IDENTIFICATION OF RIFAMPICIN RESISTANCE USING XPERT MTB/RIF AND MTBDR*plus* V2.0 FOR THE GREATER GAUTENG PROVINCE: IMPLICATIONS FOR PATIENT CARE

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DECLARATION

I, Marianne Black, declare that this Research Report is my own, unaided work. It is being submitted for the Degree of Master of Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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ABSTRACT

An ongoing challenge in the control of tuberculosis (TB) is drug resistant TB, including rifampicin resistant (RIFr) TB and multidrug resistant (MDR) TB. South Africa's current diagnostic algorithm for TB diagnosis employs the Xpert MTB/RIF (GXP) as the initial screening test for TB diagnosis and rifampicin (RIF) susceptibility, followed by submission of a second specimen for MTBDR*plus*, a line probe assay (LPA) to confirm RIFr TB and to determine isoniazid (INH) susceptibility. This study aimed to describe the distribution of *rpoB* mutation patterns as identified by LPA and GXP in Gauteng province, and compare RIF susceptibility results between LPA and GXP.

The most common *rpoB* mutation detected by LPA and GXP in Gauteng occurred at codons 530-533. LPA ΔWT2, which was mostly INH sensitive (INHs), is more prevalent in Gauteng than in other parts of the world. The LPA ΔWT3,4,8 and GXP probe B,E is a probable extensively drug resistant (XDR) TB strain prevalent in Gauteng and shows value in investigating gene regions derived from these molecular assays. The overall concordance between RIF susceptibility results was 96.42% and for the molecular codon region for RIFr results, 99.27%. There were 68 discordant RIF results over the one-year period, with a majority being, LPA RIF sensitive (RIFs), GXP RIFr. Discordant GXP RIFr results detected by delayed probe hybridisation reached statistical significance.

The management of discordant RIF susceptibility results should involve inputs from both clinician and laboratory. The laboratory may provide *rpoB* sequencing when the culture is available, report heteroresistance when appropriate, performing phenotypic RIF DST and/or MIC testing, and reviewing all results for possible GXP and LPA technical errors. The introduction of a unique LIS patient identifier is critical to identify discordant results and troubleshoot accordingly and highlights the importance of an LIS with a well-maintained central data warehouse.

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Contents

NOMENCLATURE/LIST OF ABBREVIATIONS AND SYMBOLS

CT	Cycle threshold
ΔCT	Delta CT
GXP	Xpert MTB/RIF assay
DST	Drug susceptibility testing
IUATLD	International Union against TB and Lung Disease
INH	Isoniazid
INHr	Isoniazid resistant
INHs	Isoniazid sensitive
LAM	Lipoarabinomannan
LPA	Line probe assay
MDR	Multi-drug resistance
MGIT	Mycobacteria Growth Indicator Tube
MTBC	*Mycobacterium tuberculosis* complex
MUT	LPA mutation band
RIF	Rifampicin
RIF MONO	Rifampicin monoresistance
RIFr	Rifampicin resistant
RIFs	Rifampicin sensitive
RRDR	Rifampicin resistance determining region
SA	South Africa
WHO	World Health Organization
WT	LPA wild type band
ΔWT	Missing WT
XDR	Extensively drug resistant

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1. INTRODUCTION

Tuberculosis (TB) is the leading cause of death worldwide caused by a single infectious agent, called *Mycobacterium tuberculosis* complex (MTBC). It is estimated that 10.4 million people progressed to TB disease in 2016, with 1.6 million of these dying (1).

The incidence rate of TB in South Africa (SA) is 781 per 100'000 population, ranking the country highest in the world (1). This is mainly driven by the HIV epidemic. The prevalence of HIV in the South African adult population is 17.98% (2). The risk of developing TB disease is 20-37 fold higher in those infected with HIV (3). The TB/HIV co-infection rate for 2016 was 59% (1).

The World Health Organization (WHO) has set a goal of reducing TB incidence by 4%-5% per year by 2020 to reach the first goal of the END TB Strategy (4). TB incidence is currently falling at a rate of 2% per annum; therefore additional efforts are needed to reach this goal. An ongoing challenge in the control of TB is drug resistant TB, including rifampicin resistant (RIFr) TB and multidrug resistant (MDR) TB. MDR TB is defined as resistance to at least rifampicin (RIF) and isoniazid (INH), two critical drugs used in treatment of TB. Following a national survey done in SA in 2012-2014, it was revealed that 3.4% of new TB cases and 7.1% of previously treated cases have RIFr TB or MDR TB (5). Delays in identifying RIFr TB is associated with worse clinical outcomes and increased transmission (6,7). This may, however, be improved by the rapid initial diagnosis and initiation of an effective treatment regimen early on (8).

1.1 TB diagnostic tools and drug susceptibility testing

In the past, culture (phenotypic) based identification used to be the main method for diagnosing TB and performing drug susceptibility testing (DST). Although culture-based DST remains the gold standard, it is currently not the method of choice for obtaining routine MTBC drug susceptibility results. Culture based DST is dependent on the growth rate of MTBC and its accessibility is restricted to referral centers. To overcome

the problem of the lengthy time to diagnosis and treatment initiation, molecular-based technologies (genotypic) have been developed to simultaneously diagnose TB and identify drug resistant TB, especially against the first line agents, RIF and INH (Table 1).

In 2011, the WHO recommended the use of rapid molecular diagnostics (instead of phenotypic methods) as the initial diagnostic test, to improve the time to diagnosis and obtaining drug susceptibility results (6).

Table 1. Comparison of phenotypic and genotypic diagnostic methods for the diagnosis of TB. Phenotypic tests include direct smear microscopy, culture and culture-based drug susceptibility testing. Genotypic tests include Xpert MTB/RIF and MTBDRplus V2 assays. Diagnostics have progressed from phenotypic to genotypic methods due to improvements in time to detection and sensitivity. However, culture remains the gold-standard diagnostic modality.

	Method	Detection limit (bacilli per millilitre specimen)	Specimen type	Biosafety requirement	Advantages	Disadvantages	Reference
Phenotypic methods	Direct microscopy	5000-10000	Any specimen type, pulmonary and extra-pulmonary (except blood)	Minimal risk	Cheap	• Low sensitivity (31%-80%) (especially in HIV positive patients) • Lacks information on drug susceptibility	(9–12)
	Culture	1-10	BACTEC MGIT 960 TB system: Any specimen type, pulmonary and extra-pulmonary (except blood*)	High risk	Gold standard	• Long turnaround time • Needs expertise	(13)
	Culture based drug susceptibility testing					• Needs pure culture	
Genotypic methods	Xpert MTB/RIF assay	131	• Respiratory specimens (sputum, induced sputum and gastric washings) • Selected extra-pulmonary specimens (tissue biopsies, CSF and purulent fluid)	Minimal risk	• Any staff member may be trained • Short turnaround time of 2hours • Not affected by contamination	• Expensive • Only provides rifampicin susceptibility result • Needs constant source of electricity	(14,15)
	MTBDR*plus* V2	160	• Direct testing: Respiratory specimens (sputum, induced sputum and gastric washings), selected extra-pulmonary specimens (lymph node aspirates) • Cultures from any type of specimen	Moderate to high risk	• Performed on specimens (smear positive or negative) and/or positive cultures • Provides rifampicin and isoniazid susceptibility result • Can be performed on contaminated cultures	• Expensive • Needs expertise to perform and interpret • Complex procedure, requires sophisticated laboratory infrastructure • Takes 2-3 days to perform	(16,17)

*Blood is culturable using BACTEC Myco/F Lytic medium, after which it is subcultured to BACTEC MGIT 960 TB system for DST.

New in vitro TB diagnostic assays are being developed and produced. These assays have to follow the WHO endorsement process. This entails the review of published performance data by an expert panel and recommendations made on how to use these assays (Figure 1). The current TB diagnostic and drug resistance testing assays that are endorsed by the WHO are detailed in Table 2.

Table 2. Current technologies for TB diagnosis and drug susceptibility testing endorsed by the WHO. The molecular assays listed in blue are currently in use in South Africa. The Xpert MTB/RIF assay was replaced by the improved Xpert MTB/RIF Ultra in late 2017. Table adapted from WHO Global TB Report, 2017.

	Assay (Supplier)	Principle of assay	Target genes	Reference
Microscopy	Light and light-emitting diode (LED) microscopy (diagnosis and treatment monitoring)	Microscopic examination of Ziehl-Neelson or fluorescence stained smears for diagnosis and treatment monitoring	-	(18)
	Microscopic observation drug susceptibility (MODS) test (Hardy Diagnostics, USA)	Specimens inoculated into drug-free and drug-containing liquid culture media, microscopic examination for signs of growth		(19)
Culture based technologies	Commercial liquid-based systems (eg BACTEC MGIT 960 TB System) and rapid speciation	• Bacterial growth depletes oxygen which results in fluorescence within MGIT measured with UV light • Known concentration of test drug is added to one MGIT, growth is compared with drug free MGIT	-	(12)
	Solid media (Löwenstein-Jensen (LJ) and Middlebrook 7H10/7H11 agar media	DST using 1% critical concentration method		
Molecular technologies	MTBDR*plus* (Hain Lifescience, Germany)	• LPA • Detects MTBC and resistance to RIF, INH	*rpoB, katG, inhA*	(16)
	Nipro (Nipro corporation, Japan)	• LPA • Detects MTBC and resistance to RIF, INH	*rpoB, katG, inhA*	
	MTBDR*sl* (Hain Lifescience, Germany)	• LPA • Detects MTBC and resistance to FQ and SLI agents	*gyrA, gyrB, rrs, eis*	(20)
	Xpert MTB/RIF (Cepheid, USA)	• Real-time PCR • Detects MTBC and resistance to RIF	*rpoB*	(14)
	Xpert MTB/RIF Ultra (Cepheid, USA)	• Real-time PCR • Improved sensitivity, detects MTBC and resistance to RIF	*rpoB, IS6110, IS1081*	(21)
	TB LAMP (Eiken, Japan)	• Loop-mediated isothermal amplification • Detects MTBC	Six regions of *gyrB*	(22)
Non-molecular technologies	TB-LAM (Alere, USA)	• Detects mycobacterial lipoarabinomannan (LAM) in urine	-	(23)

FQ = fluoroquinolones, SLI = second line injectable.

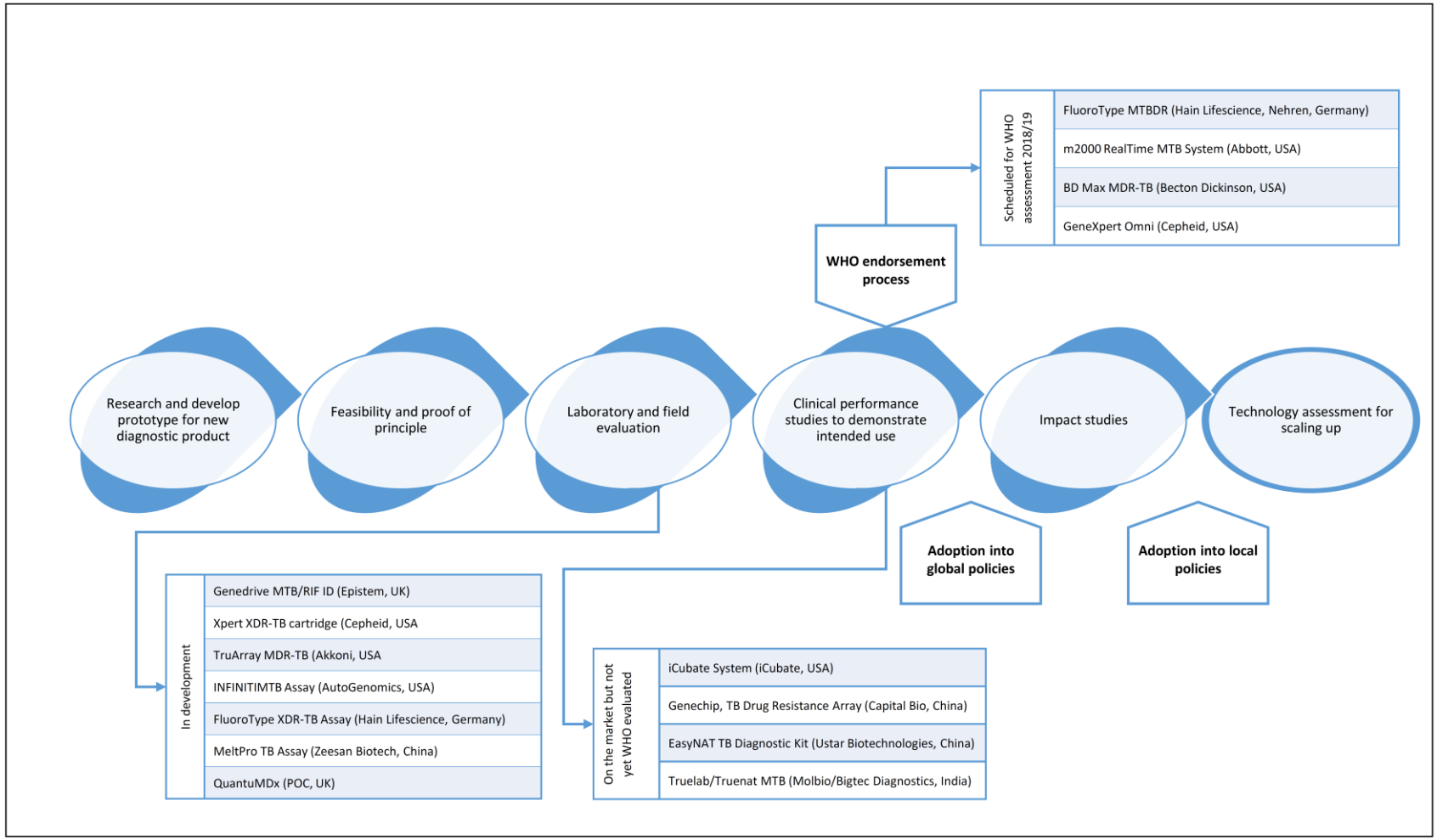


Figure 1. Stages in the development, evaluation, and adoption of new molecular diagnostic assays for TB (24). Assays under development are listed, as well as market available but not WHO evaluated assays. Assays earmarked for WHO assessment in 2018/19 are indicated.

