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A Descriptive Analysis of the Routine Use of GenoType MTBDR*sl* in a high HIV/TB Prevalent Region in South Africa

By

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Submitted in partial fulfilment of the requirements for the degree of Master of Medicine (Medical Microbiology) in the School of Laboratory Medicine and Medical Science, University of KwaZulu-Natal

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Declaration of Authorship

1. I, Dr Keeren Lutchminarain declare as follows:

That the work described in this dissertation has not been submitted to UKZN or any other institution for the purposes of an academic qualification, whether by myself or any other party.

2. That my contribution to the project is as follows:

I, Keeren Lutchminarain, did the full work on this project. This includes the literature review, protocol writing, ethics application, collection and interpretation of data, and writing of dissertation and manuscript.

I wrote the entire research protocol and dissertation with correctional assistance from my supervisors.

I solely completed the BREC application, submission, and queries.

3. That the contributions of others to the project are as follows:

My supervisor Dr Nomonde Mvelase and co-supervisor Dr Khine Swe Swe Han have both assisted with guidance on literature review, protocol layout, and corrections with the dissertation.

Miss Afsana Kajee has contributed to this project by assisting with collection of raw data.

Mr Partson Tinarwo has contributed to this project by assisting with data exploration and analysis.

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Co-Supervisor Signature: 

Date: 03 July 2020 **Amended Version: 01 Oct 2020**

Dedication:

To my son Yadav…

“Success is not final, failure is not fatal: it is the courage to continue that counts.”

Winston Churchill

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Table of contents

1. CHAPTER 1

1.1 Introduction

Tuberculosis (TB) remains the leading cause of death from a single infectious agent worldwide. In 2017, an estimated 10.0 million people developed TB disease of which two thirds belonged to eight countries namely India, China, Indonesia, the Philippines, Pakistan, Nigeria, Bangladesh and South Africa [1]. Furthermore, multidrug resistant TB (MDR-TB) was declared a global health crisis by the World Health Organization (WHO) and continues to pose a challenge. MDR-TB is defined as resistance to isoniazid (INH) and rifampicin with or without resistance to other anti-TB drugs [2]. An estimated 558,000 people developed TB resistant to rifampicin in 2017 of which approximately 82% had MDR-TB [1].

1.1.1 Defining the clinical problem

South Africa continues to feature on the WHO list of top 30 high TB burden countries. Additionally, it is one of the top 14 high burden countries with drug-resistant and HIV associated TB [1]. This high HIV/TB co-infection rate contributes to the disease burden of TB because HIV infected patients frequently present with smear negative TB or extrapulmonary TB, which is diagnostically challenging [3, 4].

In 2016, the WHO released a guideline recommending a shortened MDR-TB treatment regimen. When compared to the conventional long regimen this shortened regimen (SR) showed a successful patient outcome of 84% (95% CI 79-87) vs 62% (95% CI 53-70). These recommendations were based on observational studies from ten countries. The new recommendation states that in patients with rifampicin resistant-TB (RR-TB) or MDR-TB who were not previously treated with second-line drugs and in whom resistance to fluoroquinolones (FQ) and second-line injectable drugs (SLIDs) was excluded or considered highly unlikely, a shorter MDR-TB regimen of 9–12 months may be used instead of the longer regimens [5]. Rifampicin resistant TB is defined as resistance to at least rifampicin, with or without resistance to other drugs [2].

Given the high MDR-TB prevalence rate and its associated poor treatment outcomes in South Africa, in 2016, the South African National Department of health (NDoH) replaced the existing long conventional MDR-TB treatment regimen with the "Modified South African 9-12 Month Regimen". The modified regimen comprised the intensive (injectable) phase; kanamycin + moxifloxacin + clofazamine + ethionamide + pyrazinamide + high dose INH + ethambutol for 4-6 months followed by the continuation phase of moxifloxacin + clofazamine + pyrazinamide

+ ethambutol for a further 5 months. Only patients with RR-TB or MDR-TB (with one INH mutation only i.e. either *inhA* or *katG*) are eligible for this regimen. Therefore, to facilitate rapid triaging of patients on to the SR, there was a growing need to implement rapid molecular methods to identify resistance to FQ and SLIDs [6].

Alongside the SR recommendation, in 2016, the WHO also released a policy guidance on the use of molecular line probe assays (LPA) for the detection of resistance to second line antimycobacterial drugs. The policy states, for patients with confirmed RR-TB or MDR-TB, the GenoType MTBDR*sl* Version 2 (Hain Lifescience, Nehran, Germany) may be used as the initial test, instead of phenotypic culture based drug susceptibility testing (DST) to detect resistance to FQ and SLIDs. The GenoType MTBDR*sl* Version 2.0 is a qualitative test that offers rapid diagnosis of extensively drug resistant TB (XDR-TB) and pre-XDR-TB by detecting resistance to FQ and SLIDs on clinical specimens or cultured isolates [7]. Although the WHO has endorsed the use of this test for direct testing on smear positive and smear negative specimens, a systematic Cochrane review published by Theron *et al.* highlighted the paucity of data on the use of GenoType MTBDR*sl* Version 2.0 when used directly on smear negative specimens. Their analysis has emphasized the need for further evaluation of this assay in different geographic settings [8].

Considering both WHO guidelines (the use of molecular LPA and the SR), in 2017, NDoH implemented a nationwide revised laboratory-clinical algorithm termed Drug Resistant TB (DR-TB) Reflex testing in order to facilitate the rapid diagnosis and management of drug resistant TB in South Africa. In this algorithm, patients with newly diagnosed RR-TB by GeneXpert (GXP) (Cepheid, United States) were commenced on the SR, provided they did not have complicated extrapulmonary TB, and resistance FQ and SLIDs was unlikely. In all patients, a second sputum specimen was sent to the laboratory for DR-TB Reflex testing. DR-TB Reflex testing comprises a set of laboratory tests which include, Auramine smear microscopy, TB culture on Mycobacteria Growth Indicator Tube (MGIT) 960 system (BACTEC MGIT Becton Dickinson, USA), GenoType MTBDR*plus* (FL-LPA) (Hain Lifescience, Nehran, Germany), followed by GenoType MTBDR*sl* (SL-LPA) sequentially on the clinical specimen regardless of smear status [6]. Further DST was performed in selected isolates. Once, the results of both LPA's were available, the SR was either continued or adjusted accordingly in the respective patients.

Since the implementation of DR-TB Reflex testing in the province of KwaZulu-Natal (KZN) in January 2017, there have been no published reports on the experience of the routine use of

the SL-LPA on clinical specimens within South Africa nor in KZN specifically. In this region, smear negative TB remains a significant challenge due to the high rate of HIV associated TB (70%). The performance of diagnostic tests in programmatic settings is often inferior compared to that in controlled research settings. Given the high burden of drug resistant TB and HIV associated TB in KZN; this is an ideal region to assess the feasibility of introducing the SL-LPA as part of the DR-TB reflex algorithm. The objective is to evaluate the feasibility of the routine use of this test in a region were the prevalence of smear negative TB remains high, as well as characterize the various mutation patterns detected in this region. This will significantly contribute to our knowledge on the routine use of this assay within our population and inform further advances when designing molecular tests.

1.2 Critical Literature Review

1.2.1 Epidemiology of Tuberculosis

Despite the TB incidence rate falling at about 2% per year worldwide, according to the 2018 WHO Global TB Report, TB has still caused an estimated 1,3 million deaths globally [1]. Although TB is one of the oldest diseases known, it is still the leading cause of death by a single infectious agent and remains amongst the top 10 causes of death. In 2017, 10 million people developed active TB disease. Two thirds belonged to eight countries: India (27%), China (9%), Indonesia (8%), Philippines (6%), Pakistan (5%), Nigeria (4%), Bangladesh (4%) and South Africa (3%). South Africa remained in the top 30 high burden countries in 2018 with an estimated 500 new cases per 100 000 population. Despite WHO declaring MDR-TB a public health crisis, drug resistant TB still remains a significant challenge. In 2017, an estimated 558,000 people developed RR-TB of which approximately 82% had MDR-TB [1].

The NDoH conducted the most recent South African national drug resistant prevalence survey from 2012 – 2014. In this survey, published by Nazir *et al.*, the prevalence of MDR-TB was 2,1% (95% CI 1, 5–2,7) among new tuberculosis cases and 4,6% (3,2–6,0) among retreatment cases. Amongst MDR-TB patients, 4,9% XDR-TB and 16% had pre-XDR-TB. XDR-TB is defined as MDR-TB with resistance to any FQ as well as one or more of the three SLIDs (amikacin, kanamycin or capreomycin). Pre-XDR-TB is defined as MDR-TB with additional resistance to either a FQ or SLIDs. In this survey, prevalence of MDR-TB varied between all 9 provinces, however, KZN, had amongst the highest prevalence of patients with MDR-TB, showing an overall MDR estimate of 2,9% (95% CI 1,8-4,5) which is higher than the national average of 2,8 % [9].

Apart from the high burden of MDR-TB, KZN also has the highest burden of XDR-TB. In a study published by Kapwata *et al.*, 1027 new XDR TB cases were diagnosed throughout all 11 districts of KZN during 2011-2014. Using geospatial analysis, they observed that majority of XDR-TB was due to primary transmission [10].

1.2.2. Traditional diagnosis and susceptibility testing of TB

Historically, laboratory diagnosis of TB relied on microscopy using acid fast stains (e.g. Ziehl Neelsen) on sputum specimens to detect acid-fast bacilli (AFB). AFB are bacteria that have mycolic acids in their cell wall. These mycolic acids do not allow basic dyes e.g. crystal violet to penetrate the cell wall and therefore AFB cannot be easily viewed by the Gram staining technique. With the Auramine-O staining technique, the fluorochrome dye, Auramine-O, forms a complex with the mycolic acids in the cell wall of the organism and makes it resistant to decolourisation by acid-alcohol. The counterstain, potassium permanganate, renders surrounding tissue and debris non-fluorescent. AFB can then be visualized under the fluorescent microscope as bright yellow-green bacilli [11].

In South Africa, the traditional method to diagnose pulmonary TB was to submit two early morning sputum specimens for smear microscopy. Thereafter, these sputum specimens were cultured on liquid or solid media to isolate *Mycobacterium tuberculosis* (MTB). The advantages of AFB microscopy for initial diagnosis included low cost and high specificity in areas with a high prevalence of TB [12]. However, a major disadvantage is reduced sensitivity when the bacterial load in the specimen is less than 10,000 organisms/ml. The sensitivity is also reduced in paediatric patients and in patients with HIV/TB co-infection due to the paucibacilliary nature of TB disease in these patients [13]. Some studies have shown a further reduction in sensitivity of smear microscopy by approximately 15% in patients with HIV/TB co-infection [4]. In an effort to improve the diagnosis of TB by microscopy, in 2011, the WHO recommended that conventional fluorescence microscopy be replaced by Light Emitting Diode fluorescent microscopy (LED-FM). In a meta-analysis published by Chang *et al.*, LED-FM showed a pooled sensitivity of 66,9% (95% CI 60,5–72,7%) and pooled specificity of 96,8% (95% CI 93,1–98,6%). Importantly, the sensitivity was reduced in HIV positive population where a pooled sensitivity of 53,0% (95% CI 42,8–63,0%) and pooled specificity of 96,1% (95% CI 86,0–99,0%) was observed [14]. This reduction in sensitivity within the South African population, where the HIV/TB co-infection rate remains high gives rise to a further diagnostic

challenge. In the South African setting, a considerable number of patients have smear negative TB and therefore smear microscopy results may be falsely negative. Due to this high rate of false negative smear microscopy results, the WHO still recommends that sputum culture should be encouraged as part of the diagnostic workup in HIV infected individuals [15]. In South Africa, many patients who are smear negative at baseline are commenced on empiric TB treatment whilst awaiting culture confirmation. Empiric TB treatment is defined as the administration of TB treatment to persons being evaluated for TB who do not have laboratory evidence of TB [3].

