

# **Comparative evaluation of molecular-based detection methods for drug resistant tuberculosis**

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I, Jennifer Williams, declare that the dissertation entitled 'Comparative evaluation of molecular based detection methods for drug resistant tuberculosis' which I hereby submit to the North-West University is in compliance of the *Master of Sciences in Pharmaceutical Science* degree, is my own work and has not yet been submitted to any other university.

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## LIST OF ABBREVIATIONS

|             |                                              |
|-------------|----------------------------------------------|
| <b>AG</b>   | Aminoglycosides                              |
| <b>AMK</b>  | Amikacin                                     |
| <b>CAP</b>  | Capreomycin                                  |
| <b>CTB</b>  | Centre for Tuberculosis                      |
| <b>DR</b>   | Drug-resistant                               |
| <b>EMB</b>  | Ethambutol                                   |
| <b>FAM</b>  | Fluorescein amidites dye                     |
| <b>FQ</b>   | Fluoroquinolones                             |
| <b>INH</b>  | Isoniazid                                    |
| <b>KAN</b>  | Kanamycin                                    |
| <b>L</b>    | Litre                                        |
| <b>LMIC</b> | Low- and middle-income countries             |
| <b>LPA</b>  | Line probe assay                             |
| <b>LZN</b>  | Linezolid                                    |
| <b>MDR</b>  | Multi-drug resistant                         |
| <b>MGIT</b> | Mycobacteria growth indicator tube           |
| <b>µl</b>   | Microlitre                                   |
| <b>mL</b>   | Millilitre                                   |
| <b>MTB</b>  | <i>Mycobacterium tuberculosis</i>            |
| <b>MTBC</b> | <i>Mycobacterium tuberculosis</i> complex    |
| <b>NICD</b> | National Institute for Communicable Diseases |

|               |                                                        |
|---------------|--------------------------------------------------------|
| <b>PCR</b>    | Polymerase Chain Reaction                              |
| <b>QUADAS</b> | Qualitative assessment for diagnostic accuracy studies |
| <b>RIF</b>    | Rifampicin                                             |
| <b>ROX</b>    | Carboxyrhodamine                                       |
| <b>sl</b>     | Second line                                            |
| <b>TB</b>     | Tuberculosis                                           |
| <b>VIC</b>    | <i>Victoria</i> carboxy-fluorescein dye                |
| <b>WGS</b>    | Whole genome sequencing                                |
| <b>wt</b>     | Wild type                                              |
| <b>XDR</b>    | Extensively drug-resistant                             |

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## ABSTRACT

Tuberculosis (TB) is a globally problematic infectious disease and is currently one of the leading causes of death from any infectious disease worldwide. *Mycobacterium tuberculosis* (MTB) is notorious for developing resistance to currently available treatment, and according to the World Health Organization (WHO), 157947 cases of multidrug-resistant tuberculosis (MDR-TB) were reported globally in 2020. South Africa is classified as a high TB burden country and accounts for 6784 of the MDR-TB cases detected in 2020 (World Health Organization, 2021). This highlights the need for new and improved methods for TB drug resistance profiling. Sensitive, specific, and reproducible are some of the most important characteristics of an advanced molecular-based drug resistance profiling test. Firstly, a meta-analysis was performed to compare evidence for highly sensitive and specific *Mycobacterium tuberculosis* complex (MTBC) drug resistance profile determination. We did this to answer the question whether the Mycobacterial Growth Indicator Tube (MGIT) still serves as a good representative gold standard with all the recent advancements in molecular based diagnostics? The study outcomes were used to determine and assess sensitivity, specificity, and reproducibility of the respective drug resistance profiling kits from literature. A total of eighteen studies were identified for comparison between MTBDR*plus* V2.0 (n=9), MTBDR*sl* V2.0 (n=6) and Nipro NTM + MTBDR (n=3). Each method had related advantages. The turnaround time of the included methods are rapid compared to culture methods and the reported results yielded high accuracies especially for the MTBDR V2.0 line probe assays. Secondly, we evaluated the commercially available Hain GenoType® MTBDR*plus* and *sl* Version 2.0 kits, as well as the recently developed CapitalBio Gene Real-Time PCR Detection Kit. A total of 92 blinded and randomized MTBC DNA samples received from the Centre for Tuberculosis (CTB) at the National Institute for Communicable Diseases (NICD) and subjected to analyses by each kit. The kits were evaluated in terms of diagnostic accuracy, affordability, ease of use, turnaround time and robustness. The Hain GenoType® MTBDR V2.0 assays had the highest rates of accuracy with whole genome sequencing as reference standard. The CapitalBio Gene Real-Time PCR Detection Kit is suitable for implementation due to its affordability, rapid turnaround time and wide detection range of first-and second-line drug resistance in TB patients simultaneously, however it does require optimisation. This study determined that the Hain GenoType® MTBDR V2.0 assays are superior to the CapitalBio Gene Real-Time PCR Detection Kit when all aspects of advanced diagnostics for DR-TB in South Africa are considered.

### Key terms:

*Mycobacterium tuberculosis* complex (MTBC), drug resistance, diagnostics, genotyping, multi-drug resistant TB

## CHAPTER 1: STUDY BACKGROUND AND SCOPE

### 1.1 BACKGROUND

Tuberculosis (TB) has been documented over thousands of years and is conclusively regarded as one of the deadliest diseases in human history (Cruz-Knight & Blake-Gumbs, 2013). Although routinely curable, TB is the leading cause of death by a single infectious agent worldwide (World Health Organization, 2018). Tuberculosis has long been a global health threat even before its classification in 1882 by Dr Robert Koch (Willey *et al.*, 2011). Koch successfully isolated and characterised the causative agent of the disease as *Mycobacterium tuberculosis* or tuberculosis (TB) after the tubercles that form at the site of infection. Koch not only paved the way for future innovations into combatting this deadly disease but also made it possible to diagnose one of the most lethal diseases in human history (Willey *et al.*, 2011). The global burden has been reduced to a far less threatening size compared to when the tuberculosis infection was first described. In ancient China, tuberculosis was described as early as 2700 BC and in India as early as 3300 BC (Brown, 1942; Roberts & Buikstra, 2003; Crawford & Campbell, 2012; Barberis *et al.*, 2017). Recent studies still classify TB as the second leading cause of death by a communicable disease globally — second only to HIV (human immunodeficiency virus) — killing more than 1.3 million people annually (Drobniewski *et al.*, 2015; World Health Organization, 2018). The Global Tuberculosis Report, published annually by the World Health Organization (WHO) since 1997, contains estimates and statistics of the imputed burden caused by this persistent disease. In 2013, tuberculosis was declared a global health crisis by the WHO. Individuals from any age group and any country can contract TB from an infected host and symptoms only arise when the infection becomes active (Wei *et al.*, 2000).

Latent TB can be dormant in the respiratory system for months and even years before onset — this is due to the bacterium's slow culture rate. TB is caused by bacteria in different subgroups from the *Mycobacterium tuberculosis* complex (MTBC) and include *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. caprae*, *M. canettii*, *M. pinnipedii*, *M. mungi* and *M. microti*. These bacteria are opportunistic pathogens by nature (Willey *et al.*, 2011; Van Ingen *et al.*, 2014; King *et al.*, 2017). The body can easily eliminate bacteria from the lungs by encasing the organisms in tissue, but this is not functional when the immune system is jeopardised (Hunter, 2011). Around one quarter of the world population is said to be infected with latent TB (Suárez *et al.*, 2019). Latent-dormant TB becomes active in individuals with a compromised immune system, which makes HIV an enormous risk factor due to co-infection. This explains the correlation between a high HIV burden and

simultaneous high TB burden in South African populations (Ismail *et al.*, 2018; World Health Organization, 2018).

The weight of the tuberculosis burden in low- and middle-income countries (LMICs) is worsened by the increasing frequency of drug resistance development and direct transmission of drug resistant strains between infected individuals (Ismail *et al.*, 2018). Transmission is especially enhanced in conditions where there is low airflow and overcrowding, which are common working and living conditions in LMICs. The before mentioned risk factors explain why rural areas in Brazil, China, India, Indonesia, Nigeria, Philippines, South Africa and 14 other countries with the same or similar conditions, are amongst the most highly impacted by TB (World Health Organization, 2016; World Health Organization, 2018). Diagnosis and treatment of tuberculosis in LMICs is currently determined by the international guidelines recommended by the WHO on an annual basis (Ismail *et al.*, 2018). Drug sensitive TB (DS-TB) is curable at a success rate of 82% by the most cost-effective means, with an estimated 301 000 instances in South Africa in 2018 (World Health Organization, 2019). The global health threat is linked to drug resistant TB (DR-TB) and in worse cases, multi-drug resistant (MDR-TB) and extensively drug resistant TB (XDR-TB) (Basu, 2019). There were an estimated 11 000 instances of MDR-TB cases in South Africa in 2018, considering only 235 652 of new and relapse TB cases were notified in total. (World Health Organization, 2019).

## **1.2 PROBLEM STATEMENT**

Due to rapid mutational changes in the bacterial genome, there is a great need for development of an all-inclusive drug-resistance profiling assay (Coscolla & Gagneux, 2010). The global need is to extend the answer of active TB further and determine the specific antibiotic regime, which would by the quickest means, ultimately cure a drug-resistant TB infection. Existing drug-resistance profiling kits must be continuously evaluated to determine the path of focus for future development, especially considering that mutations have been found to be population specific (Zignol *et al.*, 2016; Osei Sekyere *et al.*, 2019). There has been considerable progress towards developing a rapid, accurate and affordable diagnostic method that encapsulates not only diagnosis of an active TB infection, but also differentiates between the resistance-conferring mutations present in the specific strain contracted by the patient. Diagnostic algorithms must stay updated to better understand the implications of certain diagnostic methods and numerically pool data for collective and accurate evidence of current trends in the field of diagnostics (Creswell *et al.*, 2019). Most advances are in the field of molecular diagnostics, discovery and improvement of numerous techniques, and implementation of those that would be most effective in all healthcare settings is made

difficult due to several reasons. Not only does the cost of equipment and consumables inhibit advancement in TB diagnostics in LMICs but also the need for space and specialized working conditions to prevent further transmission. Lack of understanding of the disease itself among individuals contributes to poor management and development of drug resistance (Willey *et al.*, 2011; World Health Organization, 2018). The lack of specific and current data on rates and occurrence of resistance also contributes to poor planning and stagnation in TB diagnostic advancements (Ismail *et al.*, 2018; Basu, 2019).

To stress the importance of diagnostic algorithms in combatting the DR-TB burden in South Africa, we aimed to conduct a meta-analysis in chapter two of this study. We focussed on molecular methods specifically endorsed by the World Health Organization. The second aim of this study was to evaluate and compare two molecular-based drug-resistance profiling kits based on leading TB diagnostic methods. The first is a line-probe assay-based kit, which is currently implemented in South Africa. The other kit is based on real-time PCR and is in its evaluation phase. In chapter three we highlight the different principles of each kit. The kits will be evaluated based on ability of identification of gene mutations conferring resistance to the standardized anti-TB drugs currently on the market, sensitivity, specificity, turnaround time, ease of use, cost per patient and difficulty of data analysis.

### **1.3 AIMS AND OBJECTIVES**

The aim of this study is to evaluate and compare molecular based methods for detection of DR-TB.

The aims were achieved by:

1. conduct a systematic review and meta-analysis of available molecular diagnostics for drug resistant TB
2. evaluate two molecular based drug resistance detection kits, namely, the CapitalBio Gene Real-Time PCR Detection Kit and both the Hain GenoType® MTBDR*plus* V2.0 and Hain GenoType® MTBDR*s*/ V2.0 line probe assays
3. evaluate suitability of the respective kits in detecting the most frequent drug-resistance conferring mutations found in strains of *Mycobacterium tuberculosis* among South African populations
4. determine and compare sensitivity, specificity, turnaround time, cost per analysis, ease of use of the kits and coupled data analysis software for result interpretation

5. compare our results from evaluation the respective kits to phenotypic drug susceptibility testing (DST) and sequencing results from the Centre for Tuberculosis (CTB) at the National Institute for Communicable Diseases (NICD)

#### **1.4 DISSERTATION OUTLINE**

**Chapter 1:** Chapter 1 (current) is an introductory chapter describing the purpose of this study and how the aim and objectives will be achieved.

**Chapter 2:** Chapter 2 comprises a meta-analysis where three rapid and recent, WHO-endorsed DR-TB kits are compared based on sensitivity, specificity, cost, and ease of use.

**Chapter 3:** Chapter 3 contains the data from evaluations performed in-house to compare two recent DR-TB diagnostic kits by using sequencing data from the NICD.

**Chapter 4:** Chapter 4 is a summative conclusion on the three abovementioned chapters where study outcome, limitations and future recommendations are given.

**Addendum 1** QUADAS-2 tool list of risk of bias and applicability questionnaire

**Addendum 2** Mutations identified within *M. tuberculosis* drug resistance-associated loci according to literature

**Addendum 3** Threshold values of the CapitalBio kit determining a positive or negative result for each resistance gene

## **CHAPTER 2: META-ANALYSIS OF LINE-PROBE ASSAYS TOWARDS REPLACING THE GOLD STANDARD FOR MULTI-DRUG- AND EXTENSIVELY-DRUG RESISTANT TUBERCULOSIS DETECTION**

### **2.1 Introduction**

Tuberculosis (TB) is a globally problematic infectious disease. An estimated 24% of the world is infected with the causative agent of TB — *Mycobacterium tuberculosis* (Cohen *et al.*, 2019). Being infected with *Mycobacterium tuberculosis* (MTB) does not necessarily cause disease. The presence of MTB where clinical symptoms are absent and an individual is not infectious, is classified as latent TB. A healthy immune system readily inactivates the MTB bacillus inhibiting the development of clinical symptoms (Coscolla & Gagneux, 2014) however, between 5% and 10% of individuals with latent TB develop an active TB infection (World Health Organization, 2020). The development of an active TB infection occurs when the immune system is compromised (Narasimhan *et al.*, 2013). *Mycobacterium tuberculosis*, a pervasive and difficult bacillus to culture and differentiate, is notorious for developing resistance to currently available treatment. According to the World Health Organization (WHO) multidrug-resistant tuberculosis (MDR-TB) has recently increased by more than 16% from 2017 to 2018. In 2018; the WHO reported more than 484 000 MDR-TB cases of which only 156 000 were started on treatment and 22% had laboratory confirmed extensively drug resistant tuberculosis (XDR-TB) (World Health Organization, 2019).

#### **2.1.1 Tuberculosis: the longest epidemic in history**

A pandemic is described as a disease or outbreak of a disease that, by transmission, is spread across an entire country and other parts of the world infecting most of the population (Porta, 2014). Tuberculosis has plagued mankind for an estimated 70 000 years (Buzic & Giuffra, 2020) and remains a public health crisis according to the World Health Organization (WHO) (World Health Organization, 2020a). According to Woodruff (2014) the high rates of transmission and deaths by TB saw a decline when streptomycin, the first antibiotic and active agent against TB, was discovered by Dr. Selman Waksman in 1943. An unfortunate consequence of the introduction of streptomycin for TB treatment, was the emergence of drug resistant TB (DR-TB) induced by long treatment regimens (Woodruff, 2014). TB was classified as a pandemic and global health crisis in 1993 and continues to be a burden (Nathavitharana & Friedland, 2015). The incidence of DR-TB is predicted to rise in some high burden countries including South Africa (Sharma *et al.*, 2017), and long treatment regimens are as concerning as the inability of certain diagnostics to detect the disease in less than four weeks. Incidence rates are not as concerning in high income countries with

95% of cases and 98% of deaths rather occurring in low- and middle-income countries (LMICs) (Grange & Zumla, 2002).

### **2.1.2 Essential diagnostics for tuberculosis**

The WHO compiled and released the first Model List of Essential *in-vitro* Diagnostics (EDL) in 2018 (Schroeder *et al.*, 2016; Moussy *et al.*, 2018). The EDL sets out guidelines and recommendations towards which diagnostic tests to use and is supported by evidence and reviews. All disease specific *in-vitro* diagnostics in the EDL reflect current global health concerns (Moussy *et al.*, 2018; World Health Organization, 2019). Moussy *et al.* (2018), mentions that including *in vitro* diagnostics (IVDs) capable of diagnosing antimicrobial resistance is a specific priority for global health reasons. This suggests an urgency for rapid, specific and sensitive tests capable of detecting not only the causative of the patient's infection but also genes or markers that indicate resistance to any antibiotics (World Health Organization, 2020). To overcome diagnostic delays, molecular methods have been widely implemented and are usually accompanied by drug susceptibility testing (DST) in the form of culture methods and/or sequencing in cases regarding multi-drug resistant (MDR) TB and extensively drug resistant (XDR) TB (Schön *et al.*, 2017). With a rising number of acquired resistance towards even the most recently developed antibiotics, e.g., bedaquiline and ethionamide, a quality and reliable diagnostic test for rapid drug resistance profiling would have a positive impact on TB prevention in LMICs (Tejashree *et al.*, 2019). Molecular diagnostic tests usually require advanced equipment and training to some extent and LMICs are not financially able to implement advancements in TB diagnostics without additional support. There are high costs related to TB prevention and control, hence the need for presenting a cost-friendly alternative diagnostic with high accuracy and rapid results. Evaluation of sensitivity gives an indication towards the assays' ability to accurately indicate positive results as positive indicating true positives (TP), i.e., identifying a diseased patient as being diseased (Šimundić, 2009; Baratloo, 2015). Similarly, specificity indicates how accurately the assay identifies negative results amongst susceptible samples indicating true negatives (TN) in a population with regards to drug resistance (Šimundić, 2009). A false positive (FP) or false negative (FN) result can impact the life of a patient and have severe consequences (Kaswa, 2014), therefore, they are core components in accuracy estimation during diagnostic kit evaluation.

### **2.1.3 Current diagnostic trends**

Diagnosis of TB starts with sputum smear microscopy (SSM); a rapid, inexpensive, and simple technique relying on direct microscopic identification of acid-fast tubercle bacteria (Desikan, 2013). Depending on patient clinical history, a patient will be submitted for SSM to

diagnose pulmonary TB (Huddart *et al.*, 2016). Patients are required to provide up to three millilitres of sputum per sample and a minimum of two samples (Médecins Sans Frontières, 2017). Sputum smear microscopy is the first and most straightforward method for TB diagnosis, which is still routinely done to distinguish TB from other bacterial infections (Huddart *et al.*, 2016). However, a low-bacilli-count in a sputum sample is usually the case in patients with human immunodeficiency virus (HIV) co-infection and in children, mainly leading to false negative results and unnecessary isolation, needless costs, and possibly incorrect treatment prescription (Drobniewski *et al.*, 2015; Bajaj & Tombach, 2017). In a study by Noori *et al.* (2016) where SSM data was collected over three years, results showed that about 12% of smear negative patients were suffering from TB. The application of SSM is arduous for sputum samples with a bacillary load below 5000 bacilli/mL and the presence of non-tubercle mycobacteria (NTM) severely compromises the specificity (Médecins Sans Frontières, 2017; Lee *et al.*, 2019). Sputum smear microscopy is either followed up by BACTEC™ MGIT™ or Xpert® MTB/RIF and MTB/RIF Ultra to detect drug resistance. Disadvantages of culture methods such as BACTEC™ MGIT™ include high contamination rates, expensive equipment, training requirements, repetitive sample processing, long turnaround times and false diagnostic results (false positives or false negatives) (Diriba *et al.*, 2017; Mahomed *et al.*, 2017). Becton, Dickinson and Company's Mycobacterial Growth Indicator Tube (MGIT)™ specifically BACTEC™ MGIT™ liquid culture system is considered to be the gold standard towards drug resistant TB detection (World Health Organization, 2020a). Culture is a highly accurate, specific, and sensitive method, which makes it the widely used reference method today. The main limitation of BACTEC™ MGIT™ is the turnaround time, which could take a minimum of two weeks and, in some cases, results can take up to eight weeks (Pfyffer *et al.*, 2012). Other conventional culture methods include Löwenstein–Jensen solid culture and BacT/Alert® culture bottles (BioMérieux, Durham, NC) liquid culture media (Mattei *et al.*, 2009; Diriba *et al.*, 2017). Phenotypic DST is routinely used to (1) detect drug resistance, (2) confirm a smear negative result when clinical symptoms are representative of TB infection, (3) detect extra-pulmonary TB and (4) distinguish between members of the MTBC and NTMs among other reasons (Médecins Sans Frontières, 2017). Suspected MDR or XDR cases are further analysed using line-probe assays (LPAs) (World Health Organization, 2020). When a diagnosis is made, a patient with drug susceptible (DS) TB will enter the standard six-month treatment regime as recommended by the WHO, whereas longer, more toxic treatment regimens are prescribed to patients with DR-TB infections (Heyckendorf *et al.*, 2018; World Health Organization, 2018).

#### **2.1.4 Diagnostic challenges caused by genetic mutations**

Conventional diagnostic methods are widely used but insufficient for diagnosis of DR-TB. Hence, the increasing development of molecular methods and cost-effective methodology (Akkaya & Kurtoglu, 2019). Currently, many molecular diagnostics exist that can accurately detect rifampicin (RIF) and isoniazid (INH) resistance in an active TB infection. These tests, however, do not encompass the entire list of resistance-conferring mutations associated with available anti-TB drugs and imperfect sensitivity and specificity estimates still exist (Coll *et al.*, 2015). According to Phelan *et al.* (2018), drug resistance in MTB is predominantly caused by mutations within the MTB genome, including single nucleotide polymorphisms (SNPs) and insertions and deletions (Phelan *et al.*, 2018). Some SNPs are still undetectable and lead to inaccurate results especially with SNPs in second-line resistance gene regions that are considered unpredictable (Tekin *et al.*, 2017; Dicks & Stout, 2019). We may assume that MTB survival could be impaired by accumulation of resistance-conferring mutations, but this is overcome by putative mechanisms that offer compensation for fitness loss (De Vos *et al.*, 2013). Among the abovementioned principles of DR-TB diagnosis and treatment, there is apparent evidence that each DR-TB case must be treated uniquely after identifying it. High-income countries have the available resources to provide a laboratory diagnosis as recommended by the WHO through clinical laboratories, whereas LMICs need to request further analysis by an external laboratory service (Davids *et al.*, 2015; Dicks & Stout, 2019). Strain-specific qualities exist within the *Mycobacterium tuberculosis* complex (MTBC) that have clinical consequences and must also be considered (Nicol & Wilkinson 2008; Moopanar & Mvubu, 2020). These are just a few challenges faced by healthcare professionals when presented with a patient who presumably has TB. Firstly, it is of utmost importance to rule out antibiotics to which a specific strain is resistant. This will dramatically improve usage of antibiotics for TB treatment further reducing the risk of developing antibiotic resistance (Eddabra & Benhassou, 2018).

#### **2.1.5 Major challenges and clinical properties of tuberculosis**

Diagnosing TB can be challenging for four main reasons; unspecific clinical properties, imperfect diagnostic detectability, drug resistance and culture time (Das *et al.*, 2018; Dicks & Stout, 2019). The most common symptom of an active TB infection is persistent coughing (more than three weeks), but this is symptomatic of a wide variety of infections of the respiratory system, including bacterial, chlamydial, fungal, parasitic, and viral (Das *et al.*, 2018; Willey *et al.*, 2017). A patient would have a persistent cough and at least one of the following symptoms: fatigue, shortness of breath, fever, night sweats, appetite loss and weight loss before being treated as a presumptive TB case (Ismail *et al.*, 2018). It is thus clinically impossible to diagnose a patient with TB before undergoing further diagnostic measures (Smith, 2003). Unlike some bacterial infections that target the pulmonary region,

tuberculosis has a very slow onset and culturing the bacteria to produce diagnostic results can take up to six weeks (Rea *et al.*, 2019). Mycobacterial culture methods incur related disadvantages. The most attractive test-and-treat strategy is not possible with culture methods and patients could develop resistance towards first-or second-line anti-TB drugs during the waiting period for the final diagnostic report (Dicks & Stout, 2019). Culture related disadvantages could be resolved by implementation of advanced point-of-care molecular diagnostics. The goal is to give an indication towards how to effectively treat a patient and alleviate symptoms arising from an infection as soon as possible. The second most important goal of diagnosis is to prevent an infected patient from spreading the disease when such is suspected (Bossuyt *et al.*, 2012). To maintain the findings of Bossuyt *et al.* (2012), the purpose of a diagnostic test is to prevent suffering and premature death when functional health is jeopardised. In South Africa, the guidelines of diagnosis and management of tuberculosis, formulated by the National Department of Health, are based on the World Health Organization Consolidated guidelines (World Health Organization, 2019). The guidelines entail identifying signs and symptoms of TB in patients at Primary Health-Care Facilities and General Hospitals and collecting sputum samples, thereafter samples are transported to Reference Laboratories for confirmation of DR-TB (Department of Health, 2019). Reports are sent within 24 hours to requesting facilities and patients are recalled based on results with a confirmatory diagnosis being made based on a second sputum sample. Patients with a positive DR-TB diagnosis are counselled, started on treatment, and hospitalized in severe cases (Department of Health, 2019).