1.2 WHO endorsed molecular tests used in SA for the simultaneous detection of MTBC and RIF susceptibility

There are currently two WHO-endorsed molecular tests used in SA for the simultaneous detection of MTBC and RIF susceptibility. The Xpert MTB/RIF (GXP) assay detects susceptibility to RIF only. MTBDR*plus*, a line probe assay (LPA), detects both RIF and INH susceptibility, and is therefore able to diagnose MDR TB. It has been suggested that RIF resistance be used to predict MDR TB, and patients diagnosed with RIFr TB may be initiated on an MDR TB treatment regimen, regardless of the INH susceptibility (25,26). However, the prevalence of RIFr TB versus MDR TB differs in the nine provinces in South Africa, as depicted by the recent South African Tuberculosis Drug-Resistance Survey of 2012-2014 (8). The ratio of MDR TB to RIF monoresistant (MONO) TB in Gauteng was 2.6:1, while in Limpopo it was 0.7:1, indicating that there were more RIF MONO than MDR TB cases in the latter province.

The gene associated with RIF resistance is the mycobacterial RNA polymerase β subunit (*rpoB*) gene. Around 96% of mutations conferring RIF resistance are confined within an 81 base pair (bp) region of the *rpoB* gene, termed the Rifampicin Resistance Determining Region (RRDR) (codon 507-533 using the *E.coli* numbering system; Figure 2) (25,27,28).

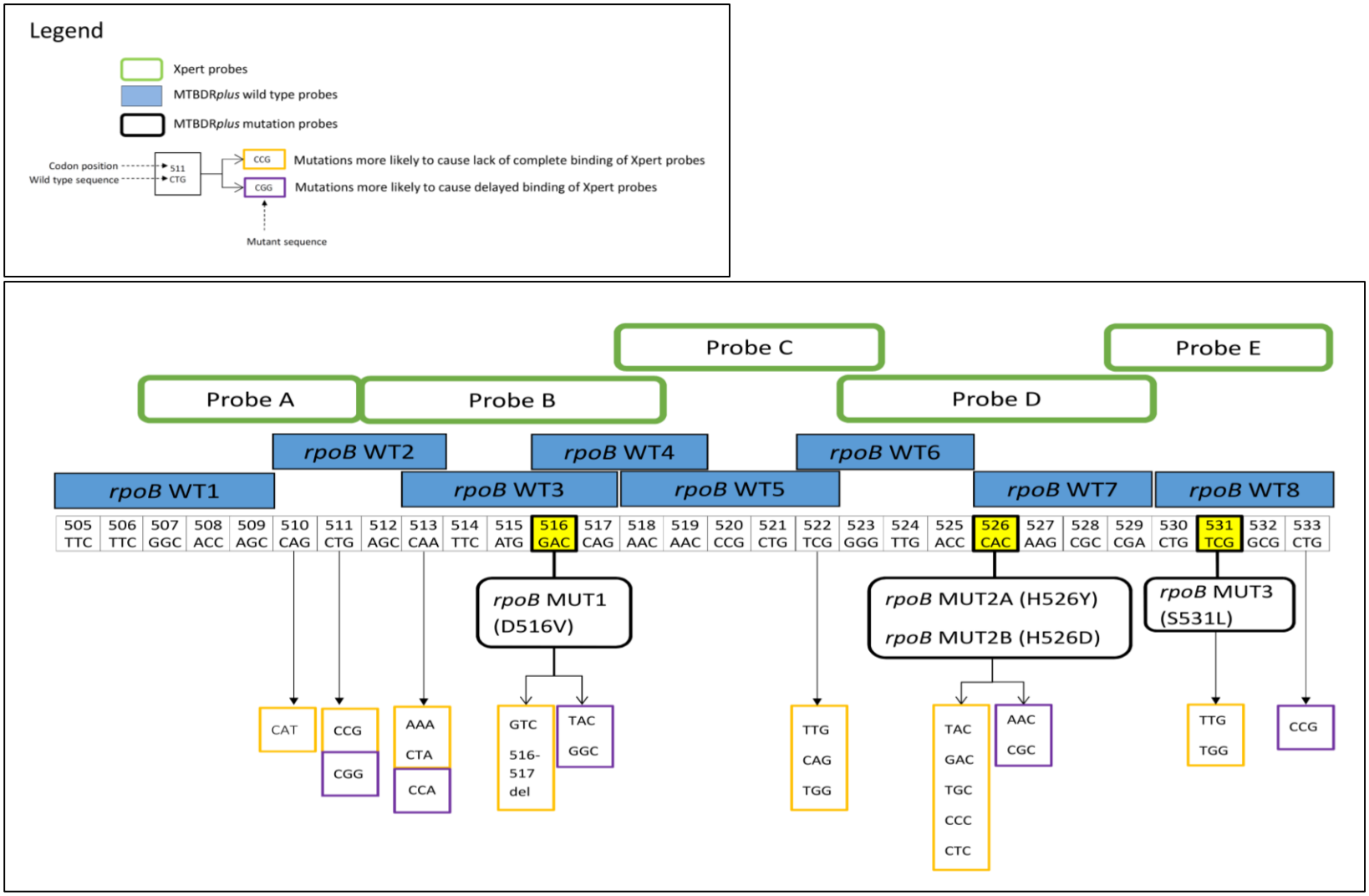


Figure 2. The Rifampicin Resistance Determining Region (RRDR). The RRDR is located in the mycobacterial RNA polymerase β subunit (rpoB) gene, codon 507-533. The codon positions along with the nucleotide sequence for MTBC are indicated. Both Xpert MTB/RIF and MTBDRplus binding probes extend across the 81-bp region. Certain probe binding positions overlap. There is an overlay between Xpert MTB/RIF probes and MTBDRplus probes. As an example, MTBDRplus WT1 and WT2 probe hybridise at the same codon region as Xpert MTB/RIF probe A, and MTBDRplus WT8 corresponds with probe E binding.

1.3 The Xpert MTB/RIF assay

The GXP assay was endorsed by the WHO in 2010, and has been used in the SA diagnostic algorithm as the initial diagnostic test since 2011 (29). The GXP uses real-time PCR for the detection of MTBC and RIF susceptibility. The assay amplifies an MTBC-specific sequence of the *rpoB* gene and uses five molecular beacon probes (Probe A to E) to detect mutations in the RRDR (30). RIF resistance is indicated by either a complete lack of hybridisation of a specific probe or by partial or delayed hybridisation. The cycle threshold (CT) value will be 0 in the case of a complete lack of binding, whereas delayed binding indicating RIF resistance is set as a ΔCT value >4 for GXP version G4. Certain mutations are more likely to cause a lack of complete binding of the probe, and others are detected by delayed or partial binding (30) (Figure 2).

The GXP is performed directly on pulmonary specimens such as sputum, induced sputum and gastric washings and selected extra-pulmonary specimens, such as tissue biopsies, fine needle aspirates, CSF and purulent fluid specimens. The laboratory turnaround time is two hours, and results are returned for patient care in a minimum of 40 hours. It requires minimal biosafety precautions and involves only three manual steps (31). Following the addition of a reagent to liquefy and inactivate the specimen, 2ml of the specimen is transferred into the cartridge and loaded into the Gene Xpert instrument.

1.4 The MTBDR*plus* assay

The MTBDR*plus* version 1 (v1) LPA was endorsed by the WHO in 2008, and has since been used in SA for the rapid diagnosis of MDR TB (17). The MTBDR*plus* version 2 (v2) was introduced in 2013 to improve performance on smear-negative specimens.

The LPA uses conventional Polymerase Chain Reaction (PCR) amplification for the detection of MTBC, RIF and INH susceptibility. Following amplification, amplicons are hybridised with specific probes which are immobilised on a strip. These hybrids, which

are labelled, undergo colourimetric development with subsequent visual detection of bands. Mutations for RIF are therefore detected by a lack of binding to wild-type probes (*rpoB*WT1-WT8), as well as by binding to specific probes for the most commonly occurring mutations (MUT1, MUT2A, MUT2B and MUT3). A lack of binding of probes are reported as missing (Δ) WT, with or without a MUT probe, e.g. ΔWT2; ΔWT8, MUT3 (Figures 3 and 4).

The LPA detects INH resistance by mutations in the two most commonly involved genes, *katG* and *inhA.* The *katG* gene has only one WT probe but two MUT probes (*katG*MUT1 and *katG*MUT2), while *inhA* has two WT probes and four MUT probes (*inhA*MUT1, *inhA*MUT2, *inhA*MUT3A and *inhA*MUT3B) (Figure 3).

The LPA is able to detect heteroresistance or mixed infections by the presence of all WT bands, but with the presence of one or more MUT bands. Heteroresistance is defined as the presence of both sensitive and resistant organisms, of the same clone, to anti-TB drugs. This drug resistance occurs faster than the spontaneous mutation rate and is driven by antibiotic selection pressure (32). Mixed infection is caused by infection with multiple strains of MTBC and is more common in endemic settings (33). Both heteroresistance and mixed infections are associated with poorer treatment outcomes (2).

Although the LPA may be performed on smear positive and negative specimens, the large majority of smear negative specimens still require a culture to obtain an interpretable susceptibility result (34). The LPA can be performed on contaminated cultures (MTBC culture mixed with yeast, bacteria or nontuberculous mycobacteria).

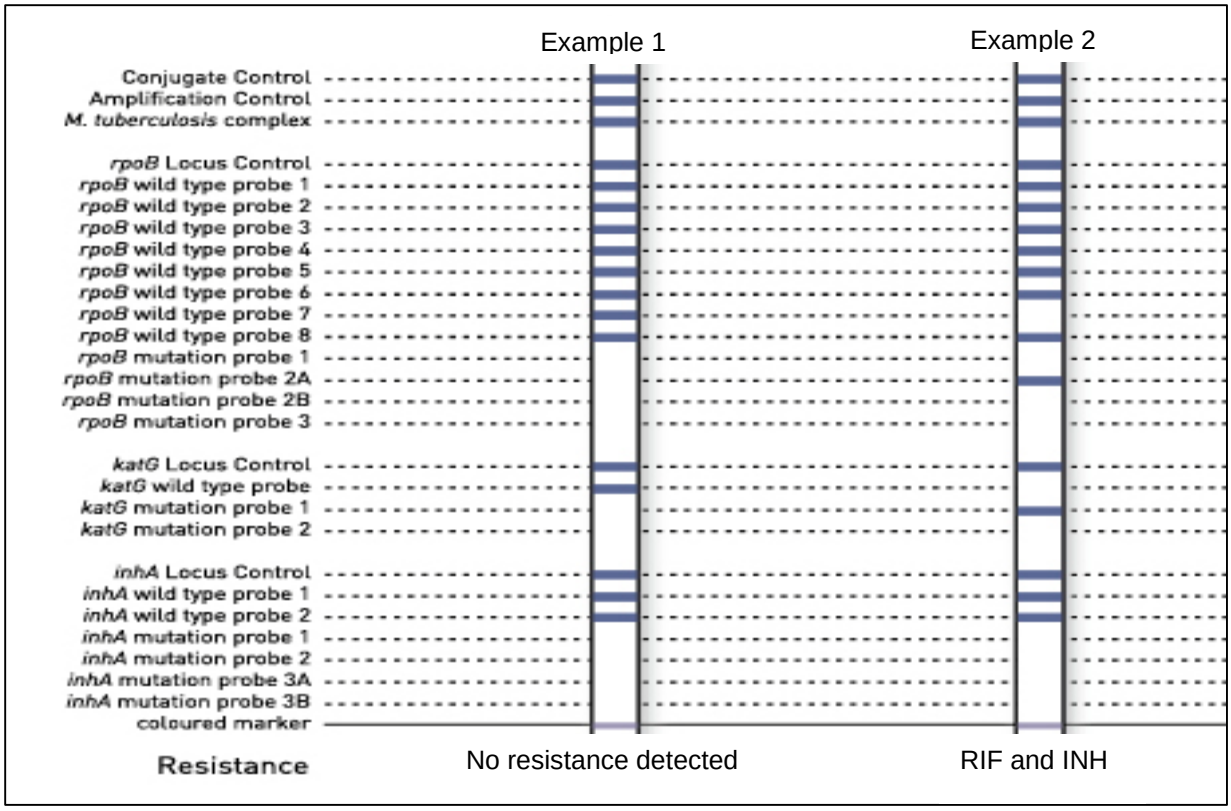


Figure 3. MTBDRplus PCR assay for the identification of MTBC and RIF and/or INH resistance. Amplicons are hybridised with specific probes which are immobilised on a strip, and undergo colourimetric development into visual bands. The presence of a band indicates the presence of the corresponding wild type (WT), or the corresponding mutation (MUT) detected. Example 1: rpoBWT, katGWT and inhAWT present with no MUT; Interpretation: rifampicin and isoniazid sensitive. Example 2: rpoBWT7 absent, rpoBMUT2A present; katGWT absent, katGMUT1 present, inhAWT present. Interpretation: rifampicin resistant, isoniazid resistant. Figure adapted from Hain Lifescience (35).

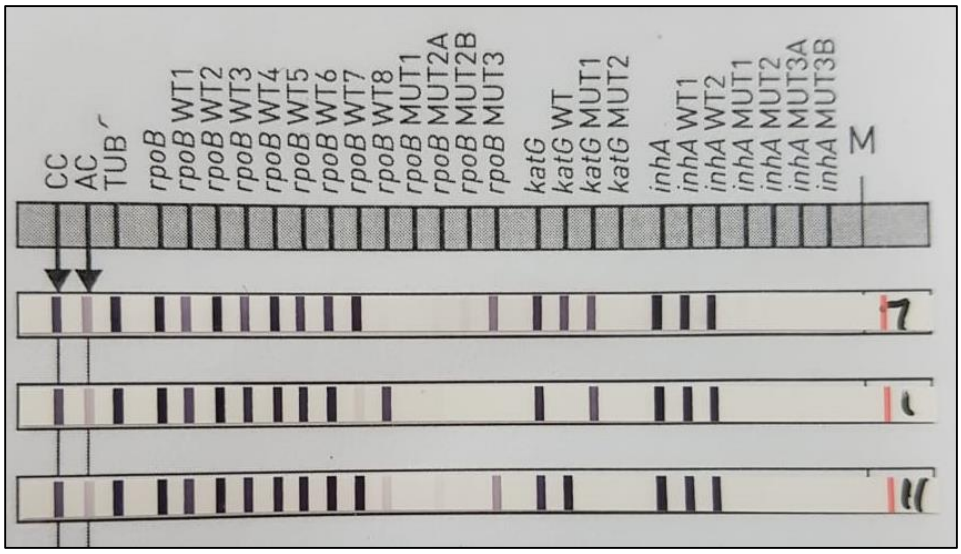


Figure 4. Example of MTBDRplus line probe strips for manual interpretation. The bands on the strip are aligned with the wild type (WT) and mutation (MUT) bands for rpoB, katG and inhA on the evaluation sheet.