Culture confirmation of viable TB bacilli using liquid media is currently the gold standard for the definitive diagnosis of MTB [16]. Liquid culture is performed in the BACTEC MGIT 960 TB System. The MGIT consists of modified Middlebrook 7H9 broth base and an enrichment supplement, MGIT OADC (oleic acid, albumin, dextrose and catalase). Before inoculation of decontaminated specimens into the MGIT tubes, MGIT PANTA is added (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) to inhibit contaminating organisms. The principle of the detection using the BACTEC MGIT system is based on the detection of fluorescence. The MGIT tube has an oxygen-quenched fluorochrome embedded in silicone at the bottom of the tube. As bacterial growth occurs, free oxygen inside the tube is utilized and replaced by carbon dioxide. When the oxygen is depleted, the fluorochrome is no longer inhibited by the oxygen and results in fluorescence within the MGIT when visualized under ultraviolet light. The intensity of the fluorescence is directly proportional to the extent of oxygen utilization [17]. The major advantage of liquid culture is the shorter time to detection (TTD) compared to solid media, as well as improved sensitivity when compared to smear microscopy. Studies have shown a TTD of approximately 12 days compared to 23 days in solid media [18]. With respect to sensitivity, studies have shown that the limit of detection (LOD) for MTB in the BD BACTEC MGIT 960 TB system is approximately 10 to 100 CFU/ml. Colony forming units (CFU) is a unit commonly used to estimate the concentration of microorganisms in a test specimen [16].

DST was originally routinely used to determine the susceptibility pattern of MTB. Determining the susceptibility patterns of various antimycobacterial agents is critical in prescribing effective drug regimens in patients with confirmed TB. The commonly used method for DST is the 1% agar proportion method. In this method, resistance to a drug is established when 1% of the total bacterial population display resistance to a specific drug[11]. DST can be performed on liquid or solid media. The preferred method in high throughput

laboratories in South Africa is liquid media using BACTEC MGIT 960 TB system. The principle of this test is that pure cultures of MTB are inoculated in a known concentration of drug. Concurrently, the same TB isolate is inoculated as a growth control with no added drug. The amount of fluorescence detected in each tube is analysed and reports a "Growth Value". In order to determine the susceptibility of the isolate, the fluorescence in the drug containing tube and the drug free tube are compared. If the isolate grows in the control tube and not in the drug containing tube, the isolate is reported as susceptible to the tested drug. If the isolate grows in both tubes, then it is considered resistant to the tested drug [17].

1.2.3 Molecular basis of drug resistance in TB

Two well-known resistance mechanisms occur in MTB:

(i) Primary (transmitted) drug resistance:

This mechanism occurs when drug resistant strains are transmitted directly to the host.

(ii) Secondary (acquired) drug resistance:

This mechanism occurs in response to inadequate antimycobacterial treatment. Within every population of TB bacilli occurring in the lung, there exists bacteria with spontaneously occurring mutations. If inadequate treatment is introduced, selection pressure occurs and the resistant subpopulations are selected out. As a result acquired resistance develops [19].

1.2.3.1 Rifampicin

The target for rifampicin is the β subunit of RNA polymerase which is encoded for by the *rpoB* gene. Majority of rifampicin resistance in MTB is caused by mutations in the *rpoB* gene. Within the *rpoB* gene, greater than 95% of these mutations occur in the 81 base pair region, which extends from codon 507 to 533 according to the *E. coli* numbering system. This mutation hotspot area is termed the rifampicin resistance-determining region (RRDR). Within the RRDR codons 526 and 531 are reported to harbour the most common rifampicin mutations. Globally, S531L mutations is reported as the most common followed by, H526Y, H526D, and D516V [20]. Rifampicin resistance has also been reported to occur outside the RRDR as well. Resistance to rifampicin usually accompanies resistance to other drugs, most commonly being INH. This makes it an ideal surrogate marker for MDR-TB [21].

1.2.3.2 Isoniazid

INH is a prodrug that requires activation by the catalase/peroxidase enzyme. The *katG* gene encodes for, this enzyme. Once activated, INH inhibits a mycobacterial protein, InhA. This protein is responsible for mycolic acid biosynthesis and encoded for by the *inhA* gene. The two main mechanisms of resistance to INH are: (i) loss of activation by the *katG* gene and, (ii) increase in expression of *inhA* gene. The commonest mutation observed is the *katG* S315T mutation. This mutation is associated with high-level INH resistance. Mutations in the *inhA* promoter region results in over expression of *inhA* gene. This mutation is associated with low-level resistance to INH and cross-resistance to ethionamide [19, 21].

1.2.3.3 Fluoroquinolones

Fluoroquinolones are bactericidal antibiotics that target the DNA gyrase enzyme. This enzyme is encoded for by the *gyrA* and *gyrB* genes and is essential for DNA replication. Majority of fluoroquinolone mutations are found in the quinolone resistance determining region (QRDR), which is a highly conserved region within *gryA* and *gyrB* genes. The most commonly occurring mutations occur at codon 90 and 94 of the *gyrA* gene. Specifically, A90V, D94G and D94H are frequent amongst clinical isolates. Mutations occurring in the *gyrB* genes are rare [19, 21].

1.2.3.4 Aminoglycosides (Second line injectable agents)

The second line injectable agents, kanamycin and amikacin are aminoglycosides and capreomycin is a cyclic polypeptide. All three drugs act by binding to the 30S subunit of the mycobacterial ribosome, which alters the 16SrRNA structure, resulting in inhibition of protein synthesis. The *rrs* gene encodes for 16SrRNA. Globally, mutations within the *rrs* gene, specifically at position 1401, are common and is associated with high-level aminoglycoside resistance. The *eis* gene encodes for acetyltransferase enzyme. Mutations in this gene are associated with low-level kanamycin resistance [19, 21].

1.2.4. Introduction of rapid molecular TB testing in South Africa

1.2.4.1 GenoType MTBDR*plus*

The first genotypic test routinely implemented in a programmatic setting in South Africa was the GenoType MTBDR*plus* Version 1 in the year 2009. In 2008, the WHO endorsed the GenoType MTBDR*plus* assay and recommended that it replace DST for INH and rifampicin

in all smear-positive and culture-positive specimens. GenoType MTBDR*plus* is a line probe assay and it is a qualitative test designed for the detection of MTB Complex (MTBC) and its resistance to rifampicin and INH. Resistance to rifampicin is detected by looking at the mutations in the RRDR region of the *rpoB* gene and resistance to INH is detected by looking at mutations in the *katG* and *inhA* promoter regions [22]. The updated version of the assay (GenoType MTBDR*plus* Version 2.0) is intended for use directly on decontaminated smear positive and smear negative pulmonary specimens as well as cultured MTB isolates. Reported sensitivity and specificity for detection of both rifampicin and INH resistance from cultured isolates were 98,1% and 98,7% respectively. However, studies show that estimated reports of sensitivity when performed directly on sputum specimens are lower. When performed directly on smear positive specimens, studies have shown an approximate sensitivity of 95% but in smear negative specimens, reported sensitivities are further reduced to approximately 65% [23]. Another study performed in Ethiopia compared the sensitivity of this test when performed directly on smear negative and smear positive clinical specimens. They also found a reduced sensitivity of 78% in smear negative specimens when compared to the 96,4% sensitivity in smear positive specimens [24].

The principle of this test is based on DNA–STRIP technology and the procedure is divided into 3 steps; (i) DNA extraction, (ii) amplification with biotinylated primers, and (iii) reverse hybridization. The membrane strips are coated with specific probes that are complementary to the amplified MTB nucleic acids present in the specimen. After chemical denaturation, the newly generated amplicons binds to the probes on the membrane. Highly specific binding is ensured by the buffer composition and specific temperature during hybridization. This binding becomes visible as coloured banding patterns when the biotin labelled amplicons bind to streptavidin labelled alkaline phosphatase. For interpretation, the banding patterns of gene locus controls, WT and MUT are compared to the conjugate and amplification control (see template below). The presence of all wild type (WT) bands and no mutation (MUT) bands indicate susceptibility. Resistance is reported when there is absence of a WT band with presence of a corresponding MUT band (defined mutation), or, the absence of WT bands only, without any corresponding MUT band (undefined mutation/resistance inferred). In certain isolates, where all WT probes and single or multiple MUT bands develop, these isolates are interpreted as resistant and are possibly due to a heteroresistant strains of MTB. The LPA is considered uninterpretable (inconclusive) if all bands of a gene locus (including the locus control) are missing.

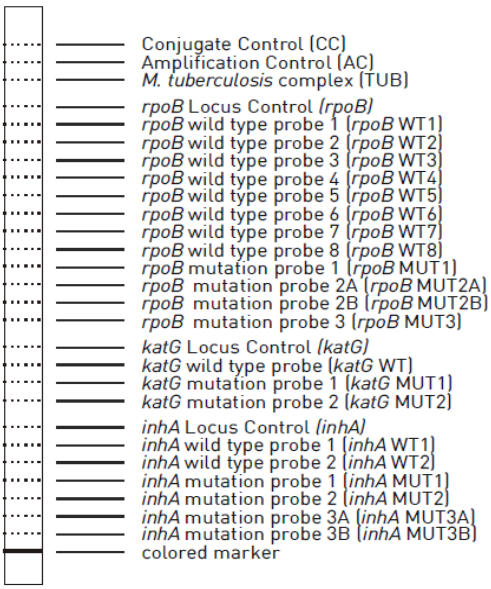

Fig.1. GenoType MTBDR*plus* interpretation template.

The main advantage of the GenoType MTBDR*plus* is the speed at which it offers diagnosis and susceptibility testing. Due to the rapid turnaround time of this test, the routine use of this assay proved to be beneficial. A study performed in a high throughput TB laboratory in Cape Town, South Africa compared traditional liquid culture and DST (using the agar proportion method on solid media) to the GenoType MTBDR*plus*. They found the use of this test reduced the time to treatment initiation in patients with MDR-TB from 80 days using conventional methods to 55 days by using FL-LPA [25].

There are several limitations to this assay:

(i) It is a procedurally complex test, which requires a special centralised molecular facility and highly trained personnel,

(ii) DNA based testing detects viable and non-viable bacteria; therefore, the test cannot be used for treatment monitoring,

(iii) The test only detects mutations that are confined to the RRDR of the *rpoB* gene, *katG* and *inhA* promoter regions. Therefore, mutations occurring outside these regions can be missed,

(iv) It detects silent mutations, which may have questionable clinical significance,

(v) Resistance may occur despite the presence of all WT bands. This may be due in the presence of a heteroresistant strain where the resistance pattern of the strain is not covered by the mutation probes in the assay [26].

1.2.4.2 GeneXpert MTB/Rif and GeneXpert MTB/Rif Ultra

With the persistently high prevalence of TB, HIV associated TB, and drug resistant TB in South Africa, it became increasingly important to implement rapid genotypic methods for detection of TB and rifampicin resistance at the time of initial patient presentation [27]. Detection of MTB using the GenoType MTBDR*plus* at initial patient presentation was impossible due to the several limitations discussed above. The most important limitations being the inability to decentralize this test because of its procedural complexity, and the need for a special molecular facility. In 2011, following the WHO recommendation for the use of GXP MTB/Rif assay, endorsing it as ‘‘the initial diagnostic test in individuals suspected of MDR-TB or HIV associated TB’’, the NDoH together with National Health Laboratory Services (NHLS), replaced conventional microscopy, culture and DST with the routine use of GXP MTB/Rif assay for the initial diagnosis of TB nationwide [28]. The GXP MTB/RIF assay offered a few advantages over smear microscopy and the GenoType MTBDR*plus*. This is largely because of its ability to rapidly diagnose MTB and detect rifampicin resistance directly on clinical specimens at a decentralized site, using real time polymerase chain reaction (PCR) technology. The principle of this real time PCR is based on molecular beacon technology. Molecular beacon probes consist of a stem loop structure with the nucleotide sequence embedded within the stem loop. There is a fluorescent dye and a quencher at the 5' and 3' end of the structure. In this test, five nucleic acid hybridization probes are used to cover the RRDR region of the *rpoB* gene. If a mutation exists in the specimen, the binding of the probe and amplicons is impaired and the beacon fails to emit fluorescence. The low limit of detection of 131 CFU/ml contributes to higher sensitivity than that of smear microscopy [29]. Laboratory evaluations of the GXP MTB/RIF assay showed a sensitivity of 98,3% on smear positive cases [30]. A study by Lombardi *et al.* found a lower GXP sensitivity of 73,1% for smear-negative culture-confirmed TB isolates [31].