#### **2.1.6 The global burden**

The difficult task of diagnosing tuberculosis has been documented for more than 100 years (Smith, 2003). Healthcare professionals and researchers alike strive towards alleviation of this common human enemy with little to no foothold in future adaptations in its genome. Eradicating tuberculosis and more importantly, drug resistance, lies in predicting clinically relevant resistance patterns (World Health Organization, 2018) and intervention within multiple sectors. This may be achieved by studying mechanisms towards genetic resistance acquisition and keeping genetic databases, e.g. ReSeqTB Consortium and CRyPTIC library, updated (Phelan *et al.*, 2019). Using available genetic databases and newly developed tools such as Resistance Sniffer, developed by Muzondiwa *et al.* (2020), may aid in TB diagnostic challenges faced worldwide. The WHO annually publishes a TB report and is in constant development of TB alleviation strategies. Worldwide, research is being conducted on anti-TB drugs, diagnostic methods for TB diagnosis, social and political approaches towards TB alleviation and community interventions (World Health Organization, 2019). In the Global TB Report published annually by the World Health Organization, it showed that more than

340 000 children under five years old were started on TB preventative treatment in 2019. In South Africa specifically, 25 000 children were started on TB preventative treatment since it was established that they were household contacts of TB patients (World Health Organization, 2019). The global efforts to alleviate the TB burden aim at reducing TB instances to 520 000 from 10.4 million and TB deaths from 1.4 million to 110 000 by 2035 compared to 2015 (World Health Organization, 2015). Major categories of intervention for TB prevention include (1) preventative treatment, (2) infection prevention and control by preventing transmission, and (3) preventative vaccination of children with the bacille Calmette-Guérin (BCG) vaccine (Velayati & Farnia, 2017).

### **2.1.7 Standards for diagnostics**

In 2006, Peeling *et al.* reported on the development of a useful criteria to serve as a benchmark for diagnostic tests for sexually transmitted diseases, namely: ASSURED – Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable. The criteria originated from extensive research by the WHO Sexually Transmitted Diseases Diagnostics Initiative (SDI) (Peeling *et al.*, 2006; Kosack *et al.*, 2017). These criteria can be applied during development of an all-inclusive, drug-resistant TB diagnostic test specifically for use in LMICs (Kosack *et al.*, 2017). The criteria were updated by Land *et al.* (2019) to produce the RE-ASSURED criteria which include additional evaluations R (real-time connectivity) and E (ease of specimen collection and environmental friendliness) for improvement of quality control during TB diagnostic strategies. The RE-ASSURED criteria could not be applied to current TB diagnostics as real-time connectivity of mobile equipment is not yet available and samples are of invasive origin (Land *et al.*, 2019). The disadvantage of the first and most straightforward diagnostic – SSM – is the low diagnostic accuracy and high rates of false negative results requiring further analysis. HIV-TB co-infected patients, extrapulmonary-TB patients and children frequently have trouble producing enough sputum for SSM leading to difficulties during sample processing and diagnosis (Sakundarno *et al.*, 2009). Culture methods remain the gold standard for drug resistance detection regardless of the high related cost, technicality, long waiting period to diagnosis and necessity for follow-up visits to deliver test results to patients (Land *et al.*, 2019). BACTEC™ MGIT™ liquid and solid culture methods are based on direct phenotypic DST at minimum inhibitory drug concentrations (in mg/L or µg/mL) included in the culture medium (Heyckendorf *et al.*, 2018). BACTEC™ MGIT™ has been the subject of meta-analyses for over thirty years since its application in clinical settings. One of the first BACTEC™ MGIT™ evaluations was performed in 1981 (Siddiqi *et al.*, 1981) based on radiometric detection of the metabolic activity of *Mycobacterium tuberculosis* and the work of Cummings *et al.* (1975). One study, Hasan *et al.* 2013, recorded 100% sensitivity and

93.3% specificity during evaluation on 421 clinical isolates. There have been reports of false negative results with BACTEC™ MGIT™ possibly due to low minimum inhibitory concentrations used during routine testing (Pang *et al.*, 2016). Until recently, the WHO has endorsed five rapid molecular diagnostic tests capable of simultaneous detection of MTB and multi-drug resistant TB (MDR-TB), Xpert® MTB/RIF and MTB/RIF Ultra by Cepheid (CA, United States), Truenat MTB and MTB Plus assays and line probe assays (LPAs) – specifically, Nipro NTM+MDRTB (RIF/INH) by Nipro Corporation (OSA, Japan) and GenoType® MTBDRplus Version 2.0 (RIF/INH) and GenoType® MTBDRs/ Version 2.0 (FLQs, KAN, AMK, CAP) by Hain Lifescience (BW, Germany) (World Health Organization, 2020a). According to the WHO operational handbook on tuberculosis, laboratory technologists not only need training and specialist expertise but also require advanced infrastructure and equipment to perform the abovementioned tests (World Health Organization, 2020a). When phenotypic characteristics are analysed, BACTEC™ MGIT™ is sufficient and can give an accurate initial diagnosis. Genotyping, on the other hand, is not possible with BACTEC™ MGIT™, which consequently requires further analysis at reference laboratories via molecular methods endorsed by the WHO (Schön *et al.*, 2017).

#### **2.1.8 Molecular diagnostics for tuberculosis**

There are many reports on the high accuracy of the Xpert® MTB/RIF cartridge and the wide application in a variety of settings due to the user-friendly nature of the design. A study by Dorman *et al.* (2018) that evaluated Xpert® MTB/RIF and Xpert® MTB/RIF Ultra, reported sensitivity and specificity of 95% and 98% respectively for Xpert® MTB/RIF but sensitivity was very low (46%) for RIF resistance detection in smear-negative samples. Xpert® MTB/RIF Ultra has improved accuracy for detecting resistance amongst these populations at 63% (Dorman *et al.*, 2018). Conventional culture methods could take eight weeks before a conclusive diagnosis can be made (Pfyffer *et al.*, 2012) compared to Xpert® MTB/RIF, which only takes about two hours (Park, 2019). Comparable to Xpert® MTB/RIF is the WHO-endorsed Truenat MTB-RIF Dx assay with a reported sensitivity of 93% and specificity of 95% for detection of MTB and RIF resistance (World Health Organization, 2020b). A recent report by Mvelase *et al.* found that among 88 559 DST analyses, 18 352 were RIF resistant and 19 190 were INH resistant infections, more than 20% of results. Evidence suggests that RIF mono-resistance and INH mono-resistance is a growing concern that must be addressed by simultaneous detection of resistance conferring mutations (Mvelase *et al.*, 2019; Malenfant & Brewer, 2021). The most recent advancements in molecular diagnostics allow for detection of more than one resistance conferring gene. The most recent WHO-endorsed LPAs have the advantage of simultaneous detection of more than one anti-TB drug. These tests include Hain GenoType® MTBDRplus and -sl, which take approximately

four hours for multi-drug resistance detection (Tekin *et al.*, 2017). Test gene identification capacity is currently limited to three genes each but encompass relevant resistance-conferring regions. These tests however, do not allow for the detection of newly approved MDR-TB management drugs that are included in longer MDR-TB regimens e.g., bedaquiline, linezolid, delamanid pyrazinamide and ethambutol and resistance to these drug have been detected previously (World Health Organization, 2019). Hain GenoType® MTBDR<sub>plus</sub> V2.0 can detect mutations among the *rpoB*, *katG* and *inhA* gene regions by targeted hybridization through the polymerase chain reaction (PCR) to 27 distinctive probes or reaction zones. Hain GenoType® MTBDR<sub>s</sub>/ V2.0 is a line-probe assay that allows for detection of second-line resistance conferring mutations within the *gyrA*, *rrs* and *gyrB* gene regions by targeting 27 different probes. Nipro Genoscholar NTM+MDRTB is a line-probe assay that detects RIF and INH resistance simultaneously by targeting regions within the *rpoB*, *katG* and *inhA* genes developed by Nipro Corporation (OSA, Japan) and is endorsed by the WHO (Nathavitharana *et al.*, 2015).

## **2.2 Aim and objectives**

The aim of this study is to find and compare recent data on commercially available molecular drug resistance testing kits for tuberculosis. The first objective of this study is to conduct a systematic literature search on recent evaluations providing diagnostic accuracy measures for DR-TB compared to phenotypic DST. The second objective is to conduct a meta-analysis of the identified academic sources to determine superiority amongst the selected diagnostic methods for diagnosing DR-TB.

## **2.3 MATERIALS AND METHODS**

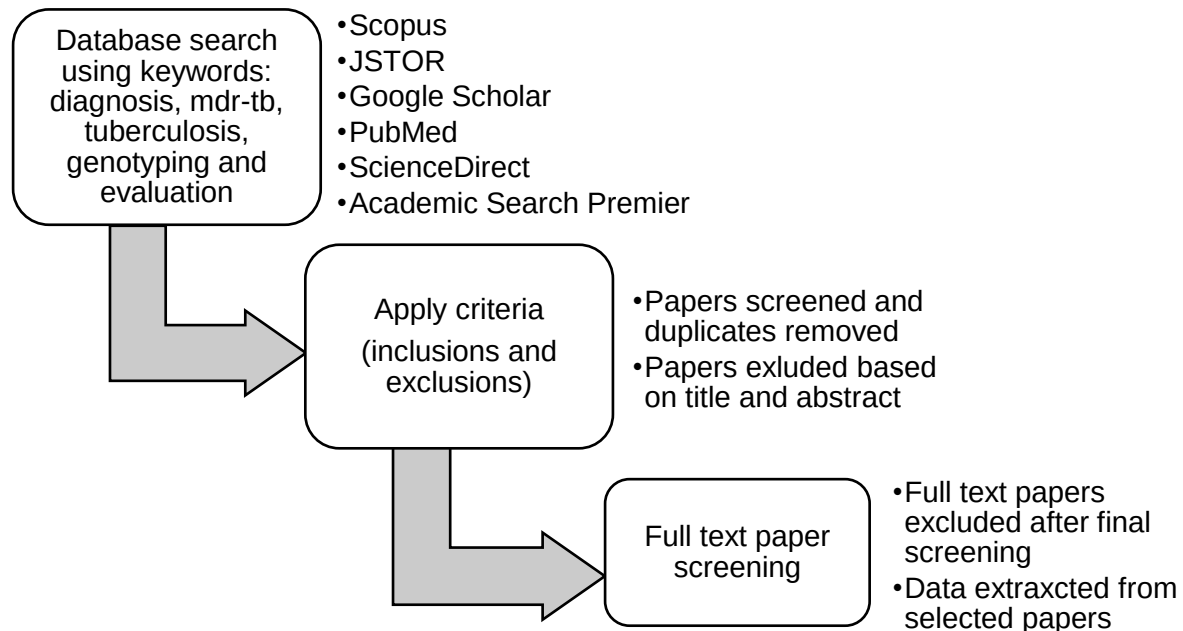
### **2.3.1 Data sources and synthesis**

A comprehensive literature search was used for this review and research articles from scientific databases such as PubMed, ScienceDirect, Academic Search Premier, Scopus and Google Scholar were included. Articles were limited to publication dates between 2012 to 2021 with emphasis on first- and second-line drug resistance detection when keywords such as diagnosis, MDR-TB, tuberculosis, genotyping and evaluation, as well as languages particularly Zulu, Xhosa, Sotho, and Afrikaans were added to the browsing engine.

#### **2.3.1.1 Study processing**

Figure 1 illustrates the literature and data processing. A custom search of five electronic literature databases was performed to identify potentially eligible studies for the review. The design is based upon keywords related to current molecular diagnostic evaluations existing for DR-TB identification. The specific search query applied was: (((diagnosis) AND (mdr-tb))

OR (tuberculosis)) AND (genotyping) AND (evaluation)). Selected sources were included in the meta-analysis after the criteria were applied. Studies were included regardless of sample type and demographics since few studies recorded patient information. We targeted diagnostic accuracy reviews and study eligibility was not blinded to publication details.



**Figure 1: Process of searching, sorting, and screening of academic database search results to identify suitable studies for meta-analysis**

### 2.3.1.2 Study selection

We screened article abstracts for studies that evaluated sensitivity and specificity of line-probe assays compared to phenotypic DST (culture), sequencing or a composite reference standard to perform this meta-analysis. Inclusion and exclusion criteria were applied to condense the dataset (Figure 1). Specific criteria for the meta-analysis were:

- (1) study must be published in a peer-reviewed scientific journal
- (2) study must have a cultured sample size higher than 50
- (3) study must be published after 2012 (as to include recent developments in TB drug resistance diagnosis)
- (4) study must evaluate a WHO-endorsed molecular kit for TB drug resistance profiling
- (5) study must be able to detect resistance to at least two anti-TB drugs simultaneously.

Only studies evaluating the second generation of Hain diagnostic line-probe assays (V2.0) were included. The first version (Hain GenoType® MTBDR*plus* V1.0) has been subjected to

numerous meta-analysis since its release in 2004 (Ling *et al.*, 2008; Drobniowski *et al.*, 2015; Nathavitharana *et al.*, 2016) and therefore was not included in our study.

### **2.3.1.3 Data extraction**

Data extraction was performed manually, and missing data calculated where possible (means, ranges, and standard deviations). Sensitivity, specificity, positive predictive value, and negative predictive values for each study, were calculated where necessary according to standard equations (Sensitivity equals true positive results divided by the sum of the true positives and false negative results; specificity equals the true negative results divided by the sum of the true negative results and false positive results) (Adam *et al.*, 2019). Data extractions are reported in the form of forest plots. Data captured for each study was manually added in a data table and included the number of true positives, true negatives, false positives, and false negatives. Sensitivity and specificity, each with a confidence interval of 95% was automatically calculated by RevMan 5.4.1 using individual study data and reviewed for any differences between the generated values and reported data. Pooling estimates was avoided due to heterogeneity concerns among selected studies. We estimated and included the proportion of true positives and true negatives for each study.

### **2.3.2 Quality of methodology and heterogeneity assessment**

To assess the quality and methodology for each included study, we performed the QUADAS-2 analysis (Quality Assessment of Studies of Diagnostic Accuracy included in Systematic Reviews-version 2 tool), SROC (summary receiver operator characteristics curves) and forest plot analysis and ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment free and Deliverable) analysis based on previous reviews and specific guidelines further explained in the following sections.

#### **2.3.2.1. Forest plot and summary receiver operator characteristics curve analysis**

Forest plots provide graphical representations of diagnostic test meta-analyses representing the effect size of each individual study and summary estimates with corresponding confidence intervals (Schild & Voracek, 2015). Corresponding confidence intervals (CI) of 95%, represented by whiskers, express the degree of uncertainty around the effect estimate. The first column of the forest plot figure indicates study identity, the following four columns represent the extracted data for each study; true positives, false positives, false negatives and true negatives respectively, thereafter the sensitivity and specificity with 95% CI estimates and lastly the graphical representation of the forest plots as histograms (Alavi *et al.*, 2020). Summary receiver operator characteristics curves were constructed to assess heterogeneity of the data using RevMan 5.4.1. Heterogeneity is visualized on a graph by

plotting the logit value of sensitivity, which is the rate of true positive results, against specificity, the rate of false positive results. The curve starts at a point where the axis values are both zero and moves in an upper-right direction to the point where both values are equal to one (Macaskill *et al.*, 2010).

#### **2.3.2.2 QUADAS-2 analysis**

Quality and heterogeneity assessment of study methodology was performed using the improved version of the Quality Assessment of Studies of Diagnostic Accuracy included in Systematic Reviews-2 tool (QUADAS-2) instrument. Using QUADAS-2, a tool validated towards study quality assessment (Whiting *et al.*, 2003), and analysis of summary receiver operating characteristics plot (SROC) curves, quality determining factors can be visualized and heterogeneity among studies detected.

The QUADAS-2 questionnaire to determine bias or applicability concerns consisted of 18 questions related to study methodology. Answers were selected based on manual interpretation of study literature and available study datasets and were limited to simplified answers; yes or no, and low, high or unclear. Questions were formulated around: risk of bias assessment during patient selection; risk of bias assessment during the evaluation of the index test; risk of bias assessment regarding reference standard conduct; risk of bias assessment about the flow and timing of a study; and applicability concerns. The complete questionnaire is available in addendum 1.

### **2.3.3 ASSURED analysis**

The ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment free and Deliverable) criteria is important in comparison of TB diagnostic tests since it represents three fundamental features: accuracy, accessibility and affordability (Peeling *et al.*, 2006). To determine the plausibility of implementing a new gold standard for tuberculosis drug resistance detection, factors influencing the decision to implement the test must be considered. Therefore the ASSURED criteria were applied to each test identified among the included sources and details of the characteristics of each test were sourced from existing literature. The RE-ASSURED criteria could not be applied as studies did not report on real-time connectivity or the method of collection of samples and instead relied on the use of previously processed and analysed samples. Each standard was applied according to the following guidelines:

#### **1. Affordable**

Affordability is influenced by a variety of factors from cost of transport to nearest testing facility, to cost of a single test for an accurate diagnosis. For our purposes, we rated less than R141 (10 USD) as highly affordable and more than R352 (25 USD) as unaffordable, or low affordability (Land *et al.*, 2019).

#### **2. Sensitive and Specific**

Currently there are no WHO recommended DR-TB diagnostic tests with a sensitivity lower than 73% and specificities no lower than 95%. A test was categorised as highly sensitive or specific if the diagnostic accuracy values ranged between 90–100% and too low for recommendation of usage when the sensitivity and specificity estimates were below 60% (World Health Organization, 2014).

#### **3. User-friendly**

User-friendly refers to how much training is needed to use each test. This is ranked from highly user-friendly to not user-friendly. A highly user-friendly test is extremely easy to use, requires minimal training and has a low risk for contamination (García-Basteiro *et al.*, 2018). Results were generated by literature search for reports on usage of the included tests by healthcare workers as few diagnostic test accuracy studies included the data. According to Land *et al.* (2019), a test is user-friendly if it requires no more than three steps to complete and if no previous knowledge regarding diagnostic testing to complete a short training course is required. Complicated protocols cannot be performed by untrained individuals and require specialised healthcare professionals with relevant qualifications at reference laboratories to perform and interpret tests.

#### **4. Rapid and robust**

The rapidness of tests can be defined on how fast the test can be performed and how long it takes to generate results. Study methods and results were screened for reports on the time taken from start to finish for each test. According to a survey by Davids *et al.* (2015) on the use of rapid tests in South Africa, primary health care providers deem a period of 24 hours as acceptable due to the option of sending the patient home without the need to wait on-site for a diagnosis. To create a point-of-care rapid test, half of the surveyed health care providers preferred a turnaround time of 5–15 minutes (Davids *et al.*, 2015). Robustness is determined by the ability of a diagnostic test to endure changes in environmental factors such as temperature, humidity and mechanical pressure without specialised storage or transport (Land *et al.*, 2019). The specific manuals of the tests were sourced and used to determine robustness by comparing shelf-life, storage conditions as well as any additional requirements that might incur related costs, such as electricity usage.

### **5. Equipment-free and Deliverable**

Ideally, a point-of-care rapid test must preclude the need for equipment but currently there are no equipment-free tests available for DR-TB diagnosis (World Health Organization, 2020b). We evaluated this characteristic by identifying tests that have minimal equipment requirements. Rapid tests that can be implemented in decentralized clinical settings without the need for purchasing additional equipment can improve testing, and therefor reduce transmission (García-Basteiro *et al.*, 2018).

## **2.4 RESULTS**

An academic database search for peer-reviewed, full-text articles evaluating drug resistance testing assays for detection of multi-drug and extensively drug resistant tuberculosis yielded limited results. Most of the articles used report on studies that occurred between 2012 to 2021 and more than 800 journal articles, and one book was retrieved. None of the included studies recorded demographic data or HIV status due to the use of blinded samples. Most samples were retrieved from TB reference laboratories and in the form of previously decontaminated samples or DNA isolates.

### **2.4.1 Details of included studies**

More than a thousand sources were identified with a total of 1341 studies identified after first screening. Of the 1341, there were 868 sources published between 2012 and 2021. A total of 18 fulfilled each of the requirements to be included in the meta-analysis (Table 1). Three recent TB drug resistance profiling assays that are WHO-endorsed were identified: Hain GenoType® MTBDR<sub>plus</sub> V2.0 (Hain Lifescience, Nehren, Germany), Hain GenoType® MTBDRs/ V2.0 (Hain Lifescience, Nehren, Germany) and Nipro Genoscholar NTM+MDRTB (RIF/INH) (Nipro, Osaka, Japan). Information on the articles selected to represent the three

respective line-probe assays in the result analysis are summarised in Table 1 and includes the location, number of samples and sample type acquired, period in which samples were collected, the target population of samples, smear status and reference standard used in each study. A total of nine studies evaluating Hain GenoType® MTBDR*plus* V2.0 were identified (Table 1) that met the inclusion criteria. The diagnostic accuracy estimates and extracted data are summarised in Figure 2 and Figure 3 for RIF and INH resistance detection respectively. Three diagnostic accuracy studies were identified for Nipro Genoscholar NTM+MDRTB (RIF/INH) and listed in Table 1. Diagnostic accuracy estimates are also summarised in Figure 2 and Figure 3 for RIF and INH resistance detection respectively.

**Table 1: Summary of selected publications based on the inclusion requirements**

| First author<br>(study ID)                   | Location                           | Sample<br>Number  <br>Type    | Period of collection of<br>samples | Population                   | Smear<br>status | Reference standard                            |
|----------------------------------------------|------------------------------------|-------------------------------|------------------------------------|------------------------------|-----------------|-----------------------------------------------|
| <b>Hain GenoType® MTBDRplus Version 2.0</b>  |                                    |                               |                                    |                              |                 |                                               |
| Barnard <i>et al.</i> , 2012                 | South Africa                       | 282   sputum                  | Unreported                         | Presumptive TB               | Both            | MGIT DST                                      |
| Chen <i>et al.</i> , 2019                    | Taiwan                             | 176   sputum                  | Unreported                         | Confirmed DR-TB              | Both            | Middlebrook 7H10 DST                          |
| Genestet <i>et al.</i> , 2020                | France                             | 274   sputum                  | Nov 2016 to Jul 2019               | Confirmed TB                 | Both            | MGIT DST                                      |
| Ghanekar <i>et al.</i> , 2018                | India                              | 130   sputum                  | Jan 2014 to May 2017.              | Presumptive TB               | Positive        | BacT/Alert                                    |
| Maningi <i>et al.</i> , 2017                 | South Africa                       | 100   culture                 | Jan to Jun 2014                    | Presumptive MDR-TB           | Both            | MGIT DST                                      |
| Meaza <i>et al.</i> , 2017                   | Ethiopia                           | 274   sputum                  | Apr to Aug 2015                    | Presumptive MDR-TB           | Both            | MGIT DST                                      |
| Nathavitharana <i>et al.</i> ,<br>2016       | Germany and South Africa           | 270   culture<br>229   sputum | Unreported                         | Presumptive TB               | Both            | Phenotypic DST                                |
| Seifert <i>et al.</i> , 2016                 | South Africa, India and<br>Moldova | 914   sputum                  | 2012 to 2013                       | Presumptive DR-TB            | Both            | MGIT DST                                      |
| Tan, 2017                                    | China                              | 4833   sputum                 | Apr 2014 to Apr 2015               | Presumptive TB               | Both            | MGIT DST                                      |
| <b>Nipro Genoscholar NTM+MDRTB (RIF/INH)</b> |                                    |                               |                                    |                              |                 |                                               |
| Mitarai <i>et al.</i> , 2012                 | Japan                              | 554   sputum                  | Jan 2005 to Dec 2009               | TB confirmed                 | Positive        | MGIT DST                                      |
| Nathavitharana <i>et al.</i> ,<br>2016       | Germany and South Africa           | 270   culture<br>229   sputum | Unreported                         | Presumptive TB               | Both            | Phenotypic DST                                |
| Rienthong <i>et al.</i> , 2015               | Thailand                           | 404   sputum                  | 2011 to 2013                       | DS-TB or DR-TB<br>confirmed  | Positive        | MGIT DST                                      |
| <b>Hain GenoType® MTBDRs/ Version 2.0</b>    |                                    |                               |                                    |                              |                 |                                               |
| Bang <i>et al.</i> , 2016                    | Lithuania                          | 53   culture                  | 2009 to 2010                       | MDR-TB confirmed             | Positive        | MGIT DST and sequencing                       |
| Brossier <i>et al.</i> , 2016                | France                             | 127   culture                 | 2005 to 2015                       | MDR-TB confirmed             | Positive        | Löwenstein-Jensen solid<br>media              |
| Gardee <i>et al.</i> , 2017                  | South Africa                       | 268   culture                 | 2012 to 2014                       | DR-TB or MDR-TB<br>confirmed | Positive        | MGIT DST                                      |
| Maningi <i>et al.</i> , 2017                 | South Africa                       | 100   culture                 | Jan to Jun 2014                    | Presumptive MDR-TB           | Both            | MGIT DST                                      |
| Tagliani <i>et al.</i> , 2015                | Italy, Sweden and Germany          | 228   sputum                  | 2009 to 2013                       | MDR-TB confirmed             | Positive        | MGIT DST or Löwenstein-<br>Jensen solid media |
| Yadav <i>et al.</i> , 2018                   | India                              | 431   sputum                  | 2017                               | MDR-TB confirmed             | Positive        | MGIT DST                                      |

\* Both: samples were included regardless of negative smear status and samples with a positive smear status were also included

All selected studies received stored samples that had been stored for more than one year. Three studies did not record the period in which the samples were received at healthcare facilities: Barnard *et al.* (2012), Chen *et al.* (2019) and Nathavitharana *et al.* (2016). All selected studies used phenotypic drug susceptibility testing (culture) as gold standard, but the selected method differed among studies, summarized in Table 1. Most studies selected BACTEC™ MGIT™ as a reference standard for diagnostic accuracy estimation in the evaluations or Löwenstein-Jensen solid media and one study by Ghanekar *et al.* (2018) selected BacT/Alert liquid culture system.