The performance characteristics for the GXP and LPA are displayed in Table 3.

Table 3. Performance characteristics of molecular diagnostics endorsed by the WHO currently in use in South Africa.

Method	Sensitivity for detecting MTBC		Sensitivity for detecting MTBC in HIV infected patients	Sensitivity for detecting rifampicin resistance	Specificity for detecting rifampicin resistance	Reference
	Smear positive specimens	Smear negative specimens				
Xpert MTB/RIF assay	98%	67%	79%	95%	98%	(36)
MTBDR*plus* v2.0	92%	57-74%	75%	97%	99%	(17) (18) (37,38)

1.5 WHO recommendations for TB diagnostic testing

The WHO recommends that the GXP be used as the initial diagnostic test, in place of smear microscopy and phenotypic culture and DST, for the diagnosis of TB and RIF resistance in the following scenarios (39):

1. All adults and children suspected of having pulmonary TB, especially those suspected of having MDR TB or living with HIV
2. All patients suspected of having TB meningitis
3. All patients suspected of having extra-pulmonary TB, where lymph node aspirate/biopsy or other tissue can be obtained from the patient

Given the high negative predictive value of the GXP (>98%), in both high- and low-prevalence settings of RIF resistance, the WHO recommends that no further testing be done to confirm the RIF susceptibility in cases where the RIF result is susceptible (39).

The positive predictive value of the GXP in predicting RIF resistance is greater than 90% in settings in which the RIFr-TB exceeds 15%. In settings with a lower prevalence of RIFr-TB, like SA, the WHO recommends doing a risk assessment of the individual

patient to decide on further management. Patients previously treated for TB have a greater likelihood of developing RIFr/MDR TB (39). Options for confirming a RIFr GXP result include repeating the GXP, or performing further DST such as culture based DST or LPA. Although the WHO recommends this approach, it also advises that national programmes design an appropriate diagnostic algorithm, taking into account their country's epidemiology, other available technologies, as well as financial and human resources.

1.6 South African TB diagnostic algorithm

South Africa's current diagnostic algorithm for TB diagnosis employs the GXP as the initial screening test for all individuals with symptoms of TB, thus replacing smear microscopy (40) (Figure 5). This is in keeping with the WHO recommendation (41). Patients are commenced on an MDR-TB treatment regimen if RIF resistance is detected by GXP. As per the TB diagnostic algorithm in SA, a second specimen is subsequently obtained from the patient, and submitted for testing using the LPA, to confirm the GXP result. This step differs from the WHO recommendation to repeat the GXP. LPA technology and expertise was available in SA prior to the introduction of the GXP. It also provides essential pharmaco-genetic information on INH. The LPA detect mutations in the *katG* gene, which is associated with high levels of INH resistance, and mutations in the *inhA* gene, which is associated with low level INH resistance as well as cross resistance to ethionamide.

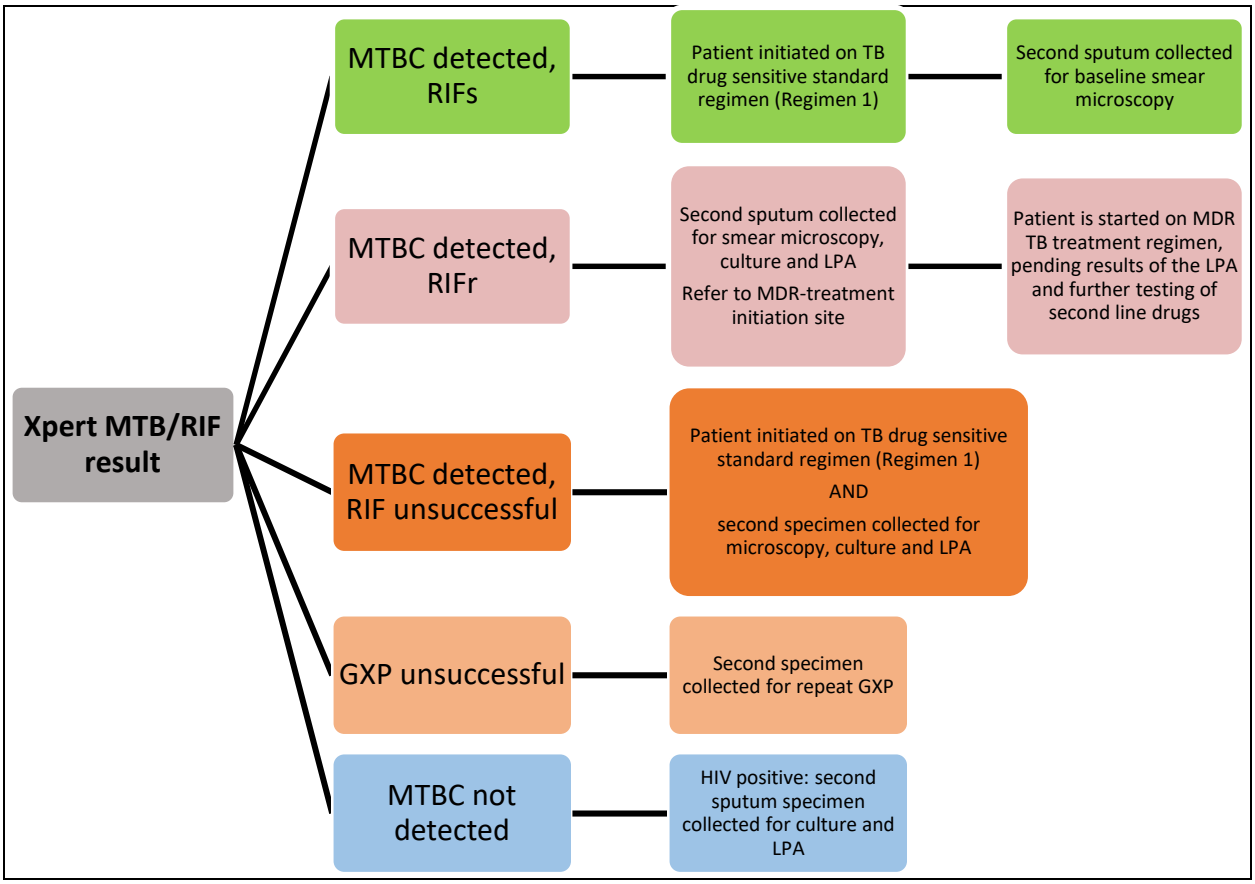


Figure 5. South African Xpert MTB/RIF assay algorithm for the diagnosis of TB and RIF susceptibility (40). Patients with a GXP RIF resistant result are referred for MDR-TB treatment initiation. A second sputum specimen is collected for smear microscopy and culture, and MTBDRplus (LPA) is performed for the confirmation of the RIF resistance and additionally, to obtain INH susceptibility. Further second-line drug susceptibility is performed for the fluoroquinolones e.g., ofloxacin, and second-line injectable drugs e.g., kanamycin. RIFs = rifampicin sensitive, RIFr = rifampicin resistant.

1.7 Rifampicin mutations detected by LPA and GXP

Both the LPA and the GXP technology make it possible to ascertain in which *rpoB* codon region the RIF mutation has occurred, as previously explained (Figure 2). This is important to know for various reasons. The performance of the LPA and GXP may vary across geographic areas, due to the circulation of different strains of MTBC (42). It is therefore important to know the local epidemiology of RIF resistant strains. Different types of mutations are also associated with different fitness costs, with differing susceptibilities to anti-TB agents within the same class.

The mutations D516V (*rpoB* MUT1) and L533P, remain susceptible to rifabutin while being resistant to RIF, and mutations S531L (*rpoB* MUT3) and H526Y (*rpoB* MUT2A) are resistant to both drugs (43). This means that certain patients with MDR TB might benefit by including rifabutin in the MDR TB regimen.

1.8 RIF mutations detected by LPA and GXP globally

The most common rifampicin mutation detected globally is in the 531 codon region (S531L). This is followed by mutations in the 516 codon (44). A study from Cape Town and a study from a mining community in Johannesburg, SA, had similar findings (45). However, a recent study in a Port Elizabeth population had contrasting results. The most common *rpoB* mutation found was in the 516 codon (56.5%), with only 17.4% occurring in the 531 codon (46). This highlights the importance of knowing the local epidemiology and its potential impact on assay performance. A study from Swaziland reported that GXP missed up to 30% of circulating strains of MDR TB in the region due to mutation I572F not being detected (42). This mutation, which falls outside of the RRDR and also was missed by the liquid-based culture DST, will only be detected by the sequencing of the *rpoB* gene. The L533P mutation was missed by the GXP in 14 specimens in a study conducted in India, even after cartridge updates to version G4 (47). The L533P mutation could not be detected by GXP in the initial analytical studies in specimens containing both RIF susceptible and RIF resistant populations (48). The L533P mutation is associated with low-level RIF resistance, which is missed by liquid-based phenotypic DST (MGIT 960 system)(49).

1.9 Discordant LPA and GXP RIF susceptibility results

Discordant GXP and LPA RIF susceptibility results pose a major management dilemma for clinicians, and are not an uncommon finding (34,50,51). There are currently no guidelines on the management of patients with discordant molecular RIF susceptibility results. Second-line treatment for drug resistant TB is expensive, lengthy and may have adverse side effects. An ineffective TB-treatment regimen may augment resistance and compromise infection control. It is therefore crucial to send a second specimen for

confirmation of the GXP RIF resistant result, as indicated in the SA TB GXP diagnostic algorithm (Figure 4).

There is currently limited data on the concordance rate between the GXP and LPA RIF susceptibility results obtained for routine patient management from South African patients (52). Only three other international studies have compared the concordance of the RIF susceptibility result between GXP and LPA (Table 4) (47,53,54). This data will inform local policy makers on whether the current assays and diagnostic algorithm are appropriate for South African patients, and might help inform future improvements of these assays.

Table 4. Studies comparing the RIF concordance between MTBDRplus and Xpert MTB/RIF assay. Discordances between the two assays are often explained by heteroresistance (presence of both a sensitive and a resistant strain). This is a known limitation of the Xpert MTB/RIF assay (52,55)

Country	**Specimens (n)**	**Concordance between MTBDR*plus* and Xpert MTB/RIF for detection of rifampicin resistance**	**Comparison of resistance profile in terms of probe characterization between MTBDR*plus* and Xpert MTB/RIF**	**Possible reasons for discrepancy**	**Reference**
India	Rifampicin mono-resistant sputum specimens (62)	64.4%	100% concordance for Probe A-D 52% concordance for Probe E	L533P mutation not detected by GXP, heteroresistance / mixed infection	(47)
	Rifampicin susceptible sputum specimens (83)	94.8%			
Bangladesh	Any rifampicin resistant sputum specimens(92)	92.4%	91.3%	Heteroresistance / mixed infection	(53)
Zimbabwe	MTBDR*plus* from cultured isolates. Xpert MTB/RIF from stored sputum specimens(39)	92.3%	Not done		(54)
South Africa	Cultured isolates (115) MDR, rifampicin monoresistant, rifampicin susceptible	99.0%	Not done	Heteroresistance / mixed infection	(52)

2. AIM OF STUDY

The aim of this study was to describe the frequency of the different rifampicin mutation patterns as detected by the Xpert MTB/RIF and MTBDR*plus* V2 across four districts in Gauteng. The rifampicin susceptibility result was compared between both assays together with the molecular hybridisation codon regions. Possible reasons for discordance were explored, as well as the implications for the national TB diagnostic algorithm.

3. STUDY OBJECTIVES

3.1 Distribution of rifampicin mutation patterns by district in the greater Gauteng, as identified by Xpert MTB/RIF and MTBDR*plus* V2.0.

3.2 Compare Xpert MTB/RIF and MTBDR*plus* v2.0 in terms of RIF resistance profile.

3.3 Determining improvements in laboratory services for patient care.

4. METHODS

4.1 Study design

This was a retrospective laboratory-based study that included all LPA results performed at the NHLS Braamfontein Mycobacteriology Referral Laboratory for patients from four districts in Gauteng: City of Johannesburg, West Rand, Ekurhuleni and Sedibeng. The study analysed data from a one-year period (01 September 2014 to 31 August 2015). LPA results from patients from the City of Tshwane were not included as these are tested at the Tshwane Academic NHLS laboratory. The patient's GXP RIF susceptibility result, which was collected and performed within four months of the LPA result, was manually linked with the LPA result via the laboratory information system (LIS). The four-month period was an arbitrary selection to maximise the linkage of GXP and LPA results and factor in delays in the collection of the second specimen for additional testing. Only valid results (susceptible or resistant) were selected to be included in the study. The GXP testing was performed at various NHLS laboratories in Gauteng.

LPA are done at centralised laboratories (such as NHLS Braamfontein Mycobacteriology Laboratory) since Biosafety Level III laboratory facilities are required for culture. GXP are tested at the nearest NHLS testing laboratory, since a GXP does not require specialised laboratory infrastructure.

4.2 MTBDR*plus*

All LPA performed at NHLS Braamfontein Mycobacteriology Referral Laboratory from 1st September 2014 until 31st August 2015 were considered for inclusion. This included tests performed directly on clinical specimens and those from positive cultures. Results were retrieved via the Corporate Data Warehouse (CDW), which was presented in an EXCEL spreadsheet.

All LPA in which MTBC was identified and an interpretable RIF result (susceptible or resistant) was obtained, were included in the analysis. Uninterpretable RIF results were excluded. Duplicate patient results were manually excluded (Figure 6).

4.3 Xpert MTB/RIF

The GXP result was manually linked with the LPA result (for the same patient) by searching the LIS using the patient's first name, surname and date of birth. When performing the search, use of the "soundex" function allows for cross-linking of possible misspelled names and surnames (single character errors), further maximising linkages of patient results. This, however, did not include all misspellings, which involve errors in more than one character. The possibility exists that two different patients may have exactly the same name, surname and date-of-birth for which LPA and GXP would have been linked in error. The GXP RIF susceptibility result was included if the specimen collection date was within four months of the LPA specimen collection date. All GXP results where MTBC was identified and an interpretable RIF result was obtained (susceptible or resistant) were included. GXP RIF unsuccessful results were excluded. The GXP RIF result, together with the probe hybridisation details, was then entered

onto the EXCEL spreadsheet (Figure 6). These GXP tests were performed in NHLS laboratories across Gauteng.

