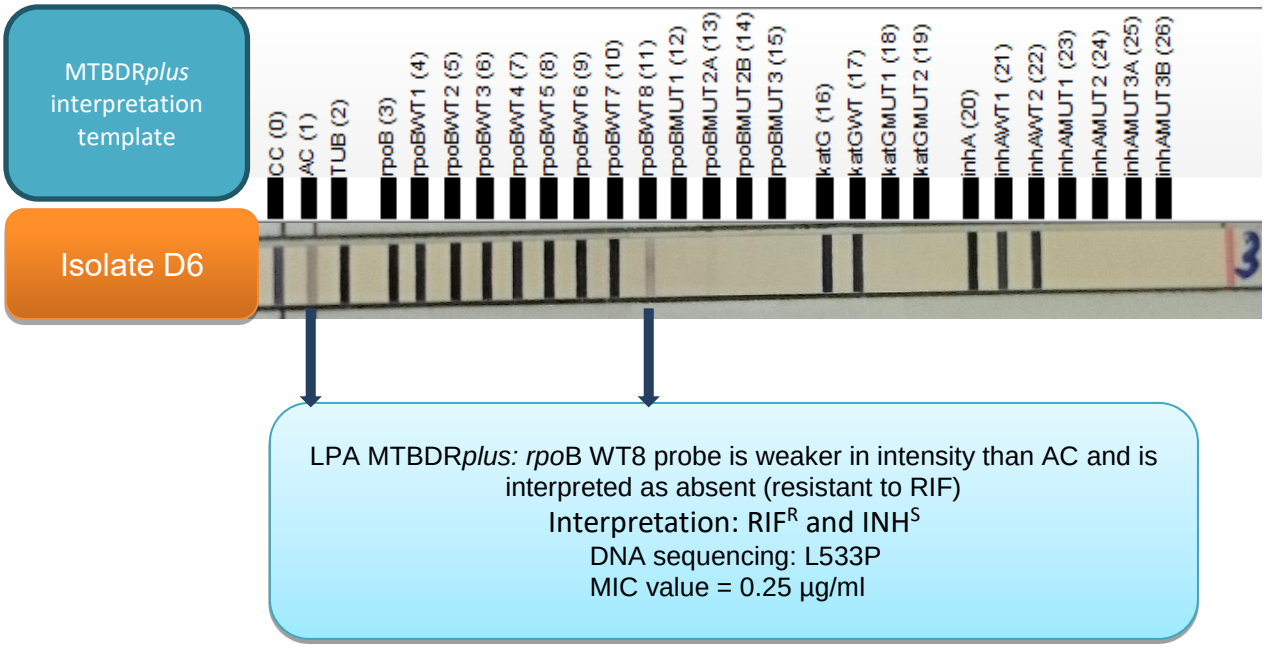


Figure 8: LPA strip of an isolate from patient D6 showing absent *rpo*B WT8 and absent MUT bands which is reported as RIF^R. DNA sequencing detected L533P. The MIC was 0.25µg/ml which is lower than the critical concentration (1µg/ml) and would be expected to test RIF susceptible by MGIT DST

*WT = wild type probe, MUT = mutation probe, RIF^R = rifampicin resistant, INH^S = isoniazid susceptible, LPA = line probe assay, MIC = minimum inhibitory concentration, AC=Amplification control, MGIT = mycobaterium growth indicator tube, DST = drug susceptibility testing

D516- / disputed mutations in codons 513 to 519

There were 43 isolates with miscellaneous mutations identified by LPA with the pattern [absent *rpo*B WT3&4 and absent MUT bands] (Figure 9). This pattern corresponds to a region of the RRDR spanning from codon 513-519. D516Y was the most frequent mutation (n=30) detected by sequencing and 29 (96.7%) had an MIC between 0.25 and 1 µg/ml. The distribution of MICs for the 29 isolates with D516Y was 0.25 µg/ml (n=10), through 0.5 µg/ml (n=15) to 1.0 µg/ml (n=4). The remaining isolate with D516Y had an MIC value above 1.0 µg/ml. The D516Y mutation has been reported previously to confer low level RIF resistance with MIC value between 0.25 µg/ml and 1.0 µg/ml (Van Deun et al., 2009, Williamson et al., 2012, Andres et al., 2014).