## **2.4.2 Data extraction and quality assessment**

This section reports on the assessment of the data extracted from literature, by using forest plot analysis, summary operator characteristic curve analysis, and the QUADAS-2 analysis. The quality of each study is determined by the respective analysis and compared, considering the risks of bias and heterogeneity.

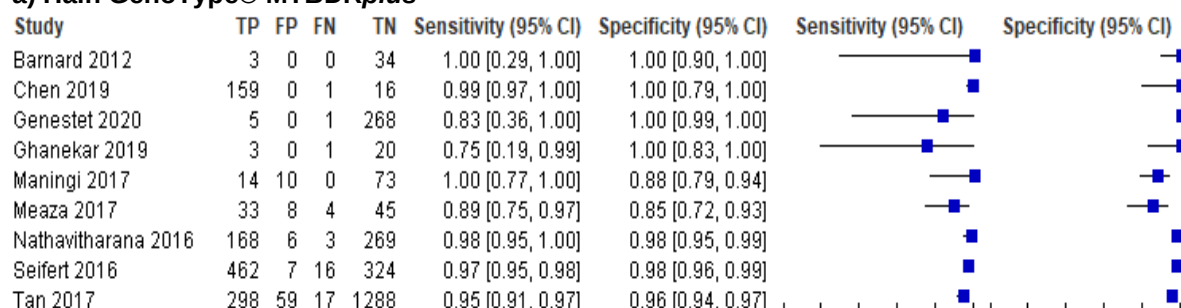
### **2.4.2.1 Forest plot and summary receiver operator characteristics curve analysis**

There were two-line probe assays identified as WHO-endorsed drug resistance detection kits, which have been implemented in Reference laboratories to detect drug resistance towards rifampicin (RIF) and isoniazid (INH) among TB infected populations. Maningi *et al.* (2017) reported 100.0% for sensitivity and 87.9% for specificity of Hain GenoType® MTBDRplus V2.0 towards RIF resistance detection but had a small sample selection compared to other studies (Table 1). The most recent study by Genestet *et al.* (2020) had no reported false positive results compared to Maningi *et al.* (2017), that had the highest relative rate of false-positive results towards detection of RIF resistance (Figure 2). Genestet *et al.* (2020) reported the highest specificity estimates among studies with 100.0% for detection towards both RIF and INH resistance but the lowest corresponding sensitivity estimates at 83.3% for RIF resistance detection and 85.7% for INH (Figure 2, 3, 6 and 7). Seifert *et al.* (2016) analysed 1128 sputum samples, the highest volume of samples analysed in a single study and reported the highest rate of false negative (FN) results with 31 false negative results for INH resistance. Seifert *et al.* (2016) also reported a specificity estimate of 99.6% (CI 97.7–100.0) for INH resistance detection during evaluation of Hain GenoType® MTBDRplus V2.0 (Figure 3).

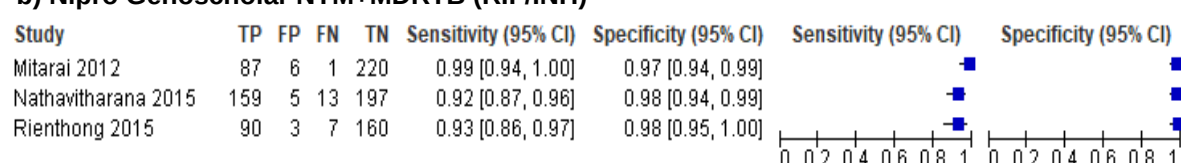
Observed in Nipro Genoscholar NTM+MDRTB (RIF/INH) results illustrated in Figure 2 (b), the highest sensitivity for RIF resistance detection is 98.8% (95%CI 93.8–99.9) reported by Mitarai *et al.* (2012) and lowest reported estimate by Nathavitharana *et al.* (2016) at 92.4% (CI 87.4–95.9) (Figure 2). A similar estimated sensitivity was observed in the study by Rienthong *et al.* (2015) at 92.7% (CI 87.4–95.9) and specificity 97.5% (CI 94.3–99.2) (Figure

2). The sensitivity towards detecting INH resistance was the lowest in the study by Mitarai *et al.* (2012), estimated at 61.5% (CI 52.9–69.7), however, the study reported that the highest sensitivity towards RIF resistance detection was estimated at 98.8% (CI 93.8–99.9).

#### a) Hain GenoType® MTBDRplus



#### b) Nipro Genoscholar NTM+MDRTB (RIF/INH)

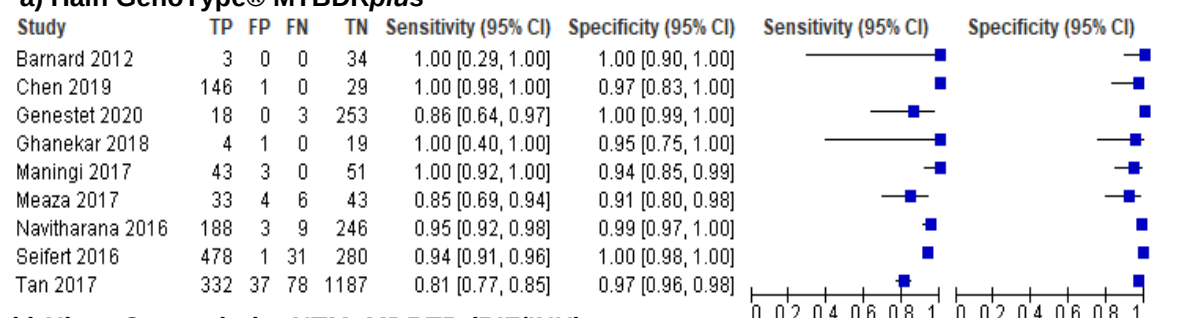


TP true positives, FP false positives, FN false negatives, TN true negatives, CI confidence

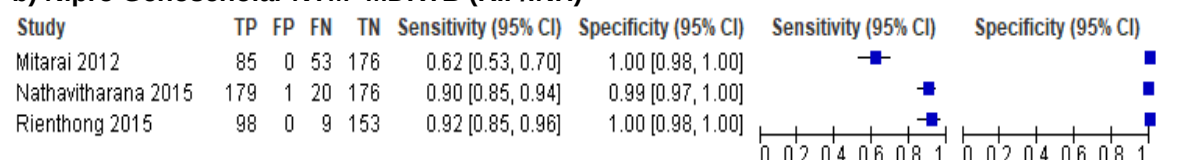
**Figure 2: Forest plots of included studies for a) Hain GenoType® MTBDRplus V2.0 and b) Nipro Genoscholar NTM+MDRTB (RIF/INH) towards rifampicin (RIF) resistance detection**

The sensitivity of Hain GenoType® MTBDRplus V2.0 towards detection of resistance to rifampicin ranged between 83.3–100.0% and specificity ranged between 84.9–100.0% (Figure 2). Sensitivity towards RIF resistance detection for Nipro Genoscholar NTM+MDRTB (RIF/INH) ranged between 92.4–98.8% and specificity between 97.3–98.1%.

#### a) Hain GenoType® MTBDRplus



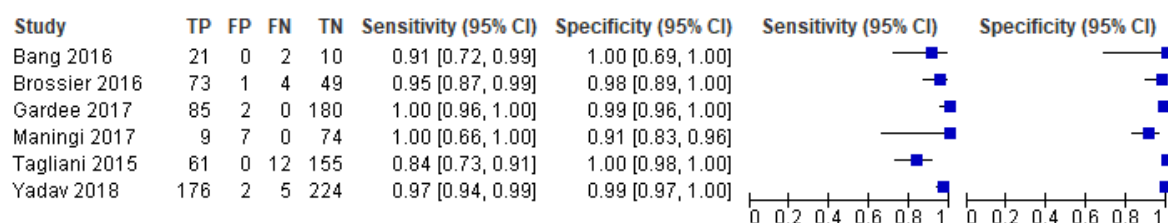
#### b) Nipro Genoscholar NTM+MDRTB (RIF/INH)



TP true positives, FP false positives, FN false negatives, TN true negatives, CI confidence interval

**Figure 3: Diagnostic accuracy results and corresponding forest plots of included studies for a) Hain GenoType® MTBDRplus V2.0, and b) Nipro Genoscholar NTM+MDRTB (RIF/INH) in isoniazid (INH) resistance detection**

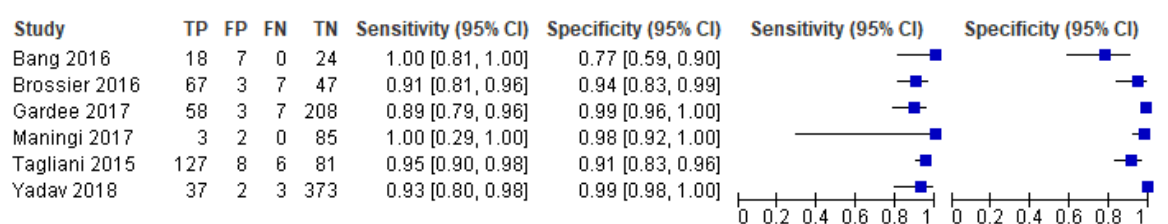
Sensitivity and specificity towards isoniazid resistance detection for Hain GenoType® MTBDR<sub>plus</sub> V2.0 ranged between 81.0–100% (standard deviation 0.07) and 91.4–100% (standard deviation 0.02) respectively. The sensitivity of Nipro Genoscholar NTM+MDRTB (RIF/INH) towards INH resistance detection ranged from 61.5–91.5% (standard deviation 0.13) and specificity between 99.4–100% (standard deviation 0.004).



TP true positives, FP false positives, FN false negatives, TN true negatives, CI confidence interval

**Figure 4: Forest plots of included studies for Hain GenoType® MTBDRs/ V2.0 diagnostic accuracy towards fluoroquinolone resistance detection**

The sensitivity of Hain GenoType® MTBDRs/ V2.0 towards fluoroquinolone (FQ) resistance detection ranged between 83.6–100% and specificity ranged between 91.4–100% (Figure 4).

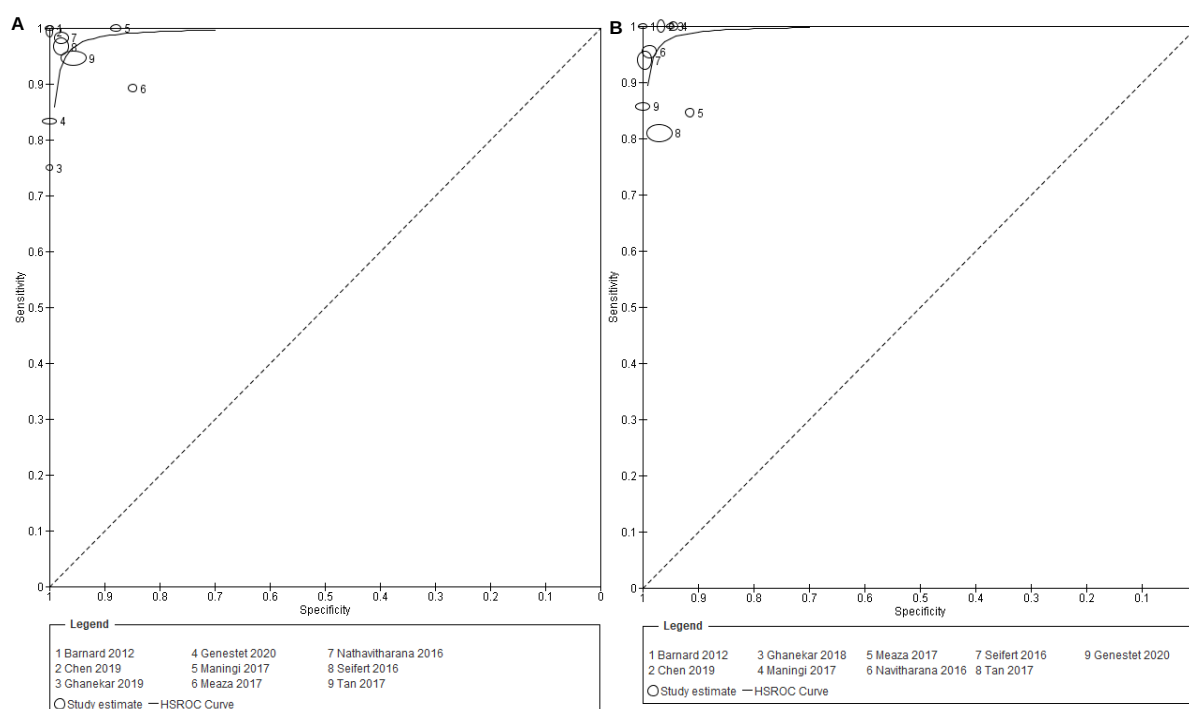


TP true positives, FP false positives, FN false negatives, TN true negatives, CI confidence interval

**Figure 5: Forest plots of included studies for Hain GenoType® MTBDRs/ V2.0 diagnostic accuracy towards aminoglycoside (AG) resistance detection**

Sensitivity towards aminoglycoside (AG) resistance detection ranged between 89.2–100.0% and specificity between 91.4–100.0% as reported in Figure 5.

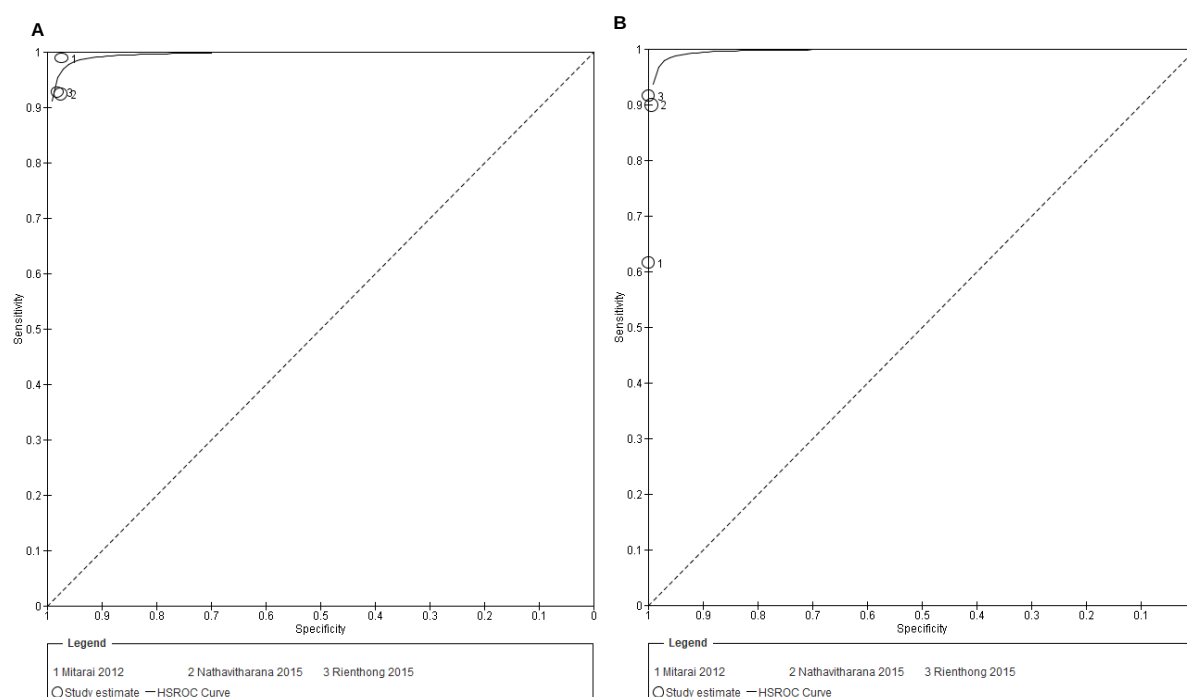
Summary receiver operating characteristics plot curves (SROC) are indicative of the relationship between sensitivity and specificity and could help determine whether heterogeneity between studies must be considered. According to Ling *et al.* (2008), SROC curves are described as shoulder-like and non-shoulder-like, where a non-shoulder-like curve is indicative of a high level of heterogeneity. Synthesis of SROC curves were included to indicate the relationship between the reported sensitivity and specificity towards detection of each respective anti-TB drug.



**Figure 6: Summary receiver operator characteristics (SROC) curve for Hain GenoType® MTBDRplus V2.0 line probe assay diagnostic accuracy towards detecting RIF (A) and INH (B) resistance among selected studies**

Overall sensitivity and specificity towards rifampicin resistance detection indicate high accuracy among evaluated diagnostic methods. Maningi *et al.* (2017) reported 100% for sensitivity and specificity of Hain GenoType® MTBDRplus V2.0 but had a small sample selection compared to other studies ( $n = 100$  culture samples). For instance, the most recent study Genestet *et al.* (2020) had no reported false positive (FP) results compared to Maningi *et al.* (2017) that had the highest reported false-positive detection rate for RIF resistance (Figure 2). Genestet *et al.* (2020) reported the highest specificity estimates amongst studies with 100% for detection towards both RIF and INH resistance but the lowest corresponding sensitivity estimates at 83.3% for RIF resistance detection and 85.7% for INH (Figure 3). Seifert *et al.* (2016) analysed the highest volume of samples (1128 sputum samples) and reported the highest rate of false negative (FN) results with 31 FN cases for INH resistance. Seifert *et al.* (2016) reported a 99.6% (95% CI 97.7–100) for INH resistance detection with Hain GenoType® MTBDRplus V2.0. For both RIF and INH shoulder-like summary receiver operating characteristics (SROC) curves were observed in Figure 3 and Figure 5 respectively. Visualized on the SROC curves are high levels of sensitivity and specificity for both RIF and INH resistance detection because the majority of studies are pooled in the top left-hand corner. The only study not on the curve for RIF resistance detection (Figure 6) is the study by Meaza *et al.* 2017 (number 6), which indicates weak correlation between the study and the group of included studies selected for analysis

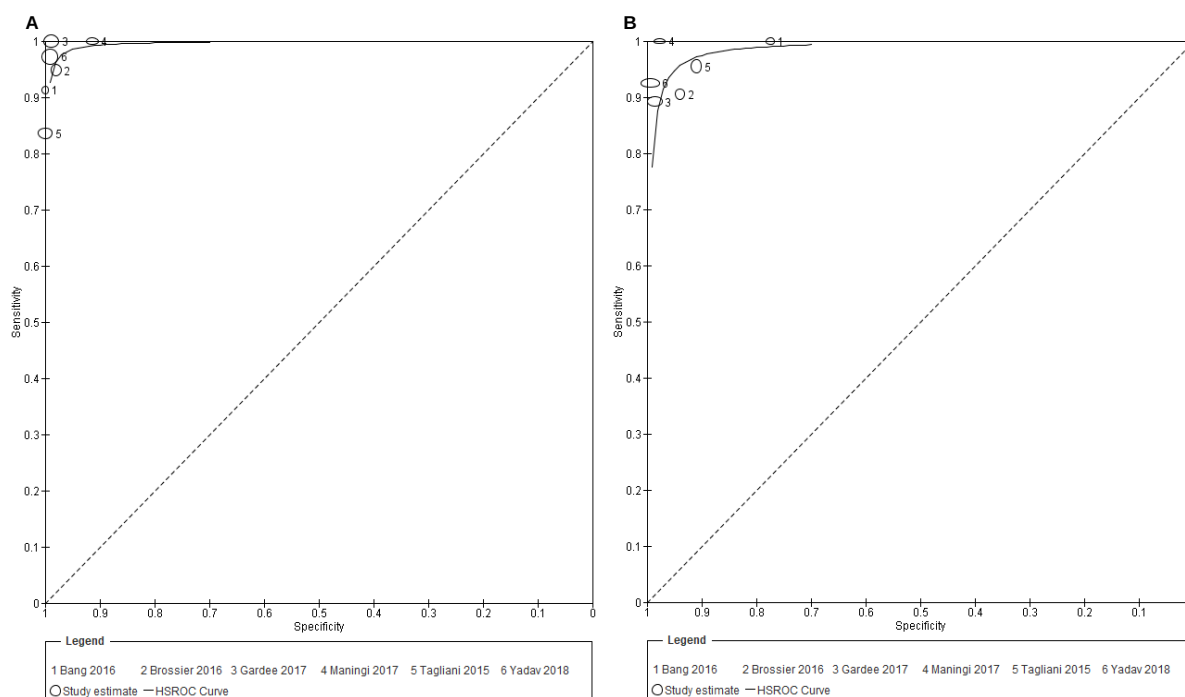
at a sensitivity and specificity of 89.1% (95% CI 75.0–97.0) and 84.9 (95% CI 72.0–93.0) respectively.



**Figure 7: Summary receiver operating characteristics plot of diagnostic accuracy of Nipro Genoscholar NTM+MDRTB (RIF/INH) towards RIF (A) and INH (B) resistance detection**

Shoulder-like curves are observed for Nipro Genoscholar NTM+MDRTB (RIF/INH) towards RIF (A) and INH (B) resistance detection in Figure 7. High accuracy estimate correlation between the included studies as depicted by the pooling of studies in the top left-hand corner of both SROC curves (Fig 7).

The pooling of the estimates in the top left-hand corner indicates strong correlation and consequently high diagnostic accuracy (Figure 6, 7 and 8). Hain GenoType® MTBDR<sub>plus</sub> V2.0 used in the studies by Ghanekar *et al.* (2018) and Meaza *et al.* (2017), are indicative of lower accuracy compared to Barnard *et al.* (2012) and Chen *et al.* (2019) studies towards RIF resistance detection, as seen in Figure 3. Overall high levels of sensitivity (>75.0%) and specificity (>84.9%) towards RIF resistance detection is observed as the majority of studies are pooled in the top left-hand corner of Figure 6 (A) and Figure 7 (A) for Hain GenoType® MTBDR<sub>plus</sub> V2.0 and Nipro Genoscholar NTM+MDRTB (RIF/INH) respectively as well as shoulder-like SROC curves in Figure 6 (A) and Figure 7 (A). Lower accuracy was observed overall for INH resistance detection, however still highly accurate, in Figure 6 (B) the study by Tan *et al.* (2017) and in Figure 7 (B) the study by Mitarai *et al.* (2012) showed the lowest accuracy when plotted on the SROC graph.



**Figure 8: Summary receiver operating characteristics plot of diagnostic accuracy for Hain GenoType® MTBDRs/ V2.0 towards FQ (A) and AG (B) resistance detection**

Tagliani *et al.* (2015) reported the lowest sensitivity estimate for fluoroquinolone (FQ) resistance detection using Hain GenoType® MTBDRs/ Version 2.0 at 83.6% (95% CI 73.4–90.3) (Figure 8). Gardee *et al.* (2017) and Maningi *et al.* (2017) reported 100% sensitivity for detection of FQ resistance. A small sample selection can potentially create high levels of uncertainty as seen with the study by Barnard *et al.* (2012) among study estimates as observed above (Figure 2 and 3). High specificity estimates are observed for all studies evaluating Hain GenoType® MTBDRs/ Version 2.0 for FQ resistance detection. Maningi *et al.* (2017) reported the lowest specificity for FQ resistance detection at 91.4% (95% CI 83.0–96.4) (Figure 4 and 8). Two studies, Maningi *et al.* (2017) and Brossier *et al.* (2016), had a small sample selection size of 90 and 127 cultured isolates respectively. Sensitivity for fluoroquinolone resistance detection ranged between 83.6–100% and specificity ranged between 91.4–100.0% as illustrated in Figure 8 (A). Sensitivity towards aminoglycoside (AG) resistance detection ranged between 89.2–100% and specificity 91.4–100% as illustrated in Figure 8 (B). Heterogeneity among studies could be attributed to study design variations and disregard of patient demographics for inclusion.

