COMPARISON OF GENEXPERT AND LINE PROBE ASSAY FOR DETECTION OF *Mycobacterium tuberculosis* AND RIFAMPICIN MONO-RESISTANCE IN KENYA

BY

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DECLARATION

I hereby declare that this thesis is my original work and has not been presented for the award of a degree in any other university or institution.

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DEDICATION

I dedicate this work to my dear wife Charity Nyakerario; our children Audrey and Aiden. To my parents Mr. and Mrs. Anderson Aricha for their support, encouragement and love during the entire study period.

ABSTRACT

Tuberculosis (TB) causes substantial human morbidity and mortality globally. About 10.4 million TB cases and 1.4 million deaths worldwide were reported in 2015. Kenya is among the 22 countries with highest TB burden. Microscopy is routinely used for *Mycobacterium tuberculosis bacilli (Mtb)* detection; however its sensitivity has been reported to be as low as 60% posing a problem for case detection. Rifampicin (RIF) is one of the major drugs for first line TB treatment; however, most patients tend to develop resistance thus making treatment a challenge. Culture method, which is the gold standard for diagnosis of *Mtb* and drug resistance, is time-consuming, limited and technically involving, thus delaying initiation treatment of TB. GeneXpert MTB/RIF and Line Probe assay (LPA) have been recommended by World Health Organization (WHO) for rapid diagnosis of *Mtb* and RIF mono resistance but their performance tend to differ from region to region. The study aimed at determining the sensitivity, specificity, positive and negative predictive values of GeneXpert MTB/RIF and LPA in *Mtb* detection; determining the sensitivity, specificity, positive and negative predictive values of GeneXpert MTB/RIF and LPA in RIF mono resistance detection and test the agreement between GeneXpert MTB/RIF, LPA with culture in *Mtb* and RIF mono-resistant detection. This was a cross-sectional study with sample size of 131 at 95% confidence level, done between November, 2016 and March, 2017. In the laboratory culture, drug susceptibility testing and molecular analysis using GeneXpert MTB/RIF and LPA was done. The sensitivity, specificity and predictive values were calculated using MGIT culture and an agreement was done by Cohen kappa values (0.01-0.20 indicating none to slight, 0.21-0.40 as fair, 0.41-0.60 as moderate, 0.61-0.80 as good and 0.81-1.0 as very good agreement). GeneXpert MTB/RIF showed sensitivity and specificity of 76% and 66% while positive and negative predictive values were 57% and 82% respectively while, LPA showed sensitivity and specificity of 98% and 71%, with positive and negative predictive values of 67% and 98% respectively in detection of *Mtb*. Regarding RIF mono-resistance, Gene Xpert MTB/RIF had a sensitivity and specificity of 33% and 96%, with positive and negative predictive values of 33% and 94% respectively, whereas LPA reported a sensitivity and specificity of 100% and 100% with positive and negative predictive values of 100% and 100% respectively. In regards to diagnosis, there was a fair agreement in GeneXpert MTB/RIF and culture (Kappa value, 0.388) with LPA and culture reporting (Kappa value, 0.628). There was a fair agreement between GeneXpert MTB/RIF and culture (Kappa value, 0.275) as compared to a very good agreement between LPA and culture (Kappa value, 1.00) for detection of RIF mono-resistance. In conclusion, LPA diagnostically outperformed GeneXpert MTB/RIF in both *Mtb* and RIF mono-resistance diagnosis and that LPA is a good alternative to culture with regards to detection of RIF mono resistance in facilities without culture. The study recommends the up-scaling of LPA for both *Mtb* detection and RIF mono resistance, and development of country specific probes for local population in *Mtb* and RIF mono resistance detection.

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LIST OF ABBREVIATIONS

BCG	:	Bacillus Calimette-Guerin
BPS	:	Buffered Phospate Saline
CDC	:	Centers for Disease Control and Prevention
DLTLD	:	Division of Leprosy, Tuberculosis and Lung Disease
DNA	:	Deoxyribonucleic acid
HIV	:	Human Immunodeficiency Virus
LJ	:	Lowenstein -Jensen
LPA	:	Line Probe Assay
MDR-TB	:	Multidrug Resistance- Tuberculosis
MTB	:	Mycobacterium tubercle
MTC	:	Mycobacterium Tuberculosis Complex
NTRL	:	National Tuberculosis Reference Laboratories
NALC	:	N-Acetyl-L-Cysteine
NAOH	:	Sodium Hydroxide
NTP	:	National Tuberculosis Program
PCR	:	Polymerase Chain Reaction
RIF	:	Rifampicin
SPSS	:	Statistical Analysis for Social Sciences
TB	:	Tuberculosis
WHO	:	World Health Organization

DEFINITION OF TERMS

Mono-resistance: Resistance to one first line anti-TB drug only

Multidrug Resistance: Resistant to at least both isoniazid and rifampicin

Poly-Resistance: Resistance to more than one first line anti-TB drug, other than both isoniazid and Rifampicin

Rifampicin Resistance: Resistance to RIF detected using phenotype without resistance to other antiTB. It includes any resistance to RIF in the form of mono resistance,

Poly-resistance, and MDR or XDR drugs

Extensively Drug Resistance (XDR) : Resistance to any fluoroquinolone and at least one of the three second line injectable drugs (capreomycin, kanamycin, and amikacin), in addition to multidrug resistance

Presumed MDR-TB: Patients who present with signs and symptoms suggestive of tuberculosis, and have been on first line TB drugs.

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CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Tuberculosis (TB) causes substantial human morbidity and mortality globally (WHO, 2016) . About 10.4 million TB cases and 1.4 million deaths worldwide were reported in 2015 (WHO, 2016). *Mycobacterium tuberculosis* bacilli *(Mtb)* are treatable with first line anti-TB drugs of Isoniazid, Rifampicin, Ethambutol and Pyrazinamide (WHO, 2014), whose mode of action is by preventing the initiation of the transcription process of *Mtb* strain (WHO, 2015). Among this drugs Rifampicin is key and it is reported to be a proxy to drug resistance. In 2015, 480,000 cases of multidrug-resistant TB (MDR-TB) and 100,000 cases of Rifampicin mono resistant TB were reported worldwide (WHO, 2016). Kenya is among the 22 countries with highest TB burden globally (WHO, 2013), with a national TB prevalence of 558 per 100,000 people as per the Kenya National TB prevalence survey 2016.