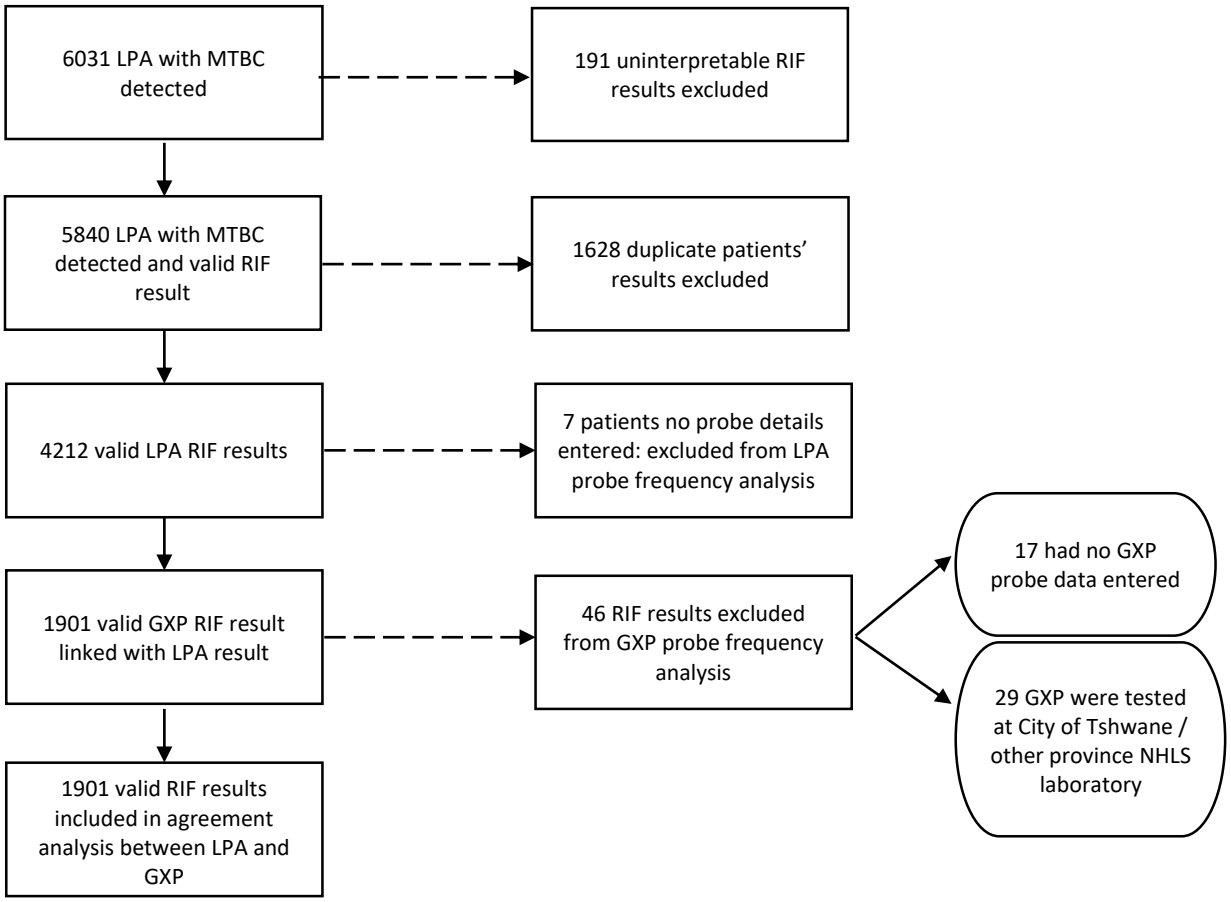


Figure 6. Study design and selection process for LPA and GXP RIF results to be included in the analysis.

5. RESULTS

5.1 MTBDR*plus* rifampicin results

In the final analysis for the LPA, 4212 valid LPA results were included. There were 1538 RIFr (rifampicin resistant) and 2674 RIFs (rifampicin susceptible) results.

Of the 1538 RIFr LPA, 877 (57%) were also INHr (isoniazid resistant), 655 (42.59%) INHs (isoniazid susceptible), and 6 (0.39%) were INH uninterpretable.

Of 1538 RIFr LPA, 1474 (95.84%) had a ΔWT, with or without a MUT band present. The remainder (57/1538) revealed heteroresistance, indicating that all WT bands were present, but with one or more MUT band detected (Figure 7, Table 5)

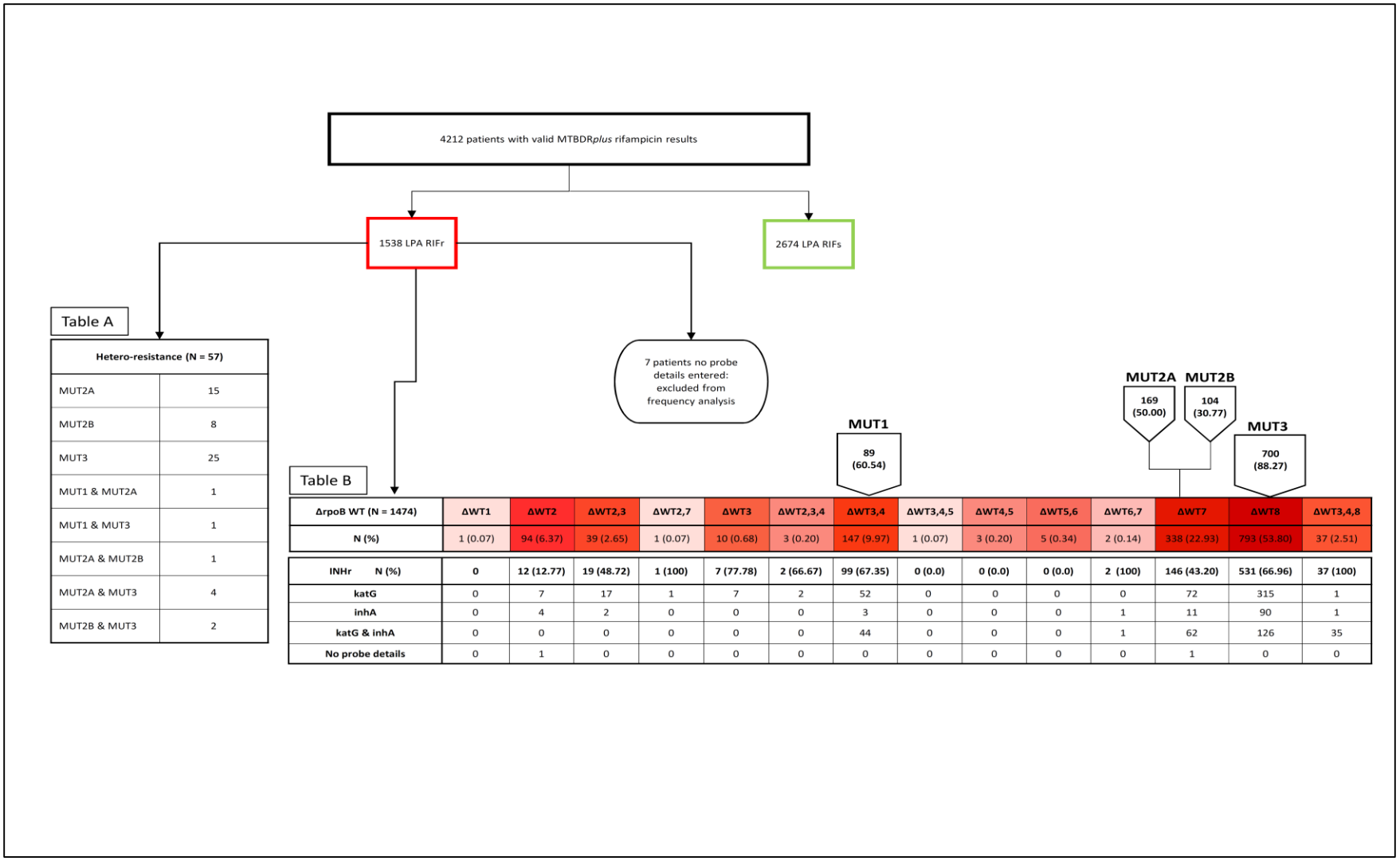


Table A

Hetero-resistance (N = 57)	
MUT2A	15
MUT2B	8
MUT3	25
MUT1 & MUT2A	1
MUT1 & MUT3	1
MUT2A & MUT2B	1
MUT2A & MUT3	4
MUT2B & MUT3	2

Table B

ΔrpoB WT (N = 1474)	ΔWT1	ΔWT2	ΔWT2,3	ΔWT2,7	ΔWT3	ΔWT2,3,4	ΔWT3,4	ΔWT3,4,5	ΔWT4,5	ΔWT5,6	ΔWT6,7	ΔWT7	ΔWT8	ΔWT3,4,8
N (%)	1 (0.07)	94 (6.37)	39 (2.65)	1 (0.07)	10 (0.68)	3 (0.20)	147 (9.97)	1 (0.07)	3 (0.20)	5 (0.34)	2 (0.14)	338 (22.93)	793 (53.80)	37 (2.51)
INHr N (%)	0	12 (12.77)	19 (48.72)	1 (100)	7 (77.78)	2 (66.67)	99 (67.35)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100)	146 (43.20)	531 (66.96)	37 (100)
katG	0	7	17	1	7	2	52	0	0	0	0	72	315	1
inhA	0	4	2	0	0	0	3	0	0	0	1	11	90	1
katG & inhA	0	0	0	0	0	0	44	0	0	0	1	62	126	35
No probe details	0	1	0	0	0	0	0	0	0	0	0	1	0	0

Figure 7. Valid MTBDRplus RIF results. The missing (Δ) WT probe frequencies are shown in colours of increasing intensity, the lowest frequency is light pink to highest frequency (dark red). The frequency of INHr corresponds to ΔrpoBWT is indicated with katG and/or inhA mutations involved. Heteroresistant RIFr results and the rpoBMUT probes involved are shown in tables A and B.

5.1.1 MTBDR*plus* frequency of rpoB wild type (ΔWT) and mutation (MUT) probes

The *rpoB*WT probe hybridisation pattern revealed that ΔWT8 was the most frequent, representing a mutation in codon region 530-533 (n=793, 53.80%) (Figure 7). The MUT3 probe hybridised in 700 of these, indicating the S531L mutation. This was followed by ΔWT7 (n=338, 22.93%), with the mutations H526Y (ΔWT7, MUT2A) and H526D (ΔWT7, MUT2B) present in 169 and 104 tests, respectively. The third commonest was ΔWT3, 4 (n=147, 9.97%), with the MUT1 probe present in 89 of these (Table 5), followed by ΔWT2 (n=94, 6.37%).

Table 5. Number and frequencies of MTBDRplus ΔrpoBWT probes and MUT probes. Heteroresistant RIFr (57) excluded.

WT probe(s) (representative codon region)	Number ΔWT N (%)	Number MUT1 present - D516V mutation (%)	Number MUT2A present - H526Y mutation (%)	Number MUT2B present - H526D mutation (%)	Number MUT3 present - S531L mutation (%)
WT1 (506-509)	1 (0.07)	0	0	0	0
WT2 (510-513)	94 (6.37)	0	0	0	0
WT2,3 (510-517)	39 (2.65)	0	0	0	0
WT2,7 (510-513; 526-529)	1 (0.07)	0	0	0	0
WT3 (513-517)	10 (0.68)	0	0	0	0
WT2,3,4 (510-519)	3 (0.20)	0	0	0	0
WT3,4 (513-519)	147 (9.97)	89 (60.54)	0	0	0
WT3,4,5 (513-522)	1 (0.07)	0	0	0	0
WT4,5 (516-522)	3 (0.20)	0	0	0	0
WT5,6 (518-525)	5 (0.34)	0	0	0	0
WT6,7 (521-529)	2 (0.14)	0	0	0	0
WT7 (526-529)	338 (22.93)	0	169 (50)	104 (30.77)	0
WT8 (530-533)	793 (53.80)	0	0	0	700 (88.27)
WT3,4,8 (513-519; 530-533)	37 (2.51)	0	0	0	0
Total	**1474**				

MTBDRplus frequency of ΔrpoBWT per district and subdistrict in Gauteng.

District: Gauteng province is divided into five municipalities, which comprise City of Johannesburg, City of Tshwane, Ekurhuleni, Sedibeng and West Rand (Figure 8). City of Tshwane was excluded from the analysis, as previously mentioned.

Subdistrict: City of Johannesburg is further divided into Regions A, B, C, D, E, F, G. Ekurhuleni is divided into North, East and South. Sedibeng consists of Emfuleni, Lesedi and Midvaal. West Rand comprises Merafong City, Mogale City and Rand West City (Randfontein and Westonaria) (Figure 8).