#### 2.4.2.2 QUADAS-2 analysis

Sample selection was consistent between studies with most receiving anonymised processed samples derived from sputum samples of patients with suspected TB or DR-TB. One study by Nathavitharana *et al.* (2016) included both culture and sputum derived sample

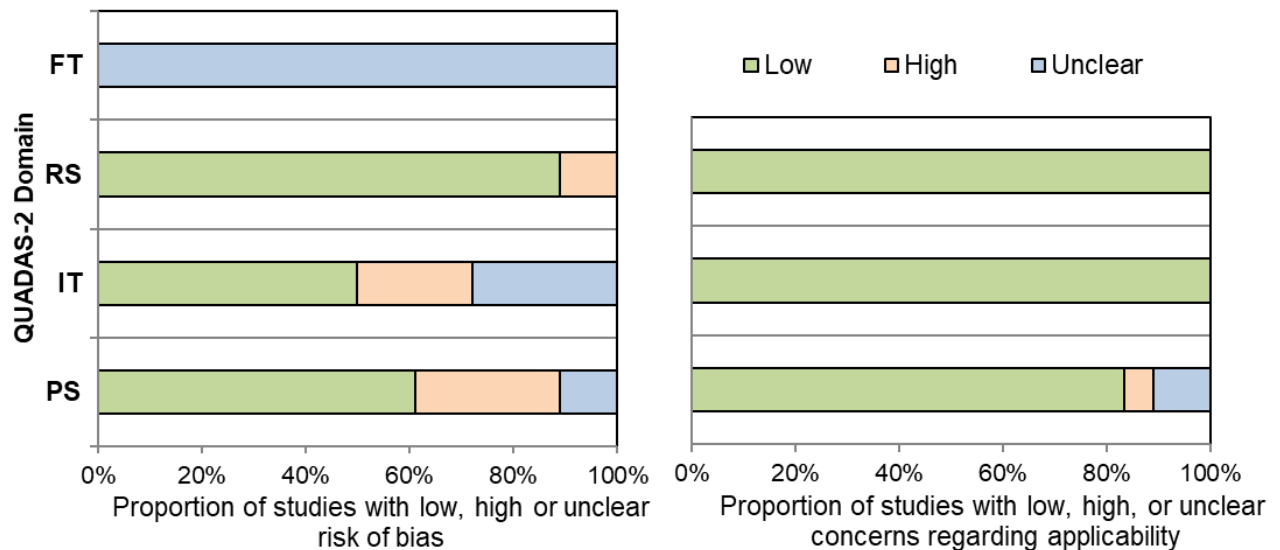
isolates (Table 1). Studies that presented high risk of bias with patient selection (PS) in Hain GenoType® MTBDR<sub>plus</sub> V2.0 and Nipro Genoscholar NTM+MDRTB (RIF/INH) evaluations include: Chen *et al.* (2017), Nathavitharana *et al.* (2016) and Seifert *et al.* (2016), whereas Rienthong *et al.* (2015) did not describe the method of patient sample selection and was declared as unclear risk of bias.

**Table 2: Risk of bias and applicability concerns for Hain GenoType® MTBDR<sub>plus</sub> V2.0 in first-line drug resistance detection to rifampicin and isoniazid**

| Study                                           | Risk of bias |    |    |    | Applicability concerns |    |    |
|-------------------------------------------------|--------------|----|----|----|------------------------|----|----|
|                                                 | PS           | IT | RS | FT | PS                     | IT | RS |
| <b>Hain GenoType® MTBDR<sub>plus</sub> V2.0</b> |              |    |    |    |                        |    |    |
| Barnard <i>et al.</i> (2012)                    | ☺            | ☺  | ☺  | ?  | ☺                      | ☺  | ☺  |
| Chen <i>et al.</i> (2019)                       | ☹            | ?  | ☹  | ?  | ☺                      | ☺  | ☺  |
| Genestet <i>et al.</i> (2020)                   | ☺            | ☺  | ☺  | ?  | ☺                      | ☺  | ☺  |
| Ghanekar <i>et al.</i> (2018)                   | ☺            | ?  | ☺  | ?  | ☺                      | ☺  | ☺  |
| Maningi <i>et al.</i> (2017)                    | ☺            | ☺  | ☺  | ?  | ☺                      | ☺  | ☺  |
| Meaza <i>et al.</i> (2017)                      | ☺            | ☺  | ☺  | ?  | ☺                      | ☺  | ☺  |
| Nathavitharana <i>et al.</i> (2016)             | ☹            | ☺  | ☺  | ?  | ☺                      | ☺  | ☺  |
| Seifert <i>et al.</i> (2016)                    | ☹            | ☹  | ☹  | ?  | ☹                      | ☺  | ☺  |
| Tan <i>et al.</i> (2017)                        | ☺            | ☺  | ☺  | ?  | ☺                      | ☺  | ☺  |
| <b>Nipro Genoscholar NTM+MDRTB (RIF/INH)</b>    |              |    |    |    |                        |    |    |
| Mitarai <i>et al.</i> (2012)                    | ☺            | ?  | ☺  | ?  | ☺                      | ☺  | ☺  |
| Nathavitharana <i>et al.</i> (2016)             | ☹            | ☺  | ☺  | ?  | ☺                      | ☺  | ☺  |
| Rienthong <i>et al.</i> (2015)                  | ?            | ☹  | ☺  | ?  | ☺                      | ☺  | ☺  |
| <b>Hain GenoType® MTBDRs/ V2.0</b>              |              |    |    |    |                        |    |    |
| Bang <i>et al.</i> (2016)                       | ?            | ?  | ☺  | ?  | ?                      | ☺  | ☺  |
| Brossier <i>et al.</i> (2016)                   | ☹            | ☹  | ☺  | ?  | ?                      | ☺  | ☺  |
| Gardee <i>et al.</i> (2017)                     | ☺            | ☺  | ☺  | ?  | ☺                      | ☺  | ☺  |
| Maningi <i>et al.</i> (2017)                    | ☺            | ?  | ☺  | ?  | ☺                      | ☺  | ☺  |
| Tagliani <i>et al.</i> (2015)                   | ☺            | ☹  | ☺  | ?  | ☺                      | ☺  | ☺  |
| Yadav <i>et al.</i> (2018)                      | ☺            | ☺  | ☺  | ?  | ☺                      | ☺  | ☺  |

PS patient selection, IT index test, RS reference standard, FT flow and timing ☺ Low Risk of bias, ☹ High Risk of bias, ? Unclear Risk of bias

Studies with risk of bias concerns regarding PS for Hain GenoType® MTBDRs/ V2.0 include Bang *et al.* (2016) with an unclear risk of bias and Brossier *et al.* (2016) with a high risk of bias due to a case-control study design and selection of MDR-TB confirmed samples. Where there is a reported unclear risk of bias regarding the index test (IT) and conduct thereof, the study design did not include a description of how the index test was performed or interpreted and studies with a high risk of bias include studies that analysed samples with the index test with previous knowledge of the outcomes for each sample as seen in Table 2 for Seifert *et al.* (2016), Rienthong *et al.* (2015), Brossier *et al.* (2016) and Tagliani *et al.* (2015). Overall, the sections of the study designs regarding the selection and interpretation of the reference standard (RS) and the RS results presented low instances of bias risk and applicability concerns as depicted in Figure 9. The two studies with high risk of bias regarding RS were Chen *et al.* (2019) and Seifert *et al.* (2016) due to previous knowledge of sample results for the reference standard. Results are summarised in Figure 9, depicting the proportion of studies with low (green), high (orange) or unclear (blue) risk of bias and applicability concerns.



**Figure 9: Summary of risk of bias and applicability concerns for all included studies evaluating anti-TB drug resistance detection LPAs. PS patient selection, IT index test, RS reference standard, FT flow and timing**

No study reported on the flow and timing (FT) of study methods from the moment of sample retrieval and processing to initial testing and comparison to reference test results, which is depicted as 100% (Figure 9). Most of the studies (90%) showed a low risk of bias regarding the conduct and interpretation of the reference standard (RS) and therefore there are no concerns regarding applicability. Half of all included studies had a low risk of bias regarding conduct and interpretation of the index test (IT) and an estimated 22% and 27% presented unclear and high risk of bias respectively, as depicted in Figure 9. However, there was no strong evidence to support applicability concerns. Regarding patient selection (PS), 60% of studies presented a low risk of bias and 11% with a high risk of bias with an estimated 29% of studies presenting high risk of bias in terms of selection of the samples used in the study.

#### 2.4.3 ASSURED analysis

Diagnostic test characteristics were summarized and used for the ASSURED analysis (Table 7). These include the drug resistance detection range, price per test for each individual test performed, turnaround time of analysis, designated setting in which tests are implemented, and whether the test can deliver results using a direct sputum sample or cultured and processed sample. Seifert *et al.* (2016) had a long list of criteria for inclusion and exclusion of patients and therefore is the only study with applicability concerns regarding patient selection as illustrated in Table 3. The failure of studies to report on flow and timing from sample acquisition to data analysis resulted in an unclear risk of bias for all selected studies as depicted in Table 3. The following studies included for Hain GenoType® MTBDRplus V2.0 represented the lowest risk of bias and applicability concerns due to one aspect of the

study designs, flow and timing, which is lacking in study design reports overall (Table 3): Barnard *et al.* (2012), Genestet *et al.* (2020), Maningi *et al.* (2017), Meaza *et al.* (2017), Tan *et al.* (2017) and for Hain GenoType® MTBDRs/ V2.0 only Gardee *et al.* (2017) and Yadav *et al.* (2018). The abovementioned studies avoided the risks by including a random or consecutive sample selection, avoiding inappropriate exclusions, interpreted results without previous knowledge of results from the index test and reference standard and did not follow a case-control study design. Line probe assays were compared to the gold standard test BACTEC™'s MGIT™ culture method, and the most widely used test in South Africa; GeneXpert.

**Table 3: Summary of characteristics of TB diagnostic tests used in ASSURED analysis**

| Diagnostic test                                | Detection and range                                                                              | Price per test (USD) | Price per test (ZAR) | Turnaround time | Designated setting   | Direct or indirect testing |
|------------------------------------------------|--------------------------------------------------------------------------------------------------|----------------------|----------------------|-----------------|----------------------|----------------------------|
| BACTEC™ MGIT™                                  | Phenotypic: RIF, INH, FQ and AG                                                                  | \$16.8               | R236.92              | ≤ six weeks     | Reference laboratory | Both                       |
| Xpert MTB/RIF and Xpert MTB/RIF Ultra          | Genotypic: <i>rpoB</i>                                                                           | \$10.0               | R 141.02             | ≤ two hours     | District laboratory  | Both                       |
| Hain GenoType® MTBDRplus V2.0 and MTBDRs/ V2.0 | Genotypic: <i>rpoB</i> , <i>katG</i> , <i>inhA</i><br><br><i>gyrA</i> , <i>rrs</i> , <i>gyrB</i> | \$8.8                | R 125.17             | ≤ four hours    | Reference laboratory | Both                       |
| Nipro Genoscholar NTM+MDRTB (RIF/INH)          | Genotypic: <i>rpoB</i> , <i>katG</i> , <i>inhA</i>                                               | \$7.9                | R 111.41             | ≥ one day       | Reference laboratory | Both                       |

Prices calculated at an exchange rate of R14.23: \$1 (14 September 2021)

The cost per test, turnaround time, setting in which the method can be performed, and whether direct and/or indirect testing is possible (Table 3), were extracted from TB diagnostic guides by the Treatment Action Group (Treatment Action Group, 2017; Treatment Action Group, 2019). Details were cross-referenced from results of included studies where reports of costs, flow and timing and ease of use of diagnostic methods and equipment requirements for implementation were mentioned. Reports on the ease of use of BACTEC™ MGIT™ liquid culture indicate that prior training is necessary notwithstanding the automated nature of result analysis due to high risks of contamination. Cepheid's Xpert MTB/RIF automated cartridges are reportedly easy to use and require minimal training beforehand whereas the conduct and manual interpretation of line probe assays, including Hain GenoType® MTBDRplus V2.0 and MTBDRs/ V2.0 and Nipro Genoscholar NTM+MDRTB

(RIF/INH) require at least two days of training in knowledgeable environments (World Health Organization, 2015a).

The ASSURED results (Table 4) correspond to data extracted from literature regarding the characteristics described as fundamental for implementation of the test in low-and middle-income (LMIC) settings and characteristics (Table 3). This includes test affordability, diagnostic accuracy (sensitivity and specificity), user friendliness of the test and interpretation of the results, whether a test is rapid and robust and whether it is equipment free and potentially deliverable in these settings.

**Table 4: ASSURED comparison of the included tests for our meta-analysis to conventional drug susceptibility testing**

| Test             | MGIT™<br>(Becton,<br>and<br>Dickinson<br>Company)                                   | Xpert® MTB/RIF<br>Ultra<br>by<br>Cepheid                                            | GenoType®<br>MTBDRplus<br>Version 2.0<br>and<br>-s/                                  | Nipro<br>NTM+MDR TB<br>(RIF/INH) by<br>Nipro<br>Corporation                           |
|------------------|-------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| Characteristic   |                                                                                     |                                                                                     |                                                                                      |                                                                                       |
| Affordable       |  |  |  |  |
| Sensitive        |  |  |  |  |
| Specific         |  |  |  |  |
| User-friendly    |  |  |  |  |
| Rapid and robust |  |  |  |  |
| Equipment free   |  |  |  |  |
| Deliverable      |  |  |  |  |

 High  Neutral  Low

Currently, diagnostic tests for TB diagnosis and more importantly drug resistance detection are expensive. The most expensive test is the gold standard test, which costs R236.9 (16 USD) per patient per test. The affordability of BACTEC™ MGIT™ is hampered by the expensive equipment requirements costing R 549 286.41 (38 950 USD) per system (FIND, 2021) for the automated radiometric detection system (BACTEC™ MGIT™ 960 System) widely used in South Africa. The most commonly used TB diagnostic test is GeneXpert, specifically the GX4 model allowing for four samples to be analysed per cartridge and one

cartridge costing R 140.7 (9.9 USD) as negotiated by the FIND global alliance for diagnostics (FIND, 2021). The most affordable tests when referring to price per test are line probe assays at R 125.17 (8.8 USD) for Hain GenoType® MTBDR<sub>plus</sub> V2.0 and MTBDRs/ V2.0 and R 111.4 (7.9 USD) for Nipro Genoscholar NTM+MDRTB (RIF/INH). Related equipment costs for Hain GenoType® MTBDR<sub>plus</sub> V2.0 and MTBDRs/ V2.0 include R 12 006.3 (851.5 USD) for a kit for 96 sample analyses and requirements of a Twincubator at R 36 686 (12601.9 USD) (FIND, 2021) and the necessity of a PCR machine, which is also used with GeneXpert. Training requirements vary among included tests. A high level of training is required for usage and interpretation of PCR analysis as with Xpert® MTB/RIF and MTB/RIF Ultra and additional training is required to perform and interpret line probe assays. BACTEC™ MGIT™ requires an almost equally high level of training due to contamination risks and risk of exposure of the user to live TB bacteria.

## **2.5. DISCUSSION**

This study aimed to compare WHO-endorsed molecular diagnostic methods, specifically line probe assays, that could potentially be implemented in middle- and low-income countries for drug resistance profiling of TB to replace culture methods as gold standard. The motivation thereof is the current lack of affordable and rapid diagnostics with high sensitivity and specificity rates for detection of some of the most frequent resistance conferring mutations. BACTEC™'s MGIT™ culture method can detect resistance to any anti-TB drug given the results are phenotypic and not indicative of the true variety of resistance genes or mechanisms present (Ahmad *et al.*, 2016).

### **2.5.1 Hain GenoType® MTBDR<sub>plus</sub> V2.0 and Nipro Genoscholar NTM+MDRTB (RIF/INH) diagnostic accuracy and performance in detecting first-line anti-TB drug resistance**

Evidence of heterogeneity, as determined by the QUADAS-2 analysis of study methodologies, can be attributed to the small sample selection of selected studies and non-randomised or non-consecutive sample selection consequently leading to high rates of uncertainty (Table 3 and Figure 9). The overall lack of reports on flow and timing of studies is concerning seeing as DNA denaturation can influence result outcomes and starts at the point of collection of the sample. To ensure the integrity of a sample is left intact, timely analysis must be performed. The lack of evidence of time was regarded as unclear risk of bias since it was observed for 100% of the studies, indicating a lack of conformance to a set standard for diagnostic accuracy reporting. The reference standard used differed among studies, but all studies used a reference standard that is highly likely to classify the target condition and therefore no prominent risk of bias or applicability concerns arose from the

reference standard conduct interpretation except in studies where the outcomes were unblinded causing a high risk of bias (Table 3). The reporting of index test usage and interpretation also varied among studies causing unclear and high levels of bias and applicability concerns especially observed in Table 3 for Seifert *et al.* (2016), which also failed to include random or consecutive samples resulting in applicability concerns for patient selection. Using random or consecutive samples without prior knowledge of the outcomes for each test results in low levels of bias and no concerns of applicability since it relates to where the tests will be implemented to diagnose patients with unknown drug resistance patterns.

The most rapid method, delivering results within two hours (Table 4), was found to be GeneXpert. The Hain GenoType® MTBDR*plus* V2.0 assay is comparable to GeneXpert with regards to turnaround time delivering results within four hours. In a study by Shah *et al.* (2013), the cost of various components of TB diagnostic tests were determined per test and compared. Estimated total costs per sample compiled from cost of consumables, equipment costs, cost of labour and overhead costs which yielded a total for each test: BACTEC™ MGIT™ solid culture at around R177.6 (12.6 USD), liquid culture at R370.8 (26.3 USD), Hain GenoType® MTBDR*plus* V2.0 at R329.9 (23.4 USD) and GeneXpert at R210.12 (14.9 USD), making GeneXpert the least expensive method. Costs that are not frequently mentioned include the cost of maintaining equipment and other quality control practices (Groessl *et al.*, 2018). GeneXpert methodology entails the lowest usage of equipment and consumables according to literature due to its cartridge design and with low related equipment costs per sample and least implicated additional costs towards wide application, it was deemed the most deliverable. Implementation of a new rapid and point-of-care test must not signal the need to appoint additional healthcare workers but instead reduce the amount of labour necessary to provide same-day results. Land *et al.* (2019) concurs that a deliverable test would require minimal organizational planning and procuring to distribute to the intended end-users. Since the widespread roll-out of GeneXpert diagnostics in South Africa in the year 2011 at about R 874 3345 (62 000 USD) per machine, the country has procured more than 300 GeneXpert diagnostic machines (Tomlinson, 2020) highly effective towards diagnosis of TB and rifampicin resistance. The related advantage of the GeneXpert machine roll-out was the improvement of infrastructure required for set-up, including an uninterruptable supply of electricity and a ventilated, climate-controlled, secure environment to perform laboratory analyses (Schmidt *et al.*, 2017). The same requirements for implementation of LPA and other molecular diagnostics without taking necessary equipment into account. The additional advantage of a wider drug resistance detection range compared to GeneXpert makes the implementation of LPAs an attractive investment for

decentralized laboratories. Further improvement of decentralized laboratories could help alleviate the workload from reference laboratories and other primary testing facilities.

### **2.5.2 Hain GenoType® MTBDRs/ V2.0 diagnostic accuracy and performance in detecting second-line anti-TB drug resistance**

The use of a line probe assay as second-line drug resistance testing method is acceptable, but it is still recommended by the World Health Organization to perform line probe assays alongside phenotypic drug susceptibility testing. The Hain GenoType® MTBDRs/ V2.0 was developed to detect resistance to aminoglycosides (kanamycin, amikacin and capreomycin) and fluoroquinolones (levofloxacin and moxifloxacin). The strips used for Hain GenoType® MTBDRs/ V2.0 cannot be altered to include a wider range of detection whereas the advantage of liquid or solid culture is that it is possible to detect phenotypic resistance to any antibiotic by adding the antibiotic to the medium. No specific gene mutations can be detected, and it is not possible to distinguish between phenotypic and/or genotypic resistance when using liquid or solid culture for drug resistance detection. The requisite to perform sequencing on selected samples to determine specific resistance patterns caused by genetic mutations arises in the case of phenotypic drug susceptibility testing. Sequencing could be required after line probe assay analysis if a sample showcases phenotypic drug resistance by culture method analysis without detectable bands present on line probe assay strips. Among the included studies that evaluated performance of the second-line drug resistance detection line probe assay, Hain GenoType® MTBDRs/ V2.0, evidence of heterogeneity can be attributed to the equally small sample selection of selected studies and non-randomised or non-consecutive sample selection (Table 3 and Figure 9). A larger sample selection allows for a higher volume of detectable mutations conferring drug resistance and could result in higher accuracy estimates if each resistance population is sufficiently represented in the sample selection. The lack of evidence of time to result analysis from start of experiments was regarded as unclear risk of bias since it was observed for 100% of the studies indicating non-inferiority when comparing flow and timing methodologies. The selected reference standard varied mostly between liquid culture and solid culture (Table 1), both highly likely to classify the target condition and one study used a composite reference standard of MGIT DST and sequencing (Bang *et al.*, 2016). No risk of bias or applicability concerns arose from the reference standard (Table 3). Index test usage and interpretation caused unclear (33%) and high levels (33%) of bias and applicability concerns among included studies. The Hain GenoType® MTBDRs/ V2.0 assay is much more rapid compared to the four to six week waiting period of MGIT DST. The costs related

to performing the Hain GenoType® MTBDRs/ V2.0 assay were estimated at R329.9 (23.4 USD), costing the same as first-line drug resistance analysis using Hain GenoType® MTBDR*plus* V2.0, which is more than BACTEC™ MGIT™ solid culture [R177.6 (12.6 USD)], and less than liquid culture [R370.8 (26.3 USD)].

### **2.5.3 Line probe assays compared to culture-based drug susceptibility testing**

In this meta-analytic study, we observed an overall lack of report on flow and timing of laboratory analyses as reported in Figure 9. Most samples used were cultured isolates originating from TB reference laboratories (Table 1). This is limiting since samples submitted for testing are usually sputum samples and thus require processing before analysis. Sample processing incurs extra costs and related training requirements. Overall sensitivity and specificity of the selected drug resistance detection kits indicated high diagnostic accuracy among the selected diagnostic tests (Figure 2, 3, 4 and 5). Sensitivity and specificity are directly linked to false positive and negative results and with a small patient selection from a specific population group, results are bottlenecked and provide a weak representation of the epidemiological trends of TB drug resistance. The affordability of diagnostic methods is key to implementation in low-income settings. A survey among primary health care providers in South Africa, revealed that no more than 200 Rand, around 16 United States Dollars (USD) is an acceptable expense per test (Davids *et al.*, 2015). To be rated as an affordable point-of-care test in a study by García-Basteiro *et al.* (2018), a rapid test for TB must cost less than four USD. For our purposes, we rated less than ten USD as highly affordable and more than 25 USD as unaffordable, or low affordability, as illustrated in Table 5. Numerous studies have been conducted on the accuracy of TB diagnostics yet there is a lack of reports on the cost of tests and components of diagnostic algorithms (De Almeida *et al.*, 2017). Comparison of cost per test is a single component of the totality of the costs related to conduct of the various diagnostic methods and do not reflect the true financial burden on patients and laboratories. Regarding price per test which a patient would expect to pay per analysis, line probe assays are the most affordable compared to other methods (Table 4), pricing at about R 112.82 (8 USD) per test compared to culture, which costs about R239.74 (17 USD) per test. Sensitivity and specificity, relating directly to diagnostic accuracy, was comparable among all included diagnostics with many reports on highly accurate results delivered from culture based and molecular based methods (Table 5). While user-friendly tests requiring no prior training or knowledge of diagnostic methods are yet to be developed, the reportedly most user-friendly method found was GeneXpert due to the single-use cartridges and automation of result analysis

comparable to automated analysis of liquid culture by BACTEC™ MGIT™ liquid culture. User-friendly methods limit exposure to samples, which could cause laboratory-based infections among healthcare professionals (World Health Organization, 2015a), making the use of culture methods the least user-friendly by design and line probe assays slightly less user-friendly than GeneXpert. The drug resistance detection range of a kit implicates the number of diagnostic decisions that can be made after one analysis. For instance, GeneXpert can indicate whether a patient has DS-TB and/or TB resistant to RIF, whereas simultaneous detection of RIF and INH resistance is made possible by multiple probes on a line probe assay such as Hain GenoType® MTBDR<sub>plus</sub> V2.0 and Nipro Genoscholar NTM+MDRTB (RIF/INH). BACTEC™'s MGIT™ culture method, although capable of phenotypic resistance detection to any approved anti-TB drug, is not capable of indicating the specific genes present in a drug resistant sample (Torrea *et al.*, 2019). The related advantages of using line probe assays include reduction of time to result analysis, cost reduction by accurate and rapid detection of a wider range of mutations per sample and improve user-friendliness compared to culture methods.