Tuberculosis detection is routinely done through microscopy in middle and low income countries, since it is cheap and simple. Microscopy sensitivity is reported as low as 60%, thus high cases missed in detection leading to continuous transmission. In addition, microscopy has low sensitivity in detection of TB in children and in extra pulmonary TB (Desikan, 2013). Bacteriological diagnosis of TB through conventional methods has shortcomings thus the emergence of nucleic acid amplification test (NAAT) as an alternative. The NAAT has been used to detect *Mtb* insertion element (IS6110) to identify *Mtb* complex (WHO, 2011). Insertion sequences (IS) forms major components of bacterial repetitive elements. IS*6110* is specific to the *M. tuberculosis* complex and has been used to detect presence of *M. tuberculosis* cells in a clinical sample. It also detects *Mtb* ribosomal ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) from both smear positive and negative sputum specimens (Niemz *et al*., 2012).

Two molecular based assays, GeneXpert MTB/RIF and Line Probe Assay (LPA) have been endorsed by World Health Organization (WHO) as alternatives to microscopy in TB detection (WHO, 2011). Preliminary studies from other areas of the world indicate that they have a better sensitivity and specificity on TB detection (WHO, 2011). GeneXpert MTB/RIF method is nucleic acid amplification test which detects deoxyribonucleic acid (DNA) of Mycobacterium

Tuberculosis complex (MTBC) and resistance to Rifampicin (RIF) caused by mutation of the beta subunit of RNA polymerase (*rpoB* gene) (Boehme, 2014). It utilizes primers in the assay that amplify the portion of the *rpoB* gene containing the 81 base pair core regions, the region is encoded with *Mtb* specific DNA sequences and mutations causing resistance (WHO, 2011). Probes are able to differentiate between the conserved wild-type sequence and mutation in the core region that are associated with resistance of Rifampicin (Helb *et al*., 2010). Line Probe Assay uses PCR/hybridization technique to detect *Mtb* while also identifying drug resistance strains to Rifampicin and Isoniazid drugs using common single nucleotide polymorphisms (SNPs). Rifampicin resistance is conferred by a diverse of mutations within a hypervariable region of the *rpoB* gene, which encodes the beta sub unit of the RNA polymerase (Lin et al., 2017). Rifampicin interacts with RNA polymerase specifically to inhibit transcription, which leads to and causes cell death, and specific mutations in *rpoB* produce drug resistance and diminishing Rifampicin binding affinity for the RNA polymerase (Mulu et al., 2017). Resistance to Isoniazid is associated with amino acid substitution in catalase peroxidase gene (*katG*) or mutation in the *inhA* promoter gene (Hirano et al., 1999). The sensitivity of GeneXpert for *Mtb* detection in South African population has been reported at 79% (Theron et al., 2016), while the sensitivity and specificity of LPA in detecting TB among South African and South American population were 81% and 100% respectively (Luetkemeyer et al., 2014).

Drug resistance TB evolves from TB which fails to respond to first line anti-TB drugs thus developing mutant genes (Freire, 2005). Culture, the diagnostic tool available for drug resistance detection is limited (Freire, 2005) and takes not less than 4 weeks to get results (Wang et al., 2011). Consequently, a large proportion of drug resistance TB cases which remain undetected lead to continuous transmission thus increased cost of treatment and management of TB (CDC, 2012). The molecular-based methods detect Mycobacterium tuberculosis nucleic acid in sputum samples and mutations associated with resistance against anti TB drugs (Helb et al., 2010). Even though GeneXpert MTB/RIF and LPA methods detect mutations causing resistant against Rifampicin, LPA also detects mutations to isoniazid drug (Helb et al., 2010).

The study aimed at determining the performance in terms of sensitivity, specificity, positive and negative predictive values of GeneXpert MTB/RIF and LPA in *Mtb* detection using liquid culture as the gold standard; determining the sensitivity, specificity, positive and negative

predictive values of GeneXpert MTB/RIF and LPA in RIF mono resistance detection using liquid culture as the gold standard and test the agreement between GeneXpert MTB/RIF, LPA with culture in *Mtb* and Rifampicin mono-resistance detection.

Performance means determination of sensitivity, specificity and predictive values which are defined: Sensitivity as the ability to correctly identify those with disease; Specificity as the ability to correctly identify those without disease; Positive predictive value as the percentage of patients with positive test who actually have the disease; Negative predictive value as the percentage of patients with negative test who do not have the disease and agreement as the measure of variable by two different assays to produce similar results

1.2 Problem Statement

TB remains a major global health problem particularly in resource-poor countries. About 10.4 million TB cases and 1.4 million deaths worldwide were reported in 2015 (WHO, 2016). In the same year, 480,000 cases of multidrug-resistant TB (MDR-TB) and 100,000 cases of Rifampicin mono resistant TB were reported. Kenya is among the 22 high TB burden countries worldwide. Tuberculosis detection is routinely done through microscopy and it targets *Mycobacterium tuberculosis* bacilli. In spite of the major efforts that are being done to increase case detection, one third of new cases are still missed out (Freire, 2005). Indeed, the sensitivity of microscopy has been found to be as low 60% thus need for a more sensitive method for TB detection (Desikan, 2013). Drug resistance TB evolves from TB which fails to respond to first line anti-TB drugs thus developing mutant genes (Freire, 2005). Culture is the diagnostic tools available for drug resistance detection (Freire, 2005), but it takes longtime to give results which gives room for resistance to develop (CDC, 2012). GeneXpert MTB/RIF and LPA have been endorsed by WHO for the rapid detection of *Mtb* and drug resistant tuberculosis despite studies showing difference in terms of performance from one region to another (Rufai et al., 2014). Despite the commissioning of the two methods by WHO, their performance has not been evaluated in Kenya, to determine their accuracy and efficiency in terms of sensitivity, specificity and predictive values in detecting *Mtb* and drug resistance.