Although the GXP may have led to more TB diagnoses, the introduction of this molecular technique still posed a challenge in our patient population due to its limited sensitivity of 66,1% (95%CI 56,4-74,9) in patients with smear negative TB [32]. The GXP MTB/Rif Ultra is an improved version of the assay. This updated version includes six nucleic acid probes; two of which bind to IS1081 and IS6110 and four probes bind to the RRDR in *rpoB* gene. Secondly, mutations are now determined by the analysis of melting temperatures. Studies have shown improved the sensitivity of this test assay in smear negative TB to 78,9 % (95%CI 70,0-86,1), however a considerable gap in sensitivity remains when compared to its performance in smear

positive clinical specimens [32]. As a result, the NDoH uses a specific algorithmic approach for the use of GXP Ultra in HIV positive patients. In this patient population, a negative GXP result cannot adequately rule out TB and a subsequent specimen for culture confirmation of viable bacilli must be obtained. Thereafter, if TB was cultured, further molecular testing using GenoType MTBDR*plus* is used for the confirmation of rifampicin and INH susceptibility. According to the GXP algorithm, in RR-TB and MDR-TB cases further DST for second line drugs is required [33].

1.2.4.3 GenoType MTBDR*sl* Version 2

In 2015, Hain Lifescience released an improved version of the GenoType MTBDR*sl* LPA Version 2. This qualitative test offers rapid detection of pre-XDR and XDR-TB. The SL-LPA allows for the identification of MTB and its resistance to FQ and SLIDs directly from smear-positive and smear negative sputum specimens, as well as cultured isolates. The principle of testing is based on DNA-STRIP technology and is similar to the GenoType MTBDR*plus*. The genetic targets included in this assay are *gyrA* and *gyrB* for FQ and the *rrs* and *eis* promoter for SLIDs [34]. The principles of interpretation are the same as for GenoType MTBDR*plus* however with a different template.

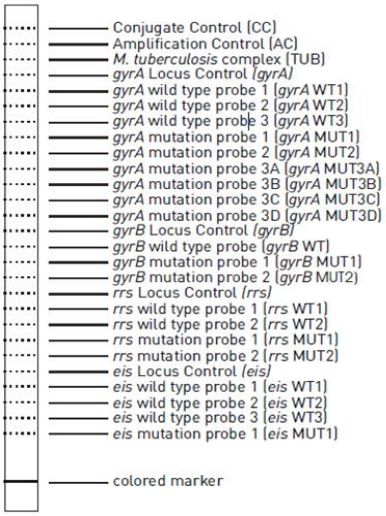

Fig.2. GenoType MTBDRsl Version 2 template.

1.2.5 Treatment of drug resistant TB

1.2.5.1 Conventional treatment of drug resistant -TB

Treatment of MDR-TB and XDR-TB has been challenging for both patients and healthcare systems worldwide. Conventional treatment regimens were of lengthy duration, approximately 18 - 24 months. These long regimens consisted of drugs that were less potent and less effective than those drugs used to treat susceptible TB. Due to the use of these less potent drugs coupled with the length of treatment duration, treatment outcomes for drug resistant TB were generally poor. Studies have shown that only 54% of MDR-TB patients and 30% of XDR-TB patients achieve cure. Secondly, prolonged use of these drugs cause multiple severe adverse effects, which include permanent hearing loss, gastrointestinal intolerance and renal impairment. Because of difficulty in getting to healthcare facilities many patients are lost to follow up resulting in non-adherence and further transmission of drug resistant strains [35].

1.2.5.2 Introduction of Shortened treatment regimens for MDR-TB

In 2016, the WHO released an updated guideline on the management of MDR-TB. The guideline states that in "patients with RR-TB or MDR-TB who were not previously treated with second-line drugs and in whom resistance to FQ and SLIDs was excluded or considered highly unlikely, a shorter MDR-TB regimen of 9–12 months may be used instead of the longer regimens". This recommendation was based on observational studies, which have shown successful patient outcomes when compared to the conventional long regimen 84% (95% CI 79-87) vs 62% (95% CI 53-70) from observational studies in 10 countries [5].

Among the ten studies that contributed to the regimen change, was the Bangladesh study published by Aung *et al*. They described 515 patients who had a success rate of 84,4% and had a bacteriologically favourable outcome. Their regimen comprised of high-dose gatifloxacin, ethambutol, pyrazinamide, and clofazimine throughout the duration, with a 4-month intensive phase of kanamycin, prothionamide, and INH. They observed that the strongest risk factor for a bacteriologically unfavourable outcome was associated with high-level gatifloxacin resistance [36]. Another trial performed in nine African countries also showed favourable outcomes in patients placed on the SR. This study showed a success rate of 82%. The regimen used in this study was formulated using the Bangladesh regimen; however, they used normal dose moxifloxacin (10-15mg/kg daily) instead of high dose gatifloxacin. They also observed

that the short regimen was effective in HIV positive patients but deaths were more frequent in this group. This study also showed that patients who had FQ resistance had an unfavourable outcome [37]. The STREAM trial, which included participants from South Africa, found that in patients with RR-TB that was susceptible to FQ and SLIDs, the shortened regimen, was statistically non-inferior to a long regimen in terms of efficacy (78,8 % compared to 79,8%) [38].

In 2017, South Africa adopted the WHO short MDR-TB regimen as "Modified South African 9-12 Month Regimen" which replaced the existing conventional regimen based on certain inclusion/exclusion criteria. The modified regimen comprises the intensive (injectable) phase; kanamycin+ moxifloxacin + clofazamine + ethionamide + pyrazinamide + high dose INH + ethambutol for 4-6 months followed by the continuation phase of moxifloxacin + clofazimine + pyrazinamide + ethambutol for a further 5 months. There are a few concerns regarding the implementation of the shortened regimen (SR) in South Africa. Most countries where these studies were performed do not have a similar prevalence of HIV and drug resistant TB. South Africa's higher prevalence of HIV and drug resistant TB may affect patient outcomes negatively. Secondly, the recent drug resistant survey shows high levels of drug resistance to ethambutol and pyrazinamide. This raises concern because these two drugs are used in the modified South African regimen in both intensive phase and continuation phase [6].

1.2.6 Implementation of Drug resistant TB reflex testing (DR-TB Reflex testing) in South Africa

With the new treatment recommendations issued by the WHO, regarding the SR for RR-TB and MDR-TB treatment, the requirement for rapid methods to detect resistance to FQ and SLIDs became necessary to rapidly triage MDR-TB and RR-TB patients into using this regimen [5]. The lengthy turnaround time of DST became inadequate in the triaging process for patients and a more rapid, genotypic means of testing became mandatory.

Therefore in order to facilitate the use of these regimens, the WHO published a policy guidance in 2016 stating, "for patients with confirmed RR-TB or MDR-TB, the SL-LPA may be used as the initial test, instead of phenotypic culture based DST to detect resistance to FQ and SLIDs" [7, 8]. These recommendations apply to the direct testing of clinical specimens regardless of smear status.

The evidence for this recommendation was based on 29 studies identified for both GenoType MTBDR*sl* Version 1 and GenoType MTBDR*sl* Version 2. Of these studies, only three studies evaluated Version 2. One study was published by Tagliani *et al.* and the other two were unpublished (FIND 2016, NICD) [7]. In view of the limited number of studies, in the 2016 policy guidance, the WHO acknowledged that data comparing direct testing on smear negative and positive specimens were sparse. Tagliani *et al*. conducted a multicenter study on smear positive clinical specimens in Europe. When compared to DST, the test shows an overall sensitivity and specificity to be 93% and 98,3% for the FQ and 88,9% and 91,7% for the SLIDs. They observed that the most common FQ mutations were *gyrA* MUT 3C (D94G), and MUT1 (A90V). They also observed that 17,5% of FQ resistant strains showed a mixture of WT and MUT strains which indicates possible heteroresistance. These strains were confirmed as resistant by DST thereby indicating that a resistant strain was present in the specimen. For SLIDs, they found that the commonest mutation was the A1401G in the *rrs* region. Heteroresistance for SLIDs was 17,5% as well and observed mainly in the *rrs* region The limitation of this study was its inability to include smear negative specimens [39].

The other two studies that guided the WHO recommendation showed that direct testing had a higher inconclusive rate on smear negative than smear positive specimens. These studies had a small specimen size with only 24 individuals. These studies were performed in a research setting where researchers reviewed thirty results only. Of these results, 6/30 (20%) showed an inconclusive rate of for direct testing on smear negative specimens [7].

A Cochrane systematic review published by Theron *et al.* concluded that further evaluation is required for the use of MTBDR*sl* Version 2.0 on smear negative specimens in different geographic settings. They highlighted that there was a paucity of data regarding direct testing on smear negative clinical specimens by the SL-LPA, and that most studies described its performance directly on cultured isolates or smear positive clinical specimens. In those studies that did analyse SL-LPA results in smear negative specimens, the specimen size was too small [8].

Gardee *et al*. evaluated the performance of the GenoType MTBDR*sl* on known clinical isolates from South Africa. Their study showed a sensitivity and specificity of 100% and 98,9% for FQ when tested against DST for ofloxacin alone and 89,2% and 98,5% for SLIDs. They also found that majority of FQ mutations were in the *gyrA* region, specifically at codon 94 and 90. The commonest mutations at codon 94 were MUT3C (D94G), MUT3A (D94A) and MUT3D (D94H). Heteroresistance for FQ was seen in 16,5% of the isolates and undefined FQ mutations

were observed in 9,4% of the isolates. These undefined isolates were confirmed as resistant by whole genome sequencing which indicated that the SL-LPA missed some mutations that are included in the assay and it also missed mutations that are not covered by the assay. The SL-LPA detected MUT1 (A1401G) in the *rrs* gene as the commonest mutation. Similar to Tagliani *et al.*, a limitation of this study was the lack of direct testing on clinical specimens and more specifically smear negative specimens [40].

Another study in China evaluated the GenoType MTBDR*sl* by comparing SL-LPA to DST and sequencing. They found the sensitivity for GenoType MTBDR*sl* for FQ and SLIDs to be 80,5% and 80,7% respectively. The predominant mutation for FQ was *gyrA* MUT3B and MUT3C (D94N and D94G). Resistance to SLIDs was predominant in the *rrs* region, specifically A1401G. C1402T in the *eis* region was observed in 10,5% of their isolates [41]. A study in India also aimed to characterize mutation patterns observed by the GenoType MTBDR*sl* Version 2 in their region. They found that among newly diagnosed RR-TB patients, 25% had displayed resistance to FQ with a predominance of the *gyrA* MUTC (42%) mutation. The most frequent *rrs* mutation was A1401G [42].

In 2017, the NDoH took both WHO recommendations together (the use of molecular line probe assays and the SR) and developed a new laboratory–clinical algorithm termed the Drug Resistant TB Reflex Testing (DR-TB Reflex). This algorithm was implemented nationally in 2017. In this algorithm, any patient with newly diagnosed RR-TB by GXP must have a second specimen sent to the laboratory for DR-TB Reflex testing.

DR-TB Reflex testing contains a set of laboratory tests that are performed directly and sequentially on the same clinical specimen. These tests include:

(i) Smear microscopy (to indicate infectiousness of the patient and for programmatic monitoring),

(ii) TB culture with the BACTEC MGIT 960 system (to enable phenotypic DST),

(iii) FL-LPA (to confirm the susceptibility pattern of rifampicin and isoniazid) followed sequentially by,

(iv) SL-LPA (to determine the presence or absence of resistance to FQ and SLIDs).

(v) DST using BACTEC MGIT 960 system on selected isolates (isolates with *katG* & *inhA* mutations, pre-XDR-TB and XDR-TB).