## 2.6 CONCLUSION

We aimed to determine the plausibility of a diagnostic testing adaptation that would place line probe assays above culture methods for drug resistance detection among patients with multi-drug and extensively drug resistant tuberculosis. There is a high likelihood of such a change in the future if further development and optimization of line probe assays continue. Line probe assays are rapid molecular methods that provide results within hours of patient admission and have been considered valuable alternatives to some diagnostic tests. Hain GenoType® MTBDR<sub>plus</sub> V2.0 and Nipro Genoscholar NTM+MDRTB (RIF/INH) showed high sensitivity and specificity estimates towards detection of first-line anti-TB drug resistance and can deliver results within one day. Hain GenoType® MTBDR<sub>s</sub>/ V2.0 showed high diagnostic accuracy in detecting second-line anti-TB drug resistance and delivers same-day results. However, these rapid molecular assays, among others, are yet to be optimized because of the possibility of a false positive or false negative result (Drobniewski *et al.*, 2015). False positive results and false negative results were present among all studies included in this analysis with all studies reporting imperfect sensitivity and specificity estimates. Among others, there were 37 false positive and 78 false negative results detected in the study by Tan *et al.* (2017), during analysis of 1634 samples for isoniazid resistance detection. The study by Mitarai *et al.* (2012) reported 53 false negative results during analysis of 314 isoniazid resistant samples. Resistance detection to first- and second-line anti-TB drugs was accurate amongst included studies given sample selection was adequate however, other sources of bias must still be considered. The reason the WHO's diagnostic policies

and recommendations do not change rapidly are because LMICs cannot afford the expenses of molecular assays currently as expensive equipment is necessary for PCR and sequencing.

The necessity to diagnose and document drug resistant TB cases and keep informed on the most recent drug-resistance-conferring mutations is pertinent. Cost reduction in genetic sequencing would see a major impact on rapid drug resistant TB diagnosis and positively impact incidence rates and decrease TB deaths (Outhred *et al.*, 2015). Genotyping is as important as phenotyping an active drug resistant TB case and to obtain highly sensitive and specific results during diagnosis, it is still recommended to use complementary methods that include both approaches (Schön *et al.*, 2017). As determined by our results, there is minimal optimization required for line probe assays to be regarded as a gold standard for drug resistance detection, however phenotypic resistance could still arise from novel mutations yet to be discovered.

# CHAPTER 3: COMPARATIVE EVALUATION OF MOLECULAR-BASED DETECTION METHODS FOR DRUG RESISTANT TUBERCULOSIS

This chapter reports on an in-house comparative evaluation of three different detection methods for drug resistant tuberculosis. The chapter is written in the format of a research article entitled “Comparative evaluation of molecular-based detection methods for drug resistant tuberculosis”.

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## ABSTRACT

South Africa is among the top five countries with the highest rate of TB burden globally. We therefore evaluated two recent molecular-based tuberculosis drug resistance testing methods and compared the results with that obtained by whole genome sequencing (WGS). The methods were based on the use of the commercially available Hain GenoType® MTBDR<sub>plus</sub> and *sl* Version 2.0 kits, which were evaluated for use in South African clinical settings, and the recently developed CapitalBio Gene Real-Time PCR Detection Kit. A total of 92 blinded and randomized *Mycobacterium tuberculosis* complex DNA samples were collected from the Centre for Tuberculosis (CTB) at the National Institute for Communicable Diseases (NICD) and subjected to analyses with each kit. The kits were evaluated in terms of sensitivity, specificity, affordability, ease of use, turnaround time and robustness. Using whole genome sequencing data as a reference standard, the Hain GenoType® MTBDR V2.0 assays performed better in South African population drug resistance screening in terms of sensitivities and specificities. The Hain GenoType® MTBDR V2.0 assays showed the highest rates of accuracy. However, the CapitalBio kit is suitable for local implementation due to its affordability, rapid turnaround time and simultaneous detection of first-and second-line drug resistance in TB DNA samples. Nevertheless, the Hain GenoType® MTBDR V2.0 assays proved to be superior when all aspects of advanced diagnostics for DR-TB in South Africa were considered.

## Keywords

*Mycobacterium tuberculosis*, drug resistance, diagnostics, molecular methods, genotyping

### **3.1 INTRODUCTION**

#### **3.1.1 Characteristics of tuberculosis infection**

The most common symptom of a tuberculosis (TB) infection is persistent coughing for more than three weeks. However, this is symptomatic of a wide variety of infections of the respiratory system (Ismali *et al.*, 2018) and more importantly a symptom of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Adewole, 2020). A patient with a persistent cough will be treated as a presumptive TB case if they present at least one of the following symptoms: fatigue, shortness of breath, fever, night sweats, appetite loss and weight loss (Ismail *et al.*, 2018). Today, a patient with these symptoms must be screened for SARS-CoV-2 and secondly, TB before either can be ruled out. It is thus clinically impossible to diagnose a patient with TB before undergoing further diagnostic measures (World Health Organization, 2021). Efforts to end the global TB burden have been hampered by the emergence of the SARS-CoV-2 pandemic. The most recent Global Tuberculosis Report, published annually by the World Health Organization, reports that progress in alleviating the global TB burden has been halted and TB mortality has increased since 2020. The number of TB cases detected between 2019 and 2020 have fallen by 18% and only 15% of people diagnosed with drug resistant TB were started on treatment (World Health Organization, 2021). Once SARS-CoV-2 infection is ruled out, sputum smear microscopy (SSM) is the go-to identification method, which is routinely done to distinguish TB from other bacterial infections (Basu, 2019). A low-bacilli-count sample is usually the case in children and patients with HIV co-infection. Determining whether a patient has TB is challenging and can lead to misdiagnosis and a false negative result (Kim *et al.*, 2019). False negative results lead to unnecessary isolation, costs, and treatment of the patient (Drobniewski *et al.*, 2015). The diagnosis and control of TB has also been compromised by the prevalence of drug resistance (Suárez *et al.*, 2019). What makes tuberculosis exceptional are its various unique virulence factors. Instead of the classic toxins, polysaccharide capsules and fimbriae, *Mycobacterium tuberculosis* relies on a specialized cell envelope, which is incomparable to both gram-positive and gram-negative bacteria (Smith, 2003). Lipids and glycolipids as well as mycolic acids on the cell surface induce a hydrophobic barrier surrounding the bacterium, which directly facilitates resistance to antimicrobials by becoming impassable (Cambau & Drancourt, 2014; World Health Organization, 2016).

#### **3.1.2 Limitations of current diagnostic tests**

Diagnostic tests that are currently available are mainly focused on diagnosing drug-sensitive (DS) TB. The World Health Organization (WHO) -endorsed BACTEC Mycobacterial Growth Indicator Tube (MGIT™) test has limited ability but can identify drug resistance to several

antibiotics. GeneXpert is more specific but expensive and can only test for drug resistance to rifampicin (RIF) (Zeka *et al.*, 2011). No rapid test exists that can detect resistance to drugs other than rifampicin and isoniazid (Treatment Action Group, 2017). It is very important that before a patient can be started on treatment, the correct and accurate diagnosis must be made. Factors that delay patient treatments — especially significant in African countries — include living in an urban area versus rural areas, seeking traditional healers first, inability to recognise symptoms, and the nature of the disease itself (Lusignani *et al.*, 2013). During a study done by Mutingwende *et al.* (2015), an affordable point-of-care (POC) TB diagnostic test was developed and evaluated. The main objective was to create a diagnostic tool with competitive advantages such as a short total assay time, minimizes biological risk and has a minimal risk for sample contamination. The final product would ultimately serve as a rapid, point of care and cost-effective community-based screening tool (Mutingwende *et al.*, 2015).

### **3.1.3 The importance of detecting drug resistance**

The global estimate of people who fall ill with drug-resistant TB each year is around 470 000. The percentage of people who die from this form of TB is estimated at 180 000 (about 38% of the total DR-TB cases)(World Health Organization, 2021). The WHO urges screening for drug susceptibility to at least rifampicin (RIF) among bacteriologically confirmed TB patients before treatment can be initiated. The WHO suggests further screening for drug resistance to second-line anti-TB drugs (fluoroquinolones and aminoglycosides) among patients with confirmed RIF resistance (World Health Organization, 2021). About 82% of people diagnosed with drug-resistant TB have an infection resistant to the most effective anti-TB drugs, rifampicin and isoniazid. The residual 18% of people with DR-TB have an infection with mono-resistance to rifampicin and related isoniazid susceptibility (World Health Organization, 2021). A patient with DS-TB will enter a standard six-month treatment regime as recommended by the WHO, whereas longer, more toxic treatment regimens are prescribed to patients with drug resistant TB infections (Zignol *et al.*, 2016; Suárez *et al.*, 2019). First-line anti-TB drugs (FLDs) include rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA) and ethambutol (EMB), which are used to treat non-MDR-TB (Nhung *et al.*, 2015). Primary transmission of rifampicin-resistant tuberculosis (RR-TB) yields drug-resistance to the most effective anti-TB drug (Ismali *et al.*, 2018). This is especially significant in South Africa where 72% of TB-infected individuals have human-immunodeficiency-virus (HIV/AIDS) co-infection (World Health Organization, 2018). Drug resistance is a complex survival mechanism that has become a global health crisis (Suárez *et al.*, 2019). Globally there were half a million detected cases of RR-TB in 2018 (World Health Organization, 2019). RR-TB has been found to be a precursor for multi-drug- and extensively drug-

resistant TB (Drobniewski *et al.*, 2015; Makhado *et al.*, 2018). Multi-drug resistant TB, resistant to the two most potent anti-TB drugs; RIF and isoniazid (INH), has reportedly been found in all regions of the world (World Health Organization, 2016; Muthaiah *et al.*, 2017). In combination with other factors, the treatment and management of drug resistant TB strains, which result in increased morbidity and mortality, would surpass the management capacity of a developing country (Zaman, 2010). There is a rapid emergence of drug-resistant TB strains worldwide (Shah *et al.*, 2007) and the WHO reported that not only multidrug-resistant TB (MDR-TB) but extensively drug-resistant TB (XDR-TB) is on the rise as well (World Health Organization, 2016). The treatment of drug-resistant TB is complex; prescribed medicine intake can be for two years with a treatment success rate of 48% as opposed to drug-sensitive TB where treatment takes six months and has a success rate of 90% (Drobniewski *et al.*, 2015). The lack of improvement in treatment outcomes are mostly due to incompletion to the correct regimens, which were given at the point of diagnosis, relapse cases and non-returning patients. Therefore, rapid diagnosis can be the solution if it is also part of a framework developed to combat treatment failure (Naik *et al.*, 2018). Diagnosis is further complicated by the high incidence of drug resistance in tubercle bacteria— virtually 20% of TB strains are resistant to at least one major TB drug globally (Dheda *et al.*, 2017).

#### **3.1.4 The need for rapid DR-TB diagnostics**

Turnaround time in a laboratory and clinical setting has become an important factor especially with modern society's obsession with time conservation (Swiner *et al.*, 2020). It is critically important to consider turnaround time when thinking of implementing a new diagnostic procedure. This is because the severity of a disease can be underrepresented by clinical symptoms of the patient (Swiner *et al.*, 2020). Rapid diagnosis has many advantages — however the results must be precise and accurate. Early diagnosis can decrease the severity of the disease and long-term recurrences, decreases mortality rates, and prevent transmission to other susceptible hosts (Furlan *et al.*, 2014). Drug-resistant TB (DR-TB) is more difficult and expensive to treat and puts healthcare workers in resource-poor settings at serious risk of infection (Dheda *et al.*, 2017).

#### **3.1.5 TB as an epidemic in South Africa**

In South Africa, more than one third of the budget for TB diagnosis and management goes towards controlling DR-TB (Pooran *et al.*, 2013; World Health Organization, 2019). Pooran *et al.* (2013) estimated that the cost per patient for management of DS-TB in South Africa was roughly R 3672 (USD 257). Furthermore, MDR-TB and XDR-TB are 26 and 103 times more expensive than the cost of managing DS-TB, respectively. Treatment costs for patients with HIV/TB co-infection are also higher and mean hospitalisation costs amount to

at least R 35 349 (2474 USD) compared to R 16 460 (1152 USD) for hospitalised TB infected patients. South Africa's sixth Millennium Development Goal; is to combat HIV/AIDS, malaria, and other diseases, including TB (United Nations Development Program, 2015). It is a national strategy aimed at TB burden alleviation. The National Strategic Plan (NSP) 2017–2022 on HIV, sexually transmitted diseases (STI's) and TB, is another national strategy aimed at alleviating the burden of TB in South Africa with specific focus on HIV-TB co-infected patients (National Strategic Plan, 2017). According to the mid-year population estimates published by Stats SA, 8.2 million South Africans are living with HIV/AIDS currently (Stats SA, 2021). Most alarmingly, 19.5% of adults aged 15–49 years are diagnosed as HIV positive (Stats SA, 2021).

The NSP strategy is closely aligned with the National Development Plan that aims to progressively improve TB prevention and cure to accelerate social and economic development (National Planning Commission, 2012). These strategies, by working together, seek to reduce the incidence of TB in South Africa by more than 30%, from 450 000 to 315 000 (National Strategic Plan, 2017). The WHO End TB Strategy, which is a global strategy targeted at TB prevention, care and control was implemented post-2015 and aims to end the global TB epidemic (World Health Organization, 2015). The targets of this strategy are to reduce TB-related deaths by 95%, decrease new cases by 90% before 2035, and to relieve the financial burden of TB management on families (World Health Organization, 2015).

### **3.1.6 Global trends in drug resistant TB**

Yearly innovation towards improved diagnosis, monitoring, and treatment to eliminate the threat of a global TB pandemic is necessary to keep up with the disease's own advances in drug resistance by adapting complicated survival mechanisms (Claiborne *et al.*, 2011). The most common resistance found is resistance to rifampicin. Rifampicin resistance is caused by mutations in the rifampicin resistance determining region (RRDR) of *rpoB*, at codons 516, 526 and 531 in the MTB gene (Claiborne *et al.*, 2011; Tajbakhsh *et al.*, 2018). Mutations in the RRDR have recently also been found to be a predictive result towards development of multi-drug resistance (Hameed *et al.*, 2017). Bedaquiline was released in December 2012 as an anti-TB drug against MDR-TB and by the end of 2014 there were already instances of bedaquiline resistance. Mutations in the Rv0678 efflux pump regulatory region of the MTB genome is responsible for acquired resistance to bedaquiline (Ioerger *et al.*, 2013). The *eis* gene, or 'enhanced intracellular survival' gene, as described by Wei *et al.* (2000), is believed to increase intracellular survival of TB bacilli in human macrophage cells. The *eis* gene is associated with resistance to the second-line anti-TB drug kanamycin and mutations are located between codons two and 402 (Chen *et al.*, 2011). Phylogenetic markers do not

contribute to conference of drug resistance. The purpose of adding phylogenetic markers as targets is to determine the lineage of the TB infection since there are seven MTBC main lineages (Rigouts *et al.*, 2016). Lineage-specific differences influence the virulence of strains and including phylogenetic analysis of these variations helps determine the strain present in a patient with TB (Coll *et al.*, 2014).

### **3.1.7 Potential advancements towards alleviating the global TB burden**

Molecular methods show enormous potential for diagnosis of MDR-TB and could prove to be rapid, sensitive, and cost-effective tools that can help alleviate the DR-TB burden. These tests are based on identification of drug-resistance conferring mutations in the bacterial genome. MDR-TB line probe assays (LPAs) are molecular based diagnostic tools that detect drug resistance by hybridization of probes with specific complementary mutations (Havumaki *et al.*, 2017). One of the most widely used LPA MDR-TB diagnostic tests endorsed by the WHO is the Hain GenoType® MTBDR V1.0. The Hain GenoType® MTBDR V1.0 kit was developed in 2008 and later updated in 2011 (Hain GenoType® MTBDR V2). The Hain GenoType® MTBDR V2.0 kit had an addition of comparable sensitivity and specificity for detection of resistance to RIF and INH and detection of MDR. The Hain GenoType® MTBDR V1.0 kit was developed to detect mutations in *rpoB*, *katG* and the *inhA* promoter associated with drug resistance (Havumaki *et al.*, 2017).

The new commercial CapitalBio Gene Real-Time PCR Detection Kit has been developed for *M. tuberculosis* drug resistance detection by means of real-time PCR and is an attractive tool. With further evaluation, this kit by the CapitalBio Corporation (2015), in combination with current TB management tools and strategies, could provide a solution towards ending the global TB crisis through effective determination of drug resistance profiles. The kit, further referred to as the CapitalBio Gene Real-Time PCR Detection Kit, or CapitalBio kit, is developed for *in vitro* diagnostic use and has specific primer and probe designs that target site mutations in five genes, which can confer drug resistance to rifampicin, isoniazid, ethionamide, fluoroquinolones, amikacin, kanamycin and capreomycin. The whole assay takes less than two hours and after the real-time PCR process, automatic software analysis provides rapid therapy advice for the specific patient (CapitalBio Corporation, 2015). These *M. tuberculosis*-specific primers are used along with TaqMan® MGB (minor groove binder) probes that were designed based on mutations of the *rpoB* and *katG* genes and regulatory region of the *inhA*, *gyrA* and *rrs* genes (CapitalBio Corporation, 2015). TaqMan® MGB probes are used to analyse single-nucleotide polymorphisms and in combination with real-time PCR, can be used to recognize different mutations. These probes are complementary to the mutant nucleic acids and upon hybridization fluorescence is emitted, which then

indicates a positive result (CapitalBio Corporation, 2015). The CapitalBio kit can detect five mutant sites associated with *rpoB* gene, which is a gene that confers resistance to RIF by interfering with drug-target binding (Trauner *et al.*, 2014). This kit can also detect one mutant site associated with the *inhA* gene (INH resistance) and two mutant sites associated with the *gyrA* gene (resistance to fluoroquinolones) (Trauner *et al.*, 2014). Furthermore, the kit can also detect three mutant sites associated with the *rrs* gene, which can be associated with STR resistance and/or resistance to aminoglycosides; kanamycin (KM), amikacin (AMK) and capreomycin (CAP) (Jnawali *et al.*, 2013). Prospects point towards sequencing, even with the related high costs, it is a clear gold standard test for drug resistance (Köser *et al.*, 2013). Mutation targeting must be considered when determining a drug resistance profile due to the scope of the Mycobacterial genome and the number of mutations already discovered to confer drug resistance (Köser *et al.*, 2013). Whole genome sequencing provides an entire profile of the genetic make-up and possible resistance factors associated with genes in the MTBC genome (McNerney *et al.*, 2017). What prohibits advancements and implementation of next generation sequencing is mainly the financial cost per test per sample and the cost of equipment. The most important is the end-line or commercial cost per sample since it determines the willingness of patients to undergo diagnostic testing (Basu, 2019). An affordable diagnostic test that can accurately detect active TB and determine the drug resistance profile of the strain on the same day that a patient is diagnosed with presumptive TB does not exist yet (Basu, 2019).

### **3.1.8 Study aim and objectives**

The aim of this study is to evaluate the commercially available Hain GenoType® MTBDR kits (VER 2.0), by Hain Lifescience (Pty) Ltd., and compare to an innovative molecular based drug resistance profiling kit in the process of validation. This was achieved by determining which kit is most effective in targeting mutations in the *Mycobacterium tuberculosis* complex (MTBC) genome compared to whole genome sequencing as a reference standard. The kits should both be able to detect mutations in target regions known to confer resistance to first- and/or second-line anti-TB drugs. The drug resistance profiles of 92 DNA samples from TB isolates received from the South African National Institute for Communicable Diseases were analysed using both kits. The new versions of the commercially available Hain GenoType® MTBDR drug resistance profiling LPAs (Hain GenoType® MTBDR*plus* Version 2.0 and Hain MTBDRs/ Version 2.0) as well as the recently developed molecular kit were selected for evaluation for use in South African clinical settings due to reports of high diagnostic accuracy and ability to detect resistance to more than one common anti-TB drug simultaneously.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Pre-evaluations and optimization**

The new assay, the CapitalBio kit, has not been tested on South African populations to date and optimization of methods was required. Preliminary evaluations and optimization of our initial protocol helped shape the final method. Eleven samples (n=11) were subjected to analysis with the CapitalBio kit. After optimizing workflow and environment, and checking specific reaction mix combinations and efficacies, the final combination of reaction mixes was decided. The 96-well plate setups for PCR amplification of samples were adjusted after pre-analyses to decrease cross-contamination risks.

### **3.2.2 Isolates of TB infected patients**

A total number of 92 blinded and randomized *Mycobacterium tuberculosis* complex (MTBC) DNA isolates collected from the Centre for Tuberculosis (CTB) at the South African National Institute for Communicable Diseases (NICD) were used in this study. The samples were routinely extracted at a reference laboratory from original sputum samples. For this study only the extracted DNA samples were used which were double-blinded before collection and transport to the DSI/NWU Preclinical Drug Development Platform (PCDDP). The study was approved by the NWU Human Research Ethics committee (NWU-00976-19-A1) (Addendum 4).

Upon receiving the DNA samples, the DNA was divided into 20µl and 30µl aliquotes and immediately stored at -80°C until being moved to 5°C in batches of 12 samples for thawing before initiation of each experiment. The DNA was divided into aliquotes to minimize freezing and thawing. Laboratory set-up included calibration of equipment and deep-clean of work environments, including thermocycler blocks. The procedures of Hain GenoType® MTBDR V2.0 were performed in two separate laboratories. Initial loading of DNA in 96-well PCR plates was performed in a biosafety II cabinet after which samples were transferred to a second laboratory for PCR amplification and hybridization steps. The procedures of the CapitalBio kit were performed in three separate stations in the laboratory as described by the main procedures before sample analysis: (1) sample preparation, (2) sample loading and (3) PCR.

### **3.2.3 Comparison of diagnostic tests**

#### **3.2.3.1 Hain Lifescience GenoType® MTBDR (plus- and second-line) Version 2.0 assay**

The line-probe-assay-based analysis of the Hain LifeScience's MTBDR*plus* and -*sl* Version 2.0 assays required 5µl of DNA per analysis, which amounted to 10µl since each of the respective kits target different gene regions. Specific characteristics, such as gene regions targeted during analysis, of Hain GenoType® MTBDR*plus* V2.0 and MTBDR*sl* V2.0 were

summarised in Table 8. Samples were subjected to PCR by loading 12 samples per experiment into microtubes and adding an amplification mixture provided in the kit. The PCR process included 22 cycles of varying temperatures (Table 1) applied to samples to amplify present DNA fragments.

**Table 5: Manufacturer recommended PCR amplification temperatures and number of cycles for Hain GenoType® MTBDR V2.0 kits**

| Hain GenoType® MTBDR V2.0 kits |                         |              |           |            |      |      |                     |
|--------------------------------|-------------------------|--------------|-----------|------------|------|------|---------------------|
|                                | Initial<br>denaturation | Denaturation | Annealing | Elongation |      |      | Final<br>elongation |
| Hain GenoType® MTBDR V2.0 kits |                         |              |           |            |      |      |                     |
| Temperature                    | 95°C                    | 95°C         | 65°C      | 95°C       | 50°C | 70°C | 70°C                |
| Time                           | 15 min                  | 30 s         | 2 min     | 25 s       | 40 s | 40 s | 8 min               |
| Cycles                         | 1                       | 20           |           | 30         |      |      | 1                   |

After PCR amplification, denaturation solution was applied to strips probed with 27 reaction zones using DNA•STRIP® technology. All procedures were followed according to the manufacturer's instructions. The hybridization buffer solution and stringent wash solution was heated to 45°C and conjugate concentrate and conjugate buffer to room temperature before starting with sample loading. Conjugate concentrate was diluted with conjugate buffer, and substrate concentrate was diluted with substrate buffer beforehand according to manufacturer's instructions. Hybridization in short, entailed dispensing denaturation buffer into wells of trays of 12 and adding 20µl of DNA amplicons to the buffer to leave at room temperature for five minutes. One millilitre (mL) of pre-warmed hybridization buffer was then added to each well and mixed. A strip was carefully placed in each well using tweezers and placed in a Twincubator to incubate for 30 minutes at 45 °C after which the hybridization buffer was removed. After removal of hybridization buffer, the strips were washed using stringent wash solution and rinse solution and diluted conjugate solution was added before incubation for 30 minutes. Strips were rinsed a second time using 1 mL of distilled water before applying diluted substrate solution to the strips to incubate protected from light for up to eight minutes until bands developed and strips were removed to stop the reaction. Time to start of hybridization was two hours.