1.3 Justification

GeneXpert MTB/RIF and LPA have been endorsed by WHO for the detection of *Mtb* and drug resistant tuberculosis. The methods appear to be good alternatives to the routine methods currently used, by having an efficient and accurate method in *Mtb* and drug resistance detection, there will be early detection of *Mtb* and Rifampicin mono-resistance, reduction in TB transmission, timely initiation of treatment hence lowering the cost of treatment, create a healthy nation with pool of manpower for the economic growth of the country. This evaluated the performance of LPA and GeneXpert MTB/RIF methods for detection of *Mtb* and Rifampicin mono resistance in terms of sensitivity, specificity and predictive values in order to advise stakeholders on their use and performance in *Mtb* and drug resistance detection. Most local hospitals need to be advised on the use of GeneXpert MTB/RIF and LPA assays, these can only be done through available empirical evidence hence reason to investigate the agreement between the two assays with culture.

1.4 Significance of the Study

The final goal is to advise the policy makers (Ministry of health) and other stakeholders on the performance of the two assays in terms of sensitivity and specificity for timely management of the disease through early detection of *Mtb* and Rifampicin mono-resistance. This has an overall impact of curbing the disease through development of country specific probes that are more sensitive and specific.

1.5. Objectives

1.5.1 Broad Objective

To compare the performance of GeneXpert MTB/RIF and Line Probe Assay for detection of *Mycobacterium tuberculosis* and Rifampicin-mono resistant at the National Tuberculosis Reference Laboratory, Kenya.

1.5.2 Specific Objectives

i. To determine the sensitivity, specificity, positive and negative predictive values of GeneXpert MTB/RIF and LPA in *Mtb* detection using liquid culture as the gold standard.

ii. To determine the sensitivity, specificity, positive and negative predictive values of GeneXpert MTB/RIF and LPA in detection of Rifampicinmono-resistance using liquid culture as the gold standard.

iii. To determine the agreement between GeneXpert MTB/RIF, LPA with culture in detection of *Mtb* and Rifampicin mono-resistance.

1.6. Research Questions

i. What is the sensitivity, specificity, positive and negative predictive values of GeneXpert MTB/RIF and LPA in *Mtb* detection using liquid culture as the gold standard?

ii. What is the sensitivity, specificity, positive and negative predictive values of GeneXpert MTB/RIF and LPA in detection of Rifampicin mono resistance using liquid culture as the gold standard?

iii. What is the agreement between GeneXpert MTB/RIF, LPA with culture in detection of *Mtb* and Rifampicin mono-resistance?

CHAPTER TWO

REVIEW OF LITERATURE

2.1 Epidemiology of Tuberculosis

Tuberculosis (TB) causes substantial morbidity and mortality in human globally (WHO, 2016). About 10.4 million TB cases and 1.4 million deaths worldwide were reported in 2015 alone (WHO, 2016). In the same year, 480,000 cases of multidrug-resistant TB (MDR-TB) and 100,000 cases of Rifampicin resistant TB were reported (WHO, 2016).

Kenya is among the 22 countries with highest TB burden globally (WHO, 2013), with a national TB prevalence of 558 per 100,000 people as reported by the Kenya National TB prevalence survey 2016.

2.2 Pathogenesis

Tuberculosis is a disease of the respiratory system caused by a bacterium called *Mycobacterium tuberculosis* (Frieden et al., 2014); (WHO, 2015) and spread through air drops (Frieden et al., 2014). The infection occurs when an active pulmonary TB from an infected individual is passed to an uninfected individual (Delogu et al., 2013). The pathogen is a symptomatic in more than 90% of the people infected with *M. tuberculosis.* There is approximately 5% risk of getting an active disease at the 18 month after initial infection and similar risk of getting infected for the remaining lifetime period (Zumla et al., 2015). At the alveoli, *Mtb* is phagocytised by alveolar macrophages thus killing the bacteria, in case the bacilli can survive this first line of defence then it starts to replicate in the macrophages, diffuse to the epithelial and endothelial cells leading to a high bacterial burden (Delogu et al., 2013). At the early steps of infection, *Mtb* can diffuse to other organs through the lymphatics and by haematogenous dissemination where it can infect other cells (Zumla et al., 2015).

2.3 Clinical Features

The clinical features of TB involve both latent and active stages. In the latent stage, Mycobacterium persist in the body without showing any symptoms, but patients are susceptible to reactivation of disease (Orme, 2014). At this stage, the granulomatous lesions calcify and

become fibrotic and are apparent on chest radiographs (WHO, 2015). During the active stage, patients experience productive cough, progressive weight loss and anaemia (Delogu et al., 2013).

2.4 Treatment

The recommended WHO current treatment of TB is six months involving use of four standard, first-lines, anti-TB drugs: Isoniazid, Rifampicin, Ethambutol and Pyrazinamide. The drugs kill the bacilli by preventing the initiation of the transcription process of *Mycobacterium tuberculosis* strain. It is also worth noting that 90% of Rifampicin strains also show resistant to isoniazid drug, a probable cause of MDR-TB (Pang et al., 2014). Rifampicin mono-resistant patients are treated with more expensive drugs and for a longer period consisting of two phases: intensive phase of eight months and continuation phase of twelve months of second line drugs.The TB drugs are classified into five groups as summarized in Table 2.1.

Table 2.1 Recommended WHO Tuberculosis Drugs (WHO, 2015).

Group	**Drugs**
1. (Oral first line)	Isoniazid, Rifampicin, Ethambutol and pyrazinamide
2.(injectable agents)	Streptomycin, Amikacin, Kanamycin and Capreomycin
3.(Fluoroquinolones)	Ofloxacin, Levofloxacin, Gatifloxacin and Moxifloxacin
4. (Oral bacteriostatic second-line)	Ethionamide, Cycloserine, and Para amino salicylic acid
5. Anti-TB drugs with limited data on efficacy and long term safety in the treatment of drug resistant	Clarithromycin, Thioacetazone, High dose isoniazid, meropenem, imepenem, Amoxicilin/clauvulanate Linezoid and clofazimine.

Mycobacterium tuberculosis bacilli are treatable with first line anti-TB drugs of Isoniazid, Rifampicin, Ethambutol and Pyrazinamide (WHO, 2014). Resistance to drugs evolves when mutations, or chromosomal replication errors, occur in genes that encode drug targets or drug metabolism mechanisms and impact the efficacy of anti-tuberculosis therapies (WHO, 2015).