Since the implementation of DR-TB Reflex testing in KZN in January 2017, there have been no published reports on the experience of the routine use of the SL-LPA directly on clinical specimens within South Africa nor in KZN specifically, where the diagnosis of smear negative TB remains challenging. The literature is clear that data regarding the use of the GenoType MTBDR*sl* Version 2 directly on smear negative clinical specimens is sparse. Additionally, the performance of diagnostic tests in programmatic settings is often inferior compared to that in controlled research settings. Given the high burden of drug resistant TB, HIV associated TB, and smear negative TB in KZN; this is an ideal region to assess the feasibility of introducing the SL-LPA routinely in the DR-TB Reflex testing algorithm.

1.3 Research questions

In South Africa, specifically the KZN province, which is a well-known high burdened HIV and drug resistant TB area, what is the diagnostic yield of the GenoType MTBDR*sl* Version 2 when performed directly on clinical specimens within a programmatic setting?

Which mutation patterns does the GenoType MTBDRsl Version 2 detect for FQ and SLIDs?

1.4 Aims

a) To determine the diagnostic yield of the GenoType MTBDR*sl* Version 2 assay when performed directly on clinical specimens.

b) To describe which mutation patterns are identified by the GenoType MTBDR*sl* Version 2 assay in KZN.

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2. CHAPTER 2

Prepared according to the Instructions for Authors of South African Medical Journal.

A Descriptive Analysis of the Routine Use of the GenoType MTBDR*sl* Version 2 in a High TB Burden Province in South Africa

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2.1 Abstract

Background. Since the implementation of shortened drug regimens for the management of drug resistant tuberculosis (TB), there is a growing need for rapid detection of resistance to second line antimycobacterial drugs. The GenoType MTBDR*sl* Version 2 allows for the rapid molecular detection of resistance conferring mutations to fluoroquinolones (FQ) and second line injectable drugs (SLIDs). Although the GenoType MTBDR*sl* is recommended for use directly on smear positive and smear negative clinical specimens, the feasibility of using this assay routinely within a programmatic setting in high HIV/TB endemic areas requires exploration.

Objectives. To assess the feasibility of routinely using the GenoType MTBDR*sl* in a high HIV/TB prevalent region and to describe the various circulating resistance patterns detected by this test in KwaZulu-Natal.

Methods. A retrospective data analysis of all GenoType MTBDR*sl* results in newly diagnosed rifampicin resistant (RR-TB)/multidrug resistant TB (MDR-TB) specimens was performed. The assays performance on direct testing of smear positive and smear negative specimens was compared. The various mutation patterns for FQ and SLIDs identified by this test was observed.

Results. Of 1873 RR-TB/MDR-TB, 37,4% were smear negative and 62,5% was smear positive. In smear negative specimens, the GenoType MTBDR*sl* showed an inconclusive rate of 61,2%, whilst amongst the smear positive specimens, an inconclusive rate of only 6,6% was observed. The commonest mutation pattern observed for FQ occurred at the *gyrA* gene at codon 90 (A90V) 61/158(38,6%) followed by the D94G mutation 31/158 (19,6%). Heteroresistance for FQ was observed in the *gyrA* gene for 6/158 (10,1%) isolates. For SLIDs, the commonest mutation occurred in the rrs region specifically A1401G, 71/108 (65,7%) followed by C1402T at 20/108 (18.5%).

Conclusion. The routine use GenoType MTBDR*sl* Version 2 assay is more feasible in smear positive specimens as compared to smear negative specimens in high HIV/TB settings. To improve future development of the assay, further studies looking at the various resistance patterns are required.

2.2 Manuscript

2.2.1 Introduction

Globally there has been a reduction in disease burden caused by tuberculosis (TB) however; TB remains the leading cause of death from a single infectious agent worldwide. In 2018, South Africa continued to feature on the World Health Organization (WHO) list of top 30 high TB burden countries and it is one of the top 14 high burden countries with drug-resistant and HIV associated TB [1]. WHO statistics show that globally an estimated 60% of the incident TB cases in 2018 were HIV infected. The high HIV/TB co-infection rate contributes to the TB disease burden because patients with co-infection are frequently associated with smear negative pulmonary TB or extrapulmonary TB, which is diagnostically challenging [2-4]. In the recent South African national drug resistant prevalence survey, the prevalence of multidrug resistant (MDR-TB) tuberculosis was 2,1% (95% CI 1,5–2,7) among new tuberculosis cases and 4,6% (3,2–6,0) among retreatment cases [5]. MDR-TB is defined as resistance to rifampicin and isoniazid (INH). In the survey, although the prevalence of MDR-TB varied between all 9 provinces, KwaZulu-Natal (KZN), had amongst the highest prevalence of patients with MDR-TB, showing an overall estimate of 2,9% (95% CI 1,8 - 4,5) which is higher than the national average of 2,8%.

Given the high MDR-TB prevalence rate and its associated poor treatment outcomes, in 2016, the South African National Department of health (NDoH) replaced the existing long conventional MDR-TB treatment regimen with the "Modified South African 9-12 Month Regimen". This was in accordance with the 2016 WHO shortened MDR-TB treatment recommendation which showed successful patient outcomes when compared to the conventional long regimen 84 %(95% CI 79-87) vs 62% (95% CI 53-70) from observational studies in ten countries [6]. The "Modified South African 9-12 Month Regimen" comprised of an injectable phase; kanamycin + moxifloxacin + clofazamine + ethionamide + pyrazinamide + High Dose INH + ethambutol for 4-6 months, followed by the continuation phase of moxifloxacin + clofazamine + pyrazinamide + ethambutol for a further 5 months [7]. The shortened regimen (SR) is only eligible for patients with rifampicin resistant TB (RR-TB) and MDR-TB; therefore the NDoH adopted the 2016 WHO policy on the use molecular line probe assays to rapidly exclude patients with extensively drug resistant TB (XDR-TB) and pre-XDR-TB [7]. XDR-TB is defined as resistance to rifampicin, isoniazid, a fluoroquinolone (FQ) and second line injectable drugs (kanamycin, amikacin or capreomycin) (SLIDs). Pre-XDR-TB is defined as resistance to rifampicin, isoniazid and either a FQ or SLIDs [8].

Considering both guidelines (the use of molecular line probe assays and SR), in 2017, nationwide implementation of a laboratory-clinical algorithm termed Drug Resistant TB (DR-TB) Reflex testing came into effect. In this algorithm, any patient with newly diagnosed RR-TB by GeneXpert (GXP) (Cepheid, United States) must have a second specimen sent to the laboratory for DR-TB Reflex testing. DR-TB Reflex testing contains a set of laboratory tests which includes the routine use of the GenoType MTBDR*sl* Version 2 line probe assay(Hain Lifescience, Nehran, Germany) (SL-LPA) to be performed directly on clinical specimens regardless of smear status [7]. The SL-LPA is a qualitative test for the identification and susceptibility testing of *Mycobacterium tuberculosis* complex (MTBC). It simultaneously detects resistance to FQ and SLIDs directly from smear positive and smear negative sputum specimens by detecting mutations in the *gyrA, gyrB, rrs* and *eis* genes respectively. This test can be performed on cultured isolates from all specimen types [9].

Since the WHO recommendation for the use of SL-LPA in 2016, only a few studies have assessed its performance on smear negative clinical specimens. A Cochrane review published by Theron *et al*. highlighted that further evaluation is required for the use of MTBDR*sl* Version 2.0 on smear negative specimens in different geographic settings. They found that

most studies described its performance directly on cultured isolates or smear positive clinical specimens. For those studies that did analyse SL-LPA in smear negative specimens, the specimen size was too small [4]. Additionally, the 2016 WHO policy acknowledges the results generated from the SL-LPA V2 directly from smear negative specimens have a higher inconclusive rate than smear positive specimens [10]. The lack of data regarding direct testing of smear negative clinical specimens is particularly concerning in a high HIV/TB prevalent country, like South Africa. Studies conducted in KZN and other parts of Africa have confirmed that TB is often undetected among HIV-infected patients because they are likely to present with smear negative pulmonary TB [11, 12]. Therefore, the WHO still recommends that sputum culture should be encouraged as part of the diagnostic workup in HIV infected individuals [13].

Since the implementation of DR-TB Reflex testing in KZN in January 2017, there have been no published reports on the experience of the routine use of the SL-LPA on clinical specimens within South Africa nor in KZN specifically, where the incidence of smear negative TB remains high. The performance of diagnostic tests in programmatic settings is often inferior compared to that in controlled research settings. Given the high burden of drug resistant, TB and HIV associated TB in KZN; this is an ideal region to assess the feasibility of introducing the SL-LPA as part of the DR-TB reflex laboratory algorithm. Therefore, the objective is to evaluate the feasibility of routine use of the SL-LPA when performed directly on clinical specimens, as well as characterize the various mutation patterns in an area with high TB/HIV prevalence. This will significantly contribute to our knowledge on the routine use of this assay within the KZN population.

2.2.2 Methods

Study design

This is retrospective observational study of laboratory results acquired from specimens received for DR-TB reflex testing from January – December 2018 (Figure: 1).

Study setting

Since implementation of DR-TB Reflex testing in 2016, for all newly diagnosed patients with RR-TB diagnosed by GXP, a second sputum specimen was submitted to the South African National Accreditation System (SANAS) accredited KZN provincial TB reference laboratory for DR-TB Reflex testing. Whilst awaiting results of the DR-TB reflex testing, patients were commenced on the SR regimen, provided they did not meet any of the exclusion criteria.

Exclusion criteria for the SR include: (i) History of previous second line drugs ≥ 1 month; (ii) Complicated extra-pulmonary TB; (iii) Both INH mutations; (iv) Contact with XDR/ Pre XDR pateints.

DR-TB Reflex testing contains a set of laboratory tests which include, Auramine smear microscopy, TB culture on Mycobacteria Growth Indicator Tube (MGIT) 960 system (BACTEC MGIT Becton Dickinson, USA), GenoType MTBDR*plus* (FL-LPA) (Hain Lifescience, Nehran, Germany), followed by GenoType MTBDR*sl* Version 2 sequentially on the same clinical specimen regardless of smear status [7]. The FL-LPA was performed to confirm the resistance to rifampicin (detected by GeneXpert) and test for isoniazid susceptibility. The SL-LPA was performed to detect susceptibility to FQ and SLIDs, which classified patients as MDR-TB, pre-XDR or XDR-TB. In specimens that showed inconclusive results for either FL-LPA or SL-LPA, the respective test was repeated on the corresponding cultured isolate. Once the mutations from both line probe assays were analysed, further phenotypic drug susceptibility testing (DST) using 1% agar proportion method was performed on selected isolates. These isolates included those that had double INH mutations (i.e. both *katG* and *inhA* mutations), pre-XDR-TB and XDR-TB. DST for moxifloxacin (high and low levels), capreomycin and linezolid were performed on those selected cultured isolates (Figure 1).

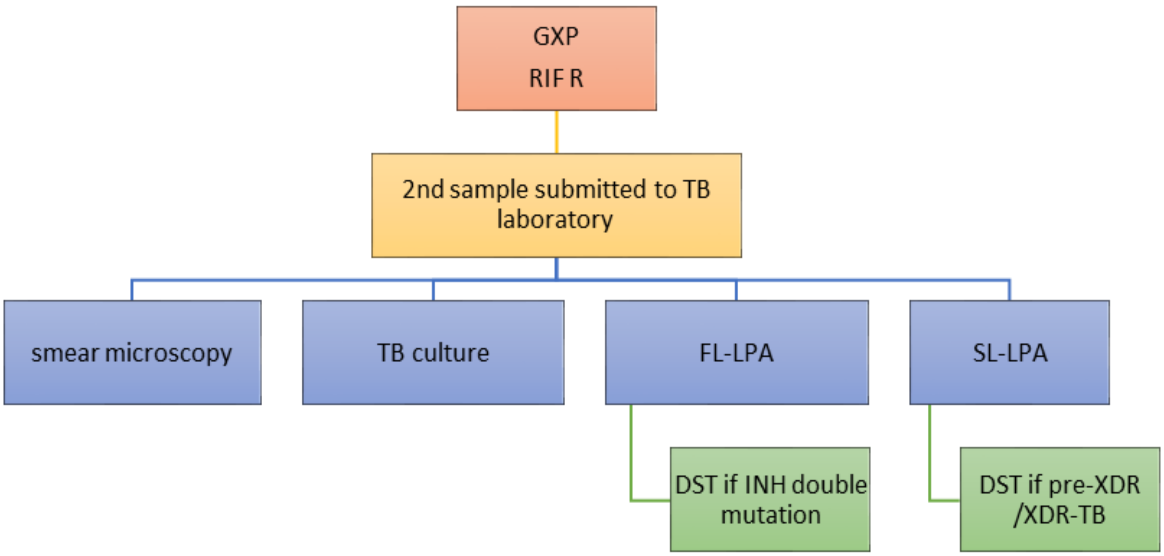

Figure: 1. DR-TB Reflex testing algorithm. GXP: GeneXpert MTB/RIF; RIF R: rifampicin resistant; FL-LPA: first line-line probe assay; SL-LPA: second line-line probe assay; DST: phenotypic

susceptibility testing; INH: Isoniazid; XDR-TB: Extensively drug resistant TB; INH double mutation: both *katG* and *inhA* mutations.