The entire hybridization process on 12 samples was completed in two hours and analysis of results performed within one hour immediately after hybridization. A template card was provided in the kit that was aligned with the respective locus control bands. A locus control band on the strips indicated the gene regions of respective DNA loci and reaction zones related to reaction success were considered. Three reaction zones or bands on the strips related to test validity: the conjugate control band, amplification control band and

*Mycobacterium tuberculosis* complex control band. The presence of a small line or band, developed after hybridization, indicated a positive result for each region. A negative result was indicated by the absence of a band at the sites of the loci on the strip. The presence of a band at the conjugate control site indicated the efficiency of the reaction, where the amplification control band indicated whether binding of conjugate to substrate was successful and *Mycobacterium tuberculosis* complex control band developed where amplicons strictly from strains of MTB complex were present. Where conjugate control band, amplification control band and MTB complex control band forming was unsuccessful, the reaction result was unevaluable. Signal strength varied and the amplification control band can be weak or disappear completely when a result was positive due to multiple competing single reactions on a single strip. In the case of drug sensitive samples, all wild type probes would be present, and any deviation was then classified as drug resistant, usually accompanied by a band formed at respective mutation sites on the strip. Bands with a signal strength as intense as or stronger than the amplification control were all considered positive. The same principles applied to Hain GenoType® MTBDRs/ V2.0 where the difference is that Hain GenoType® MTBDRs/ V2.0 strips had 27 reaction zones relating to drug resistance patterns indicating resistance to the second-line anti-TB drugs summarised in Table 8. Results were reviewed and bias surfaced. Result bias was resolved by a third review of the strips and banding patterns, considering bands faded during storage time.

### **3.2.3.2 The CapitalBio Gene Real-Time PCR Detection Kit**

The CapitalBio kit is a PCR-based kit and can analyse mutations in the multiple gene regions reported in Table 8 simultaneously in one single experiment. The kit's reaction mixtures contained probes linked to specific fluorescent dyes and channels, fluorescein amidites (FAM) dye and *Victoria* carboxy-fluorescein dye (VIC). Carboxyrhodamine (ROX) dye was used as a passive reference dye. The reaction mixtures contain dyes that tag oligonucleotide probes during PCR amplification of DNA fragments to produce curves of strength of light emission over cycle number on linear graphs. The assay procedure to analyse nine samples, in short, involved preparing the appropriate volume of reagents before initiation of any experiment. All reagents and solutions were thawed and centrifuged briefly before use. A positive and negative control for each reaction mixture was included for each experiment. We included a positive and negative control for each experiment. The positive control was a sample with a known resistance pattern, as determined by whole genome sequencing at the NICD Centre for Tuberculosis. The negative control was distilled water. A 25µl aliquot of each reaction mixture was placed into Bio-Rad Microseal® Semi-Skirt 96-well PCR plates™ for PCR amplification. A 5µl DNA sample was added to each well. Care was taken to prevent bubbles in the wells.

Samples were then subjected to PCR amplification following the manufacturer's recommended PCR temperatures and cycles (Table 2).

**Table 6: Manufacturer recommended PCR amplification temperatures and number of cycles for the CapitalBio Gene Real-Time PCR Detection Kit**

| The CapitalBio Gene Real Time PCR Detection Kit |                         |              |           |            |      |      |                     |
|-------------------------------------------------|-------------------------|--------------|-----------|------------|------|------|---------------------|
|                                                 | Initial<br>denaturation | Denaturation | Annealing | Elongation |      |      | Final<br>elongation |
| The CapitalBio kit                              |                         |              |           |            |      |      |                     |
| Temperature                                     | 50°C                    | 95°C         | 95°C      | 63°C       |      |      | 95°C 63°C           |
| Time                                            | 2 min                   | 10 min       | 15 s      | 50 s       |      |      | 15 s 50 s           |
| Cycles                                          | 1                       | 1            | 5         |            |      | 40   |                     |
| Hain GenoType® MTBDR V2.0 kits                  |                         |              |           |            |      |      |                     |
| Temperature                                     | 95°C                    | 95°C         | 65°C      | 95°C       | 50°C | 70°C | 70°C                |
| Time                                            | 15 min                  | 30 s         | 2 min     | 25 s       | 40 s | 40 s | 8 min               |
| Cycles                                          | 1                       | 20           |           | 30         |      |      | 1                   |

Samples were analysed on an ABI7200 PCR machine (Applied Biosystems, California, USA). The fluorescence level at the 40<sup>th</sup> PCR cycle was used to determine the result using relative normal fluorescence (Rn) as indicator. Relative normal fluorescence Rn is calculated by dividing the fluorescence of the reporter dye (the dye attached to the specific probes) by the fluorescence of the passive reference dye, whereas Delta Rn ( $\Delta Rn$ ) can be determined using any given cycle number after subtracting the baseline average Rn of the plotted values (Thermo Fisher Scientific, 2016). Each threshold value indicates a cut-off point where results are interpreted and determine whether a mutation is present or absent in each sample. Resistance to rifampicin was determined by a positive result detected using reaction mixture A and reaction mixture B and determining the value presented by the curves formed by fluorescent channel FAM for *rpoB* at the 40<sup>th</sup> cycle (cut-off). Resistance to isoniazid was determined by determining the value at the cut-off for the VIC fluorescent channel in reaction mixture A and B indicating mutations of *katG* and *inhA*.

### 3.2.4 Data analysis

To determine the sensitivity (rate of true positives), specificity (rate of true negatives), and positive and negative predictive values of each test we used standard equations as described by Parikh *et al.*, 2008:

$$\text{Sensitivity} = \frac{\text{True positive results}}{\text{True positive results} + \text{False negative results}}$$

$$\text{Specificity} = \frac{\text{True negative results}}{\text{True negative results} + \text{False positive results}}$$

$$\text{Positive predictive value} = \frac{\text{True positive results}}{\text{True positive results} + \text{False positive results}}$$

$$\text{Negative predictive value} = \frac{\text{True negative results}}{\text{True negative results} + \text{False negative results}}$$

### 3.2.5 ASSURED analysis

To compare key characteristics of each kit, the ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment free and Deliverable) criteria were applied. The criteria were developed by the World Health Organization (WHO) Sexually Transmitted Diseases Diagnostics Initiative to aid in identification of tests suitable for implementation in settings where resources could be limited (World Health Organization, 2021). In developing countries routine diagnostic tests are performed at centralized laboratories which are easily accessible to communities nearby whereas distant communities must travel long distances to receive a diagnosis (Peeling *et al.*, 2006). The criteria were implemented by timing index test duration from initiation to result analysis, comparing current prices related to implementation of each test, determining the number of steps to result analysis, and comparing accuracy (sensitivity and specificity). According to Kosack *et al.* (2017), the ASSURED criteria can serve as a standard for decision-making purposes when the implementation of a new diagnostic test is considered. The criteria were applied according to the following guidelines adapted from Land *et al.* (2019):

#### 1 Affordable

The use, conduct, and implementation of a diagnostic test incurs related costs. Nevertheless, the FIND initiative sets guidelines on kit and component pricing (FIND, 2021). For a test to be considered for implementation in a low-and-middle income country (LMIC), it must be economical. According to Land *et al.* (2019), 10 USD is accepted as affordable for a single tuberculosis diagnostic test at the point-of-care (POC) setting. For comparison purposes, less than R141 (10 USD) per test was labelled as high affordability and more than R352 (25 USD) per tests as low affordability. The costs related to each kit were considered because it determines the willingness of consumers to procure the kit or seek more affordable alternatives. Costs included in the calculation of total costs to the consumer were

the commercial costs of the kits themselves, all equipment needed to perform the steps in each procedure and the cost of consumables used per sample was calculated.

## **2 Sensitive and Specific**

A diagnostic accuracy value of 90% implies that ten out of every hundred patients could receive a misdiagnosis. To avoid misdiagnosis, a test must have a high diagnostic accuracy. Diagnostic accuracy is determined by sensitivity and specificity and both estimates are regarded as high when the values are above 90% and low if the values are below 60% (World Health Organization, 2014).

## **3 User-friendly**

The user-friendliness of a diagnostic test refers to level of training required where tests consisting of two to three steps are regarded as highly user-friendly according to guidelines by Land *et al.* (2019). We regarded a protocol of more than ten steps requiring advanced techniques as complex. According to García-Basteiro *et al.* (2018), highly user-friendly tests are simple and safe to use. Turnaround time was predicted from existing reports and then determined on-site during each evaluation. Turnaround time is determined by the time related to each procedure to produce the final result. Sample processing and DNA extraction were performed by the NICD Centre for Tuberculosis and the time needed for these steps was not included in our analysis, but it is mentionable that sample preparation is a timely process where automatic DNA extraction systems are not available. The total turnaround time includes interpretation of the results as per manufacturer's instructions for each respective kit.

Ease of use was determined by considering the environmental requirements in which the assays were performed and whether training is required to be able to use the assay. Ease of use was also influenced by factors such as necessity and duration of required training, number of pipetting steps per sample, ease of following protocols and procedures as per manufacturer's instructions and whether separate environments were required for certain steps of the assays, e.g., to be performed in biosafety cabinets.

## **4 Rapid and robust**

Turnaround time is an important determinant of diagnostic test performance. Rapid tests are considered to deliver results within 60 minutes from sample acquisition (Land *et al.* 2019). To maintain the findings of Davids *et al.* (2015) in a survey regarding rapid TB screening in South Africa, a period of 24 hours was regarded as the maximum acceptable time to diagnosis. The optimal turnaround time of 5–15 minutes was regarded as rapid for a point-of-care TB diagnostic test (Davids *et al.*, 2015). To determine whether a test was rapid and robust, we considered the turnaround time of each test for rapidness. To evaluate

robustness, we considered storage instructions and expiry date as maintained by the standards used in the study by Land *et al.* (2019). Manufacturing also relates to robustness of a kit as it must be reliable enough to ensure consistency regarding the quality of the consumables produced for multiple batches (Kosack *et al.*, 2017).

## **5 Equipment-free and Deliverable**

Current DR-TB diagnostic tests are all equipment-based (World Health Organization, 2020b). To evaluate the last criterion, we considered equipment and consumable needs for each diagnostic test. Ideally a diagnostic test for low resource settings must be portable and able to run without the need for an electricity source (Land *et al.* 2019). As maintained by Land *et al.* (2019) delivery of a diagnostic kit refers to management processes involved in procuring the kit with regards to organizational structures that need to be developed and established to steer implementation of the kit.

## **3.3 RESULTS AND DISCUSSION**

### **3.3.1 Blinded microbial DNA isolates from TB infected patient sputum**

The NICD Centre for Tuberculosis provided 50 µl of high quality, purified DNA suspended in elution buffer extracted using the MagNA Pure automatic DNA extraction instrument. A total of 92 samples were received between October 2018 and January 2019. Concentrations of the sample DNA were above 0.8 ng/µl as determined by The NICD Centre for Tuberculosis. Sample characteristic data, determined using whole genome sequencing (WGS), was made available once the last experiment was performed. No demographic data was made available as samples were completely blinded. The WGS was used to compare the kits with one another. All experiments and result analyses were performed between January 2019 and April 2019.

### **3.3.2 Pre-evaluations and optimization**

Five DNA samples with a concentration range between 0.8–2.7 ng/µl, extracted by the NICD Centre for Tuberculosis from sputum samples of TB infected patients, were received (Table 7). This was to determine the compatibility of the equipment and determine whether optimization is necessary beforehand. All targets were amplified except for the *gyrA* gene in samples S2, S3, S4 and S5 during real-time PCR. Data was released by the NICD-CTB when the experiments were concluded on the first five samples. We suspected a faulty probe design or that the probe did not encompass all variations of the gene for populations in South Africa. Another six high quality DNA samples were received specifically selected from a group of fluoroquinolone-resistant samples known to have at least one mutation in the

*gyrA* gene (Table 8). The results were marginally more promising, but it was still evident that the probe for the Asp94His mutation fails to bind during amplification. This issue could not be solved by our own means. However, it is possible to synthesize a probe that would specifically target Asp94His by redesigning the probe using population-specific samples and determining a personalized threshold value. This process of optimization would require hundreds (n=~400) of samples within South Africa's demographics to establish a new threshold or design a new probe for Asp94His, this is due to population-based reference ranges (Phillips, 2009). It would be up to the kit manufacturers to establish a new threshold for diagnosis of fluoroquinolone resistance to this specific *gyrA* gene mutation.

**Table 7: Table of results from pre-evaluations and optimization for the CapitalBio Gene Real-Time PCR Detection Kit**

| Reaction mix | Sample | FAM result | VIC result | Result indication                      | Antibiotic resistance detected | Sanger sequencing                        | Concordance with sequencing |
|--------------|--------|------------|------------|----------------------------------------|--------------------------------|------------------------------------------|-----------------------------|
| A            | S1     | -          | -          | Wild type                              | susceptible                    | -                                        | Yes                         |
|              | S2     | +          | +          | <i>rpoB</i> mutant, <i>katG</i> mutant | RIF, INH                       | Asp435Val, Ser6Tyr, Ser315Tyr            | Yes                         |
|              | S3     | +          | -          | <i>rpoB</i> mutant                     | RIF                            | Ser450Leu, Arg463Leu                     | Yes                         |
|              | S4     | -          | -          | Wild type                              | susceptible                    | His445Asp                                | No                          |
|              | S5     | +          | +          | <i>rpoB</i> mutant, <i>katG</i> mutant | RIF, INH                       | Ser450Leu, Asn31le, Ser315Thr            | Yes                         |
| B            | S1     | -          | -          | Wild type                              | susceptible                    | -                                        | Yes                         |
|              | S2     | -          | -          | Wild type                              | susceptible                    | -                                        | Yes                         |
|              | S3     | -          | -          | Wild type                              | susceptible                    | -                                        | Yes                         |
|              | S4     | +          | -          | <i>rpoB</i> mutant                     | RIF                            | -                                        | No                          |
|              | S5     | -          | -          | Wild type                              | susceptible                    | -                                        | Yes                         |
| C            | S1     | -          | -          | Wild type                              | susceptible                    | -                                        | Yes                         |
|              | S2     | -          | +          | <i>embB</i> mutant                     | RIF, INH, EMB                  | -                                        | No                          |
|              | S3     | -          | -          | Wild type                              | susceptible                    | -                                        | Yes                         |
|              | S4     | -          | -          | Wild type                              | susceptible                    | -                                        | Yes                         |
|              | S5     | -          | +          | <i>embB</i> mutant                     | RIF, INH, EMB                  | -                                        | No                          |
| D            | S1     | -          | -          | Wild type                              | susceptible                    | -                                        | Yes                         |
|              | S2     | -          | -          | Wild type                              | susceptible                    | Glu21Gln, Asp94His, Ser95Thr, Gly668Asp  | No                          |
|              | S3     | -          | -          | Wild type                              | susceptible                    | Glu21Gln, Ser95Thr                       | No                          |
|              | S4     | -          | -          | Wild type                              | susceptible                    | Glu21Gln, Ser95Thr, Gly247Ser, Gly668Asp | No                          |
|              | S5     | -          | -          | Wild type                              | susceptible                    | Glu21Gln, Ser95Thr, Gly247Ser, Gly668Asp | No                          |
| E            | S1     | -          | -          | Wild type                              | susceptible                    | -                                        | Yes                         |
|              | S2     | +          | -          | <i>rrs</i> mutant                      | AG                             | C492T                                    | Yes                         |
|              | S3     | -          | -          | Wild type                              | susceptible                    | -                                        | Yes                         |
|              | S4     | -          | -          | Wild type                              | susceptible                    | -                                        | Yes                         |
|              | S5     | -          | -          | Wild type                              | susceptible                    | -                                        | Yes                         |

**Table 8: Table of results from the second pre-evaluation and optimization for the CapitalBio Gene Real-Time PCR Detection Kit**

| Reaction mix | Sample no. | FAM result | VIC result | Result indication  | Antibiotic resistance detected | Sanger sequencing | Concordance with sequencing |
|--------------|------------|------------|------------|--------------------|--------------------------------|-------------------|-----------------------------|
| D            | S6         | -          | -          | Wild type          | susceptible                    | Asp94His          | No                          |
|              | S7         | +          | -          | <i>gyrA</i> mutant | resistant                      | Asp94Gly          | Yes                         |
|              | S8         | -          | -          | Wild type          | susceptible                    | Asp94Asn          | No                          |
|              | S9         | +          | -          | <i>gyrA</i> mutant | resistant                      | Asp94Gly          | Yes                         |
|              | S10        | -          | -          | Wild type          | susceptible                    | Asp94His          | No                          |
|              | S11        | +          | -          | <i>gyrA</i> mutant | resistant                      | Asp94Asn          | Yes                         |

With analysis of resistance gene regions *rpsL* and *rrs*, the fluorescent dye VIC was not involved in detection of resistance (Table 7). Reaction mixture D and E do not require VIC for determining the presence of resistance-conferring *rpsL* and *rrs* mutations. Wild type indicates a negative result as no mutation is present (drug sensitive).

### 3.3.3 Comparative evaluations

The diagnostic accuracy of each respective kit was determined by the number of true positive results, false positive results, true negative results, and false negative results. Data analysis comprised of using these estimates to determine sensitivity, specificity, positive predictive value, and negative predictive value of each respective kit. The results are summarised in Table 9 reports on the determined first-line resistances using the selected kits against the reference standard of the whole genome sequence (WGS). The ASSURED criteria was applied to compare the kits based on important characteristics as described by Land *et al.* (2019).

The diagnostic accuracy for each respective assay towards first-line anti-TB drug resistance (rifampicin and isoniazid) is reported in Table 14. The genes related to first-line drug

resistance are *rpoB*, *katG* and *inhA* and mutations at specific sites on the DNA loci confer drug resistance to rifampicin and isoniazid. The target regions of the CapitalBio kit are the same gene regions targeted by the LPA, however detection is based on PCR amplification of the target mutations (Table 15). The mutations are related to first-line resistance with the means to detection not accompanied by detection of missing wild types. Susceptibility is determined by the absence of a mutation in the targeted regions and is then described as a wild type. Drug resistance was detected by analysing banding patterns of mutations and missing wild type bands on Hain GenoType® MTBDR strips. The CapitalBio kit and Hain GenoType® MTBDR kits were compared based on diagnostic performance towards identifying rifampicin resistance and isoniazid resistance by targeting the respective gene regions that confer resistance to these first-line anti-TB drugs. We successfully detected 67 rifampicin resistant samples (Table 14) with the Hain GenoType® MTBDR*plus* V2.0 kit. There were four false positives, three with a mutation of Leu430Pro and one with a mutation of Asp435Tyr (Table 9). Among the 92 samples, 21 were rifampicin susceptible according to LPA results (Table 14). The related sensitivity and specificity estimate of Hain GenoType® MTBDR*plus* towards detection of rifampicin resistance were 94.4% and 100%, respectively. The CapitalBio kit successfully detected 63 rifampicin resistant samples and 18 rifampicin susceptible samples with three false positive results and eight false negative results (Table 14). The estimated sensitivity and specificity of CapitalBio towards detection of rifampicin resistance were 88.7% and 85.7%, respectively (Table 14). With regards to isoniazid resistance detection, both *katG* and *inhA* were analysed for resistance conferring mutations (Table 14). The Hain GenoType® MTBDR*plus* V2.0 kit detected 51 of 52 isoniazid resistant samples, where resistance was conferred by a mutation in the *katG* gene region, specifically mutations of Ser315Asn and Ser315Thr (Table 9). The sensitivity and specificity of Hain GenoType® MTBDR*plus* towards detection of resistance conferring mutations in the *katG* gene region were 98.1 and 100%, respectively (Table 14). Detection of mutations in the *inhA* gene region, which is also related to isoniazid resistance, yielded 35 true positive results, 6 false negative results, 51 susceptible results and no false negatives for Hain GenoType® MTBDR*plus* (Table 14). The resulting sensitivity and specificity estimates were 100% and 89.5%, respectively (Table 14). Using the CapitalBio kit, we detected 39 isoniazid resistant samples targeting the *katG* gene region and 37 susceptible samples (Table 14). Of the 92 samples analysed for isoniazid resistance using the CapitalBio kit, there were three false positive results and 13 false negative results (Table 14). The sensitivity and specificity estimates for the CapitalBio kit analysis towards isoniazid resistance were 75% and 92.5%, respectively (Table 14). The CapitalBio kit successfully detected five isoniazid resistance conferring mutations in the *inhA* gene region and failed to detect an additional 30 resistant samples. There were 13 false positive results and 44 true negative results indicating

isoniazid susceptibility during analysis of the *inhA* gene region with the CapitalBio kit (Table 14). The resulting sensitivity and specificity estimates were 14.3% and 77.2%, respectively (Table 14).

**Table 9: Results of first-line resistance detection using the Hain GenoType® MTBDR V2.0 LPA and the CapitalBio kit compared to WGS data**

| Banding pattern on Hain MTBDR strips | Isolates | Banding pattern on Hain MTBDR strips | Mutations by DNA sequencing | Phenotypic drug susceptibility | Mutations detected by the CapitalBio kit | Isolates | Mutations by DNA sequencing | Phenotypic drug susceptibility |
|--------------------------------------|----------|--------------------------------------|-----------------------------|--------------------------------|------------------------------------------|----------|-----------------------------|--------------------------------|
| <b>Rifampicin – <i>rpoB</i></b>      |          |                                      |                             |                                |                                          |          |                             |                                |
| WT1, WT8, MUT3                       | n=3      | WT1, WT8, MUT3                       | Ser450Leu                   | Resistant                      | MUT1A                                    | n=21     | Asp435Val                   | Resistant                      |
| WT2                                  | n=2      | WT2                                  | Leu430Pro                   | Resistant                      | MUT1A                                    | n=3      | Asp435Tyr                   | Resistant                      |
| WT3, WT4, MUT1                       | n=23     | WT3, WT4, MUT1                       | Asp435Val                   | Resistant                      | MUT1A                                    | n=2      | His445Tyr                   | Resistant                      |
| WT3, WT4                             | n=9      | WT3, WT4                             | Asp435Tyr                   | Resistant                      | MUT1A                                    | n=3      | Ser450Leu                   | Resistant                      |
| WT3, WT8                             | n=1      | WT3, WT8                             | Asp435Gly, Leu452Pro        | Resistant                      | MUT1A                                    | n=2      | wt                          | Susceptible                    |
| WT7, MUT2A                           | n=4      | WT7, MUT2A                           | His445Tyr                   | Resistant                      | MUT1A, MUT1B                             | n=15     | Ser450Leu                   | Resistant                      |
| WT7, MUT2B                           | n=2      | WT7, MUT2B                           | His445Asp                   | Resistant                      | MUT1A, MUT1B                             | n=1      | Asp435Gly                   | Resistant                      |
| WT7                                  | n=2      | WT7                                  | His445Asn                   | Resistant                      | MUT1A, MUT1B                             | n=2      | Asp435Gly, Leu452Pro        | Resistant                      |
| WT7                                  | n=1      | WT7                                  | His445Leu                   | Resistant                      | MUT1A, MUT1B                             | n=1      | Asp435Val                   | Resistant                      |
| WT8, MUT3                            | n=15     | WT8, MUT3                            | Ser450Leu                   | Resistant                      | MUT1A, MUT1B                             | n=1      | His445Asn                   | Resistant                      |
| WT8, MUT3                            | n=1      | WT8, MUT3                            | Ser450Leu, Gln409Arg        | Resistant                      | MUT1A, MUT1B                             | n=2      | His445Asp                   | Resistant                      |
| WT8                                  | n=1      | WT8                                  | Asp435Gly, Leu452Pro        | Resistant                      | MUT1A, MUT1B                             | n=1      | His445Leu                   | Resistant                      |
| MUT3                                 | n=1      | MUT3                                 | His445Tyr                   | Resistant                      | MUT1A, MUT1B                             | n=3      | His445Tyr                   | Resistant                      |
| MUT3                                 | n=1      | MUT3                                 | Ser450Leu                   | Resistant                      | MUT1A, MUT1B                             | n=4      | Leu430Pro                   | Resistant                      |

wt = wild type

**Table 9 (continued): Results of first-line resistance detection using the Hain GenoType® MTBDR V2.0 LPA and the CapitalBio kit compared to WGS data**

| Banding pattern on Hain MTBDR strips | Isolates | Banding pattern on Hain MTBDR strips | Mutations by DNA sequencing | Phenotypic drug susceptibility | Mutations detected by the CapitalBio kit | Isolates | Mutations by DNA sequencing | Phenotypic drug susceptibility |
|--------------------------------------|----------|--------------------------------------|-----------------------------|--------------------------------|------------------------------------------|----------|-----------------------------|--------------------------------|
| <b>Rifampicin – <i>rpoB</i></b>      |          |                                      |                             |                                |                                          |          |                             |                                |
| MUT2B                                | n=1      | MUT2B                                | His445Asp                   | Resistant                      | MUT1B                                    | n=1      | His445Asp                   | Resistant                      |
| -                                    | n=3      | -                                    | Leu430Pro                   | Resistant                      | MUT1B                                    | n=1      | Leu430Pro                   | Resistant                      |
| -                                    | n=1      | -                                    | Asp435Tyr                   | Resistant                      | MUT1B                                    | n=2      | Asp435Tyr                   | Resistant                      |
| -                                    | n=21     | -                                    | wt                          | Susceptible                    | MUT1B                                    | n=1      | wt                          | Susceptible                    |
|                                      |          |                                      |                             |                                | -                                        | n=5      | Asp435Tyr                   | Resistant                      |
|                                      |          |                                      |                             |                                | -                                        | n=1      | His445Asn                   | Resistant                      |
|                                      |          |                                      |                             |                                | -                                        | n=1      | Ser450Leu                   | Resistant                      |
|                                      |          |                                      |                             |                                | -                                        | n=1      | Ser450Leu, Gln409Arg        | Resistant                      |
|                                      |          |                                      |                             |                                | -                                        | n=18     | wt                          | Susceptible                    |
| <b>Isoniazid – <i>katG</i></b>       |          |                                      |                             |                                |                                          |          |                             |                                |
| WT, MUT1                             | n=45     | WT, MUT1                             | Ser315Thr                   | Resistant                      | MUT                                      | n=38     | Ser315Thr                   | Resistant                      |
| WT, MUT2                             | n=1      | WT, MUT2                             | Ser315Thr                   | Resistant                      | -                                        | n=1      | Ser315Asn, Ser315Ile        | Resistant                      |
| MUT1                                 | n=3      | MUT1                                 | Ser315Thr                   | Resistant                      | MUT                                      | n=11     | Ser315Thr                   | Resistant                      |
| -                                    | n=1      | -                                    | Ser315Ile, Ser315Asn        | Resistant                      | -                                        | n=1      | Ser315Asn                   | Resistant                      |
| WT                                   | n=2      | WT                                   | Ser315Asn                   | Resistant                      | MUT                                      | n=3      | wt                          | Susceptible                    |
| -                                    | n=40     | -                                    | wt                          | Susceptible                    | -                                        | n=38     | wt                          | Susceptible                    |
| <b>Isoniazid – <i>inhA</i></b>       |          |                                      |                             |                                |                                          |          |                             |                                |
| WT1, MUT1                            | n=12     | WT1, MUT1                            | C-15T                       | Resistant                      | MUT                                      | n=5      | C-15T                       | Resistant                      |
| WT2, MUT3B                           | n=5      | WT2, MUT3B                           | T-8A                        | Resistant                      | -                                        | n=17     | G-17T                       | Resistant                      |
| MUT1                                 | n=1      | MUT1                                 | C-15T                       | Resistant                      | -                                        | n=8      | C-15T                       | Resistant                      |
| WT1                                  | n=6      | WT1                                  | wt                          | Susceptible                    | -                                        | n=5      | T-8A                        | Resistant                      |
| WT1                                  | n=17     | WT1                                  | G-17T                       | Resistant                      | MUT                                      | n=13     | wt                          | Susceptible                    |
| -                                    | n=51     | -                                    | wt                          | Susceptible                    | -                                        | n=44     | wt                          | Susceptible                    |

wt = wild type

Similarly, the diagnostic accuracy for each respective assay towards detecting resistance to second-line anti-TB drugs (aminoglycosides and fluoroquinolones) are also reported in Table 10 and Table 11. The gene regions where mutations are targeted by Hain GenoType® MTBDRs/ V2.0 are *eis* and *gyrB*, which confer resistance to aminoglycosides and fluoroquinolones respectively. The CapitalBio kit can be used to detect mutations in the *embB* and *rpsL* genes regions for second-line anti-TB drug resistance detection to ethambutol and streptomycin respectively (Table 11). We could only compare resistance detection to second-line anti-TB drugs in the *gyrA* and *rrs* gene regions as Hain GenoType® MTBDRs/ V2.0 and the CapitalBio kit do not examine the same gene regions for resistance to the second-line anti-TB drugs fluoroquinolones and aminoglycosides (Table 10 and Table 11). Second-line resistance mutations in the specific gene regions: *gyrA*, *gyrB*, *rrs* and *eis*, and missing wild type bands in these regions, are described as MDR-TB when detected. Banding patterns on Hain MTBDR strips in these gene regions are analysed to detect resistance to fluoroquinolones and aminoglycosides (Table 10).