Suboptimal treatment and poor adherence to drug therapies contribute to resistance. If the right dose is not taken for the correct length of time, some of the bacteria may survive, multiply and adapt themselves to the environment under low dosage thus developing resistance to the antibiotic it was subjected to and more other antibiotics (Pillay et al., 2007). At the 81 base pair core regions, it is encoded with DNA sequences of the conserved wild-type sequence and mutation in the core region that are associated with resistance of Rifampicin (Helb *et al*., 2010) Among the first line ant TB drugs Rifampicin is key and it is reported to be a proxy to multidrug resistance, when combined with isoniazid. Resistance is conferred by a diverse of mutations within a hypervariable region of the *rpoB* gene, which encodes the beta sub unit of the RNA polymerase (Lin et al., 2017). Rifampicin interacts with RNA polymerase specifically to inhibit transcription, which leads to and causes cell death, and specific mutations in *rpoB* produce drug resistance and diminishing Rifampicin binding affinity for the RNA polymerase (Mulu et al., 2017). Resistance to Isoniazid is associated with amino acid substitution in catalase peroxidase gene (*katG*) or mutation in the *inhA* promoter gene (Hirano et al., 1999).

2.5 Tuberculosis Detection

A key component of TB control and management program is rapid detection of TB (WHO, 2014). Laboratory detection of *Mtb* is through microscopy, culture and molecular-based techniques of GeneXpert MTB/RIF and Line Probe Assay (LPA). Microscopy is used for routine diagnosis especially in countries with limited resources; it is easy to perform and takes shorter time to give results but it is unreliable in detecting extra pulmonary and pediatric TB (Desikan, 2013). It has reported sensitivity of as low as 60% as per the Kenya National prevalence survey 2016.

Culture remains the gold standard for *Mtb* diagnosis although it is technically involving and takes not less than 4 weeks to give results (Zhao et al., 2011). It involves both solid and liquid methods. The solid medium is used to enhance the growth of *Mtb* and inhibit the growth of other microorganisms. However, the results take long and may thus provide opportunities for continued transmission and death (Cambau et al., 2000). Liquid culture is automated and exploits the florescence of an oxygen sensor to detect growth of mycobacterium in culture; it is 10% more sensitive and has a reduced turnaround time compared to solid culture.

GeneXpert MTB/RIF is based on nucleic acid amplification to test TB using polymerase chain reaction and was approved by WHO in 2010 (WHO, 2011). Although it has been introduced for use in many countries in the world, its sensitivity and specificity tend to differ from one region to another thus need for country specific probes (Rufai et al., 2014).

The LPA, based on strip technology is used to detect TB (Lin et al., 2017). The method involves three processes: DNA extraction, multiplex PCR amplification, and reverse hybridization (Singh et al., 2017). Studies have shown LPA having a high sensitivity and specificity for smear positive culture positive samples in adults as compared to pediatric populations. This can be associated with the specimen quality (Meaza et al., 2017) ; LPA has reported sensitivity and specificity of LPA among South African and South American was 81% and 100% respectively (Luetkemeyer et al., 2014), similarly in India two studies showed a sensitivity and specificity of 96% and 99% respectively in 248 smear positive pa tients (Raizada et al., 2014). Even though GeneXpert MTB/RIF and LPA methods have been endorsed by WHO in detecting *Mtb*, they have not been evaluated in the Kenyan set up to determine their performance by use of country specific probe in detection of *Mtb*.

2.6 Tuberculosis Resistance

Resistance develops as results of either under dose of first line anti-TB drugs (CDC, 2012) or poor adherence to TB medication (Pillay et al., 2007), leading to development of resistant *Mtb* strains. Globally, about 3 % of all newly diagnosed patients have Multi-Drug Resistance Tuberculosis with the highest proportion in subjects who have previously received anti-tuberculosis treatment (Sharma & Mohan, 2006). World Health Organization and International Union against Tuberculosis and Lung Disease survey in 35 geographic sites indicated that primary resistance to at least one drug is around 10.7 % (Irfan et al., n.d.). The prevalence of MDR among new cases was found to be 0.4% in Tanzania, 0.7 in Kenya and 4.4% in Uganda, and among recurrent cases was 3.9% in Tanzania, 8.5% in Kenya and 17.7% in Uganda (Kidenya et al., 2014). In a study done in Nairobi in 2011, on the drug resistance patterns, 30.2% of *Mycobacterium* isolates were resistant to isoniazid, 11.6% streptomycin, 4.5% to ethambutol 1.4% to Rifampicin, 10.4 % to pyrazinamide and 0.7% were multidrug resistant (Ndung'u et al., 2012).

According to the WHO there are various types of resistance to tuberculosis namely mono-resistance (resistance to one first line anti-TB drug only), poly-resistance (resistance to more than one first line ant-TB drug, other than both isoniazid and Rifampicin), Rifampicin resistance (resistance to Rifampicin detected using phenotypic or genotypic methods) and Multidrug-resistant, resistance to at least Rifampicin (RIF) and isoniazid (INH) both being the major anti-tuberculosis drugs (WHO, 2015). And also resistance due to cross-resistance, which refers to the situation where treating a patient with a first drug confers changes in the physiology of the bacteria that reduce efficacy of a second drug, unrelated drug that may be administered at a later time (WHO, 2015)

Drug-resistant strains of *Mycobacterium tuberculosis* present a great challenge to global tuberculosis (TB) control efforts (WHO, 2009). Multidrug-resistant (MDR) strains of *M. tuberculosis*, defined as resistant to rifampicin (RIF) and isoniazid (INH), are difficult to diagnose and are complicated and expensive to treat. Furthermore, fewer treatment options are available for patients who contract or develop extensively drug-resistant (XDR) TB, which is MDR and has gained additional resistance to a fluoroquinolone and at least one of the second-line injectable antibiotics kanamycin (KAN), amikacin (AMK), or capreomycin (CAP) (WHO, 2009).

The molecular-based methods detect mutations associated with resistance against anti TB drugs. It utilizes primers in the assay that amplify the portion of the *rpoB* gene containing the 81 base pair core regions, these region is encoded with *Mtb* specific DNA sequences and mutations causing resistance to rifampicin (WHO, 2010). Probes are able to differentiate between the conserved wild-type sequence and mutation in the core region that are associated with resistance of Rifampicin (Lawn et al., 2011). RIF-resistance is associated with mutations in RRDR 81 bp region with Probe E related mutations (codon 531and 533) as the most common *rpo*B genetic mutation with sequence 511 as the least. The assay automates sample processing, nucleic acid amplification, and detection of the target sequences (CDC, 2016). On the other hand, The Line Probe Assay uses PCR/hybridization technique to detect RIF as well as Isoniazid (INH) resistance due to mutations in *rpoβ*, and *inhA* and *katG* genes. (Rufai et al., 2014). The methods having reported different results in various populations due to different circulating strains of Mtb, there is need for country specific probes in order to improve on diagnostic performance.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

The study was carried out at the National Tuberculosis Reference Laboratory (NTRL) in Nairobi, Kenya. The laboratory is the only referral public facility equipped with refrigerated centrifuges, Biosafety Cabinet II (BSCII), automated BACTEC 960 system and molecular assays where comprehensive diagnosis of the TB and drug susceptibility testing are done. It has capacity of processing an average of 9,000 samples per year.