From the remaining 13 isolates, five had D516F and seven had either double mutation or deletions of nucleotides in more than one codon. All of these 12 isolates had MICs of >1.0 µg/ml. The D516F mutation has been reported previously to have MIC >1.0 µg/ml and isolates with either deletions of nucleotides or double mutation involving codon 516 have also been reported to have MIC >1.0 µg/ml (Berrada et al., 2016) and thus lead to high level RIF resistance.

There was only one isolate that harboured the D516G mutation and it had an MIC ≤0.0625 µg/ml (susceptible). This particular mutation has previously been reported to confer low level RIF resistance with an MIC value 0.5 µg/ml (Ho et al., 2013).

Overall, of the 43 MTB isolates with this particular LPA pattern, one D516G was RIF^S; 29 had low level RIF^R MIC (all of which were D516Y) and 13 (30%) had high level RIF^R (of which 7 were double mutations / deletions).

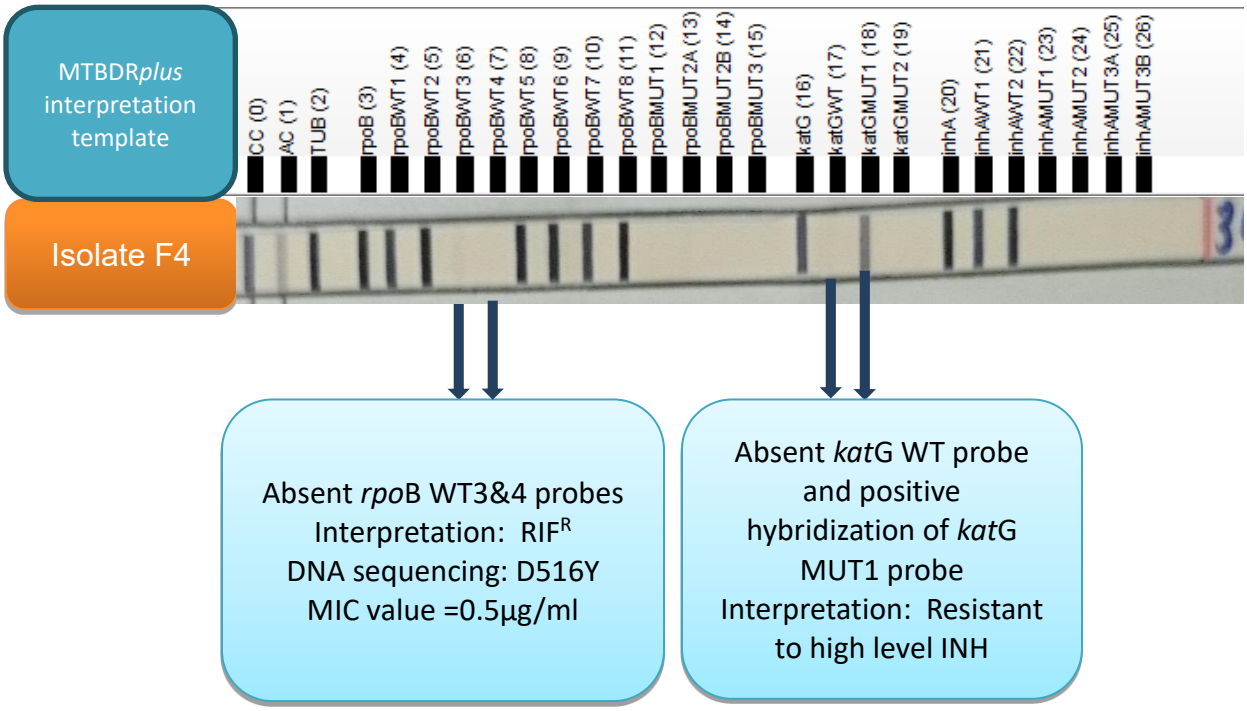


Figure 9: LPA strip of an isolate from patient F4 showing absent *rpo*B WT3&4 probes and absent MUT bands as well as absent *kat*G WT probe and *kat*G MUT1 probe present and therefore reported as MDR. DNA sequencing showed D516Y mutation and MIC value was ≤0.5µg/ml which is lower than the critical concentration (1 µg/ml); would be expected to test RIF^S by MGIT DST.