**Table 10: Summary of results generated by sample analysis with the Hain GenoType® MTBDR LPA compared to WGS data regarding detection of resistance to second-line anti-TB drugs**

| Banding pattern on Hain MTBDR strips  | Isolates | Mutations by DNA sequencing | Phenotypic drug susceptibility |
|---------------------------------------|----------|-----------------------------|--------------------------------|
| <b>Fluoroquinolones – <i>gyrA</i></b> |          |                             |                                |
| WT2, MUT1                             | n=2      | Ala90Val                    | Resistant                      |
| WT2, MUT2                             | n=2      | Ser91Pro                    | Resistant                      |
| WT3, MUT3A                            | n=3      | Asp94Ala                    | Resistant                      |
| WT3, MUT3B, MUT3C                     | n=1      | Asp94Asn, Asp94Gly          | Resistant                      |
| WT3, MUT3C                            | n=15     | Asp94Gly                    | Resistant                      |
| WT3, MUT3C                            | n=1      | Asp94Ala                    | Resistant                      |
| WT3, MUT3D                            | n=1      | Asp94His                    | Resistant                      |
| WT3                                   | n=1      | Asp94Tyr                    | Resistant                      |
| WT3                                   | n=1      | Asp94Phe                    | Resistant                      |
| MUT3C                                 | n=4      | Asp94Gly                    | Resistant                      |
| MUT3C                                 | n=1      | Ala90Val                    | Resistant                      |
| MUT3C                                 | n=1      | wt                          | Susceptible                    |
| -                                     | n=59     | wt                          | Susceptible                    |
| <b>Fluoroquinolones – <i>gyrB</i></b> |          |                             |                                |
| WT, MUT1                              | n=1      | wt                          | Susceptible                    |
| -                                     | n=91     | wt                          | Susceptible                    |
| <b>Aminoglycosides – <i>rrs</i></b>   |          |                             |                                |
| WT1, MUT1                             | n=8      | A1401G                      | Resistant                      |
| WT1, MUT1                             | n=10     | A1401G, A514C               | Resistant                      |
| WT1, MUT1                             | n=12     | A514C, A1401G               | Resistant                      |
| -                                     | n=1      | A514C                       | Susceptible                    |
| -                                     | n=12     | C492T                       | Susceptible                    |
| -                                     | n=2      | C517T                       | Susceptible                    |
| -                                     | n=47     | wt                          | Susceptible                    |
| <b>Aminoglycosides – <i>eis</i></b>   |          |                             |                                |
| -                                     | n=92     | wt                          | Susceptible                    |

wt = wild type

The Hain GenoType® MTBDRs/ V2.0 kit successfully detected 32 fluoroquinolone resistant samples by analysing mutations in the *gyrA* gene region (Table 14). Of the 92 samples analysed, we detected 59 fluoroquinolone susceptible samples but also detected one false positive. The sensitivity and specificity of Hain GenoType® MTBDRs/ towards fluoroquinolone resistance detection were 100% and 98.3%, respectively (Table 14). A total of 25 samples had mutations conferring fluoroquinolone resistance in the *gyrA* gene region according to analysis with the CapitalBio kit (Table 12). We also detected seven false positives and seven false negatives with 53 samples determined to be susceptible (Table 14). The related sensitivity and specificity estimates were 78.1% and 88.3%, respectively. Analysis of 92 samples with no mutations in the *gyrB* region that confer resistance to fluoroquinolones yielded a total of 91 true negative results and one false positive result (Table 14). The specificity towards detection of mutations in the *gyrB* gene region was estimated at 98.9% and sensitivity could not be calculated due to the lack of true positive samples (Table 14).

**Table 11: Summary of results generated by sample analysis with the CapitalBio kit compared to WGS data regarding detection of resistance to second-line anti-TB drugs**

| Mutations detected by the CapitalBio kit | Isolates | Mutations by DNA sequencing | Phenotypic drug susceptibility |
|------------------------------------------|----------|-----------------------------|--------------------------------|
| <b>Fluoroquinolones – <i>gyrA</i></b>    |          |                             |                                |
| MUT                                      | n=19     | Asp94Gly                    | Resistant                      |
| MUT                                      | n=3      | Ala90Val                    | Resistant                      |
| MUT                                      | n=2      | Asp94Ala                    | Resistant                      |
| MUT                                      | n=1      | Asp94Asn, Asp94Gly          | Resistant                      |
| -                                        | n=2      | Asp94Ala                    | Resistant                      |
| -                                        | n=1      | Asp94His                    | Resistant                      |
| -                                        | n=1      | Asp94Phe                    | Resistant                      |
| -                                        | n=1      | Asp94Tyr                    | Resistant                      |
| -                                        | n=2      | Ser91Pro                    | Resistant                      |
| MUT                                      | n=7      | wt                          | Susceptible                    |
| -                                        | n=53     | wt                          | Susceptible                    |
| <b>Aminoglycosides – <i>rrs</i></b>      |          |                             |                                |
| MUT                                      | n=8      | A1401G                      | Resistant                      |
| MUT                                      | n=10     | A1401G, A514C               | Resistant                      |
| MUT                                      | n=12     | A514C, A1401G               | Resistant                      |
| MUT                                      | n=1      | A514C                       | Susceptible                    |
| MUT                                      | n=2      | C517T                       | Susceptible                    |
| MUT                                      | n=3      | C492T                       | Susceptible                    |
| MUT                                      | n=8      | wt                          | Susceptible                    |
| -                                        | n=9      | C492T                       | Susceptible                    |
| -                                        | n=39     | wt                          | Susceptible                    |
| <b>Aminoglycosides – <i>rpsL</i></b>     |          |                             |                                |
| MUT                                      | n=2      | Lys43Arg                    | Resistant                      |
| MUT                                      | n=3      | Lys88Arg                    | Resistant                      |
| -                                        | n=1      | Lys43Arg                    | Resistant                      |
| MUT                                      | n=25     | wt                          | Susceptible                    |
| -                                        | n=61     | wt                          | Susceptible                    |
| <b>Ethambutol – <i>embB</i></b>          |          |                             |                                |
| MUT                                      | n=25     | Met306Ile                   | Resistant                      |
| MUT                                      | n=5      | Met306Val                   | Resistant                      |
| -                                        | n=1      | Gln497Arg                   | Resistant                      |
| MUT                                      | n=31     | wt                          | Susceptible                    |
| -                                        | n=30     | wt                          | Susceptible                    |

wt = wild type

With regards to aminoglycoside resistance detection, Hain GenoType® MTBDRs/ V2.0 targets the *rrs* and *eis* gene regions (Table 14). Analysis of mutations in the *rrs* gene region, yielded 30 true positive results and 62 true negative results (Table 14) with a resulting 100% sensitivity and 100% specificity (Table 14). We successfully detected 30 aminoglycoside resistant samples by analysing the *rrs* gene region with the CapitalBio kit (Table 14) accompanied by a total of 48 true negative results. The remaining samples were detected as 14 false positives resulting in an estimated 100% sensitivity and 77.4% specificity (Table 14). The additional gene region targeted using the CapitalBio kit is the *rpsL* gene region where mutations could confer resistance to streptomycin. We detected a total of five true positive results, 25 false positive results, one false negative and 61 true negative results (Table 14). Sensitivity was estimated at 83.3% and specificity at 70.93% for the CapitalBio

kit towards detection of mutations in the *rpsL* gene region (Table 14). The Hain GenoType® MTBDRs/ V2.0 kit was successful in detecting the total 92 samples as true negative for resistance to aminoglycosides when targeting the *eis* gene region (Table 14). Sensitivity could consequently not be determined, due to the lack of true positives, which are essential towards estimation of sensitivity (Parikh *et al.*, 2008). However, the specificity is determined as 100% (Table 14). Whole genome sequencing confirmed these findings by reporting that all samples were wild types when analysing the *eis* gene region (Table 14).

We included analysis of the additional gene regions for the respective kits since it is advantageous to determine a comprehensive diagnosis (Table 9). What makes the CapitalBio kit an attractive diagnostic assay is the simultaneous detection of resistance to first-line and second-line anti-TB drugs. The gene region targets that are not synonymous between the two assays were not included in the assay comparison, but the reported results could provide valuable information especially in MDR-TB cases. For instance, ethambutol is used in conjunction with other anti-TB drugs and is commonly prescribed as part of first-line treatment plans (Pawar *et al.*, 2019). During this analysis, we detected 30 ethambutol resistant DNA samples however there were 31 false positive results and one false negative (Table 14). Additionally, 61 ethambutol susceptible samples were detected (Table 14).

### **3.4 ASSURED analysis**

The ASSURED analysis was implemented based on criteria adapted from Land *et al.* (2019). The ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment free and Deliverable) criteria was first described by the World Health Organization (WHO) Sexually Transmitted Diseases Diagnostics Initiative (World Health Organization, 2021) and Kosack *et al.* (2017) maintains that the ASSURED criteria are important to apply before implementation of a new diagnostic test can be considered.

Equipment and consumables, as well as the estimated costs of both the Hain GenoType® kits, and the CapitalBio kits are given in Table 12 and Table 13, respectively. Key features regarding the principles of each kit, gene regions targeted, turnaround time, result analysis, equipment requirements and ease-of-use in terms of steps to results analysis and training requirements are summarised in Table 16. The main difference are the principles on which the respective kits are based, the CapitalBio is a real-time PCR based kit and the Hain GenoType® kits are line probe assay based, however samples must be PCR amplified before hybridization to probed strips with LPA analysis.

### 3.4.1 Affordable

We compared the costs related to possible implementation of the respective kits in resource constrained settings where equipment and consumables would need to be procured and determined a total cost per sample per analysis based on this assumption. Virtually, the costs are imperfect due to factors that were not taken into account during this analysis, for example, the cost per hour for a healthcare worker that would be required to do the analysis.

The Hain GenoType® MTBDR*plus* V2.0 including GenoLyse, contains enough buffer solution and membrane strips to evaluate and interpret 96 samples. The commercial cost of one 96-unit kit is R 12 581 (860 USD) excluding tax. The Hain GenoType® MTBDR*s*/ V2.0 is identical in cost and it is also a 96-unit kit, which can be used to determine second-line resistance. Expensive equipment is required to perform Hain GenoType® MTBDR V2.0 assays. The thermocycler used was provided by the laboratory where analyses were performed however, we included an estimated cost for the Quantstudio-3 real-time PCR system (Table 12) and maintenance and calibration of equipment could result in extensive costs over a long period of time. The Hain Lifescience Twincubator was used to perform the hybridization steps. We were able to rent the Twincubator from Hain Lifescience at R350 per month.

**Table 12: Summary of required equipment and consumables not included in the Hain Lifescience GenoType® MTBDR kits**

| Equipment and consumables                                                                               | Estimated cost (excluding VAT)        |
|---------------------------------------------------------------------------------------------------------|---------------------------------------|
| Real-time thermal cycler (PCR) machine                                                                  | R 547 390 (35 774 USD)                |
| Twincubator                                                                                             | R 38 000 (2483 USD)                   |
| Twincubator (rent)                                                                                      | R 1400 (R350 per month) (95 USD)      |
| Centrifuge (>10000 rpm)                                                                                 | R 21 268 (1390 USD)                   |
| Pipettes (10–100µl and 100–1000µl)                                                                      | R 4921 each (321 USD)                 |
| Hain GenoType® MTBDR <i>plus</i> V2.0/Hain MTBDR <i>s</i> / V2.0 kits for analysis of 96 samples (each) | R 12 581 (860 USD)                    |
| Pipette tips with filters (~800 used)                                                                   | R1515 (99 USD) (one pack of 960 tips) |
| Bio-Rad Microseal® Semi-Skirt 96-well PCR plates™ (up to 200µl capacity) package of 25                  | R 808 (52 USD)                        |
| <b>Total</b>                                                                                            | <b>R 6540 (459 USD) per sample</b>    |
| <b>Total (excluding thermocycler, centrifuge and pipettes):</b>                                         | <b>R 155 (10 USD)</b>                 |

Prices calculated at an exchange rate of R14.23: \$1 (14 September 2021)

Costs for this study were alleviated by a gift of five kits for our analysis by the CapitalBio Corporation (Peking, China). However shipment to South Africa was required and amounted to R 2366 (162 USD) per kit.

**Table 13: Summary of required equipment and consumables not included in CapitalBio Gene Real-Time PCR Detection Kit**

| Equipment and consumables                                                                                        | Estimated cost                            |
|------------------------------------------------------------------------------------------------------------------|-------------------------------------------|
| Applied Biosystems GeneAmp PCR System 7500                                                                       | R 660 811 (44 500 USD)                    |
| Centrifuge (>10000 rpm)                                                                                          | R 21 268 (1390 USD)                       |
| Pipettes (1–10µl and 10–100µl)                                                                                   | R 4921 each (321 USD)                     |
| CapitalBio Gene Real-Time PCR Detection Kit for analysis of 24 samples [analysis of n=96 samples]                | R 7066 (480 USD)<br>[R 28 264 (1986 USD)] |
| CapitalBio Gene Real-Time PCR Detection Kit shipment to South Africa (n=3) [approximate transport price per kit] | R 7100 (481 USD)<br>[R 2366 (166 USD)]    |
| Fluorescent dyes                                                                                                 | R 3195 each (208 USD)                     |
| Bio-Rad Microseal® Semi-Skirt 96-well PCR plates™ (up to 200µl capacity)                                         | R 808 (52 USD)                            |
| Pipette tips with filters (~580 used)                                                                            | R 910 (62 USD)                            |
| Total                                                                                                            | R 7306 (513 USD) per sample               |
| Total (excluding thermocycler, centrifuge and pipettes):                                                         | R 444 (31 USD)                            |

Prices calculated at an exchange rate of R14.23: \$1 (14 September 2021)

The equipment requirements to perform the steps for each diagnostic kit result in high related costs. The CapitalBio Gene Real-Time PCR Detection Kit required an Applied Biosystems GeneAmp (ABI) PCR System 7500 to the value of an estimated R 660 811 (44 500 USD). Before the target genes in each sample could be amplified, samples were processed using pipettes to the value of R 4921 (321 USD) each and pipette tips, whereas about 580 tips were used to the value of R 910 (62 USD). Final processing steps required centrifugation using a centrifuge (>10000 rpm) valued at R 21 268 (1390 USD). We loaded the samples into Bio-Rad Microseal® Semi-Skirt 96-well PCR plates™ valued at R 808 (52 USD) for a package of 25 plates. The fluorescent dyes used for the real-time PCR reaction were valued at R 3195 (208 USD) each. The kit itself contains enough consumables to perform analysis of 24 samples and costs R 7066 (480 USD) per kit (Table 13). The kits were manufactured by the CapitalBio Corporation (Peking, China) and required shipment to South Africa that involved a R 7100 (481 USD) shipment fee due to the requirement of cold-chain shipment to preserve kit integrity.

Other factors that contribute to expenses related to assay procedures include equipment calibration, warranties, and servicing, as well as waste disposal of consumables (e.g., pipette tips and buffer solutions) that cannot be discarded in standard waste bins. The most

important is the end-line or commercial cost per sample since it determines the willingness of patients to undergo diagnostic testing (Basu, 2019).

### 3.4.2 Sensitive and specific

To determine the sensitivity and specificity of the kits, we used standard equations, as previously described by Parikh *et al.* 2008. This was achieved using the total number of true positives divided by the sum of the total number of true positives and total number of false negative results detected during analysis. Specificity was determined by dividing the total number of true negative results by the sum of the total number of true negative results and total number of false positive results (Table 14).

Overall, the kits performed well with low numbers of false positives and false negatives regarding resistance detection to first-line anti-TB drugs rifampicin and isoniazid (Table 14). The accuracy estimates of the Hain GenoType® MTBDR kit consequently determined superiority above the CapitalBio kit. Specific mutations in the gene regions targeted relate to drug resistance and can be detected by both line probe assays, and PCR based methods (Table 10 and Table 11).

**Table 14: Summary of diagnostic accuracy results of the CapitalBio, Hain GenoType® MTBDR*plus* V2.0, and Hain GenoType® MTBDR*s*/ V2.0 kits compared to the reference standard, WGS, for detection of drug resistances of rifampicin, isoniazid, fluoroquinolones, aminoglycosides (general, kanamycin, and streptomycin) and ethambutol. Corresponding target genes are included. Diagnostic accuracies are reported as true positives (TP), false positives (FP), True negatives (TN), false negatives (FN), sensitivity, specificity, positive- and negative predictive values. All estimates are reported as percentages (%)**

|                        | True Positive (TP) |             |             | False Positive (FP) |             |             | True Negative (TN) |             |             | False Negative (FN) |             |             | Sensitivity (TP/TP+FN) |             |             | Specificity (TN/TN+FP) |             |             | Positive predictive value (TP/TP+FP) |             |             | Negative predictive value (TN/TN+FN) |             |             |
|------------------------|--------------------|-------------|-------------|---------------------|-------------|-------------|--------------------|-------------|-------------|---------------------|-------------|-------------|------------------------|-------------|-------------|------------------------|-------------|-------------|--------------------------------------|-------------|-------------|--------------------------------------|-------------|-------------|
|                        | RIF                | INH         |             | RIF                 | INH         |             | RIF                | INH         |             | RIF                 | INH         |             | RIF                    | INH         |             | RIF                    | INH         |             | RIF                                  | INH         |             | RIF                                  | INH         |             |
|                        | <i>rpoB</i>        | <i>katG</i> | <i>inhA</i> | <i>rpoB</i>         | <i>katG</i> | <i>inhA</i> | <i>rpoB</i>        | <i>katG</i> | <i>inhA</i> | <i>rpoB</i>         | <i>katG</i> | <i>inhA</i> | <i>rpoB</i>            | <i>katG</i> | <i>inhA</i> | <i>rpoB</i>            | <i>katG</i> | <i>inhA</i> | <i>rpoB</i>                          | <i>katG</i> | <i>inhA</i> | <i>rpoB</i>                          | <i>katG</i> | <i>inhA</i> |
| CapitalBio kit         | 63                 | 39          | 5           | 3                   | 3           | 13          | 18                 | 37          | 44          | 8                   | 13          | 30          | 88                     | 75          | 14          | 86                     | 93          | 77          | 96                                   | 93          | 28          | 69                                   | 74          | 60          |
| MTBDR <i>plus</i> V2.0 | 67                 | 51          | 35          | 0                   | 0           | 6           | 21                 | 40          | 51          | 4                   | 1           | 0           | 94                     | 98          | 100         | 100                    | 100         | 90          | 100                                  | 100         | 85          | 84                                   | 97          | 100         |
|                        | FQ                 | AG          |             | FQ                  | AG          |             | FQ                 | AG          |             | FQ                  | AG          |             | FQ                     | AG          |             | FQ                     | AG          |             | FQ                                   | AG          |             | FQ                                   | AG          |             |
|                        | <i>gyrA</i>        | <i>rrs</i>  |             | <i>gyrA</i>         | <i>rrs</i>  |             | <i>gyrA</i>        | <i>rrs</i>  |             | <i>gyrA</i>         | <i>rrs</i>  |             | <i>gyrA</i>            | <i>rrs</i>  |             | <i>gyrA</i>            | <i>rrs</i>  |             | <i>gyrA</i>                          | <i>rrs</i>  |             | <i>gyrA</i>                          | <i>rrs</i>  |             |
| CapitalBio kit         | 25                 | 30          | 7           | 14                  | 53          | 48          | 7                  | 0           | 78          | 100                 | 88          | 77          | 78                     | 68          | 88          | 100                    |             |             |                                      |             |             |                                      |             |             |
| MTBDR <i>s</i> / V2.0  | 32                 | 30          | 1           | 0                   | 59          | 62          | 0                  | 0           | 100         | 100                 | 98          | 100         | 97                     | 100         | 100         |                        |             |             |                                      |             |             |                                      |             |             |

**Table 14 (continued): Summary of diagnostic accuracy results of the CapitalBio, Hain GenoType® MTBDR*plus* V2.0, and Hain GenoType® MTBDR*s*/ V2.0 kits compared to the reference standard, WGS, for detection of drug resistances of rifampicin, isoniazid, fluoroquinolones, aminoglycosides (general, kanamycin, and streptomycin) and ethambutol. Corresponding target genes are included. Diagnostic accuracies are reported as true positives (TP), false positives (FP), True negatives (TN), false negatives (FN), sensitivity, specificity, positive- and negative predictive values. All estimates are reported as percentages (%)**

|                       | True Positive (TP) |     | False Positive (FP) |                   | True Negative (TN) |     | False Negative (FN) |                   | Sensitivity (TP/TP+FN) |     | Specificity (TN/TN+FP) |                   | Positive predictive value (TP/TP+FP) |     | Negative predictive value (TN/TN+FN) |                   |
|-----------------------|--------------------|-----|---------------------|-------------------|--------------------|-----|---------------------|-------------------|------------------------|-----|------------------------|-------------------|--------------------------------------|-----|--------------------------------------|-------------------|
| MTBDR <i>s</i> / V2.0 | <i>embB</i>        | EMB | <i>rpsL</i>         | AG (streptomycin) | <i>embB</i>        | EMB | <i>rpsL</i>         | AG (streptomycin) | <i>embB</i>            | EMB | <i>rpsL</i>            | AG (streptomycin) | <i>embB</i>                          | EMB | <i>rpsL</i>                          | AG (streptomycin) |
|                       | <i>gyrB</i>        | FQ  | <i>eis</i>          | AG (kanamycin)    | <i>gyrB</i>        | FQ  | <i>eis</i>          | AG (kanamycin)    | <i>gyrB</i>            | FQ  | <i>eis</i>             | AG (kanamycin)    | <i>gyrB</i>                          | FQ  | <i>eis</i>                           | AG (kanamycin)    |
|                       | 0                  | 0   | 1                   | 0                 | 91                 | 92  | 0                   | 0                 | –                      | –   | 99                     | 100               | –                                    | –   | 100                                  | 100               |
|                       |                    |     |                     |                   |                    |     |                     |                   |                        |     |                        |                   |                                      |     |                                      |                   |
|                       |                    |     |                     |                   |                    |     |                     |                   |                        |     |                        |                   |                                      |     |                                      |                   |
|                       |                    |     |                     |                   |                    |     |                     |                   |                        |     |                        |                   |                                      |     |                                      |                   |
|                       |                    |     |                     |                   |                    |     |                     |                   |                        |     |                        |                   |                                      |     |                                      |                   |
|                       |                    |     |                     |                   |                    |     |                     |                   |                        |     |                        |                   |                                      |     |                                      |                   |
| CapitalBio kit        | 30                 | 5   | 31                  | 25                | 30                 | 61  | 1                   | 1                 | 97                     | 83  | 49                     | 71                | 49                                   | 17  | 97                                   | 98                |

### 3.4.3 User-friendly

Training and a basic knowledge of molecular methods was required to be able to perform the Hain GenoType® MTBDR assays and the CapitalBio kit procedures. With regards to training on the use of the Hain GenoType® MTBDR assays, a Hain Lifescience technician provided a one-day theory and practical training course. Training was supplied with purchase of the Hain GenoType® MTBDR assays however the cost could amount to R 6000 (409 USD) for a minimum of six participants and the reagents and strips used during training must be supplied out of the kits received. The entire procedure from sample amplification to hybridization entails 17 steps (two PCR steps, 14 hybridization steps and the last step is result analysis) (Table 15). Time from sample thawing to PCR 96-well loading is half an hour for 12 samples and includes adding sample DNA to amplification mixtures, briefly mixed by vortex and centrifuging briefly to prevent bubble forming in the tubes. Regarding training on the use of the CapitalBio kit, training was provided on the result analysis software and use of the QuantStudio™ 3 thermocycler. Training consisted of a one-day theory and practical training session provided by a ThermoFisher Scientific representative. The CapitalBio procedure entails ten steps to result interpretation (Table 15), which is less than the steps required to perform the Hain GenoType® LPAs, however each step entails a more tedious approach towards sample loading and analysis. Methods were performed in a level two (II) biosafety cabinet, as recommended by the kit manufacturers, to ensure the safety of healthcare professionals during sample handling and prevent sample contamination. Both kits were performed with ease after training on the usage of the kit, the kit components and the result analysis software. The CapitalBio kit requires installation and set-up of analysis software to be able to interpret results whereas the Hain GenoType® LPA results are interpreted manually. The analysis software of CapitalBio does not determine the results, results are rather presented visually as a graph where manual interpretation is then necessary to identify a positive or negative result. The advantage of the software-based analysis of CapitalBio results is the longevity of the results whereas the Hain GenoType® LPA strips deteriorate and cannot be used for re-interpretation of results.