Laboratory Procedures

All sputum specimens received for DR-TB Reflex testing were decontaminated using the N-acetyl-l-cysteine sodium hydroxide (NALC-NAOH) method [14]. Thereafter 0,5ml of the decontaminated sputum were inoculated in BACTEC MGIT 960 tubes with PANTA (Polymyxin B: 6000 units; amphothericin B: 600µg; nalidixic acid: 2400µg, trimethoprim: 600µg and azlocillin: 600µg) and loaded into the BACTEC 960 machines. From the same decontaminated specimen, another 10µl was removed using a sterile loop and heat fixed on the slide. Auramine stains were performed using the Auramine Auto-stainer [14].

GenoType MTBDR*sl* Version 2

After performing culture and smears, FL-LPA and SL-LPA were performed sequentially, directly on the same decontaminated specimen. The SL-LPA was performed in accordance with the manufacturer's guideline. First, DNA was extracted, then amplified, followed by hybridization. Each strip of the assay has 27 probes, which target specific regions of *gyrA, gyrB, rrs,* and *eis* promoter regions in MTBC. Mutations detected in the *gyrA* and *gyrB* genes confer resistance to FQ, and resistance to SLIDs is detected by mutations in the *rrs* and *eis* genes. Interpretation of these reaction zones was in accordance to manufacturer's guideline [15]. The presence of all wild type (WT) bands and no mutation (MUT) bands indicate susceptibility. Resistance was reported when there was absence of a WT band with presence of a corresponding MUT band (defined mutation), or the absence of WT bands only, without any corresponding MUT band (undefined mutation/resistance inferred). In certain isolates, where all WT probes and a MUT band developed, or multiple MUT bands developed, this isolate was interpreted as resistant due to heteroresistance. The SL-LPA was defined as uninterpretable (inconclusive) if all bands of a gene locus (including the locus control) were missing. In some specimens where MTBC band was present on the FL-LPA but absent on SL-LPA, this was reported as inconclusive.

Data analysis

Data of all results for both line probe assays and Auramine smears from January –December 2018 was collected. These results were obtained from the National Health Laboratory Service (NHLS) laboratory information system, which contains all electronic laboratory results.

Duplicate specimens were removed using patients' demographic data (name, surname and date of birth). Once the results were de-duplicated, all FL-LPA results that showed RR-TB and MDR-TB were filtered. All rifampicin susceptible and FL-LPA inconclusive results were excluded. (Figure: 2)

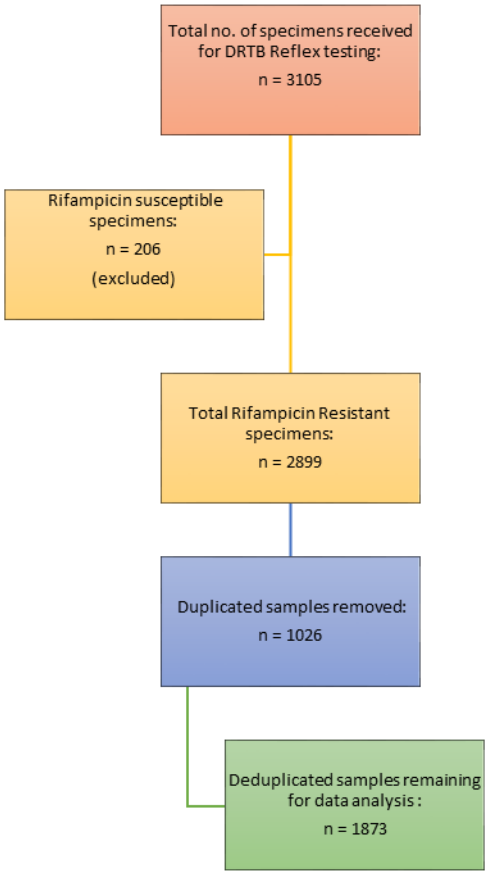

Figure: 2. Flow diagram of specimens included in the study.

All the RR-TB and MDR-TB results were divided into two arms according to smear status i.e. smear positive arm and smear negative arm. In each arm, the percentage of results that showed conclusive and inconclusive results by SL-LPA were calculated. From all conclusive SL-LPA results, the proportion was MDR-TB, pre-XDR-TB and XDR-TB were calculated. Once all the pre-XDR-TB and XDR-TB results were found, the original SL-LPA strips were read to identify which mutation pattern was detected for FQ and SLIDs. Thereafter the frequency at which each mutation pattern occurred was calculated.

For de-duplication and data analysis, R software (R Core Team) was used first, and then exported data to Microsoft Excel (Version 2019) for further exploration.

2.2.3 Results

A retrospective data analysis of GenoType MTBDR*sl* and corresponding smear results on 1873 LPA MDR/Rif resistant clinical specimens over 1 year (January – December 2018) was performed.

Of the 1873 clinical specimens, 37,4% (701/1873) were smear negative and 62,5% (1172/1873) were smear positive. Of the smear negative specimens, 67,2 % (471/701) had inconclusive SL-LPA results (Table 1). Of those 471 inconclusive results, in 269 results, the SL-LPA did not detect MTBC despite the FL-LPA showing a prominent MTBC band.

In the smear negative arm, 32,8% (230/701) showed clear banding patterns with 77,4% (178/230) MDR-TB, 14,8% (34/230) pre-XDR-TB and 7,8% (18/230) XDR-TB rapidly diagnosed.

For smear positive specimens, 6,6 % (77/1172) had inconclusive results. The remaining 93,4% (1095/1172) showed clear banding patterns with 84,9% (930/1095) MDR-TB, 10,1 % (111/1095) pre-XDR-TB and 4,9 % (54/1095) XDR TB rapidly diagnosed.

Overall, for MDR/Rif resistant clinical specimens, 29,3% (548/1873) had inconclusive results where majority of inconclusive results belonged to smear negative specimens. MTBC and its respective susceptibility pattern was clearly identified in 70,7% (1325/1873) of the specimens. In total the SL-LPA facilitated rapid diagnosis of pre-XDR and XDR in 7,7 % (145/1873) and 3.8 % (72/1873) respectively (Figure: 3).

AFB Smear on MDR isolates	**SL-LPA Inconclusive n (%)**	**SL-LPA Conclusive n (%)**	**Total n (%)**
Negative	471 (67,2)	230 (32,8)	701 (37,4%) ($p<0.01$)
Positive	77 (6,6)	1095 (93,4)	1172 (62,5%)
Total Results	**548**	**1325**	**1873**

Table: 1. GenoType MTBDR*sl* results stratified according to smear status.

Table 1 shows GenoType MTBDR*sl* results stratified according to smear status. Smear negative specimens had significantly more inconclusive results compared to smear positive specimens ($P<0.01$).

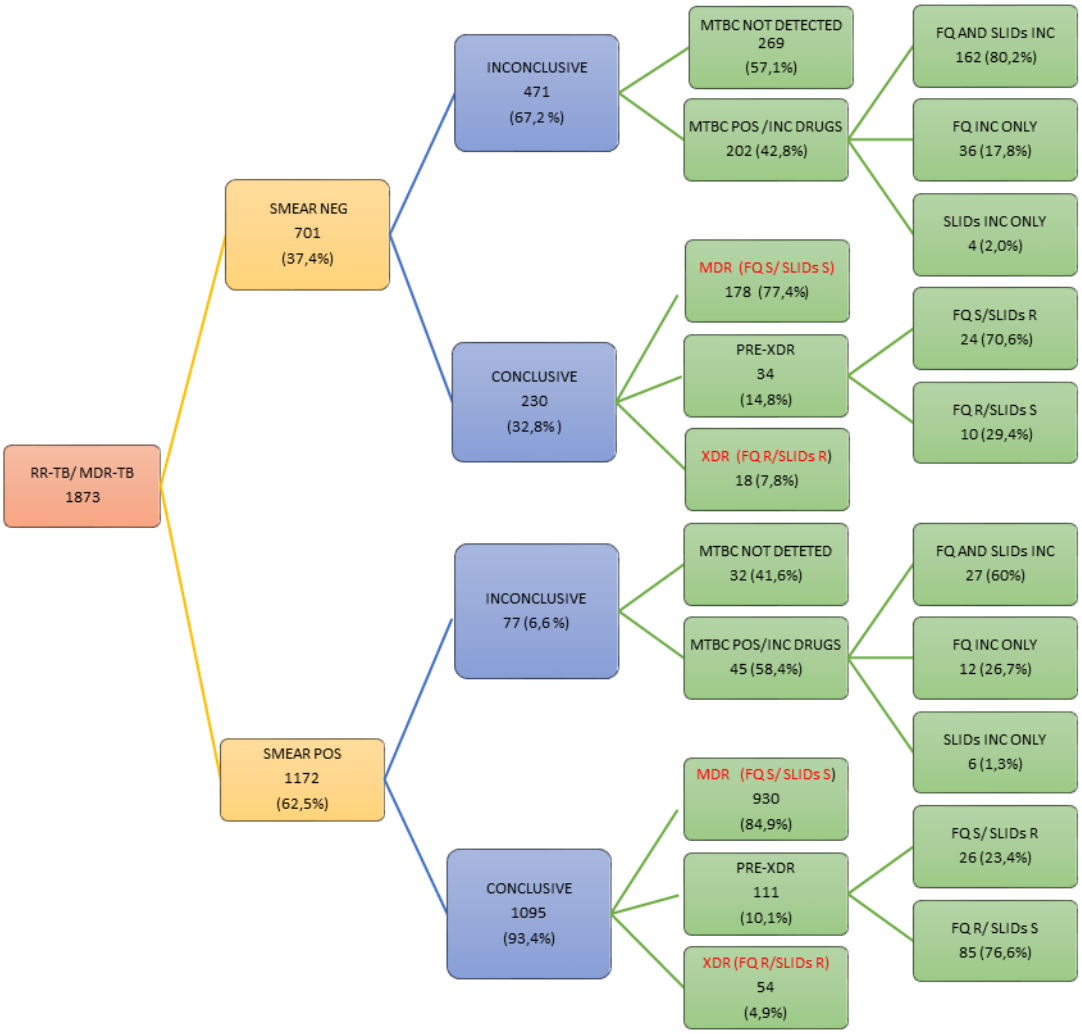

Figure: 3. Analysis of GenoType MTBDR*sl* Version 2 results according to smear results. RR-TB: Rifampicin resistant TB; MDR TB: Multi-drug resistant TB; Smear Pos: Auramine smear positive; MTBC: Mycobacterium tuberculosis complex; INC: Inconclusive; XDR-TB: Extensively drug resistant TB; FQ: Fluoroquinolones; SLIDs: Second line injectable drugs; R: Resistant; S: Susceptible.

Figure 3 shows that the SL-LPA did not detect the TUB band in 57.1% of inconclusive isolates. Amongst smear negative inconclusive results, 80,2% showed inconclusive results for both FQ and SLIDs.