*WT = wild type probe, MUT = mutation probe, RIF^R = rifampicin INH = isoniazid, MDR = multi drug resistant, LPA= line probe assay, MIC= minimum inhibitory concentration, MGIT = mycobacterial growth indicator tube, DST = drug susceptibility testing

Of the remaining 16 isolates that had miscellaneous *rpo*B mutations, the majority had an MIC >1.0 µg/ml (n=14) and two had MICs of 0.5 and 1.0 µg/ml.

There were eight isolates that were identified by LPA as absent *rpoB* WT2&3 bands and absent MUT bands that by sequencing exhibited *rpo*B mutations Q513P (n=3), Q513 (n=2), del511-514 (n=1) and del513-515 (n=1). All of these had MICs of >1.0 µg/ml and one isolate with a triple mutation L511P/M515V/V581A had an MIC of 1.0 µg/ml.

There were two isolates identified by LPA as absent *rpo*B WT5&6 and absent MUT bands that exhibited the S522L *rpo*B mutation and had an MIC >1.0 µg/ml. Isolates with the S522L mutation have been reported to be resistant at 1.0 µg/ml using MGIT DST (Jamieson et al., 2014).

One isolate had absent *rpo*B WT4 and absent MUT bands and exhibited the deletion del517,518 with an MIC of 0.5 µg/ml.

The remaining five isolates were identified by LPA as absence of two or more *rpo*B WTs and absent MUT bands: absent *rpo*B WT2,3&4 (mutations Q510L & D516Y); absent *rpo*BWT3,4&8 (mutations D516G &L533P); absent *rpo*B WT3,4&5 (deletion del 515-518); absent *rpo*B WT4,5 (deletion del518) and absent *rpo*B WT7&8 (mutations H526N & L531W).

Isolates with an LPA pattern of two or more absent *rpo*B WT bands and absent MUT bands all had an MIC ≥1.0 µg/ml. Although the exact same patterns/combinations of double mutation have not been described elsewhere, isolates with other double *rpo*B mutations have been shown to have variable MICs (Jamieson et al., 2014).

In our setting most (93.3%) of isolates identified by the LPA as miscellaneous *rpo*B mutations with 2 or more absent *rpo*B WT bands and no MUT bands had an MIC >1.0µg/ml. The one exception was an LPA pattern with absent *rpo*B WT3&4 bands (and MUT band). When the LPA detects double (miscellanous) *rpo*B mutations, it may be

helpful for the laboratory to report that the mutations detected almost always confer high level RIF resistance.

Since the proportion of miscellaneous *rpo*B mutations is 11.3% and 78.7% of these have MICs below 1 µg/ml, we can estimate that 9% of all RIF resistance detected by molecular tests in our setting will not result in high level RIF resistance, and be expected to give a discordant susceptible result with phenotypic DST using the MGIT method. Some questions remain unanswered and require further study, including the clinical significance of each of these miscellaneous *rpo*B mutations, whether they should be reported routinely, at the point of diagnostic testing (i.e. when the LPA indicates that it is not a high confidence RIF resistant mutation (by virtue of there being an absent MUT band when there is absence of a WT band)), and what TB regimen is best suited to the isolate – specifically whether rifampicin should be included in the regimen and if so, at what dose.

Limitations of the study

In this study, Xpert was performed on the first specimen whilst LPA and sequencing were performed on the culture isolate from the second specimen. We did not sequence directly from sputum for Part 1 of the study, as the specimen that was used for Xpert testing was not available by the time that discordance was detected. It is therefore possible that we may have under-called cases of mixed infection which has been described as occurring with high frequency in our setting (Warren et al., 2004). Sequencing applied directly to sputum may have detected mixed infection with resistant and susceptible strains and this would account for more of the discordance between Xpert and LPA.