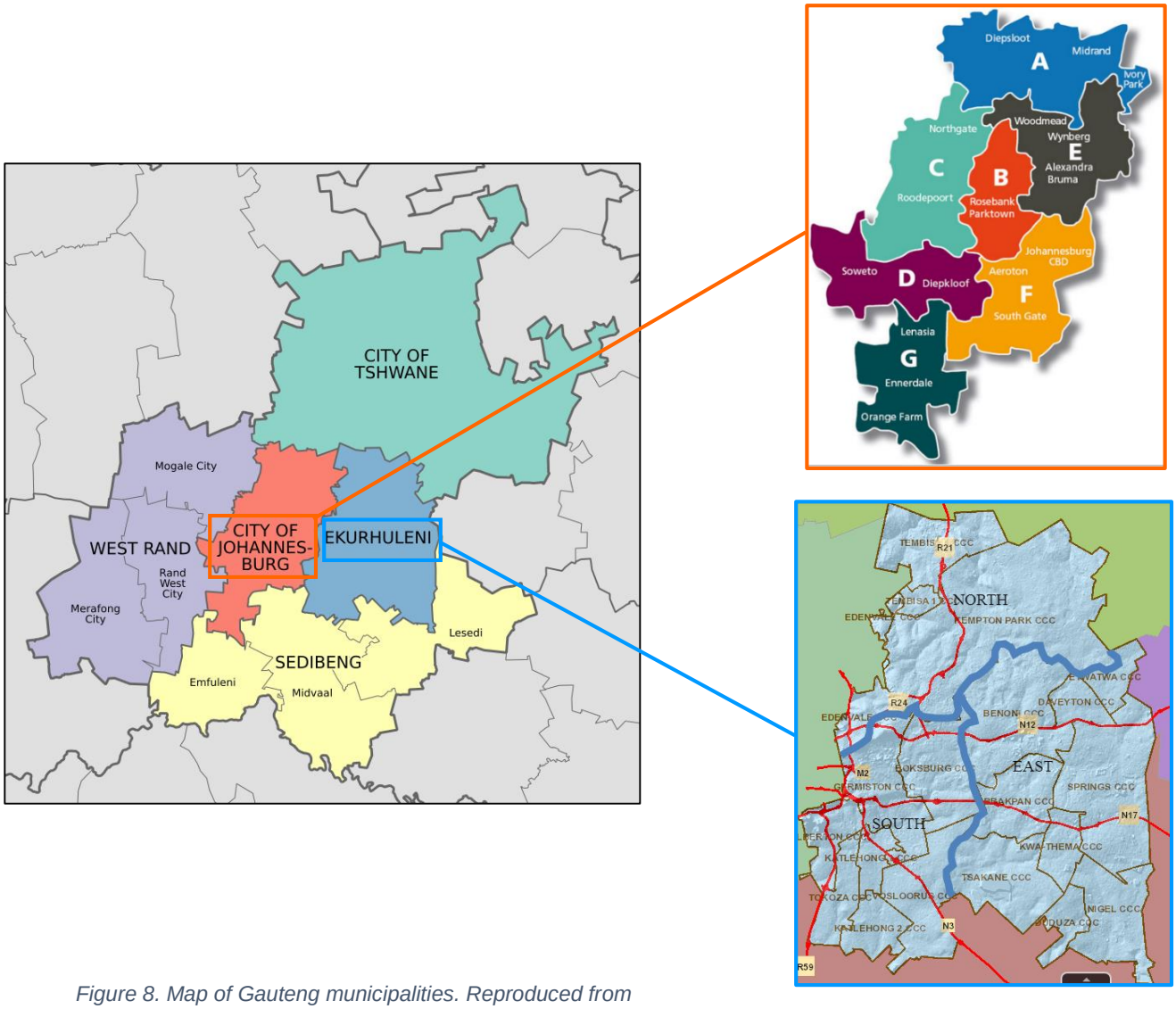


Figure 8. Map of Gauteng municipalities. Reproduced from https://en.wikipedia.org/wiki/List_of_municipalities_in_Gauteng, https://www.jra.org.za/jra-info/regional-map, https://gis.ekurhuleni.gov.za/mapviewer/.

Gauteng district:

From the 1474 patients with RIFr LPA, 871 (59.09%) were from the City of Johannesburg, 333 (22.59%) from Ekurhuleni, 142 (9.63%) from Sedibeng and 128 (8.68%) from the West Rand (Table 6). When analysing by subdistricts, it was clear that the City of Johannesburg Region E and F carried the biggest burden of RIFr TB disease with 305 (20.69%) and 307 (20.83%) patients, respectively. This was followed by Ekurhuleni East with 144 (9.77%) patients (Table 7).

Table 6. Number of MTBDRplus ΔrpoBWT per district.

District	ΔWT1/ N (%)	ΔWT2/ N (%)	ΔWT2,3 /N (%)	ΔWT2,7/ N (%)	ΔWT3 /N (%)	ΔWT2,3,4/ N (%)	ΔWT3,4/ N (%)	ΔWT3,4,5/ N (%)	ΔWT4,5 /N (%)	ΔWT5,6 /N (%)	ΔWT6,7 /N (%)	ΔWT7 /N (%)	ΔWT8/ N (%)	ΔWT3,4,8/ N (%)	Total/ N (%)
Johannesburg	0 (0)	49 (3.32)	18 (1.22)	1 (0.07)	9 (0.61)	3 (0.20)	99 (6.72)	1 (0.07)	3 (0.20)	3 (0.20)	1 (0.07)	219 (14.86)	440 (29.85)	25 (1.70)	**871 (59.09)**
Ekurhuleni	1 (0.07)	33 (2.24)	9 (0.61)	0 (0)	1 (0.07)	0 (0)	28 (1.90)	0 (0)	0 (0)	2 (0.14)	0 (0)	69 (4.68)	181 (12.28)	9 (0.61)	**333 (22.59)**
Sedibeng	0 (0)	8 (0.54)	10 (0.68)	0 (0)	0 (0)	0 (0)	11 (0.75)	0 (0)	0 (0)	0 (0)	0 (0)	22 (1.49)	90 (6.11)	1 (0.07)	**142 (9.63)**
West Rand	0 (0)	4 (0.27)	2 (0.14)	0 (0)	0 (0)	0 (0)	9 (0.61)	0 (0)	0 (0)	0 (0)	1 (0.07)	28 (1.90)	82 (5.56)	2 (0.14)	**128 (8.68)**
Total	**1 (0.07)**	**94 (6.38)**	**39 (2.65)**	**1 (0.07)**	**10 (0.68)**	**3 (0.20)**	**147 (9.98)**	**1 (0.07)**	**3 (0.20)**	**5 (0.34)**	**2 (0.14)**	**338 (22.93)**	**793 (53.80)**	**37 (2.51)**	**1474**

Gauteng Subdistricts:

Table 7. Number of MTBDRplus ΔrpoBWT per subdistrict.

Sub District	ΔWT1 /N (%)	ΔWT2/ N (%)	ΔWT2,3 /N (%)	ΔWT2,7/ N (%)	ΔWT3 /N (%)	ΔWT2,3,4 /N (%)	ΔWT3,4 /N (%)	ΔWT3,4,5/ N (%)	ΔWT4,5 /N (%)	ΔWT5,6 /N (%)	ΔWT6,7 /N (%)	ΔWT7 /N (%)	ΔWT8 /N (%)	ΔWT3,4,8 /N (%)	TOTAL /N (%)
Johannesburg A	0 (0)	3 (0.20)	0 (0)	1 (0.07)	0 (0)	0 (0)	0 (0)	1 (0.07)	0 (0)	0 (0)	0 (0)	11 (0.75)	7 (0.47)	0 (0)	**23 (1.56)**
Johannesburg B	0 (0)	5 (0.34)	2 (0.14)	0 (0)	0 (0)	0 (0)	10 (0.68)	0 (0)	1 (0.07)	1 (0.07)	0 (0)	39 (2.65)	39 (2.65)	1 (0.07)	**98 (6.65)**
Johannesburg C	0 (0)	0 (0)	1 (0.07)	0 (0)	0 (0)	0 (0)	2 (0.14)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.07)	4 (0.27)	0 (0)	**8 (0.54)**
Johannesburg D	0 (0)	9 (0.61)	2 (0.14)	0 (0)	2 (0.14)	1 (0.07)	14 (0.95)	0 (0)	1 (0.07)	0 (0)	0 (0)	30 (2.04)	52 (3.53)	4 (0.27)	**115 (7.80)**
Johannesburg E	0 (0)	8 (0.54)	6 (0.41)	0 (0)	4 (0.27)	0 (0)	42 (2.85)	0 (0)	1 (0.07)	0 (0)	0 (0)	59 (4.00)	170 (11.53)	15 (1.02)	**305 (20.69)**
Johannesburg F	0 (0)	22 (1.49)	5 (0.34)	0 (0)	3 (0.20)	2 (0.14)	29 (1.97)	0 (0)	0 (0)	2 (0.14)	1 (0.07)	77 (5.22)	161 (10.92)	5 (0.34)	**307 (20.83)**
Johannesburg G	0 (0)	2 (0.14)	2 (0.14)	0 (0)	0 (0)	0 (0)	2 (0.14)	0 (0)	0 (0)	0 (0)	0 (0)	2 (0.14)	7 (0.47)	0 (0)	**15 (1.02)**
Ekurhuleni East	1 (0.07)	17 (1.15)	5 (0.34)	0 (0)	0 (0)	0 (0)	13 (0.88)	0 (0)	0 (0)	1 (0.07)	0 (0)	32 (2.17)	74 (5.02)	1 (0.07)	**144 (9.77)**
Ekurhuleni North	0 (0)	3 (0.20)	0 (0)	0 (0)	0 (0)	0 (0)	9 (0.61)	0 (0)	0 (0)	0 (0)	0 (0)	15 (1.02)	40 (2.71)	0 (0)	**67 (4.55)**
Ekurhuleni South	0 (0)	13 (0.88)	4 (0.27)	0 (0)	1 (0.07)	0 (0)	6 (0.41)	0 (0)	0 (0)	1 (0.07)	0 (0)	22 (1.49)	67 (4.55)	8 (0.54)	**122 (8.28)**
Sedibeng - Emfuleni	0 (0)	8 (0.54)	10 (0.65)	0 (0)	0 (0)	0 (0)	9 (0.61)	0 (0)	0 (0)	0 (0)	0 (0)	17 (1.15)	75 (5.09)	1 (0.07)	**120 (8.14)**
Sedibeng - Lesedi	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (0.14)	0 (0)	0 (0)	0 (0)	0 (0)	3 (0.20)	12 (0.81)	0 (0)	**17 (1.15)**
Sedibeng - Midvaal	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (0.14)	3 (0.20)	0 (0)	**5 (0.34)**
West Rand - Merafong City	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (0.27)	0 (0)	0 (0)	0 (0)	0 (0)	9 (0.61)	23 (1.56)	0 (0)	**36 (2.44)**
West Rand - Mogale City	0 (0)	3 (0.20)	1 (0.07)	0 (0)	0 (0)	0 (0)	1 (0.07)	0 (0)	0 (0)	0 (0)	1 (0.07)	11 (0.75)	43 (2.92)	2 (0.14)	**62 (4.21)**
West Rand - Randfontein	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (0.20)	4 (0.27)	0 (0)	**7 (0.47)**
West Rand - Westonaria	0 (0)	1 (0.07)	1 (0.07)	0 (0)	0 (0)	0 (0)	4 (0.27)	0 (0)	0 (0)	0 (0)	0 (0)	5 (0.34)	12 (0.81)	0 (0)	**23 (1.56)**
Total	**1 (0.07)**	**94 (6.38)**	**39 (2.65)**	**1 (0.07)**	**10 (0.68)**	**3 (0.20)**	**147 (9.97)**	**1 (0.07)**	**3 (0.20)**	**5 (0.34)**	**2 (0.14)**	**338 (22.93)**	**793 (53.80)**	**37 (2.51)**	**1474**

City of Johannesburg:

There were two particular differences noted in the number of Δ*rpob*WT for City of Johannesburg: Johannesburg Region E had 15 (1.02%) ΔWT3,4,8, which was higher than other subdistricts in Gauteng. Johannesburg Region F had 22 (1.49%) ΔWT2, of which 18 (81.82%) were RIFr INHs by LPA.

Ekurhuleni:

In Ekurhuleni East and South, ΔWT2 was the third most common ΔWT seen (n=30), whereas it was the fourth most common ΔWT seen in other districts of Gauteng. The INH was susceptible by LPA in 27 (90%) of these. There were 8 (0.54%) ΔWT3,4,8 in Ekurhuleni South (Table 7).

Sedibeng:

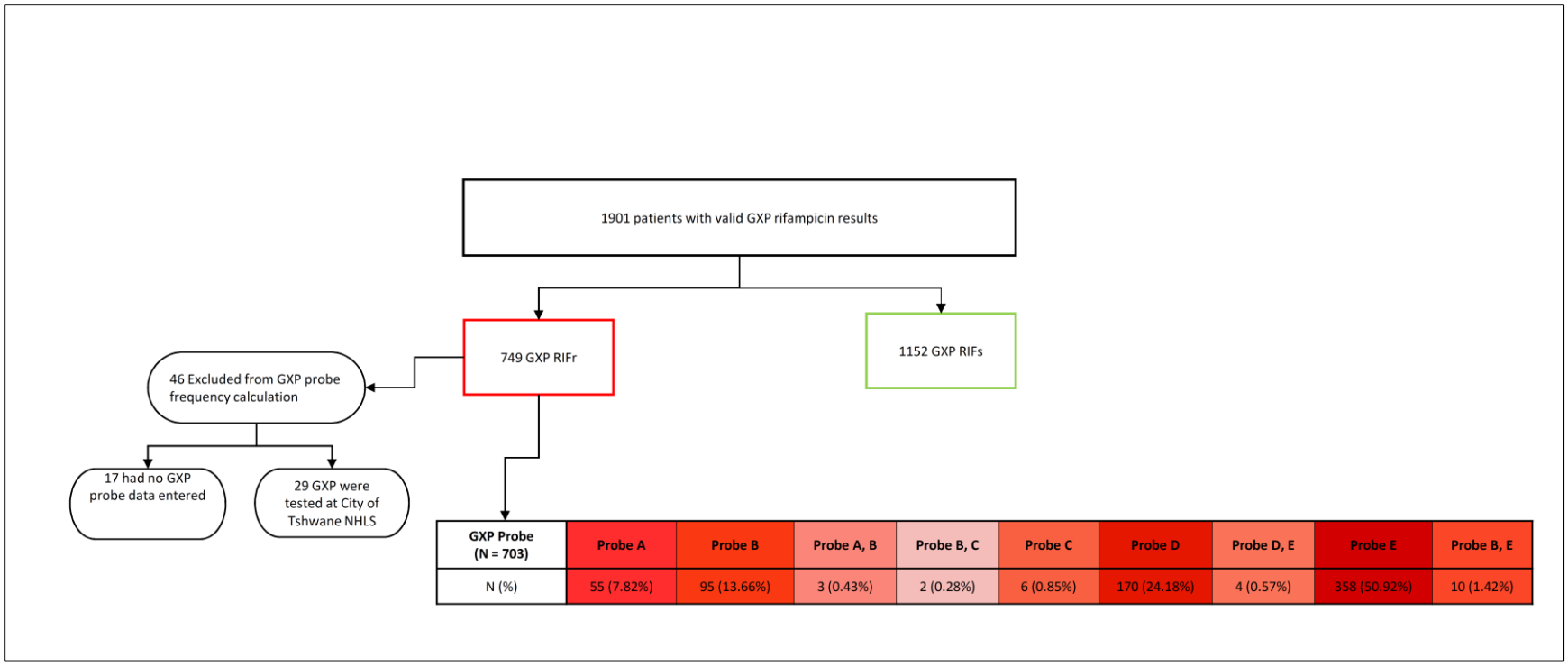
In the Emfuleni district of Sedibeng, ΔWT2,3 (n=10, 0.65%) was the third most common ΔWT seen (Table 7). This is 7.04% of the total number of RIFr (n=142) seen in Sedibeng (Table 6).