3.2 Study Population

Patients presumed to have MDR-TB including; return after defaulting, failed retreatment, failed first line treatment and both smear positive and negative relapses. The patients samples from both public and private diagnostic facilities were collected, packaged and transported to NTRL-Kenya by registered courier between November, 2016 to March, 2017.

3.3 Sample Size

The number of subjects was determined using Fisher's formula;

$n = (z)^2 p(1-p)/d^2$

n = sample size

z = standard normal deviate at 95 % confidence level = (1.96)

p = Estimated re-current MDR-TB prevalence (8.5%) (Kidenya et al., 2014)

d = Degree of precision at 5 %

$$n = \frac{(1.96 * 1.96 * 0.085(1-0.085)}{0.05*0.05}$$

n=119 samples

The sample size was increased by 10% up to 131 to cater for lost to follow up.

3.4 Study Design

A cross-sectional study design was used. Systematic random sampling was done from sputum samples received from various diagnostic facilities across Kenya. On average 30 samples were received at NTRL-Kenya during each day. For a study period of 5 months an average of 3 samples were needed on each day hence an interval of 10.

3.5 Inclusion Criteria

Sputum samples of suspected MDR-TB patients received at NTRL-Kenya from various diagnostic facilities across Kenya were included in the study.

3.6 Exclusion Criteria

Sputum samples from patients who were newly diagnosed with *Mtb* and sensitive to first line drugs

3.7 Sample Collection and Transportation

The suspected MDR-TB patients were given sputum containers and instructed on how to collect sputum sample and bring them back to the facility. The sputum sample together with the patient request form was packed in a standard triple packaging container and an ice bag inserted to keep the sample cool transportation to NTRL-Kenya by registered courier for culture, drug susceptibility testing and molecular analysis.

3.8 Sample Reception and Processing

At NTRL the sputum samples together with the culture request form (**Appendix I**) were received and checked for completeness of culture request form, sample labeling and leakage. Those accepted were given laboratory request form (**Appendix II**) and number for processing. All sputum samples with complete information and package integrity were decontaminated from contaminants (overgrowth of another bacterium present in the sputum specimen, which can potentially mask the presence of *Mycobacterium tuberculosis*.) using the *N*-acetyl-l-cysteine-sodium citrate-NaOH (NALC-NaOH) method (Morcillo et al., 2008). Samples were decanted following centrifugation at 3000g for 15 minutes, and the pellets were re-suspended to make 3 ml using phosphate buffer solution. Several aliquots from each processed sample were prepared as follows and stored at 4°C; 0.5 ml to perform Florescent microscopy, 0.5ml MGIT960 culture, 0.5 ml for GeneXpert MTB/RIF, 0.5 ml for LPA. The 1 ml of the remaining aliquot sample was stored at −80°C as back up. All samples that were not accepted for culture were sterilized by autoclaving and incineration.

3.8.1. Liquid Culture and Drug Susceptibility Testing

3.8.1.1. Sample incubation

A 500-µl aliquoted sample were incubated at 37 ^{o}C in Mycobacterium growth indicator tubes (MGIT) in the BACTEC machine (BACTEC^{TM} MGIT^{TM} 960 System, Series 1300 A2, Becton Dickson and Company, MA, USA) for a maximum of 42 days (6 weeks) from initial incubation date (**Appendix lll**). The MGIT tubes in the BACTEC machine were flagged by green light for no growth and red light for growth of Mycobacterium, on the front drawer of the BACTEC MGIT 960 machine.

3.8.1.2. Confirmation of Mycobacterium tuberculosis

Before drug susceptibility was performed, the tuberculosis bacilli were confirmed by: Plating of Brain Heart Infusion agar with the culture of *Mycobacterium tuberculosis*, staining the isolates with Zeil-Neelsen for microscopic examination and carrying out capillia test. This is antigen/antibody test which targets the presence of MTP64 antigen found in strains of Mycobacterium tuberculosis complex.

3.8.1.3. Drug Susceptibility testing

A stock solution for drug susceptibility testing was prepared by pipetting 4 millilitres of sterile distilled water into avail of lypholized drug powder of streptomycin, isoniazid, rifampicin, and Ethambutol (SIRE). A working solution was prepared by pipetting 0.1 millilitres of the stock solution and adding into MGIT tubes to obtain a drug concentration in the medium for streptomycin as 0.1 microgram per milliliter, isoniazid 1.0 microgram per milliliter, rifampicin 40 milligram per millilitre and Ethambutol 0.1 microgram per millitre. Growth control tubes (GCs) were included which contained growth supplement but no drugs. The working bacterial suspension for these control tubes were prepared by pippeting 0.1millilitresl of the already prepared bacilli suspension and 10 ml of sterile saline (Lawson *et al*., 2010). To all control tubes, 0.5 millilitres of Growth Control working solution was added into labeled drug free Mycobacterium growth indicator tubes, and to all the other drugs containing tubes labeled S (streptomycin), I (isoniazid), R (rifampicin) and E (ethambutol), 0.5millilitreof the bacilli stock solution was added and all the 5 MGIT tubes were put into a carrier rack and placed into the BACTEC MGIT 960 instrument.