Sequencing was not performed on the comparator group for Part 1 of the study due to resource limitations. This would have confirmed that there was a mutation present in the comparator group, which would be expected to be in the region of the probes determined by Xpert and LPA. However, since the probes involved in resistance for both the Xpert and the LPA corresponded in every case (e.g. if mutation was in probe E for Xpert, it was in WT8 band for LPA), we are of the opinion that the comparator group does represent a true resistant group to compare with the false resistant cases.

A limitation of Part 2 of the study is the fact that we have no supporting clinical outcome data to inform the clinical significance of the miscellaneous *rpo*B mutations. We suggest

report on the low-level RIF resistant mutations but more data is required to inform their clinical relevance.

4.2 CONCLUSIONS

The high prevalence of MDR-TB worldwide necessitates the availability of rapid and accurate drug susceptibilty testing methods. The test method should not only have a rapid turnaround time, be amenable to high throughput and be available at an affordable cost but it should also be accurate for detecting resistance in MTB in all settings including high burden developing countries.

Rapid molecular test methods such as GenoType MTBDR*plus* LPA and Xpert MTB/RIF are very good at screening for MDR-TB. However, it is important for the end-user of the result to know that neither of these rapid molecular diagnostic methods nor phenotypic methods are perfect in predicting MTB drug susceptibility (Rigouts et al., 2013, Van Deun et al., 2015, Van Deun et al., 2009). In cases of discordant RIF susceptibility results between different diagnostic methods, DNA sequencing, if available, should be performed to obtain the predicted resistance result, based on an accurate identification of genotype. However, this tool is not routinley available in low resource settings where the burden of TB disease is greatest.

In cases where Xpert is used as a screening tool for MDR-TB it is recommended by the WHO that another test method is used to confirm RIF^R (WHO, 2011). We recommend, that in a case of RIF resistance detected by an Xpert MTB/RIF test that is due to probe delay with a ΔCt max value of between 4.1 and 4.9, double probe delay or with an abnormal graph curve, the RIF resistant result should not be released to clinicians. The result should be changed to RIF inconclusive and the Xpert test should be repeated on a second specimen from the patient. Xpert MTB/RIF parameters (probe Ct values, bacterial load, probes involved and graph curves) should be checked before reporting an Xpert MTB/RIF RIF^R result to clinicians. In a case of Xpert / LPA discordance where no error on the part of Xpert is detected, the LPA strip result must be checked thoroughly and repeated prior to releasing LPA result. The submission of multiple specimens for repeat Xpert and LPA is useful to determine which result is in error, as well as to prove mixed infection which can cause discordant results, but comes with additional costs. Specimen mix-up could be the cause of discordance and should be ruled out by repeating the LPA and / or by submitting another sample for repeat Xpert and LPA.

Mixed MTB infection is not an uncommon cause for discordant rifampicin Xpert / LPA results and though it was detected at a low frequency in this study, this may be a due to a limitation of the methodology, as it is thought to occur at moderate to high frequency in high TB burden setttings (Warren et al., 2004, Shamputa et al., 2006, Folkvardsen et al., 2013, Zetola et al., 2014). It is difficult to rule out or in mixed infection and depends on multiple specimens being submitted (for now; until a better diagnostic is developed and proven to be reliable) and that perhaps WGS on direct specimens will assist in determining the exact prevalence of it in newly diagnosed TB cases. More studies determining its frequency and optimal diagnostic tools that can detect heteroresistance accurately are required.

Arising from this study is a laboratory based guideline that is now used within NHLS TB laboratories detailing steps on how to detect possible false RIF^R results by Xpert MTB/RIF and on how to troubleshoot discordant Xpert RIF^R and LPA RIF^S results (Appendix A).

As new tests are implemented and experience is gained with their use in routine laboratories, more information becomes available that may be useful to share with other laboratories using the same test. The same information may assist manufacturers in improving their assays. A new cartridge, Xpert Ultra will be implemented in the near future, in South Africa. Ultra promises a lower limit of detection of MTB (15.6 CFU/ml), and improved RIF resistance detection (Chakravorty et al., 2017). Hain Lifesciences have developed an updated technology for the LPA called the FluoroType MTBDR assay(de Vos M, 2017). It is hoped that the discordance for rifampicin between Xpert and LPA is minimised once new assays are in routine use, but ongoing monitoring and evaluations are important and routine laboratories are best placed to determine this.