West Rand:

The West Rand reflected the overall Gauteng number of ΔWT, with ΔWT8 the most common (n=82, 5.56%), followed by ΔWT7 (n=28, 1.90%), ΔWT3,4 (n=9, 0.61%) and then ΔWT2 (n=4, 0.27%) (Table 6).

5.2 GXP rifampicin results

A total of 1901 valid GXP RIF results were successfully linked with the LPA result. Of these, 749 (39.40%) were RIFr and 1152 (60.60%) were RIFs. A total of 17 RIFr GXP had no probe details entered on the LIS, and 29 RIFr GXP were from City of Tshwane; so these were excluded from the probe frequency analysis (Figure 9).



GXP Probe (N = 703)	Probe A	Probe B	Probe A, B	Probe B, C	Probe C	Probe D	Probe D, E	Probe E	Probe B, E
N (%)	55 (7.82%)	95 (13.66%)	3 (0.43%)	2 (0.28%)	6 (0.85%)	170 (24.18%)	4 (0.57%)	358 (50.92%)	10 (1.42%)

Figure 9. Valid GXP RIF results. The GXP probe frequencies are shown in colours of increasing intensity, the lowest frequency is light pink to highest frequency (dark red)

5.2.1 GXP *rpoB* probe frequencies

Probe E was the most common probe involved in GXP RIFr (n=358, 50.92%). This was followed by Probe D (n=170, 24.18%) and Probe B (n=95, 13.66%) (Table 8).

Table 8. Number and frequencies of GXP RIFr probes.

GXP probe (representative codon region)	Number GXP (%)
A (507-511)	55 (7.82)
B (512-518)	95 (13.66)
A and B (507-518)	3 (0.43)
B and C (512-523)	2 (0.28)
C (518-523)	6 (0.85)
D (523-529)	170 (24.18)
D and E (523-533)	4 (0.57)
E (529-533)	358 (50.92)
B and E (512-518; 529-533)	10 (1.42)
Total	**703 (100)**

GXP rpoB probe frequencies per district and subdistrict

District:

Table 9. Number of GXP rpoB probes per district in Gauteng.

District	A N (%)	B N (%)	A and B N (%)	B and C N (%)	C N (%)	D N (%)	D and E N (%)	E N (%)	B and E N (%)	Total N (%)
Johannesburg	32 (4.55)	49 (6.97)	3 (0.43)	1 (0.14)	5 (0.71)	92 (13.09)	2 (0.28)	151 (21.48)	6 (0.85)	335 (47.65)
Ekurhuleni	16 (2.28)	23 (3.27)	0 (0)	0 (0)	1 (0.14)	37 (5.26)	1 (0.14)	102 (14.51)	3 (0.43)	180 (25.60)
Sedibeng	5 (0.71)	12 (1.71)	0 (0)	1 (0.14)	0 (0)	19 (2.70)	1 (0.14)	60 (8.53)	0 (0)	98 (13.94)
West Rand	2 (0.28)	11 (1.56)	0 (0)	0 (0)	0 (0)	22 (3.13)	0 (0)	45 (6.40)	1 (0.14)	80 (11.38)
Total	**55 (7.82)**	**95 (13.51)**	**3 (0.43)**	**2 (0.28)**	**6 (0.85)**	**170 (24.18)**	**4 (0.57)**	**358 (50.92)**	**10 (1.42)**	**703**

From the 703 (36.98%) patients with GXP RIF resistant results included in the probe frequency analysis, 335 (47.65%) were from the City of Johannesburg, 180 (25.60%) from Ekurhuleni, 98 (13.94%) from Sedibeng and 80 (11.38%) from the West Rand (Table 9).

The distribution between the subdistricts is, however, different from that of LPA. City of Johannesburg Region D carries the biggest burden (n=124, 17.64%), followed by Sedibeng – Emfuleni (n=82, 11.66%), Johannesburg Region F (n=77, 10.95%), Ekurhuleni East (n=73, 10.38%) and Ekurhuleni South (n=68, 9.67%) (Table 10).

Subdistricts:

The differences or the presence of a particularly rare probe (or combination of probes) in the subdistrict, compared to the average numbers of GXP probes involved in GXP RIFr for Gauteng, is highlighted.

Table 10. Frequency of GXP probe per subdistrict in Gauteng

Sub District	A N (%)	B N (%)	A and B N (%)	B and C N (%)	C N (%)	D N (%)	D and E N (%)	E N (%)	B and E N (%)	Total N (%)
Johannesburg A	3 (0.43)	0 (0)	0 (0)	1 (0.14)	0 (0)	4 (0.57)	0 (0)	10 (1.42)	0 (0)	**18 (2.56)**
Johannesburg B	4 (0.57)	6 (0.85)	0 (0)	0 (0)	1 (0.14)	22 (3.13)	0 (0)	19 (2.70)	0 (0)	**52 (7.40)**
Johannesburg C	0 (0)	1 (0.14)	0 (0)	0 (0)	0 (0)	2 (0.28)	0 (0)	7 (1.00)	0 (0)	**10 (1.42)**
Johannesburg D	11 (1.56)	24 (3.41)	0 (0)	0 (0)	2 (0.28)	32 (4.55)	1 (0.14)	52 (7.40)	2 (0.28)	**124 (17.64)**
Johannesburg E	2 (0.28)	4 (0.57)	0 (0)	0 (0)	1 (0.14)	6 (0.85)	0 (0)	16 (2.28)	1 (0.14)	**30 (4.27)**
Johannesburg F	8 (1.14)	9 (1.28)	3 (0.43)	0 (0)	1 (0.14)	17 (2.42)	1 (0.14)	35 (4.98)	3 (0.43)	**77 (10.95)**
Johannesburg G	4 (0.57)	5 (0.71)	0 (0)	0 (0)	0 (0)	9 (1.28)	0 (0)	12 (1.71)	0 (0)	**30 (4.27)**
Ekurhuleni East	7 (1.00)	8 (1.14)	0 (0)	0 (0)	1 (0.14)	16 (2.28)	0 (0)	41 (5.83)	0 (0)	**73 (10.38)**
Ekurhuleni North	2 (0.28)	4 (0.57)	0 (0)	0 (0)	0 (0)	12 (1.71)	0 (0)	24 (3.41)	0 (0)	**42 (5.97)**
Ekurhuleni South	7 (1.00)	11 (1.56)	0 (0)	0 (0)	0 (0)	9 (1.28)	1 (0.14)	37 (5.26)	3 (0.43)	**68 (9.67)**
Sedibeng - Emfuleni	5 (0.71)	10 (1.42)	0 (0)	1 (0.14)	0 (0)	16 (2.28)	1 (0.14)	49 (6.97)	0 (0)	**82 (11.66)**
Sedibeng - Lesedi	0 (0)	2 (0.28)	0 (0)	0 (0)	0 (0)	3 (0.43)	0 (0)	9 (1.28)	0 (0)	**14 (1.99)**
Sedibeng - Midvaal	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (0.28)	0 (0)	**2 (0.28)**
West Rand - Merafong City	0 (0)	2 (0.28)	0 (0)	0 (0)	0 (0)	8 (1.14)	0 (0)	12 (1.71)	1 (0.14)	**23 (3.27)**
West Rand - Mogale City	2 (0.28)	4 (0.57)	0 (0)	0 (0)	0 (0)	6 (0.85)	0 (0)	21 (2.99)	0 (0.14)	**33 (4.69)**
West Rand - Randfontein	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (0.57)	0 (0)	3 (0.43)	0 (0.14)	**7 (1.00)**
West Rand - Westonaria	0 (0)	5 (0.71)	0 (0)	0 (0)	0 (0)	4 (0.57)	0 (0)	9 (1.28)	0 (0.14)	**18 (2.56)**
Total	**55 (7.82)**	**95 (13.51)**	**3 (0.43)**	**2 (0.28)**	**6 (0.85)**	**170 (24.18)**	**4 (0.57)**	**358 (50.92)**	**10 (1.42)**	**703**

City of Johannesburg:

The uncommon Probe C, of which only 6 (0.85%) were detected in Gauteng, was found in 5 patients (83.33%) from the City of Johannesburg (Table 9). The uncommon combination of Probe B and E was seen in 6 patients (60%) from this district (there were only 10 in Gauteng). In Region B, Probe D (n=22, 3.13%) was more commonly involved in GXP RIFr than Probe E (n=19, 2.70%) (Table 10).

Ekurhuleni:

Probe B (n=11, 1.56%) was more common than Probe D (n=9,1.28) in Ekurhuleni South. The combination of B and E was seen in three patients from Ekurhuleni South (Table 10).

Sedibeng:

Probe E was very common in Sedibeng – Emfuleni, with 60% of all GXP RIFr patients in this subdistrict (n= 82) showing Probe E involved in RIFr (Table 10).

West Rand:

In Westonaria, Probe B (n=5, 0.71%) was more common than Probe D (n=4, 0.57%), although the numbers were small (Table 10).

5.3 Agreement between MTBDR*plus* and GXP rifampicin result

The number of patients included in the agreement analysis was 1901.

The agreement between the LPA and the GXP RIF result was 96.42% (kappa = 0.9245, z score = 40.34).

A total of 698 (36.72%) patients had both an LPA and GXP RIFr susceptibility result (Figure 10). Of these 698 patients, 688 (98.57%) had probe details entered for both GXP and LPA for the molecular codon region comparison. The agreement between the LPA and the GXP molecular region was 99.27% (n=683/688).

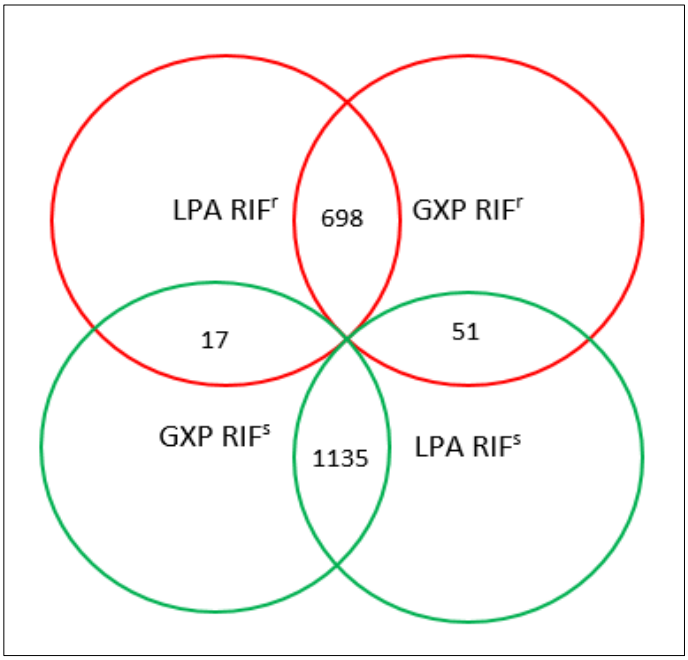


Figure 10. Comparison between rifampicin susceptibility results for GXP and LPA. Red circles indicate rifampicin resistant results, green circles indicate rifampicin sensitive results. Different colour circles overlapping indicate discordant results. RIF = rifampicin, RIFr = rifampicin resistant, RIFs = rifampicin sensitive.

There were 68 (3.58%) discordant RIF susceptibility results. This included 17 LPA RIFr but GXP RIFs (25%), and 51 LPA RIFs but GXP RIFr (75%) (Figure 10). The

discordance was mostly found in *rpoB* codon region 529-533. This is represented by the WT8 region for the LPA (n=10, 58.82%) and probe E for the GXP (n=19, 41.30%) (Figure 11).

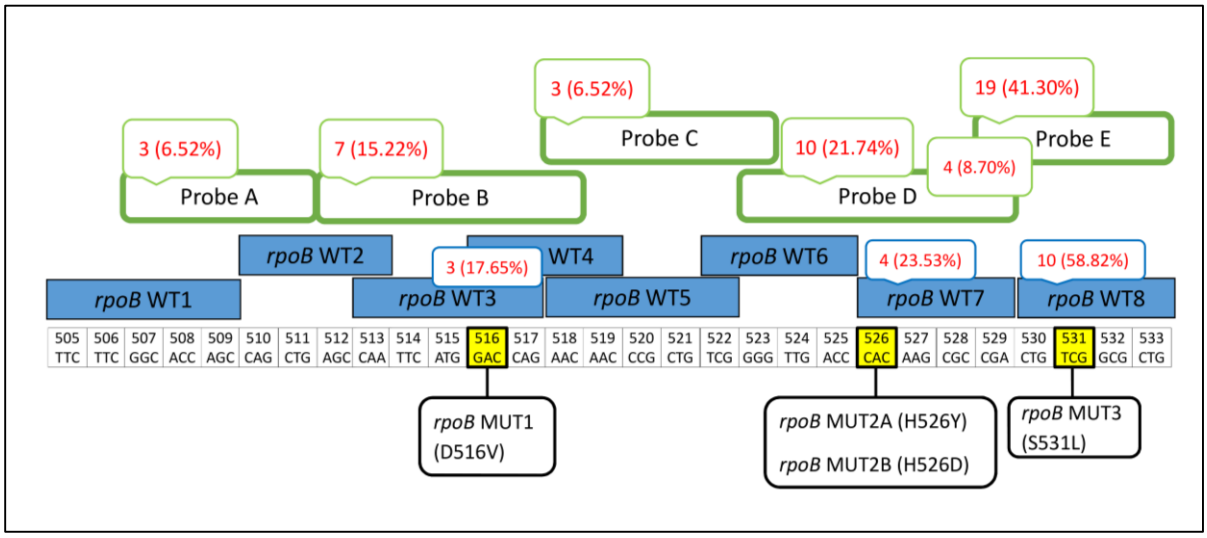


Figure 11. RRDR with the frequencies of the discordant GXP (green boxes) and MTBDRplus (blue boxes) indicated, according to the probe region it was detected in. For GXP there were four that involved both Probe D and E, and for MTBDRplus there were three that involved both WT3 and WT4 regions.

The 68 discordant RIF susceptibility results are summarized in Table 11. The LPA were performed from a total of 50 cultured specimens and the rest (n=18) were performed on clinical specimens.

Table 11. Characteristics of discordant rifampicin susceptibility results. LPA were performed either directly from the clinical specimen or from the culture. Smear quantification reported using the WHO/IUATLD reporting scale.