3.8.1.4. Susceptibility interpretations

When the growth control tubes reached a growth unit of 400 or more the instrument indicates that the test was complete and the Bactec equipment machine interprets the results as resistant or susceptible. The tubes were removed from the machine after being scanned and printed. The final pattern of susceptibility testing was manually interpreted per sample as fully susceptible, mono resistant, poly resistant or multidrug resistant

3.8.2. Line probe assay

The LPA, based on strip technology was used to diagnose TB and detect RIF as well as Isoniazid (INH) resistance due to mutations in *rpoβ*, and both *inhA* and *katG* genes. The method involved three processes: DNA extraction, multiplex PCR amplification, and reverse hybridization. Mycobacterial DNA was extracted using a GenoLyse kit (Hain Lifescience, Nehren, Germany)-based manual method. Polymerase chain reaction (PCR) was performed using pre-made amplification mixes (amplification mix A and amplification mix B) that contained all the necessary components. Hybridization was performed using an automated GT Blot 48 device (Hain Lifescience, Nehren, Germany), and the results were interpreted based on the operating manual provided by the manufacturer (**Appendix IV)**. The DNA strip was removed from the tube and marked as per the number of samples. It was then added to each well containing 20 microliters of corresponding amplified DNA sample with coloured part facing up. The well was placed in the twincubator and hybridization procedure was initiated. Hybridization occurred by pre-warming the hybridization buffer to 45°C in water bath for 15 minutes in the twincubator machine (Hain Life Science GmbH, Nehren, Germany). Denaturing solution, 20 microliters was pipetted to each of the tray that was used, and then 1 ml of rinse solution added per well and incubated for 1 minute. The well was removed and rinsed with a rinse solution. One millilitre of the conjugate was added into each well, then incubated for 30 min, removed and washed with rinse solution. Finally, 1 ml of the substrate was added into the well and incubated for 10 min and then washed twice with distilled water. The strips were left to dry and results scanned and interpreted by the Hain Life Science GmbH, Nehren, Germany machine (**Appendix V**).

3.8.3. GeneXpert MTB/RIF

The GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA) test was performed as per the manufacturer's instruction, **Appendix VI**. Aliquots of decontaminated samples were taken out of

4°C storage, together with the sample reagent buffer containing NaOH and isopropanol were mixed at the ratio of 1:3 followed by incubation at room temperature for 15 min. Two milliliters of the sample was then transferred into the GeneXpert MTB/RIF cartridge (Cepheid, Sunnyvale, CA) and loaded into the GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA). The results were generated after two hours, reported as either *Mycobacterium tuberculosis* negative or positive, or whether those positive were RIF susceptible or resistant.

3.9. Ethical Considerations

Ethical approval for this study was obtained from Amref Health Africa Ethics and Scientific Review Committee with approval reference number **ESRC P256/2016** (**Appendix VII**). The samples were assigned unique study codes to ensure participant anonymity and delinked with the patient maintaining age and sex as the only socio-demographic data. The study did not alter the original results in any way. There were no risks involved in the study and the results obtained were released to the referring facility for patient management.

3.10 Data Processing and Analysis

The sensitivity, specificity and predictive variables for GeneXpert MTB/RIF and LPA for detection of *Mtb* and RIF mono-resistance were calculated using MGIT culture as the gold standard. Kappa statistic was used to separately show the agreement in TB and RIF mono resistant detection between GeneXpert MTB/RIF versus culture LPA versus culture. Cohen kappa interpretation as (0.01-0.20 to indicate none to slight, 0.21-040 as fair, 0.41-0.60 as moderate agreement, 0.61-0.80 as good agreement, and 0.81-1.0 as very good agreement). All the data was analyzed by STATA version 13.

3.11 Study Limitation

The study used a design which failed to separate results based on smear results thus impacting the interpretation.

CHAPTER FOUR

RESULTS

One hundred and thirty one sputum samples of patients suspected to have drug resistant TB were picked and subjected to GeneXpert MTB/RIF, LPA and Culture. This included 10% adjustment to cater for *Mycobacterium* other than *M. tuberculosis*, contaminated by overgrowth of other bacterium present in the sputum specimen, the invalid samples (no bands on the strip), those that had errors resulting from cartridge or machine malfunction and temperature instability and the ones with indeterminate results whereby the test could not accurately determine if the bacteria is resistant to RIF or not. Out of the 131, 58(44%) were salivary, 31(24%) blood stained and 42(32%) muco purulent.

4.1. Demographic Characteristic of Study Participants

Of the 131 samples, 84 (64.10%) were female and 47 (35.90%) male. The mean age was 39 years and the age range interval of 10 years with age group 25-30 having many cases of suspected drug resistant TB ; Table 4.1.

Table 4.1: Demographic characteristics of study participants

	Range	**Number (%)**
Age (years)	5 - 14	2 (1.53)
	15 - 24	14 (10.69)
	25 - 34	37 (28.24)
	35 - 44	34 (25.95)
	45 - 54	27 (20.61)
	55 - 64	13 (9.92)
	65 - 74	2 (1.53)
	75 - 84	2 (1.53)
Gender	**Male**	47 (35.90)
	Female	84 (64.10)

4.2. Detection of *Mycobacterium tuberculosis*

To assess the diagnosis performance, 131 samples were analyzed. Culture method diagnosed 49(37.40%) as positive for TB and 82(62.60%), GeneXpert MTB/RIF method diagnosed 65 (49.60%) to be positive for *Mtb* while 66(50.38%) were negative for *Mtb* and LPA diagnosed 72 (55%) to be positive with 59 (45%) being negative for *Mtb*, **Table 4.2.1**. For GeneXpert MTB/RIF, the sensitivity and specificity was 76% and 66% while positive and predictive values were 57% and 82% respectively. For LPA the sensitivity and specificity was 98% and 71% respectively, while the positive and negative predictive value were 67% and 98% respectively as shown; **Table 4.2.2**

Table 4.2.1 Two by two table on detection of *Mycobacterium tuberculosis* (N=131)

		TRUE disease status	**Culture MTB**		
			Negative	**Positive**	**Total**
GeneXpert MTB	Test result	Negative	54	12	66
		Positive	28	37	65
		Total	82	49	131
LPA MTB	Test result	Negative.	58	24	82
		Positive.	1	48	49
		Total	59	72	131

Genexpert: True positive 37, false positive 12, true negative 54 and false negative 28

LPA: True positive 48, False positive 24, true negative 58 and false negative 1

Table 4.2.2 Calculated sensitivity, specificity and predictive values on detection of *Mycobacterium tuberculosis*

	[95% Confidence Interval]	
	Culture and GeneXpert	**Culture and LPA**
Sensitivity	76%	98%
Specificity	66%	71%
Positive predictive value	57%	67%
Negative predictive value	82%	98%