Probe	Wild type Probe	Mutation Probe	Corresponding Mutation	Total Number n(%)
gyrA(n 152)	WT2 Absent	No mutation	Undefined	17(10.7)
		MUT 1	A90V	61 (38,6)
		MUT 2	S91P	10 (6.3)
	WT3 Absent	No mutation	Undefined	1(0.63)
		MUT 3A	D94A	2(1.2)
		MUT 3B	D94N / D94Y	13(8.2)
		MUT 3C	D94G	31(19.6)
		MUT 3D	D94H (rare mutation)	2(1.2)
	No WT Absent	MUT 1	HR	4(2.5)
		MUT 3B	HR	1(0.6)
		MUT 3C	HR	10(6.3)
gyrB(n=6)	WT Absent	Nil	Undefined	5(3.1)
	No WT Absent	MUT 2	HR	1 (0.63)
*rrs (*n=105*)*	WT 1 Absent	MUT 1	A1401G	71(65.7)
		MUT 2	Unknown	3 (2, 7)
		No mutation	C1402T	20(18.5)
	WT 2 Absent	No mutation	Undefined	6(5.5)
	No WT Absent	MUT 1	HR	2(1.9)
eis(n=3)	No WT Absent	MUT 2	HR	3(2.8)
	WT 1 Absent	No mutation	G-37T	1 (0, 92)
	WT2 Absent	MUT 1	C-14T	1(0, 92)
		No mutation	C-12T / G-10A	1(0, 92)

Table: 2. GenoType MTBDR*sl* hybridization patterns observed for fluoroquinolones and second line injectable agents. WT: Wild type; MUT: Mutation; HR: Heteroresistance.

Table 2 shows the commonest mutation in gyrA region was A90V and the commonest mutation for rrs region was A1401G

When characterizing mutations, the original SL-LPA strips for all XDR (72), FQ pre-XDR (95) and SLID pre-XDR (50) were read. This would have resulted in 167 FQ banding patterns (95+72) and 122 SLID resistant patterns (72+50). Unfortunately, the banding patterns for 9 FQ and 14 SLID results had faded and were uninterpretable during retrospective analysis. Therefore, only 158 FQ mutations and 108 mutations for SLIDs were analysed.

For the FQ, 152 mutations were observed in the *gyrA* region and only six mutations in the *gyrB* region. A summary of FQ and SLIDs mutations are described in Table 2. Majority of defined mutations occurred at codon 90 (A90V) 61/158 (38,6%) followed by the D94G mutation 31/158 (19,6%). Of note, 16/158 (10,1%) isolates showed heteroresistance. For the *gyrB* region, 5/6 (3,1%) mutations were undefined. Altogether, undefined FQ mutations were noted for 23/158 (14,5%) of the isolates.

For SLIDs, 108 mutation patterns observed in total, 105 in the *rrs* region and only 3 in the *eis* region. In the *rrs* region the commonest mutation was rrs MUT1 (A1401G), 71/108 (65,7%) followed by C1402T at 20/108 (18,5%). Heteroresistance was observed in five isolates (4,6%).

2.2.4 Discussion

Following the WHO endorsement of the GenoType MTBDR*sl* Version 2, this test has gained popularity in high TB burden countries and high throughput TB laboratories to rapidly assess drug resistance to FQ and SLIDs. To our knowledge, since the introduction of the DR-TB Reflex testing clinical-laboratory algorithm in South Africa, this is the first study, which describes the use of this test under programmatic conditions in South Africa. In this study, the data analysis showed that of 1873 MDR/RR-TB, smear positive TB was predominant (62,5%). This percentage of smear positive TB is slightly higher when compared to another retrospective study performed in Western Cape, South Africa. In that study, data over a 6 year period showed that of 36,219 cases of pulmonary TB, 19,195 (53 %) were sputum smear positive at the time of diagnosis [16]. However, in this study, the smear negative rate of 37,4% is in keeping with other studies which have reported that among patients who have HIV/TB co-infection, sputum smear negative rates ranged from 24% to 61% [12].

The SL-LPA performed well in the smear positive arm by showing valid and conclusive results for 93,4% of the specimens whilst showing inconclusive results for only 6,5% of the specimens. The limit of detection for SL-LPA in clinical specimens is 150 CFU/ml, therefore the high percentage of conclusive results is expected seeing that approximately 5000-10000 CFU/ml of bacteria is required in smear positive sputum specimens [9]. Although smear positive specimens predominated, a large proportion of specimens were smear negative (37,4%). In the smear negative arm, the SL-LPA showed inconclusive results in 67,1% of the specimens and interpretable results in 32,8% of the specimens. This high rate of inconclusive results in smear negative specimens is in keeping with the WHO policy

guidance, where they acknowledged that although data comparing direct testing on smear negative and positive specimens were sparse, two small studies showed that direct testing had a higher inconclusive rate on smear negative than smear positive specimens. These studies had a small specimen size with only 24 individuals. Additionally, these studies were performed in a research setting where researchers reviewed thirty results only, and of these results, 6/30 (20%) showed an inconclusive rate of for direct testing on smear negative specimens [10].

Although there are no published reports from South Africa regarding the routine use of GenoType MTBDR*sl* Version 2 in a programmatic setting, in the recent Rifampicin resistant clinical guideline released by the NDoH, the DR-TB Reflex testing laboratory algorithm was revised based on experience from high throughput laboratories. This guideline now recommends, for smear negative specimens the FL-LPA is performed routinely but SL-LPA will only be performed on smear positive or culture positive specimens [8].

Furthermore, this study found that among the smear negative inconclusive results, where the FL-LPA detected MTBC, the sequential SL-LPA failed to detect MTBC in 57,1% (269/471) of isolates. This is an unexpected finding seeing that the same DNA extraction kit is used for both tests. There are no reports thus far comparing the sequential testing of FL-LPA and SL-LPA, however, Tomassicchio *et al*. performed a study, in which they used a similar sequential approach to diagnosing MDR, pre-XDR and XDR TB using GenoType MTBDR*sl* Version 1. In their study, they found that the GenoType MTBDR*sl* Version 1 showed a lower overall sensitivity in detecting MTBC and associated resistance when compared to GenoType MTBDR*plus* Version 1 for both smear positive and smear negative specimens [17].

The performance characteristics of the MTBDR*sl* Version 2 has been assessed in only a few studies. Tagliani *et al*. conducted a multicentre study on smear positive clinical specimens in Europe. When compared to DST, the test showed an overall sensitivity and specificity to be 93% and 98,3% for the FQ and 88,9% and 91,7% for the SLIDs. However, this study did not include any smear negative specimens [18]. Gardee *et al.* evaluated the performance of the GenoType MTBDR*sl* Version 2 by comparing it to phenotypic susceptibility testing on known clinical isolates from South Africa. Their study showed a sensitivity and specificity of 100% and 98,9% for FQ when tested against ofloxacin alone and 89,2% and 98,5% for SLIDs. The limitation of this study was the lack of direct testing on clinical specimens and more specifically smear negative specimens [19]. Similarly, Yadav *et al.* studied the diagnostic accuracy of the GenoType MTBDR*sl*. In their study, the SL-LPA showed a high

sensitivity and specificity when compared to DST for levofloxacin 97, 2% (95% CI 93,5 – 99,1) and 99,1% (95%CI 97-99,9) respectively. The sensitivity and specificity for kanamycin was 92,5% (95% CI 79,6 -98,4) and 99,5% (95%CI 98,1-99,9). However, a limitation in their study was the lack of direct testing on smear negative specimens as well [20].

In this study, the SL-LPA enabled rapid and accurate diagnosis of 1108 (59%) cases of MDR/Rif resistant TB, 145 (7,7%) cases of Pre-XDR TB and 72 (3,8%)cases of XDR TB overall. This enabled clinicians to rapidly triage patients into their respective drug resistant treatment regimens. Interestingly, among all pre-XDR isolates, (taking both arms together), more specimens showed FQ resistance as compared to resistance to SLIDs (65,5% vs 34,5%). Similarly, this higher frequency of FQ resistance was reported in another study in India [21]. In the KZN setting, seeing that these were all newly diagnosed patients, this is of particular concern because it highlights the possibility of primary transmission of drug resistant TB strains, which has been described in recent studies in South Africa, specifically KZN [22]. FQ resistant strains could also be a result of widespread use of FQ for other infectious diseases [22]. Furthermore, when it comes to the use of the shortened MDR-TB drug regimen, one study showed that FQ resistance was a key factor for a bacteriologically unfavourable outcome [23]. The latest drug resistant prevalence survey shows an estimated resistance of 1,2% (95% CI 2,2 -3,6) to ofloxacin in new cases and 1,5 % (95 CI 3,8 -6,7) in retreatment cases [5]. Future studies in South Africa assessing the frequency of FQ resistance is important especially since FQ have been introduced as the backbone of the new shortened MDR-TB treatment regimen.

The most frequent mutations associated with FQ occurred in the *gyrA* region with 38,6% at codon 90 (A90V) and 30,3% at codon 94 (D94G, D94N/D94Y, D94A, D94H). This differs slightly with other published data from South Africa, India and China where studies have shown a predominance of mutations occurring at codon 94 and 90 respectively [19, 20, 24]. In comparison with Gardee *et al*., this study reported a comparable frequency of S91P (6,3%) mutations when compared to their finding of 9,8%, however the rare in silico D94H mutation was considerably lower (1,2% vs 10,6%) [19]. Regarding resistance to SLIDs, the predominant mutation was *rrs* MUT1 (A1401G) 71 (65,7%) followed by C1402T 20 (18,5%). This was consistent with other published data [18, 19]. Only 3/108 specimens displayed resistance in the *eis* promoter region. The intention of the improved version of this test was to increase detection of resistance by the addition of the *gyrB* and *eis* genes;

however, this study indicates that mutations in the *gyrB* and *eis* promoter regions only contributed to approximately only 4% of resistance.

Heteroresistance occurs when there is co-existence of susceptible and resistant TB strains in the same specimen. It can be due to transmission of an infection with two different strains, or the development of resistance in patients with susceptible TB that were inadequately treated. This study showed that heteroresistance was noted with both FQ and SLIDs however, it was more predominant for FQ in the *gyrA* region 16/158 (10,5%). All these heteroresistant strains were identified by the presence of all WT bands plus a positive MUT band. These specimens were reported as resistant, however the clinical relevance of these heteroresistant specimens are largely unknown. Gardee *et al*. observed FQ heteroresistance in 16,5% of ofloxacin resistant strains [19]. Heteroresistance is not unexpected in high TB/HIV endemic areas where high transmission rates may occur. A study conducted by Warren *et al*. in Cape Town South Africa showed that multiple infections are not infrequent, due to high rate of re-infection in a high incidence setting [25].

For undefined mutations, resistance was inferred for both FQ and SLIDs. The majority of these mutations were seen in the *gyrA* region, specifically with an absent WT2 and no MUT bands appearing 17/158 (10,7%). Undefined *gyrA* mutations were further explored in another South African study. In that study, resistance was confirmed by DST and whole genome sequencing [18]. This highlights the limitation of the SL-LPA whereby the mutation causing resistance, is not covered by the mutation probes included in the assay [9, 19]. In these undefined mutations, whole genome sequencing may be beneficial to inform the mutations that are circulating within South Africa. Whole genome sequencing may also contribute to our knowledge on the clinical significance of these undefined mutations, as well as inform the design of future molecular tests.

A limitation in this study was that we did not compare and confirm the resistance patterns detected by the GenoType MTBDR*sl* results with DST. This was either due to contamination of several cultured isolates or no growth from clinical specimens. Secondly, we did not confirm undefined mutations with DST or WGS. Another limitation is that the mutation profile for the isolates studied belonged to one province only and did not include other areas within the country.

In conclusion, the GenoType MTBDR*sl* Version 2 is a valuable assay for the rapid and early diagnosis of pre-XDR and XDR-TB directly from smear positive specimens. However, when

performed directly on smear negative specimens, the inconclusive rate is much higher. In this study, based on the poor performance of the GenoType MTBDR*sl* on smear negative samples, it is recommended that, the SL-LPA should be performed on smear positive clinical specimens only. For smear negative specimens, the SL-LPA should be performed on the positive cultured isolates only in order to increase the rate of conclusive results. The clinical implications of this will be a longer turnaround time (TAT) when compared to processing directly on the clinical specimens. Despite the longer TAT, these patients should be commenced on SR as smear negative pre-XDR and XDR-TB only constitute a small amount (2.8%) among RR cases in this study. Further studies looking at the diagnostic accuracy in smear negative specimens in different geographic settings are required. In view of the revised NDoH treatment guideline where injectable free regimen is now recommended, rapid diagnostic tests require updating because the clinical significance of mutation patterns in SLIDs is now questionable.