The majority of the miscellanous *rpo*B mutations that we detected (61.2%) had MICs ranging between 0.125 and 1.0 µg/ml which is regarded as low level RIF^R resistance that would be missed by MGIT DST. A futher 17.4% had MIC ≤0.0625 µg/ml which is regarded as RIF^S; these would be expected to be correctly called as RIF^S by MGIT DST. Laboratories that utilize MGIT or other liquid based phenotypic DST would interpret these isolates as RIF^S at a critical concentration of 1 µg/ml, thus leading to a discordant result between the molecular test (Xpert and / or LPA) and the phenotypic test result. It is

important to further determine the frequency of these misceallanous *rpo*B mutations in more settings, and to determine the clinical significance of each of them (Sirgel et al., 2013).

The LPA is useful in that it can be used to rule out a high level RIF^R ("undisputed") *rpo*B mutation and can indicate a likely disputed *rpo*B mutation that would be expected to give a RIF^S result by MGIT DST. Laboratories using the LPA must in conjunction with the end-user of the result determined whether to add to the report about whether a high level or low level RIF resistance mutation has been detected by the LPA. At the present time, the LPA package insert states that whenever there is an absent WT band, even in the case of an absent MUT band, the RIF result should be reported as resistant. There is no way that the end-user knows whether the *rpo*B mutation detected by the LPA confers high or low level RIF resistance.

In cases where a non-high level RIF^R mutation is detected by the genotypic test, and where alternative drug options are not available, or are contra-indicated, it may be useful to add high dose rifampicin (Ho et al., 2013) or rifabutin to the drug regimen (Sirgel et al., 2013).

A database has been created from the results obtained in this study that lists specific *rpo*B mutations and their corresponding MIC value (Appendix B). This can be used as a reference in regional laboratories to provide clinicians with the frequency of particular *rpo*B mutations per LPA pattern detected as well as the likely level of RIF resistance that can be expected for each mutation. More studies describing miscellaneous *rpo*B mutations in different regions are required as there seems to be some geographical variation in the distribution of the *rpo*B mutations (Ioerger et al., 2010, Georghiou et al., 2016). In the future performing MIC testing will be helpful in compiling individualized treatment regimens.

The question of whether to report the likely *rpo*B mutation when a disputed, low level RIF resistance mutation is expected needs to be answered by clinical studies where patient who are infected with MTB harbouring these mutations are followed up for clinical outcomes of cure and relapse, in relation to the drug regimen used. The optimal drug regimen for these isolates is not known and further studes are required to inform this.

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6. APPENDICES

6.1 Appendix A

Guideline for TB laboratories to investigate possible false RIF^R by Xpert and discordance with a subsequent RIF^S by LPA

1. All RIF^R results by Xpert should be checked prior to releasing the result. During Xpert review check the Xpert parameters for a possible false RIF^R result.
   a. Probe delay where the ΔCt max value is between 4.1 and 4.9
      - Report RIF inconclusive, repeat Xpert on second specimen
   b. Double probe delay (specifically delayed hybridization of probes D and E)
      - Report RIF inconclusive, repeat Xpert on second specimen
   c. An abnormal graph fluorescence curve
      - Report RIF inconclusive, repeat Xpert on second specimen

2. If a RIF^S result by LPA is reported, but this is discordant with an already-reported Xpert result of RIF^R

   a. Check the Xpert readout and graph (as point 1 above)
   b. If Very Low MTB bacterial load was detected by Xpert, then it is likely false RIF^R by Xpert
   c. Note which probe is involved (Ct=0 or delay >4) in the Xpert RIF^R result
      - If for example the RIF^R result by Xpert was due to probe dropout or probe delay of probe D, it is expected that the LPA has an absent *rpo*B WT7 band. The LPA WT7 band may be equal or darker in intensity than the AC band (especially if the AC bands of most strips in the batch are very light) but intensity of WT7 should be compared to intensity of all the other *rpo*B WT bands. If it is lighter than the others *rpo*B WT bands it is possible that it should be interpreted as absent rather than present (and hence resistant rather than susceptible for RIF)
   d. Check LPA result again and repeat if indicated
      - Obtain the LPA strip if possible. If not possible, obtain a photo of the strip (in a case where Genoscan was in use). Look again at the intensity of all the *rpo*B WT bands. Although the LPA package insert states that comparison of intensity of the band is