Patient number	Date of MTBDR*plus* specimen	MTBDR*plus* rifampicin result (probe)	Specimen/Culture		Date of GXP specimen	GXP rifampicin result (probe)	GXP semi-quantitative result	GXP probe Drop Out (DO) /Delay (ΔCT>4) (DE)	ΔCT value
			Specimen – Smear quantity	Culture – TTP (d)					
1	11-08-2014	R (ΔWT8)		11	07-04-2014	S			
2	27-08-2014	R (ΔWT8, MUT3)	1+		18-07-2014	S			
3	02-09-2014	R (ΔWT7, MUT2A)		13	18-08-2014	S			
6	22-09-2014	R (MUT2A)		7	17-09-2014	S			
7	36-09-2014	R (ΔWT8, MUT3)		26	23-06-2014	S			
8	30-09-2014	R (WT8 Equivocal)	scanty		08-05-2014	S			
12	17-10-2014	R (ΔWT8)		21	28-07-2014	S			
17	28-10-2014	R (ΔWT3,4)		9	23-01-2015	S			
32	09-01-2015	R (ΔWT8 MUT3)		2	12-09-2014	S			
38	04-02-2015	R (ΔWT3,4)		32	18-02-2015	S			
41	13-02-2015	R (MUT2B)		14	14-02-2015	S			
53	11-05-2015	R (ΔWT3,4)		14	16-04-2015	S			
54	14-05-2015	R (MUT3)	1+		13-02-2015	S			
56	25-05-2015	R (ΔWT8, MUT3)		15	30-04-2015	S			
61	11-06-2015	R (ΔWT8, MUT3)		9	04-06-2015	S			
65	15-07-2015	R (MUT2A)		16	07-07-2015	S			
66	17-07-2015	R (MUT3)	1+		01-04-2015	S			
29	12-12-2014	S		21	14-10-2014	R (A)	low	DO	
39	05-02-2015	S	3+		03-02-2015	R (A)	medium	DO	
40	10-02-2015	S		16	04-02-2015	R (A)	Very low	DE	4.4
5	16-09-2014	S		11	19-08-2014	R (B)	medium	DE	4.5
10	02-10-2014	S	3+		30-09-2014	R (B)	high	DE	4.1
35	26-01-2015	S		8	21-01-2015	R (B)	high	DE	4.3
46	31-03-2015	S	3+		29-03-2015	R (B)	high	DE	5.4

47	01-04-2015	S		0	13-02-2015	R (B)	high	DO	
52	20-04-2015	S		23	09-04-2015	R (B)	Very low	DE	11.5
60	10-06-2015	S		8	03-06-2015	R (B)	high	DO	
22	13-11-2014	S		14	03-11-2014	R (C)	Very low	DO	
37	02-02-2015	S		15	21-01-2015	R (C)	Very low	DE	4.8
50	15-04-2015	S		41	08-04-2015	R (C)	Very low	DO	
15	27-10-2014	S	2+		23-10-2014	R (D)	low	DE	10.9
16	28-10-2014	S		11	23-10-2014	R (D)	low	DE	6.1
25	26-11-2014	S		9	22-11-2014	R (D)	low	DE	5
30	17-12-2014	S		6	09-12-2014	R (D)	medium	DE	4.7
49	15-04-2015	S		14	07-04-2015	R (D)	medium	DE	4.7
51	15-04-2015	S		12	23-02-2015	R (D)	Very low	DE	7.1
55	15-05-2015	S		11	12-05-2015	R (D)	Very low	DO	
57	27-05-2015	S		38	05-05-2015	R (D)	high	DO	
63	30-06-2015	S		20	26-06-2015	R (D)	Not entered	DO	
6	25-09-2014	S	1+		16-05-2014	R (D)	medium	DO	
4	12-09-2014	S		5	04-09-2014	R (E)	high	DO	
9	02-10-2014	S		15	26-09-2014	R (E)	Very low	DE	8.5
11	06-10-2014	S		24	11-09-2014	R (E)	Very low	DE	5.6
13	21-10-2014	S		22	14-10-2014	R (E)	Very low	DE	4.1
14	24-10-2014	S		14	21-10-2014	R (E)	Very low	DE	4.4
21	12-11-2014	S	scanty		06-11-2014	R (E)	Very low	DO	
23	21-11-2014	S		12	18-12-2014	R (E)	Very low	DO	
24	26-11-2014	S		11	21-11-2014	R (E)	Very low	DE	4.1
33	14-01-2015	S		13	07-01-2015	R (E)	low	DO	
34	23-01-2015	S		16	07-01-2015	R (E)	Very low	DO	
42	23-02-2015	S		10	26-01-2015	R (E)	Very low	DE	5.6
44	10-03-2015	S		36	17-12-2014	R (E)	low	DO	
45	10-03-2015	S		18	06-01-2015	R (E)	low	DO	
48	09-04-2015	S		6	02-04-2015	R (E)	Very low	DO	

58	02-06-2015	S		32	26-05-2015	R (E)	Very low	DE	4.7
59	04-06-2015	S		12	02-06-2015	R (E)	Very low	DE	4.1
62	30-06-2015	S		18	09-06-2015	R (E)	Very low	DE	7.1
64	07-07-2015	S	2+		26-06-2015	R (E)	high	DE	4.2
67	22-07-2015	S		21	25-06-2015	R (E)	Very low	DE	4.3
18	29-10-2014	S	3+		22-10-2014	R (D,E)	medium	DO	
28	09-12-2014	S		6	05-12-2014	R (D,E)	medium	DO	
36	28-01-2015	S	3+		13-01-2015	R (D,E)	medium	DO	
43	27-02-2015	S	2+		22-10-2014	R (D,E)	Very low	DO	
19	31-10-2014	S	1+		22-10-2014	R, no values	medium		
20	06-11-2014	S	1+		29-10-2014	R, no values	Very low		
26	27-11-2014	S		13	26-11-2014	R, no values	medium		
27	05-12-2014	S	1+		02-12-2014	R, no values	Not entered		
31	08-01-2015	S	2+		06-01-2015	R, no values	high		

R=resistant; S=susceptible; d = days; TTP=time to positivity; ΔCT= difference between maximum and minimum probe CT value.

All TB RIF susceptibility results for patients with discordant RIF results were reviewed by searching the LIS to explore possible reasons for discordance (Table 12,13).

Table 12. Discordant rifampicin results: MTBDRplus RIF resistant, GXP RIF sensitive and possible explanation for discordance. n = 17

MTBDR*plus* rifampicin resistant, GXP sensitive	**Time between MTBDR*plus* and GXP specimen collection**	**Review of all rifampicin susceptibility results**	**Possible explanation**
Patient 1	4 months	Repeat LPA = R	New TB episode
Patient 3, 6, 41, 54, 65 and 66	N/A	Both WT and MUT bands detected by LPA.	Heteroresistance / mixed infection
Patient 56	25d	Repeat LPA and GXP also discordant RIF results	
Patient 2	39d	Repeat GXP = R	Possible false GXP sensitive / mixed infection
Patient 8	4 months	WT8 equivocal on LPA (smear was scanty), previous LPA = S	Possible false LPA resistant (poor quality strip due to insufficient DNA) OR new TB episode
Patient 17	3 months	LPA and GXP = R performed two months before LPA	Possible false GXP sensitive OR new TB episode
Patient 32	4 months	GXP = R performed three months after LPA	New TB episode
Patient 38	14d	LPA = S done two months before	Possible false LPA resistant
Patient 7, 12, 53, 61		No other results found	

R = Rifampicin Resistant; S = Rifampicin Susceptible

Table 13. Discordant rifampicin results: MTBDRplus RIF sensitive, GXP RIF resistant and possible explanation for discordance. n = 50.

MTBDR*plus* sensitive, GXP resistant	**Time between MTBDR*plus* and GXP specimen collection**	**Review of all rifampicin susceptibility results**	**Possible explanation**
Patient 4, 5 , 11, 13, 14, 21, 23, 24, 29, 33, 34, 35, 44, 48, 50, 51, 52, 55, 57, 58, 59, 60, 63, 67		No other results found.	
Patient 9	6d	Repeat LPA = R	Possible LPA false sensitive
Patient 64	11d	Repeat LPA repeated thrice and GXP = R	
Patient 10	2d	Repeat LPA and GXP = S performed in same month	Possible false GXP resistant
Patient 15	4d	Repeat LPA twice = S	
Patient 16	5d	Repeat LPA = S	
Patient 18	7d	Repeat LPA twice = S	
Patient 22	10d	Repeat GXP = S	
Patient 19	9d	No values entered for GXP probes. Repeat LPA = S.	Possible transcription error when entering GXP result into LIS.
Patient 20	7d	No values entered for GXP probes. No other results found.	
Patient 26	1d	No values entered for GXP probes. Repeat GXP = S.	
Patient 27	3d	No values entered for GXP probes. No other results found.	
Patient 31	2d	No values entered for GXP probes. Repeat GXP = S.	
Patient 25	4d	GXP = S performed 5 months before	Mixed infection
Patient 28	4d	Two GXP probes did not hybridise (D,E). Repeat GXP = S.	Possible false GXP resistant
Patient 36	15d	Two GXP probes did not hybridise (D,E). Repeat LPA = S.	
Patient 43	4 months	Two GXP probes did not hybridise (D,E). Repeat GXP and LPA = S.	
Patient 30	8d	Repeat GXP = S	
Patient 37	12d	Repeat GXP = S	
Patient 40	6d	Repeat GXP = S	
Patient 42	28d	Repeat GXP = S	
Patient 49	8d	All repeat LPA thrice and GXP = S	
Patient 39	2d	Repeat LPA twice = R (ΔWT2)	Possible ΔWT2 missed on LPA.
Patient 45	2 months	All repeat LPA and GXP = R	Laboratory contamination event: Report was amended to “Inconclusive”
Patient 46	2 d	Repeat LPA and GXP also discordant results.	Heteroresistance / mixed infection.
Patient 47	2 months	LPA = S performed same day	New infection / Possible false GXP resistant
Patient 62	21 d	Repeat LPA = S	

R = Rifampicin Resistant; S = Rifampicin Susceptible; d = days

5.4 Comparison of GXP probe details for the concordant versus discordant GXP RIF resistant

The number of GXP RIFr that were discordant with the LPA RIF, detected by delayed (DE) binding of the probe, were higher than GXP RIFr that was concordant with the LPA RIF result (47.06% versus 6.59%) ($p < 0.001$). Most (58.33%) of the ΔCT values of the discordant GXP RIFr were between 4.1-4.9, compared to concordant GXP RIFr (15.56%). The median ΔCT values were also much lower for the discordant GXP RIFr (4.7 versus 7). The involvement of multiple probes was more frequent in the discordant GXP RIFr (8.89% versus 2.20%) ($p = 0.055$) (Table 14).

Table 14. Comparison of discordant GXP RIFr versus concordant GXP RIFr results. Only 45 from the 51 discordant GXP RIFr and 683 from the 698 concordant GXP RIFr had probe details entered on LIS.

GXP	45 discordant GXP RIF resistance (%)	683 concordant GXP RIF resistance (%)	p value*
RIF resistance by delayed hybridisation (ΔCT>4)	24 (47.06%)	45 (6.59%)	p<0.001
ΔCT value 4.1-4.9	14 (58.33%)	7 (15.56%)	p = 0.910
Median ΔCT value	4.7	7	p = 0.333
Multiple probes involved	4 (8.89%) (Probe D & E)	15 (2.20%)	p = 0.055

***Differences between concordant and discordant GXP RIF were calculated using the chi squared test for categorical variables and the Wilcoxon rank-sum test for continuous variables. P values are two-sided, p<0.05 considered statistically significant.

6. DISCUSSION

6.1 Frequencies of *rpoB* mutations detected by LPA and GXP

The performance of LPA and GXP may vary in different geographic areas, due to differences in circulating MTBC strains (42). The development of new diagnostic tools, new drug targets, or regimens depends on the availability of information regarding resistance conferring mutations and their frequency in different geographical areas (56). It is therefore important to know the local epidemiology of RIF resistant strains.

In this study, 53.80% of LPA RIFr occurred in the *rpoB* codon region 530-533 (ΔWT8), with 88.27% having the S531L (MUT3) mutation (ΔWT8, MUT3). This mutation has also been reported as the most common in studies performed in SA, as well as other countries (44,45). This was followed by 22.93% of LPA RIFr detected in codon region 526-529 (ΔWT7), with mutation H526Y (ΔWT7, MUT2A) present in 50% and H526D (ΔWT7, MUT2B) present in 30.77% of these. By LPA, the third most common mutated codon region was 513-517 (ΔWT3,4) (9.97%) with mutation D516V (ΔWT3,4, MUT1) present in 60.54% of these (Figure 7, Table 5). These results are in agreement with other studies in different geographic areas worldwide (45).

The most common GXP probe involved in RIFr was probe E (50.92%), followed by probe D (24.18%), and probe B (13.66%). This coincides with the results for LPA RIFr as these probes correspond to the regions covered by WT8, WT7 and WT2, WT3,4 respectively (Figure 2).

6.2 Frequencies of *rpoB* mutations detected by LPA and GXP per district in Gauteng

Although Gauteng province is the smallest province in terms of land area in SA, it has the largest population with 12 272 263 residents. Over four million of these (4 434 827) reside in the City of Johannesburg. This is followed by 3 178 470 in Ekurhuleni, 916 484 in Sedibeng, and 820 995 in West Rand (57). The City of Johannesburg had the most RIFr patients by LPA (n=871, 59.05%) and GXP (n=335, 47.65%) followed by Ekurhuleni LPA (n=333, 22.59%) and GXP (n=180, 25.60%).

The *rpoB* codon region 510-513 (WT2) seems to be rarely involved in RIF resistance in other studies (<3%) (42,56). An *rpoB* mutation detected by LPA ΔWT2 was present in 6.37% in this study (the fourth most common ΔWT), of which 87.23% were INHs by LPA. The overlapping GXP probe A was involved in 7.82% of rifampicin resistance. This was predominantly observed for LPA results obtained in Johannesburg Region F (ΔWT2 n=22), Ekurhuleni East (n=17) and South (n=13). It may indicate a cluster of a

specific strain of MTBC with ongoing transmission in these geographic areas. Although the number of ΔWT2 did not change during the one-year period, ongoing surveillance of the frequency of ΔWT2 is necessary to see if this changes over time.