Furthermore, more studies looking at the various patterns of resistance in different geographic areas is required. This will inform future development of the test to include relevant mutation probes thereby improving the overall detection of resistance.

Acknowledgement:

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Conflict of interest:

All authors declared no conflict of interest

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2.4 Appendices

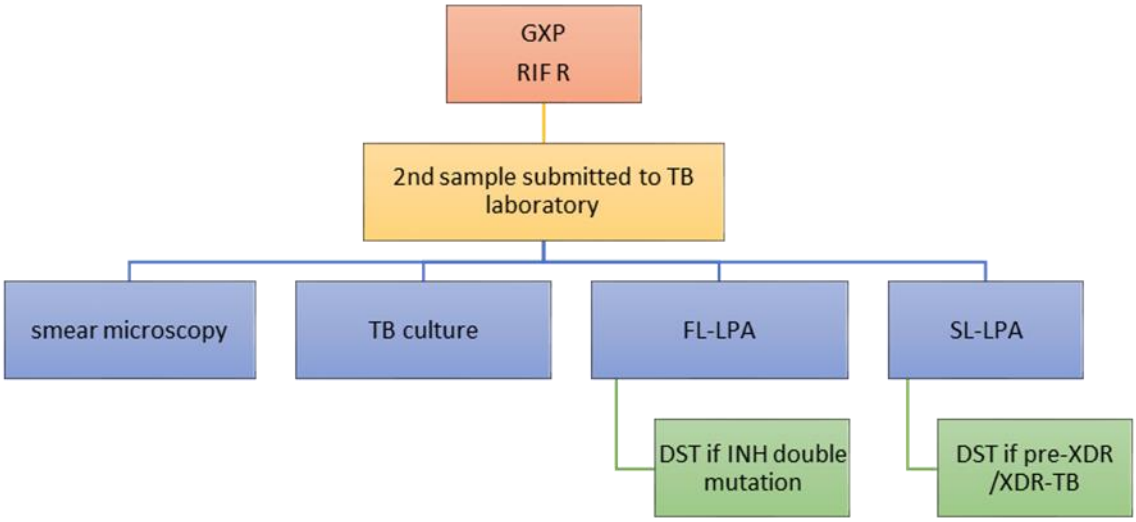


Figure: 1. DR-TB Reflex testing algorithm. GXP: GeneXpert MTB/RIF; RIF R: rifampicin resistant; FL-LPA: first line-line probe assay; SL-LPA: second line-line probe assay; DST: phenotypic susceptibility testing; INH: Isoniazid; XDR-TB: Extensively drug resistant TB

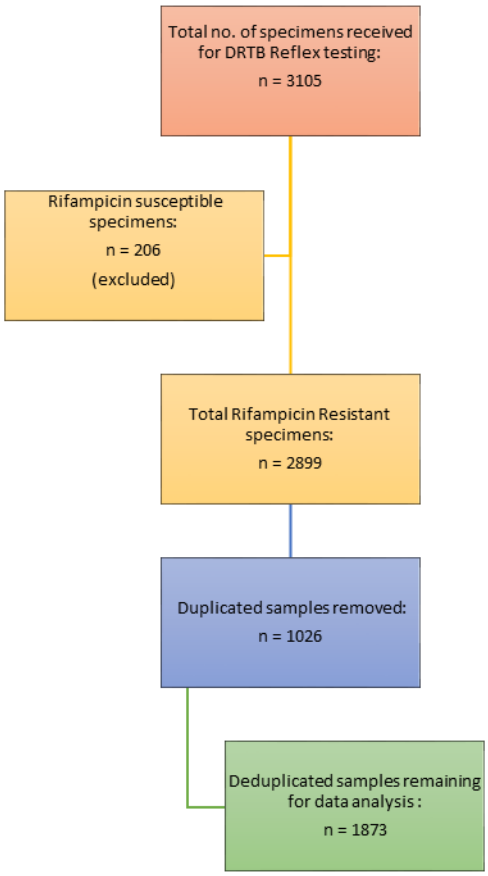
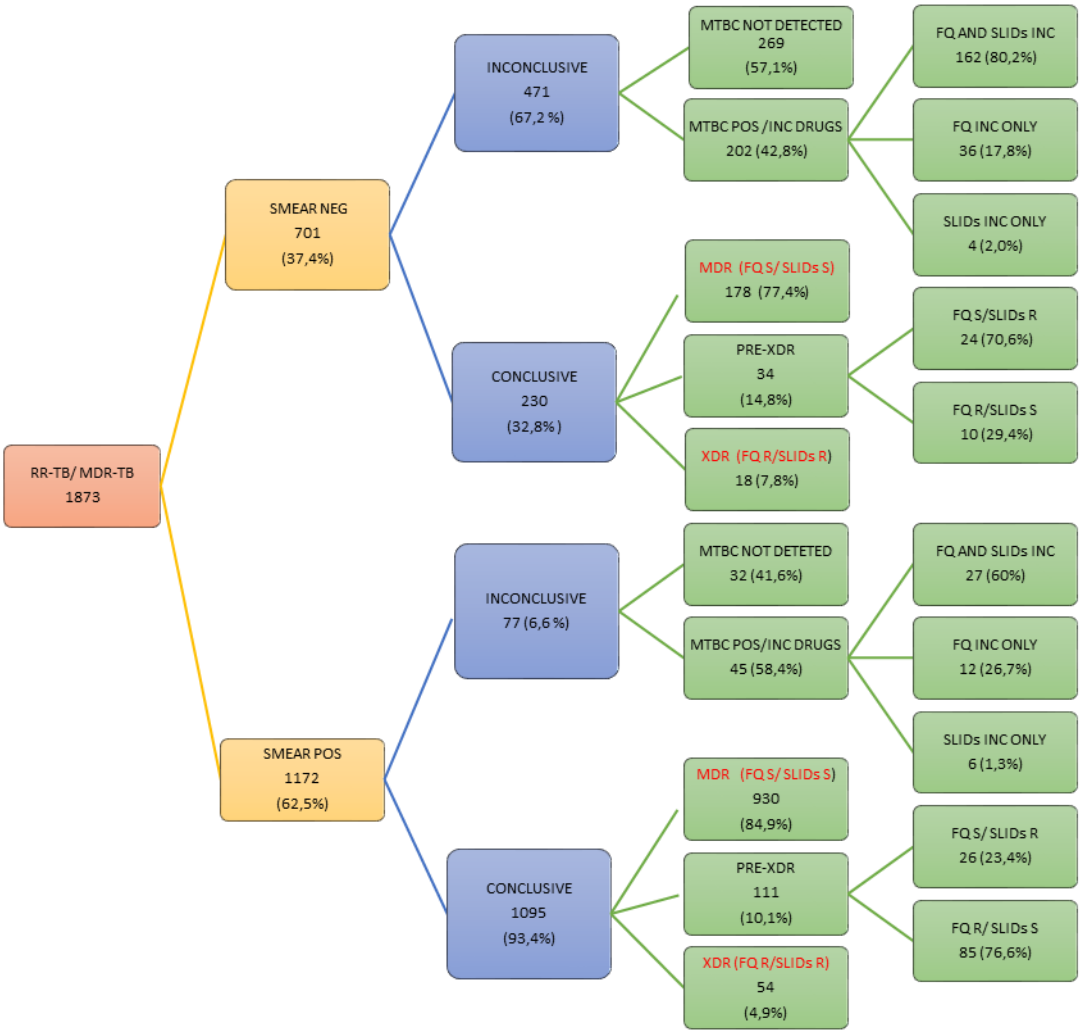


Figure: 2. Flow diagram of specimens included in the study.



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		MUT 3C	D94G	31(19.6)
		MUT 3D	D94H (rare mutation)	2(1.2)
	No WT Absent	MUT 1	HR	4(2.5)
		MUT 3B	HR	1(0.6)
		MUT 3C	HR	10(6.3)
gyrB(n=6)	WT Absent	Nil	Undefined	5(3.1)
	No WT Absent	MUT 2	HR	1 (0.63)
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		MUT 2	Unknown	3 (2, 7)
		No mutation	C1402T	20(18.5)
	WT 2 Absent	No mutation	Undefined	6(5.5)
	No WT Absent	MUT 1	HR	2(1.9)
eis(n=3)	No WT Absent	MUT 2	HR	3(2.8)
	WT 1 Absent	No mutation	G-37T	1 (0, 92)
	WT2 Absent	MUT 1	C-14T	1(0, 92)
		No mutation	C-12T / G-10A	1(0, 92)

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3. References & Appendices

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3.2 Appendices

3.2.1 Appendix 1: BREC approval letter

3.2.2 Appendix 2: NHLS data approval letter

3.2.3 Appendix 3: GCP certificate

3.2.4 Appendix 4: MMED protocol submitted to BREC

3.2.5 Appendix 5: Turnitin report

Appendix 1: BREC approval letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

24 June 2020

Dr Keeren Lutchminarain (9900105)
School of Lab Med & Medical Sc
Medical School

Dear Dr Lutchminarain,

Protocol reference number: BREC/00001020/2020
Project title: Feasibility of routine use of the Genotype MTBDRsl Version 2 assay as part of the Drug resistant TB Reflex Testing (DR-TB) algorithm within the Kwazulu Natal (KZN) central TB laboratory
Degree Purposes: MMed

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 24 June 2020. Please ensure that outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is subject to national and UKZN lockdown regulations dated 5th June 2020 which is available on the BREC website (http://research.ukzn.ac.za/Libraries/BREC/Proposed_UKZN_BREC_revision_to_research_constraints_anticipating_change_to_Level_3_lockdown.sflb.ashx).

This approval is valid for one year from 24 June 2020. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (If applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 14 July 2020.

Yours sincerely

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
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Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

Appendix 2: NHLS data approval letter

Department of Microbiology
Inkosi Albert Luthuli Central Hospital
800 Vusi Mahlaba (Bellair) Road, Mayville, 4091
Private Bag X03, Mayville, 4058
Tel: +27 (031) 240 2770 Fax: +27 (031) 240 2786

28 May 2020

Dear Dr Samuel

RE: Research site permission letter: Request for use of DR-TB Reflex testing data generated by the central TB Laboratory at IALCH

I am registered as a post-graduate student at the University of Kwazulu Natal (Student No: 9900105) for my MMed (Microbiology) degree. The protocol that I have written and submitted to BREC is as follows:

Feasibility of routine use of the Genotype MTBDRs/ Version 2 assay as part of the Drug resistant TB Reflex Testing (DR-TB) algorithm within the Kwazulu Natal (KZN) central TB laboratory

Protocol Ref No: BREC/00001020/2020 (Status: Provisionally approved)

Principal Investigator: Dr Keeren Lutchminarain
MMed Supervisor: Dr Nomonde Mvelase
MMed Co-Supervisor: Dr Khine Swe Swe Han

The study that we are embarking upon is analysis of retrospective data generated from samples sent to the central TB lab at IALCH for DR-TB Reflex testing. These samples were sent routinely as part of the revised national laboratory algorithm for TB testing. The retrospective data analysis will be from samples obtained from 1 January - 31 December 2018.

I humbly request your permission to use this data for my study, which is for degree purposes.

Thanking you in advance.

Dr Keeren Lutchminarain
Pathologist (Medical Microbiology)

29/05/2020
HOD
Dr. Khine Swe Swe - Than
Medical Microbiology Dept.