4.3. Detection of RIF mono-resistance

To detect RIF mono-resistance, 37 samples that were positive by the three methods of LPA, GeneXpert MTB/RIF and culture. LPA diagnosed 3 samples as RIF resistant and 34 as RIF sensitive, GeneXpert MTB/RIF diagnosed RIF resistance in 2 samples and 35 samples as RIF sensitive. On the other hand, culture recorded RIF resistance in 3 samples and 34 as RIF sensitive, **Table 4.3.1**. For GeneXpert MTB/RIF the sensitivity and specificity was 33% and 94% while positive and predictive values were 33% and 94% respectively. For LPA the sensitivity and specificity was 100% and 100% respectively while the positive and negative predictive value were 100% and 100% respectively as shown; **Table 4.3.2.**

Table 4.3.1Two by two table on RIF mono-resistance detection (N=37)

			Culture MTB		
		TRUE disease status	**RIF sensitive**	**RIF Resistant**	**Total**
GeneXpert MTB	**Test Result**	**RIF. sensitive**	32	2	34
		RIF. resistant	2	1	3
		Total	34	3	37
LPA MTB	**Test Result**	**RIF. sensitive**	34	0	34
		RIF. resistant	0	3	3
		Total	34	3	37

RIF= Rifampicin

Genexpert: True positive 1, false positive 2, true negative 32 and false negative 2

LPA: True positive 3, False positive 0, true negative 34 and false negative 0

Table 4.3.2 Calculated sensitivity, specificity and predictive values on detection of RIF mono-resistance detection

	Culture and GeneXpert [95% Confidence Interval]	Culture and LPA
Sensitivity	33%	100%
Specificity	94%	100%
Positive predictive value	33%	100%
Negative predictive value	94%	100%

4.4. Test of agreement between GeneXpert MTB/RIF, LPA with culture

To test for agreement, 131 samples were analyzed for *Mtb* detection and 37 for RIF mono resistance detection. The LPA method diagnosed 72 (55%) samples as positive for *Mtb* with 59 (45%) being negative. In contrast, GeneXpert MTB/RIF diagnosed 65 (49.60%) to be positive for *Mtb* while 66(50.38%) were negative, **Table 4.4.1**. There was a fair agreement in GeneXpert MTB/RIF and culture (Kappa value, 0.388), LPA and culture (Kappa value, 0.628) for *Mycobacterium tuberculosis.* There was a fair agreement between GeneXpert MTB/RIF and culture *(*Kappa value, 0.275) as compared to a very good agreement between LPA and culture *(*Kappa value, 1.00) for detection of RIF mono-resistance, **Table 4.4.2.**

Table 4.4.1 Kappa values on detection of *Mycobacterium tuberculosis*

		TRUE disease status	Culture MTB			Kappa	p. value
			Negative	Positive	Total	Kappa Value	
GeneXpert MTB	**Test result**	Negative	54	12	66	0.388	0.001
		Positive	28	37	65		
		Total	82	49	131		
LPA MTB	**Test result**	Negative.	58	24	82	0.628	0.001
		Positive.	1	48	49		
		Total	59	72	131		

Table 4.4.2 Kappa values on detection of Rifampicin mono resistance

		TRUE disease status	Culture MTB Sensitive	Rifampicin	Total	Kappa Value	Kappa	P value
GeneXpert MTB	**Test result**	**Sensitive**	32	2	34		0.275	0.001
		Rifampicin	2	1	3			
		Total	34	3	37			
LPA MTB	**Test result**	**Sensitive**	34	0	34		1.0	0.095
		Rifampicin	0	3	3			
		Total	34	3	37			

Cohen kappa interpretation (0.01-0.20 to indicate none to slight, 0.21-040 as fair, 0.41-0.60 as moderate agreement, 0.61-0.80 as good agreement, and 0.81-1.0 as very good agreement)

CHAPTER FIVE

DISCUSSION

Resurgence and rapid spread of TB and drug resistant *M. tuberculosis* over recent years (CDC, 2016) raises an urgent need to find an efficient rapid assay for diagnosis and detection of drug resistant TB. No study has been carried out in Kenya to compare the performance of both GeneXpert and LPA even though the performances of these tests have been shown to vary from region to region. In this study, the performance of GeneXpert MTB/RIF and LPA in detecting TB and Rifampicin mono-resistance was compared to that of liquid culture as the gold standard.

5.1 Detection of Tuberculosis

For GeneXpert MTB/RIF, the diagnostic sensitivity and specificity was 76% and 66% while positive and negative predictive values were 57% and 82% respectively, as compared to culture. The diagnostic sensitivity agreed with other studies. For example, the sensitivity of GeneXpert MTB/RIF was 79% in a South African population (Theron et al., 2016). Similarly, (Zhao et al., 2011) found sensitivity of between 68% -100% in several studies done on an Asian population. In addition (Boehme et al., 2014) recorded 79% in sampled samples from Peru, India and South Africa. This study reported low specificity as compared to other studies, for example in China there was specificity of 98% (Shiying et al., 2017) and 93.10% in India. The variation may have resulted from the different circulating strains of *Mtb* which varies from region to region (Rufai et al., 2014). The study used commercial probes which were not region specific hence variation in specificity.

Line Probe Assay detected TB with a sensitivity and specificity of 98% and 71% respectively, and positive and negative predictive value of 67% and 98% respectively. Other studies also showed a similar trend. In India a study showed a sensitivity of 96% (Raizada et al., 2014). Similarly, a study in high risk MDR-TB population in Taiwan reported a specificity 65.70% (Lin et al., 2017). From the above, it is observed that LPA reported low specificity but on further interrogation of the results the method has an ability of 98.40% in picking the true negative among those without disease. It can therefore be concluded that it's reliable method for *Mtb* detection.

Predictive values gives more insight on the test results used for diagnostic accuracy (Trevethan, 2017). With all factors being constant positive predictive values increases with increase in prevalence of the condition on one hand while, on the other hand negative predictive value decreases with increase in prevalence (Parikh et al., 2008). In the current study GeneXpert and LPA reported a fair positive predictive value as compared to high negative predictive value reported by the same assays. The test is based on nucleic acid amplification and was able to pick more positive cases than the gold standard which had an impact of reducing the true positives thus reduced PPV.

5.2. RIF mono-resistance detection

Rifampicin forms the backbone of first line anti-tuberculosis chemotherapy (WHO, 2015). Rifampicin resistant has been used as an indicator of multi-drug resistant tuberculosis (Pang et al., 2014) . There is need to explore more for a more efficient methods of diagnosing Rifampicin mono resistance, as it is key to the management of drug resistance TB.