An interesting mutation pattern, involving both *rpoB* codon region 513-519 and 530-533 (ΔWT3,4,8) was present in 2.51% of patients (n=37). The LPA INH susceptibility result was resistant in all of these. The LPA ΔWT3,4,8 were mainly from the City of Johannesburg (n=25) and Ekurhuleni South (n=8). Fourteen were patients admitted to Sizwe hospital, which is a referral center for complicated MDR TB and XDR TB patients in Gauteng. The second-line testing was available for 22 of these patients, all of whom were resistant to both second line drugs (fluoroquinolones and second-line injectable agents). There were ten patients with GXP RIFr involving both probe B and E, which corresponds with the LPA ΔWT3,4,8 codon regions. These were also mainly from the City of Johannesburg (n=6) and Ekurhuleni (n=3). Nine patients had both LPA ΔWT3,4,8 and GXP Probe B, E. This may indicate an XDR TB strain that is recognisable by this type of LPA and GXP probe pattern and an opportunity to intervene with infection prevention and control practices.

6.3 Heteroresistance / Mixed infections

Heteroresistance for RIF was detected in 57 patients by LPA (Figure 6). Heteroresistance or mixed infection may occur in >50% of patients in hyper endemic settings, and is highly underestimated. It is currently not standard practice to include in the laboratory report the presence of RIFr heteroresistance as detected by the LPA. It should be reported, as the treatment regimen of the patient may be adapted to include rifampicin (2). The GXP is not able to report the presence of heteroresistance or mixed infection. The sensitivity of the GXP for detecting RIFr is also lower when a patient has multiple clones present in the specimen, due to the proportion of mutant DNA that is required for detecting resistance (60-99%). This also depends on the type of *rpoB* mutation present in the particular specimen (48), and is further complicated by the pressure of the antimicrobial regimen that the patient may already have initiated (58).

6.4 Agreement between LPA and GXP molecular *rpoB* codon regions

The 99.27% agreement between the molecular codon region for LPA and GXP RIFr susceptibility results reaffirm the South African algorithm whereby GXP RIFr patients have a second specimen submitted for LPA. This is also in accordance with the WHO guidelines to confirm GXP RIFr susceptibility results in areas with a <15% prevalence of RIFr TB, with another GXP, DST or LPA (39).

6.5 Agreement between LPA and GXP RIF susceptibility results

The overall concordance between all RIF susceptibility results for the two molecular assays was 96.42%. This is similar to other studies from Asia and Southern Africa (47,52–54). There were 68 discordant RIF susceptibility results from the 1901 LPA and GXP that could be matched (3.58%) (Table 12).

The 68 discordant RIF susceptibility results consisted of 17 LPA RIFr and GXP RIFs, and 51 LPA RIFs and GXP RIFr (Table 11). This likely reflects the GXP RIFr diagnostic algorithm, for only GXP RIFr had a follow up specimen sent for a confirmatory LPA, and not GXP RIFs. A Cape Town study found similar results with the majority of discordance due to GXP RIFr and LPA RIFs results (51).

The discordant results were mostly from LPA performed from cultured isolates (n=50/68). The strain composition may be altered in the cultured specimen from what was present in the initial clinical specimen, for various reasons. One of the strains may be underrepresented in the clinical specimen or the growth rate may vary between the different strains, so that one may outgrow the other and go undetected. The susceptibility to decontamination procedures may differ between the strains or the differences in the tendency to clump may eventually alter the culture composition. Therefore the culture might not reflect the clonal complexity that was present in the

original clinical specimen (58,59). These reasons have to be taken into consideration when assessing whether a patient might have a possible mixed infection or heteroresistance present in a specimen as a possible cause for the discordant results.

6.6 Exploring possibilities for discordant GXP and LPA RIF results

TB RIF susceptibility results for the patients with discordant RIF susceptibility results were reviewed by searching the LIS to explore possible reasons for discordance (Table 9, Table 10).

Although the lack of *rpoB* sequencing for the discordant specimens prevents definitive conclusions, the possible reasons for discordance are summarized in tables 12 and 13. The most common reasons identified were:

1. Mixed infection or heteroresistance.
2. Poor quality of the LPA strip which makes manual interpretation difficult, particularly when performed directly on the clinical specimen. The sensitivity of the LPA increases with the bacillary burden (60).
3. Possible false GXP RIFr result. Certain characteristics from the GXP RIFr probe analysis were identified.

    a) Discordant GXP RIFr by delayed hybridisation of the probe was detected in 47.06% versus 6.59% in concordant GXP RIFr.
    b) The majority (58.33%) of the ΔCT value was between 4.1-4.9 for the discordant GXP RIFr, with a median of 4.7 versus 7 for the concordant GXP RIFr (Table 14).
    c) Four discordant GXP RIFr susceptibility results were due to multiple probes not hybridising (D and E). Repeat LPA or GXP on all of these patients were RIF susceptible.
    d) From the above, only the discordant GXP RIFr by delayed hybridisation reached statistical significance.

Other authors have also analysed the characteristics of GXP probe binding to elucidate possible identifiable factors for discordant results. GXP RIFr by delayed hybridisation and a ΔCT value of 4-4.9 was associated with discordant GXP results (50,51).

e) Transcription errors. Three of the five patients with initial discordant GXP RIFr (RIFr was manually entered on the LIS without any probe details provided) had repeat LPA or GXP RIFs results. This may be prevented by ensuring good laboratory practice.

6.7 The INH susceptibility results

The INH was resistant in 57% of the RIFr by LPA. This varied according to the LPA WT region (Figure 6). The recent TB Drug Resistance Survey (2012-2014), performed by the National Institute for Communicable Diseases estimated that for every RIF mono-resistant TB case, there were 2.6 as many MDR TB cases in Gauteng (61). It also noted, with concern, an increase in rifampicin mono-resistant TB since the previous survey was done in 2001-2002.The ratio for this study was 1.33:1 RIFr INHr: RIFr INHs cases.

The rifampicin mono-resistance figure in this study might be slightly lower since the LPA misses around 10% of INH resistance, and the phenotypic- or culture-based DST for INH was not captured for this study. The INH genotypic susceptibility result is needed in the case of GXP RIFr for the second-line regimen. It is essential to know if there is a *katG* and/or *inhA* mutation in order to decide on the inclusion of INH and/or ethionamide in the treatment regimen.

6.8 Management of discordant RIF susceptibility results

There are currently no guidelines that provide a standardised approach to the troubleshooting of discordant GXP and LPA RIF susceptibility results. Clinicians are faced with a treatment dilemma in such situations. Patients with discordant results are likely to be initiated on sub-optimal treatment regimens. Troubleshooting and resolution

of discordant RIF results require timeous communication between the clinician and the clinical microbiologist.

The clinical microbiology laboratory may employ several methods as part of both the detection and troubleshooting of discordant GXP and LPA results. These include DNA sequencing of the *rpoB* gene, which will also detect mutations outside the RRDR, and detect mixed strains (62). Patients' previous TB susceptibility results should be reviewed and the presence of heteroresistance, when detected by the LPA, should be reported. Technical issues, such as poor quality of the LPA strip, may lead to errors in interpretation. False GXP RIFr secondary to delayed binding of the probe, ΔCT value < 5, and multiple probes failing, should also be considered as reasons for discordant results. Manual entering of GXP RIF susceptibility results on the LIS should be avoided, as transcription errors may occur.

Phenotypic DST may assist in cases of well described RIFr conferring mutations. This is especially true for the S531L mutation and some mutations found in codon 513 and 526, with more than 90% of resistance mutations detected by phenotypic methods found at these positions. Mutations at other positions may cause only low-level, but clinically relevant, rifampicin resistance, and may not be detected when testing with the automated MGIT 960 system at the critical concentration of 1µg/ml (49,63).

The clinician's responsibility when dealing with GXP and LPA discordance includes assessing the patient's clinical condition and response to therapy as well as the history of previous TB disease and previous results, to determine if the patient is at an increased risk of RIFr TB or mixed infections. Repeat specimens must be sent to the laboratory for repeat GXP and LPA.

6.9 Implications for TB diagnostic algorithm and patient care

The current South African TB diagnostic algorithm (Figure 4), whereby a GXP RIFr result is followed by submission of a second specimen and a confirmatory LPA, is justified. This is illustrated by the number of discordant RIF susceptibility results (n=68/1901, 3.58%) seen in this study. Knowledge of the INH susceptibility is also essential for the second-line regimen. The limitation is that GXP RIFs is not followed by a confirmatory LPA, so these discordances (GXP RIFs, LPA RIFr) will mostly be missed in the current algorithm (there were only 17 GXP RIFs, LPA RIFr seen in this study).

Costs currently limit the confirmation of all GXP RIF susceptibility results. This may need re-evaluation as the RIFs, INHr cases may go undetected in the current SA TB diagnostic algorithm. INH mono-resistant TB is associated with poor treatment outcomes (64). If the INHr is undiagnosed, the patient will be on monotherapy (RIF) during the continuation phase of standard therapy, which may augment further resistance. The INH mono-resistance rate has increased from 2.7% (2001-2002) to 4.9% (2012-2014) according to a recent South African TB drug resistance survey (61).

Only 1901 from the 4212 valid LPA RIF results could be matched with a valid GXP RIF result. Although this study was not designed to calculate the number of GXP RIFr that had a second specimen sent for LPA (GXP negative, unsuccessful and RIF inconclusive were excluded), the need for a unique identifier is critical for proper patient care and follow up. A decision to send two clinical specimens upfront (as is currently the practice in the Western Cape Province) will limit the difficulty in obtaining a second specimen when the GXP is RIFr. The LPA could then automatically be performed on the second specimen when the GXP is RIFr.

All laboratories that perform and interpret the LPA should review previous TB susceptibility results for each patient before authorising RIF results, in order to identify

and troubleshoot discordance. A comment should be made on the laboratory report in the case of discordance to guide further patient management.

All laboratories that perform GXP and TB LPA need to have access to standardized guidelines on how to troubleshoot discordant RIF susceptibility results. A guideline advising clinicians on the management of patients with discordant GXP and LPA RIF results is also critical to optimising patient outcomes.

South Africa has recently (2017) introduced the improved version of the GXP assay, called the Xpert MTB/RIF Ultra, with improved sensitivity for diagnosing TB (Table 15). The sensitivity for identifying RIF resistance has remained the same, but the reliability of detecting mutations conferring RIF resistance has been improved by employing post-PCR melting curve analysis (22).

Table 15. Xpert MTB/RIF compared to Xpert MTB/RIF Ultra in a recent eight country non-inferiority accuracy study.

Method	Sensitivity for detecting MTBC		Sensitivity for detecting MTBC in HIV infected patients	Sensitivity for detecting rifampicin resistance	Specificity for detecting rifampicin resistance	Reference
	Pooled	Smear negative specimens				
Xpert MTB/RIF assay	82.9%	44.5%	75.5%	95.5%	99.4%	(65)
Xpert MTB/RIF Ultra	87.8%	61.3%	87.8%	94.4%	99.7%	

The number of discordant GXP Ultra and LPA RIF results will have to be monitored post introduction, as well as whether the GXP Ultra is more robust in detecting RIF resistance, when compared to the GXP assay. A current drawback of reporting GXP Ultra results is that the LIS does not support the identification of the particular probe involved in GXP Ultra RIFr, which makes it difficult to compare the molecular codon region with that of the LPA codon region. This will have to be improved in future updates of the LIS software and the GXP software.

6.10 The future for TB diagnostic assays in South Africa

Molecular platforms that already exist in SA include the GeneXpert (Cepheid) and Abbott RealTime platforms. Promising molecular technologies for the diagnosis and identification of drug resistance include the GXP XDR cartridge (for second-line testing), Abbott m2000 RealTime MTB system (for RIF and INH susceptibility) and the Flourotype MTBDR (Hain Lifescience; for RIF and INH susceptibility). Two of these (Abbott m2000 RealTime MTB system and Flourotype MTBDR) are marked for WHO assessment in the near future (2018/19; Figure 1), and may solve the problems that are associated with the complexity of the MTBDR*plus*.

The urine lipoarabinomannan (LAM) is endorsed by the WHO for the diagnosis of TB in HIV infected individuals in two scenarios: those who are seriously ill or in those where TB is suspected and the CD4 <100 cells/ml (24). The urine LAM may be used in addition to GXP or on its own if the patient is unable to produce sputum. A specimen should still be obtained whenever possible for TB culture and subsequent drug susceptibility testing. The advantage of the urine LAM is point of care testing. It will supplement the current TB GXP diagnostic algorithm in the population mentioned before.

6.11 Study limitations

There are several limitations to this study.

1. There was no gold standard such as sequencing or phenotypic DST with which to compare discordant RIF susceptibility results.

2. Due to the non-use of a unique identifier, less than half of the LPA RIF results could be linked with a valid GXP RIF result, even after an extensive manual search by name, surname and date of birth. A unique identifier is necessary to improve on this shortcoming.

3. The GXP and LPA were performed on different specimens. This is the testing algorithm followed in SA (except for Western Cape province). These were the actual results on which the patient management was based (GXP RIFr is followed by obtaining a second specimen for LPA confirmation).

4. Findings are relevant to Gauteng province and although both assays are used extensively across SA, these results may not be representative across all geographic areas in SA.

7. CONCLUSION

The most common *rpoB* mutation detected by LPA and GXP in the four districts in Gauteng occurred in the 530-533 codon region, which is also the most common codon region involved in RIFr worldwide. LPA ΔWT2, which was mostly INHs, is more prevalent in Gauteng than in other parts of the world. The LPA ΔWT3,4,8 and GXP probe B,E is a probable XDR TB strain prevalent in Gauteng and shows value in investigating gene regions derived from these molecular assays.

The overall concordance between all RIF susceptibility results and for the molecular codon region for RIFr results was very good (96.42% and 99.27%). Over the one-year period there were 68 discordant RIF susceptibility results out of the 1901 matched LPA and GXP. The majority were GXP RIFr, LPA RIFs. Certain characteristics were identified, such as GXP RIFr detected with delayed probe hybridisation and ΔCT value < 5, multiple GXP probes not hybridising, mixed infection or heteroresistance and transcription errors. However, this data only reached statistical significance in discordant GXP RIFr detected by delayed hybridisation.

The troubleshooting of discordant RIF susceptibility results should involve both the clinician and the clinical microbiologist. This includes taking a proper history, performing a thorough clinical examination and obtaining more clinical specimens from the patient

for repeat GXP and LPA. The laboratory can assist with troubleshooting of discordant results by reporting heteroresistance when detected, performing phenotypic RIF DST, submitting the culture for *rpoB* sequencing and reviewing of results for possible GXP and LPA technical errors. The introduction of a unique LIS patient identifier is critical to identify discordant results and troubleshoot accordingly and highlights the importance of a LIS with a well-maintained central data warehouse. These are recommendations to tackle the problem of discordant GXP and LPA results in a high-burden TB setting.

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9. APPENDICES

Ethics clearance certificate