Approved
29/5/20

1 Modderfontein Road, Sandringham, Johannesburg, South Africa
Prof Eric Buch Dr Kemani Chetty
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Appendix 3: GCP certificate

Certificate of Attendance

Keeren Lutchminarain

Good Clinical Practice and Informed Consent Training
Sponsored by
Division of Microbiology & Infectious Diseases, NIAID, NIH
International Clinical Studies Support Center, FHI 360

Durban, South Africa
29-30 November 2018

Robin Mason
ICSSC Contract Officer's Representative
NIH/NIAID/DMID

Loice A. Magaria, BSN, MPH, CCRA
Clinical Research Associate II
FHI 360

NIH

ICSSC International Clinical Studies Support Center

fhi360 THE SCIENCE OF IMPROVING LIVES

Appendix 4: MMED protocol submitted to BREC

Mmed Protocol Final BREC submission

Principal Investigator: Dr. Keeren Lutchminarain (MBBcH –Wits; FCPath Microbiology)

Project Title: Feasibility of routine use of the Genotype MTBDR*sl* Version 2 assay as part of the Drug resistant TB Reflex Testing (DR-TB) algorithm within the Kwazulu Natal (KZN) central TB laboratory

Background Literature Review and Introduction

Globally there has been a reduction in disease burden caused by tuberculosis (TB) however; TB remains the leading cause of death from a single infectious agent worldwide. In 2017, an estimated 10.0 million people developed TB disease of which two thirds belonged to eight countries namely India, China, Indonesia, the Philippines, Pakistan, Nigeria, Bangladesh and South Africa. Furthermore, multidrug resistant TB (MDR-TB), a global health crisis, continues to pose a challenge. An estimated 558,000 people developed TB resistant to rifampicin (RR-TB) in 2017 of which approximately 82% had multidrug resistant TB (MDR-TB) [1].

In 2018, South Africa continued to feature on the WHO list of top 30 high TB burden countries. Additionally, it is one of the top 14 high burden countries with drug-resistant and HIV associated TB [1]. In the most recent national drug resistant prevalence survey, the prevalence of MDR tuberculosis was 2.1% (95% CI 1·5–2·7) among new tuberculosis cases and 4.6% (3·2–6·0) among retreatment cases. The prevalence of MDR TB has varied between all 9 provinces, however, the province of KwaZulu-Natal(KZN),has amongst the highest prevalence of patients with MDR-TB, showing an overall MDR estimate of 2.9% (95%CI 1.8 -4.5) which comes in higher than the national average of 2.8% [43]. In 2012, there were 6630 new cases of laboratory confirmed MDR TB and 754 cases of laboratory confirmed extensively drug resistant TB (XDR-TB) cases, making KZN the province with the highest MDR and XDR TB burden in South Africa at the time[44].

Historically, within South Africa, laboratory diagnosis of TB relied on smear microscopy and subsequent culture on liquid or solid media in selected cases. Conventional culture based drug susceptibility testing (DST) was primarily used to confirm MDR and XDR TB. The traditional method of initial TB diagnosis using smear microscopy has several limitations, the

most important being a lack of sensitivity[27]. Reported sensitivities of the improved Light Emitting Diode(LED) microscopy are between 70 and 78% [45, 46].The sensitivity of smear microscopy is further reduced by approximately 15% in patients with HIV co-infection [4]. This reduction in sensitivity within the South African population gave rise to a further diagnostic challenge in view of the high HIV/TB co-infection rate where a considerable number of patients have smear negative TB. The most recent WHO statistics shows that an estimated 60% of the incident TB cases in 2018 were HIV infected [1] .

Due to this high prevalence of TB, TB/HIV co-infection and drug resistant TB in SA, it became increasingly important to implement rapid genotypic methods for detection of TB and rifampicin resistance at the time of initial patient presentation[27]. Therefore, in 2011, following the World Health Organization strong recommendation for the use of Xpert, endorsing it as ‘‘the initial diagnostic test in individuals suspected of MDR-TB or HIV associated TB’’, the South African Department of Health(NDoH) together with National Health Laboratory Services(NHLS), rolled out the use of GeneXpert nationwide[28].The GeneXpert MTB/RIF assay proved superior to smear microscopy for initial diagnosis of MTB. This is largely because of its ability to diagnose MTB and detect Rifampicin resistance on unprocessed clinical specimens, using real time PCR technology. The low limit of detection of 131 CFU/ml greatly improved the sensitivity of the assay and contributed to higher sensitivity than that of smear microscopy. Laboratory evaluations of the GeneXpert MTB/RIF assay showed a sensitivity of 98.3% on smear positive cases [30].

Although the GeneXpert MTB Rif Assay may lead to more TB diagnoses, the introduction of this molecular technique still posed a challenge in our patient population due to its limited sensitivity of 66.1% (95%CI ,56.4,74.9) in patients with smear negative TB[32]. The GeneXpert MTB Ultra was designed to improve the sensitivity of the assay in smear negative TB to 78.9 %(95%CI, 70.0, 86.1), however a considerable gap in sensitivity remains when compared to its performance in smear positive clinical specimens[32]. As a result, the NDoH uses a specific algorithmic approach for the use of GeneXpert in HIV positive patients. In this patient population, a negative GeneXpert result cannot adequately rule out TB and a subsequent specimen for culture confirmation of viable bacilli must be obtained. Thereafter, if TB was cultured, further molecular testing using Genotype MTBDR*plus* is used for the

confirmation of rifampicin and isoniazid susceptibility. In RR-TB and MDR-TB cases further drug susceptibility testing (DST)for second line drugs is required.[33]

Traditionally in South Africa, phenotypic DST for second line drugs was performed on liquid and solid media using the BACTEC MGIT 960 Automated mycobacterial detection system (BD Biosciences Becton, Dickinson and Company; USA) and the agar proportion method respectively. With the new recommendations issued by the WHO, regarding a shortened regimen (SR) for MDR-TB treatment, the requirement for rapid second line DST for fluoroquinolones (FQ) and second line injectable drugs(SLID) became necessary to rapidly triage MDR and RR-TB patients who could benefit from the SR , into using this regimen[5]. The lengthy turnaround time of phenotypic DST became inadequate in the triaging process for patients and a more rapid, genotypic means of testing became mandatory[6].

In 2015, Hain Lifescience released a new version of the Genotype MTBDR*sl* line probe assay Version 2 (SL LPA V2) (Hain Lifescience, Nehran, Germany). This assay offers rapid detection of pre-XDR and XDR TB. The GenoType MTBDR*sl* VER 2.0 (SL LPA V2) is a qualitative test for the identification of MTB and its resistance to FQ and SLID from smear-positive or smear negative sputum specimens and cultured isolates. The genetic targets included in this assay are *gyrA* and *gyrB* for FQ and the *rrs* and *eis* promoter for SLID[34]. Following a Cochrane systematic review, the WHO published a new recommendation in 2016 stating, for patients with confirmed RR-TB or MDR-TB, the Genotype MTBDR*sl* may be used as the initial test, instead of phenotypic culture based DST to detect resistance to FLQ and SLID[7, 8]. There are only a few studies to assess the performance characteristics of the MTBDRsl Version 2. Tagliani et al. conducted a multicenter study on smear positive clinical specimens in Europe. When compared to phenotypic susceptibility testing, the test shows an overall sensitivity and specificity to be 93% and 98.3% for the FLQ and 88.9% and 91.7% for the SLID. However, this study was limited in its ability to include smear negative specimens[39]. Gardee et al. evaluated the performance of the Genotype MTBDR*sl* on known clinical isolates from South Africa. Their study showed a sensitivity and specificity of 100% and 98.9% for FQ when tested against ofloxacin alone and 89.2% and 98.5% for SLID. Here again, a limitation of the study was the lack of direct testing on clinical specimens and more specifically smear negative specimens.[40] Additionally, studies published in the 2016 WHO

policy acknowledges that the results generated from the SL LPA V2 directly from smear negative specimens have a higher inconclusive rate than smear positive specimens[7].

Taking both the shortened MDR treatment and the use of the SL LPA V2 WHO recommendations into consideration, the NDoH and together with relevant stakeholders incorporated these recommendations into clinical practice by developing a revised laboratory – clinical algorithm. South Africa adopted the WHO short MDR-TB regimen as "Modified South African 9-12 Month Regimen" which replaced the existing conventional regimen based on certain inclusion/exclusion criteria. The modified regimen comprises the intensive (injectable) phase; Kanamycin (Km) + Moxifloxacin (Mfx) + Clofazamine (Cfz) + Ethionamide (Eto) +Pyrazinamide (Z) + High Dose Isoniazid (Hh) + Ethambutol (E) for 4-6 months followed by the continuation phase of Mfx + Cfz + Z + E for a further 5 months. In order for the successful triage of RR-TB and MDR TB patients, the mutation patterns for INH, Mfx and Km must be known so treatment can be tailored accordingly. The simultaneous national roll out of the SL LPA V2 grants the laboratory and the clinicians knowledge of these mutations. Together with the use of the SR and the roll out of the Genotype MTBDRsl Version 2, a new laboratory – clinical algorithm termed the Drug Resistant TB Reflex Testing (DR-TB Reflex) was implemented nationally mid-2016. Part of this algorithm includes the use of Genotype MTBDR*plus* followed by Genotype MTBDR*sl* sequentially on clinical specimens regardless of smear status [6].

Since the implementation of DR-TB Reflex testing in KZN in January 2017, there have been no published reports on the experience of the routine use of the SL LPA V2 on clinical specimens within South Africa nor in KZN specifically, where the incidence of smear negative TB remains high. The performance of diagnostic tests in programmatic settings is often inferior compared to that in controlled research settings. Given the high burden of drug resistant TB and HIV associated TB in KZN, this is an ideal region to assess the feasibility of introducing the SL LPA V2 in the DR-TB reflex algorithm. Therefore, evaluating the performance of the SL LPA V2 in the DR-TB reflex algorithm, in an area with high TB/HIV prevalence, will significantly contribute to our knowledge as to how feasible the routine use of this assay is within our population.

Aim of the research project

The overall goal of this research project is to assess the feasibility of routine use of the SL LPA V2 in DR-TB Reflex testing within the KZN central TB laboratory, where a fair proportion of smear negative cases of TB are observed.

The objective of this research is to describe the experience of the routine use of the SL LPA V2 within the DR-TB Reflex testing algorithm in the KZN central TB culture laboratory by looking at:

1) The yield of MTBDRsl when used directly on smear positive compared to smear negative clinical specimens.
2) Describe the mutation patterns detected by the MTBDRsl V2.
3) Describing how often partially inconclusive results were observed.

Methods

Study design

The study is a retrospective observational study using laboratory data for January – December 2018.

Study setting

In all newly diagnosed adults and children with RR-TB diagnosed by GXP, a second sputum specimen is submitted to the SANAS accredited provincial TB reference laboratory for DR-TB Reflex testing. This testing comprises a "super-set" of tests, which include smear microscopy, TB culture, both Genotype MTBDRplus(FL LPA) and Genotype MTBDRsl(SL LPA V2) line probe assays, as well as phenotypic susceptibility testing, using agar proportion method, in selected isolates where resistance is detected on both LPA results. Both line probe assays are performed irrespective of smear status. The first line LPA is performed to confirm the resistance to Rifampicin and test for Isoniazid susceptibility. The second line LPA is performed to detect mutations in FQ and SLID, which classifies patients as pre-XDR or XDR TB. Once the mutations from both line probe assays are analyzed, further phenotypic DST using agar proportion method is performed on all isolates showing double INH mutations (i.e. *katG* and *inhA*), all pre-XDR and XDR isolates. Phenotypic DST for Ofloxacin,

Moxifloxacin (high and low levels), Capreomycin and Kanamycin were performed on those selected cultured isolates.

Laboratory Procedures

Smear microscopy: is performed using the Auramine O staining method and in accordance with the standard operating procedure.

TB Culture: MTB isolation from clinical specimens is performed routinely using the automated BACTEC mycobacteria growth indication tubes (MGIT).

DNA Extraction: DNA was extracted from clinical specimens using a commercially available kit, Genolyse (Hain Lifescience) and was used in accordance to the manufacturer's guideline

Genotype MTBDRsl assay: Amplification and hybridization of DNA extracted from clinical specimens were performed according to the manufacturer's guideline.

DST: Phenotypic susceptibility testing was performed using 1% agar proportion method on Middlebrook Media (7H10) for FQ (Ofloxacin and Moxifloxacin) and SLID (Kanamycin and Capreomycin).

Data collection

Collection of all test results will be obtained from the laboratory information system (LIS) which contains all electronic laboratory results.

Data analysis

Data analysis will be performed using Microsoft Excel and Epi Info software.

Ethical consideration

No patient data and no consent, routine data analysis

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