made only with the AC band, in some cases of false RIF^S results by LPA, the AC band is particularly light, and the WT2 / WT7 band in particular is more intense than the AC band; however, the WT2/7 band may be lighter than the adjacent *rpo*B WT bands in a case where Xpert has detected resistance due to probe delay / dropout in probes A / D respectively

e. If there is no obvious questionable *rpo*B WT bands, consider repeating the LPA

f. Batches of LPA should be checked for any indication of contamination; specimen / culture mix up i.e. laboratory contamination# I should be ruled out prior to reporting of any results

3. All previous and current results of a patient should be checked prior to reporting results to clinicians;
    a. Actively look for all previous molecular (Xpert and LPA) results on the patient, as well as any phenotypic DST results
    b. Examine the probe patterns and graphs of each Xpert and LPA result to make sure no technical errors occurred
    c. Multiple specimens / isolates with repeatedly discordant results may be an indication of true mixed infection

4. Repeat testing

    a. If interpretation of all performed tests seems correct according to the probe patterns expected, and the Xpert graphs look normal, repeat testing is recommended:
    b. LPA should be repeated as not always possible to repeat Xpert on the same specimen at the time of discordance and repeating the Xpert on culture may be considered
    c. If the Xpert parameters indicate likely false RIF resistance, the Xpert should be repeated on a second sample that should be submitted (a comment must be added to original Xpert report)
    d. If repeat testing gives the same result, further tests may be done, if available
        - Phenotypic DST (MGIT method most commonly available); the limitations of particularly liquid based phenotypic should be considered i.e. MGIT DST may miss disputed *rpo*B mutations that confer low level RIF resistance
    e. If the Xpert and LPA results are not altered after all results are checked, the isolate (on which LPA was performed) may be referred for *rpo*B sequencing

6.2 Appendix B

Table 11: Relationship between *rpo*B mutations and MIC values (MGIT 960 EpiCenter)

	MIC (µg/ml)	MGIT 960 with EpiCenter					no. of isolates
	RIF Susceptible	Low level RIF^R				High level RIF^R	
*rpo*B mutations detected	≤0.0625	0.125	0.25	0.5	1	>1.0	Total
L511P	23	27	3	0	0	0	53
H526N	6	10	3	0	0	0	19
L533P	0	2	10	12	3	0	27
D516Y	0	0	10	15	4	1	30
H526L	0	0	0	1	6	4	11
S531W	0	0	0	0	0	4	4
D516F	0	0	0	0	0	5	5
D516G	1	0	0	0	0	0	1
Q513P	0	0	0	0	0	3	3
Q513K	0	0	0	0	0	2	2
S522L	0	0	0	0	0	2	2
H526R	0	0	0	0	0	1	1
S531C	1	0	0	0	0	0	1
S531F	0	0	0	0	0	1	1
S531Q	0	0	0	0	0	1	1
L511P/D549E	0	1	0	0	0	0	1
D516G/I572M	0	0	0	0	0	1	1
H526N/S531W	0	0	0	0	0	1	1
Q510L/D516Y	0	0	0	0	0	1	1
D516Y/I572M	0	0	0	0	0	1	1
D516G/L533P	0	0	0	0	0	1	1
L511P/M515V/V581A	0	0	0	0	1	0	1
del 518	0	0	0	0	0	1	1
del517/518	0	0	0	1	0	0	1
del 514/515	0	0	0	0	0	1	1
del 514/515/516	0	0	0	0	0	3	3
del 515/516/517	0	0	0	0	0	1	1
del 513/514/515	0	0	0	0	0	1	1
del 515/516/517/518	0	0	0	0	0	1	1
del 511/512/513/514	0	0	0	0	0	1	1
Total	31	40	26	29	14	38	178

MIC = minimum inhibitory concentration, MGIT = mycobacterial growth indicator tube, RIF^S = Rifampicin susceptible, RIF^R = Rifampicin resistant