### 3.4.4 Rapid and robust

Both kits can detect mutations in the gene regions: *rpoB*, *katG*, *inhA*, *gyrA* and *rrs* whereas the Hain GenoType® MTBDRs/ V2.0 assay includes analysis of *gyrB* and *eis*, and CapitalBio includes *embB* and *rpsL* gene region analysis as reported in the results. The turnaround time of both kits are less than 4 hours (Table 15). Both kits required high quality, extracted DNA which was amplified according to the instructions from manufacturers of the kits. The steps to result analysis of 12 samples with the Hain GenoType® assays were

performed in approximately three hours for first-line resistance detection (three gene regions) and second-line resistance detection (four gene regions) respectively. Result analysis was performed in 30 minutes; however, review of results must be considered to confirm correct interpretation and lower the risk of bias. The steps leading up to result interpretation with the CapitalBio kit were performed in less than two hours and comprises both first- and second-line anti-TB drug resistance detection (six gene regions), nevertheless result analysis was a time-consuming process. To determine a positive or negative result with the result analysis software of the ABI7500 thermocycler, there were two thresholds to consider per gene region targeted. There were five reaction mixtures and two thresholds per mixture which led to a total time of approximately three to four hours before results for both first- and second-line anti-TB drug resistance could be determined.

The storage requirements of the Hain GenoType® kit components were to store the consumables used for hybridization at 2–8°C and the amplification solutions at -20°C. Repeated freezing and thawing of amplification mixtures could damage the integrity of the constituents and was avoided by dividing mixtures into batches and thawing the minimum requirements for each 12-sample analysis. The CapitalBio kit components could be stored at 2–5°C for up to five days, however kits were stored at -20 °C as soon as possible. Both kits were packaged by the respective manufacturers with attention to preservation of component integrity. Mixtures or components were placed in carefully designed packaging that could endure shipment and prevent leakages.

#### **3.4.5 Equipment free and deliverable**

The required equipment for each respective kit was described during cost analysis and summarised in Table 15. The equipment costs are quintessential to consider with regards to deliverability of a diagnostic assay as diagnostic solutions must be cost-effective to be considered for implementation in LMICs. Most national reference laboratories possess a thermocycler for molecular diagnosis and consequently allows for kit implementation by more cost-effective means. Both kits required the use of a thermocycler during kit procedures. The amplification step of the Hain GenoType® assays were performed using a Quantstudio-3 real-time PCR system and the CapitalBio kit required the use of an older thermocycler, the ABI7500 system, as it was the model used to establish diagnostic thresholds that determine the results. Deliverability could be improved by collaboration within multiple sectors related to diagnostic kit selection and implementation. The Hain GenoType® assays were currently being widely used in South Africa and results in a highly deliverable option that has previously been recognised (Havumaki *et al.*, 2017). The CapitalBio kit is novel and has not been evaluated in performance on anti-TB drug

resistance detection within South African populations previously. This hampers deliverability as funding and strategical approaches would be required to implement a new diagnostic in South Africa.

**Table 15: Summary of characteristics of each respective assay with regards to principles, gene regions targeted, turnaround time, result interpretation, equipment required, and number of steps involved**

| Kit                                                | CapitalBio                                                                                                                                                                                                               | Hain GenoType® MTBDRplus & -sl V2.0                                                                                                                                                                                                            |
|----------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Principle</b>                                   | Real-time PCR, specific primer and probe designs, TaqMan® MGB probes are used to analyse single-nucleotide polymorphisms, fluorescence labelling by FAM & HEX                                                            | Line probe assays, molecular based diagnostic tools, detect drug resistance by hybridization of probes with specific complementary mutations, DNA Strip technology                                                                             |
| <b>Gene regions targeted</b>                       | <i>rpoB</i> (rifampicin),<br><i>katG</i> and <i>inhA</i> (isoniazid),<br><i>embB</i> (ethambutol),<br><i>gyrA</i> (fluoroquinolones),<br><i>rpsL</i> (streptomycin),<br><i>rrs</i> (amikacin, kanamycin and capreomycin) | Hain MTBDRplus V2.0: <i>rpoB</i> (rifampicin),<br><i>katG</i> and <i>inhA</i> (isoniazid)<br>Hain MTBDRsl V2.0: <i>gyrA</i> and <i>gyrB</i> (fluoroquinolones),<br><i>rrs</i> (amikacin, kanamycin and capreomycin),<br><i>eis</i> (kanamycin) |
| <b>Turnaround time</b>                             | 10 samples in two hours                                                                                                                                                                                                  | 12 samples in 3 hours                                                                                                                                                                                                                          |
| <b>Interpretation of results</b>                   | ABI 7500 analysis software, $\Delta$ RN threshold value, CT value                                                                                                                                                        | Visualised band patterns on strips are interpreted by either manual comparison with a printed template or read & analysed in GenoScan reader                                                                                                   |
| <b>Equipment</b>                                   | Applied Biosystems GeneAmp PCR system 7500, vortex, centrifuge, incubator                                                                                                                                                | Twincubator, vortex, centrifuge, QuantStudio™ 3 thermocycler                                                                                                                                                                                   |
| <b>Number of steps (including result analysis)</b> | 10 steps                                                                                                                                                                                                                 | 17 steps                                                                                                                                                                                                                                       |
| <b>Training</b>                                    | One to two days (depending on previous experience)                                                                                                                                                                       | One to two days training (depending on previous experience)                                                                                                                                                                                    |

Each characteristic was taken into consideration to determine which kit complies to the ASSURED criteria and more importantly, which is the most accurate towards anti-TB drug resistance detection.

**Table 16: ASSURED comparison of GenoType® MTBDR*plus* and -*s*/ Version 2.0 to the CapitalBio kit**

| Test             | GenoType® MTBDR <i>plus</i><br>and - <i>s</i> / Version 2.0                         | The CapitalBio kit                                                                  |
|------------------|-------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|
| Characteristic   |                                                                                     |                                                                                     |
| Affordable       |    |    |
| Sensitive        |    |    |
| Specific         |    |    |
| User-friendly    |    |    |
| Rapid and robust |   |   |
| Equipment free   |  |  |
| Deliverable      |  |  |

 High  Neutral  Low

To compare key characteristics of the diagnostic tests part of our evaluation, we applied the ASSURED criteria (Table 16). By comparison, the Hain GenoType® MTBDR V2.0 assays are more affordable, however these assays require advanced equipment to perform. This is the same for BACTEC™ MGIT™ system (Becton, Dickinson and Company) and the CapitalBio kit which also comprise extensive equipment-based techniques. The ASSURED table is based on the outcomes of the guidelines described and corresponding values of high, neutral, or low were matched with the related diagnostic kit characteristic.

### 3.5 CONCLUSION

In this study, a total number of 92 sample isolates were analysed using three respective kits of which the CapitalBio Gene Real-Time PCR Detection Kit can detect resistance to not only to the first-line anti-TB drugs rifampicin and isoniazid but second-line anti-TB drugs aminoglycosides and fluoroquinolones simultaneously. Results showed high accuracy, however false positive results and false negative results were prevalent during analysis with each kit. Previous studies have also reported mutations conferring resistance to first- and second-line anti-TB drugs that were undetected by Hain GenoType® LPAs (Maschmann *et al.*, 2013). The sample size of this study is small when considering the genetic variations among MTB strains and the various drug resistance mechanisms they harbour. The first time the complete genome sequence of *Mycobacterium tuberculosis* was reported was in 1998 by Cole *et al.* where an estimated 4.41 million single base pairs were sequenced to reveal an approximate 4000 genes (Cohen *et al.*, 2019). Including a larger number of samples, categorised provincially, will lead to establishment of mutational patterns among populations and provide critically relevant data for healthcare professionals (Zignol *et al.*, 2016).

Bias may arise during result interpretation and can lead to false positives or false negative results (Basu, 2019), this is applicable for both the CapitalBio kit and the Hain GenoType® MTBDR assays. This is due to the manual nature of result interpretation in both kits. Contamination is a risk factor for both assays due to repetitive pipetting steps in the methods. The CapitalBio kit is more affordable but transport costs from an international manufacturer to South Africa must be considered. Primer and probe designs are easily accessible on online databases and could aid in the development of a new probe to targeted undetected mutations, especially since disease prevalence and distribution of resistance-conferring genes are country-specific (Sekyere *et al.*, 2019). Comparative evaluations can produce informative results that will positively impact the current state of diagnostics in South Africa. Rapid molecular testing is the most attractive tool for diagnosis of TB but needs optimization and further development to decrease current costs. The genetics behind drug resistance are not yet fully understood or categorised and therefore whole-genome sequencing cannot replace phenotypic DST at present (Köser *et al.*, 2013). Line-probe assays provide many advantages above phenotypic DST and could minimize diagnostic treatment delays if implemented into advanced diagnostic algorithms.

## CHAPTER 4: STUDY CONCLUSION

### 4.1 STUDY OUTCOMES

This study was based on the current challenges faced worldwide to detect, treat, and cure tuberculosis. The history of tuberculosis (TB) and more importantly, drug resistant TB (DR-TB) has been documented for decades yet there are still many diagnostic challenges for this disease. The constant improvement of anti-TB drugs also mediates future adaptations towards these drugs by the *Mycobacterium Tuberculosis* complex (MTBC). To end a global problem, there must be intervention in the form of rapid and advanced diagnostics. The goal is to move away from conventional drug susceptibility testing (DST) which is still routinely used to date. This step is supported by this study in part because results indicated that a lack of sample uniformity could influence interpretations. A high bacterial load could provide a higher quality sample. The potency of the disease is not linked to bacterial load and thus a larger sputum sample is necessary for analysis in certain cases. Providing a large amount of sputum is not always possible. Patients with underlying health-issues and patients under the age of five years are part of a group with difficulty producing sputum. Furthermore, certain genes have been reported to be silent mutations that do not contribute to phenotypic resistance towards anti-TB drugs (Rigouts *et al.*, 2018).

The evaluated methods in chapter two yielded promising results with the Hain GenoType® MTBDR kits being the most attractive option and easily implementable due to the already widespread use of these kits in South African settings. Both versions of the Hain GenoType® MTBDR V2.0 assays have been widely implemented in South Africa and are well known among healthcare professionals (Treatment Action Group, 2017). Implementing a novel kit in low- and middle-income countries could prove to be challenging since equipment and infrastructure needs to be advanced for diagnosis of tuberculosis. The CapitalBio kit evaluated in chapter 3 delivered potentially impactful results, however optimisation is necessary before it could be implemented in South African settings where there is a high burden of TB and related drug resistance. The other challenge is to train healthcare professionals in all settings where the kit will be implemented to ensure high quality results. To ensure integrity of DNA is left virtually unchanged from the moment of extraction of the sample, is to create a point-of-care diagnostic system and prevent unnecessary shipment of samples. Extracting DNA using the kit suggested by the same manufacturer of the drug resistance profiling kit ensures buffer homogeneity.

## 4.2 STUDY LIMITATIONS

There are certain aspects of each diagnostic kit that could contribute to bias during analyses. The manual nature of interpretation, even with the accompanying analysis software, had related concerns. One of the limitations of this study is attributed to the complexity of DR-TB, the mechanisms behind observed resistances and epidemiological differences regarding the existence of different strains. To fully evaluate a diagnostic kit, one must consider the country profile and prevalence of mutations per population group. The prevalence of mutations among a population could provide preliminary assumptions that could guide a pre-diagnosis. This is because TB can only be transmitted from one infected person to another and thus the country profile is an important tool to track the spread of DR-TB. Blinding patient history for the purpose of this study was necessary but in clinical settings patient history is pertinent.

Difficulties with DNA probes that correspond to resistance-conferring genes proved to be a notable limitation since this could cause false negative results. All evaluations could only be performed once on the selected samples due to the small volume and low concentration of DNA per sample.

## 4.3 FUTURE RECOMMENDATIONS

A meta-analytic study towards comparing molecular methods for TB drug resistance determination would benefit from the following guidelines:

- Clearly describe sources of bias that could arise during the study. The QUADAS 2 method has previously described sources of bias among studies and provides easily interpretable measures of heterogeneity

A comparative study towards evaluating molecular methods for drug resistant TB would benefit from the following recommendations:

- Use a sample volume larger than 50µl. Alternatively use higher quality DNA with high concentrations which can be diluted for respective evaluations
- Connect with a national entity within the country of origin of samples to firstly assess TB epidemiology within the selected population group and draw a hypothesis
- Include optimization steps to ensure consistent workflow before main evaluations start

- Design new primers and/or probes if found to be the causative of false positive or false negative results
- Visit reference laboratories to get a clear idea of the diagnostic algorithm mainly used for the selected population group and how samples are handled up to the point of diagnosis

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## ADDENDUM 1

**Table 1:** QUADAS-2 tool list of risk of bias and applicability questionnaire

### QUADAS-2 tool: Risk of bias and applicability judgments

#### Domain 1: Patient selection

##### A. Risk of bias

Describe methods of patient selection:

- |                                                            |                |
|------------------------------------------------------------|----------------|
| • Was a consecutive or random sample of patients enrolled? | Yes/No/Unclear |
| • Was a case-control design avoided?                       | Yes/No/Unclear |
| • Did the study avoid inappropriate exclusions?            | Yes/No/Unclear |

|                                                       |                           |
|-------------------------------------------------------|---------------------------|
| Could the selection of patients have introduced bias? | RISK:<br>LOW/HIGH/UNCLEAR |
|-------------------------------------------------------|---------------------------|

##### B. Concerns regarding applicability

Describe included patients (prior testing, presentation, intended use of index test and setting):

|                                                                               |                              |
|-------------------------------------------------------------------------------|------------------------------|
| Is there concern that the included patients do not match the review question? | CONCERN:<br>LOW/HIGH/UNCLEAR |
|-------------------------------------------------------------------------------|------------------------------|

#### Domain 2: Index test(s) (if more than 1 index test was used, please complete for each test)

##### A. Risk of bias

Describe the index test and how it was conducted and interpreted:

- |                                                                                                       |                |
|-------------------------------------------------------------------------------------------------------|----------------|
| • Were the index test results interpreted without knowledge of the results of the reference standard? | Yes/No/Unclear |
| • If a threshold was used, was it pre-specified?                                                      | Yes/No/Unclear |

|                                                                             |                           |
|-----------------------------------------------------------------------------|---------------------------|
| Could the conduct or interpretation of the index test have introduced bias? | RISK:<br>LOW/HIGH/UNCLEAR |
|-----------------------------------------------------------------------------|---------------------------|

##### B. Concerns regarding applicability

|                                                                                                       |                              |
|-------------------------------------------------------------------------------------------------------|------------------------------|
| Is there concern that the index test, its conduct, or interpretation differ from the review question? | CONCERN:<br>LOW/HIGH/UNCLEAR |
|-------------------------------------------------------------------------------------------------------|------------------------------|

#### Domain 3: Reference standard

##### A. Risk of bias

Describe the reference standard and how it was conducted and interpreted:

- |                                                                                                       |                |
|-------------------------------------------------------------------------------------------------------|----------------|
| • Is the reference standard likely to correctly classify the target condition?                        | Yes/No/Unclear |
| • Were the reference standard results interpreted without knowledge of the results of the index test? | Yes/No/Unclear |

|                                                                  |       |
|------------------------------------------------------------------|-------|
| Could the reference standard, its conduct, or its interpretation | RISK: |
|------------------------------------------------------------------|-------|

|                                                                                                                                                        |                              |
|--------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------|
| have introduced bias?                                                                                                                                  | LOW/HIGH/UNCLEAR             |
| B. Concerns regarding applicability                                                                                                                    |                              |
| Is there concern that the target condition as defined by the reference standard does not match the review question?                                    | CONCERN:<br>LOW/HIGH/UNCLEAR |
| <b>Domain 4: Flow and timing</b>                                                                                                                       |                              |
| A. Risk of bias                                                                                                                                        |                              |
| Describe any patients who did not receive the index test(s) and/or reference standard or who were excluded from the 2x2 table (refer to flow diagram): |                              |
| Describe the time interval and any interventions between index test(s) and reference standard:                                                         |                              |
| • Was there an appropriate interval between index test(s) and reference standard?                                                                      | Yes/No/Unclear               |
| • Did all patients receive a reference standard?                                                                                                       | Yes/No/Unclear               |
| • Did patients receive the same reference standard?                                                                                                    | Yes/No/Unclear               |
| • Were all patients included in the analysis?                                                                                                          | Yes/No/Unclear               |
| Could the patient flow have introduced bias?                                                                                                           | RISK:<br>LOW/HIGH/UNCLEAR    |

## ADDENDUM 2

**Table 1:** Mutations identified within *M. tuberculosis* drug resistance-associated loci among isolates, each locus serves as a resistance marker (Bakula *et al.*, 2016; Tuberculist, 2004)

| Drug                       | Locus                                        | Common mutations                                                   |
|----------------------------|----------------------------------------------|--------------------------------------------------------------------|
| RIF                        | <i>rpoB</i> (81 bp region)                   | Ser531Leu<br>His526Tyr<br>Asp516Val                                |
| INH                        | <i>katG</i> (codon 315)                      | Ser315Thr                                                          |
|                            | <i>inhA</i> ( <i>inhA</i> regulatory region) | C(-15)T, T(-8)C                                                    |
| ETH                        | <i>inhA</i> ( <i>inhA</i> regulatory region) | C(-15)T, T(-8)C                                                    |
| EMB                        | <i>embB</i>                                  | Met306Val<br>Met306Ile<br>Pro832Ser                                |
| AMK                        | <i>rrs</i> (A1401G)                          | Single base substitutions; 1401(A→G), 1402 (C→T or A), 1484 (G→T)  |
| KAN                        | <i>rrs</i> (A1401G)                          | Single base substitutions; 1401(A→G), 1402 (C→T or A), 1484 (G→T)  |
|                            | <i>eis</i>                                   | Between codons 2–402                                               |
| STR                        | <i>rpsL</i>                                  | Lys88Arg<br>Ribosomal protein S12                                  |
| CAP                        | <i>rrs</i> (A1401G)                          | Single base substitutions; 1401 (A→G), 1402 (C→T or A), 1484 (G→T) |
| CIP/OFX (fluoroquinolones) | <i>gyrA</i> (conserved region)               | Between codons 67–106 (mostly 90 and 94)                           |

### ADDENDUM 3

**Table 1:** Threshold values of the CapitalBio kit determining a positive or negative result for each resistance gene

| Reaction mix | Gene        | Detection channel | $\Delta Rn$ threshold value | Result   |
|--------------|-------------|-------------------|-----------------------------|----------|
| <b>A</b>     | <i>rpoB</i> | FAM               | $\geq 0.080$                | Positive |
|              |             |                   | $< 0.080$                   | Negative |
|              | <i>katG</i> | VIC               | $\geq 0.084$                | Positive |
|              |             |                   | $< 0.084$                   | Negative |
| <b>B</b>     | <i>rpoB</i> | FAM               | $\geq 0.102$                | Positive |
|              |             |                   | $< 0.102$                   | Negative |
|              | <i>inhA</i> | VIC               | $\geq 0.037$                | Positive |
|              |             |                   | $< 0.037$                   | Negative |
| <b>C</b>     | <i>gyrA</i> | FAM               | $\geq 0.020$                | Positive |
|              |             |                   | $< 0.020$                   | Negative |
|              | <i>embB</i> | VIC               | $\geq 0.030$                | Positive |
|              |             |                   | $< 0.030$                   | Negative |
| <b>D</b>     | <i>rpsL</i> | FAM               | $\geq 0.040$                | Positive |
|              |             |                   | $< 0.040$                   | Negative |
| <b>E</b>     | <i>rrs</i>  | FAM               | $\geq 0.080$                | Positive |
|              |             |                   | $< 0.080$                   | Negative |

21 August 2019

## RESEARCH ETHICS COMMITTEE LETTER OF DECISION: NO RISK

Based on the review by the North-West University Health Research Ethics Committee (NWU-HREC) on 21/08/2019, the NWU-HREC hereby clears your study as a no risk study. This implies that the NWU-HREC grants its permission that, provided the general conditions specified below are met, the study may be initiated, using the ethics number below.

**Study title: Comparative evaluation of molecular based detection methods for drug resistant tuberculosis**

**Principal Investigator/Study Supervisor/Researcher: Dr W Pheiffer**

**Student: J Williams - 24249300**

**Ethics number:**

|             |   |   |              |   |   |   |   |      |   |   |        |   |   |   |
|-------------|---|---|--------------|---|---|---|---|------|---|---|--------|---|---|---|
| N           | W | U | -            | 0 | 0 | 9 | 7 | 6    | - | 1 | 9      | - | A | 1 |
| Institution |   |   | Study Number |   |   |   |   | Year |   |   | Status |   |   |   |

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation;  
A = Authorisation

**Application Type: Single study**

**Commencement date: 21/08/2019**

**Risk:**

**No Risk**

### General conditions:

*The following general terms and conditions will apply:*

- *The commencement date indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-HREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research;*
  - *withdraw or postpone clearance if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the NWU-HREC or that information has been false or misrepresented;*
    - *submission of the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-HREC can be contacted for further information via [Ethics-HRECAppl@nwu.ac.za](mailto:Ethics-HRECAppl@nwu.ac.za) or 018 299 1206*

The NWU-HREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-HREC for any further enquiries or requests for assistance.

Yours sincerely,



Digitally signed  
by Prof Petra  
Bester  
Date: 2021.09.14  
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NWU-HREC Chairperson



Digitally signed by  
Gordon Wayne  
Towers  
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Head of the Faculty of Health Sciences Ethics Office for Research, Training and Support

Current details: (13210572) G:\My Drive\My Documents 20190227\NWU-HREC\NWU-HREC\_Approval Letters\No Risk studies-LoD\2019\_LoD\9.1.5.4.3\_LOD\_NWU-00976-19-A1\_20210906.docm  
6 September 2021

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