The GeneXpert MTB/RIF sensitivity and specificity for Rifampicin mono resistance detection was 33% and 94%, while positive and negative predictive values were 33% and 94% respectively. Whereas the specificity agreed with similar studies, for example a study in South Africa recorded specificity of 94% respectively for GeneXpert MTB/RIF (Theron et al., 2016). Similarly, in an Asian population, there was a pooled specificity of between 68% -100% (Pang et al., 2016). It was notable that the study reported lower sensitivity. Previous studies have suggested reasons for these, for example need for probe specific in different regions (Rufai et al., 2014). The difference in the circulating strains maybe the reason for the low sensitivity in the current study.

The sensitivity of Rifampicin mono resistance of LPA observed in this study corresponded with other studies. For example, from both South African and South American population, the sensitivity and specificity was 92% and 97% (Luetkemeyer et al., 2014). Equally, (Lin et al., 2017) working at high risk MDR- TB set up in Taiwan reported a sensitivity of 96%. In addition, a study in New Delhi recorded a sensitivity and specificity of 97.6% and 94.4% (Ritu et al., 2012) equally study in South Africa reported sensitivity and specificity of 97.7% and 91.8% respectively (Tomasicchio et al., 2016) In other studies done in East African countries, the

sensitivity and specificity of Rifampicin mono resistance detection was 96.4% and 100% among smear positive samples samples in Ethiopia (Meaza et al., 2017) and 100% and 96.1% among smear positive population in Uganda (Albert et al., 2010).

5.3 Test of agreement between GeneXpert MTB/RIF and LPA with culture

Agreement is the measure of a variable by two different assays to produce similar results (Flight et al., 2015). When comparing a new medical assay quality with the gold standard, test of agreement forms an important consideration (Zaki et al., 2012). Kappa values are used to report on agreement with Cohen kappa suggesting an interpretation of 0.01-0.20 to indicate none to slight, 0.21-040 as fair, 0.41-0.60 as moderate agreement, 0.61-0.80 as good agreement, and 0.81-1.0 as very good agreement (McHughs, 2012).

In the current study, it is reported that a fair agreement between GeneXpert MTB/RIF and culture (Kappa Value, 0.388) while LPA and culture recorded a good agreement (Kappa Value, 0.628). Similarly there was a fair agreement between GeneXpert, MTB/RIF and culture *(*Kappa Value, 0.275) for Rifampicin mono resistance detection while, on the other hand there was a very good agreement between LPA and culture *(*Kappa Value, 1.00) for detection of RIF mono-resistance. The current study reported similar to what was reported in India, of 100% between MGIT 960 and LPA results (Rufai et al., 2014), equally LPA reporting the same with the conventional DST at 96% in New Delhi (Yadav et al., 2013).

CHAPTER SIX

SUMMARY, CONCLUSION, RECOMMENDATIONS AND SUGGESTIONS FOR FUTURE RESEARCH

6.1 Summary

Overall, the present study showed, first, that GeneXpert MTB/RIF reported slightly reduced sensitivity, specificity and predictive values than LPA on TB detection when compared to liquid culture. Second, regarding Rifampicin mono-resistance, Gene Xpert MTB/RIF had a sensitivity, specificity and predictive values lower than LPA. Lastly, the study reported a fair agreement in both *Mtb* and Rifampicin detection by GeneXpert MTB/RIF on one hand and culture on the other. While, LPA reported a good agreement for *Mtb* detection while on Rifampicin mono-resistance it reported a very good agreement with culture.

6.2 Conclusion

1. LPA diagnostically outperformed GeneXpert MTB/RIF.
2. Regarding Rifampicin mono-resistance, LPA outperformed GeneXpert MTB/RIF.
3. LPA is a good alternative to culture with regards to detection of Rifampicin mono resistance in facilities without culture and DST.

6.3 Recommendation

1. The study recommends the up-scaling of LPA, for TB detection.
2. The study recommends LPA for detection of Rifampicin mono resistance.
3. Development of specific probes for the local population in *Mtb* and RIF mono resistance.

6.4 Suggestions for Future Studies

1. Comparing the performance of GeneXpert MTB/RIF and LPA based on sample quality, for example salivary and blood stained.
2. Conducting a follow up study to determine whether false positive samples with GeneXpert and LPA eventually develop *Mtb* and RIF mono-resistant.
3. Sequencing of the rpoB gene to understand more on RIF mono resistance.

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APPENDICES

APPENDIX I. Culture Request Form

CULTURE REQUEST FORM

Ministry Of Public Health and Sanitation

Division of Leprosy, Tuberculosis and Lung Disease

CULTURE REQUEST FORM

Study ID……..Reg No …… OP/IP No…….

Age……Sex…….Facility…….

Date of collection……..

County …….

Examination requested: smea☐ Culture ☐ Sensitivity ☐

Type of subject: Sputum smear positive relapse ☐

Sputum smear-negative relapse ☐

Return after defaulting ☐

Failure 1-st line treatment ☐

Failure re-treatment ☐

HIV status: pos ☐ Neg ☐ Not Done ☐

Previous treatment:

2RHZE/4RH from: Date …….to…….

2RHZE/6HE from: Date …….to……..

2SRHZE/IRHZE/5RHE from: Date……..to…….....

Other (specify) from: Date…….....to……..

Date of last treatment……..

APPENDIX II. Laboratory Request Form

Ministry of Public Health and Sanitation

Division of Leprosy, Tuberculosis and Lung Disease

LABORATORY REPORT

National Tuberculosis Reference Laboratory

Direct Smear Report (*WHO/IUATLD reporting format*)

0	No seen in 300 fields	o	☐
1-9	AFB seen in 100 fields, actual number……		☐
10-99	AFB seen in 100 fields	+	☐
1-10	AFB seen /field in at least 50 fields	++	☐
> 10	AFB/field in at least 20 field	+++	☐

Culture Report

Mode of culture: MGIT 960 ⬭ Lowenstein Jensen ⬭

Confluent growth ☐

>100 colonies ☐

20-200 colonies ☐

1-19 colonies ☐

Contaminated ☐

First Line Drugs			Second Line Drugs		
Sensitivity Testing:	Sensitive	Resistance		Sensitive	Resistant
Isoniazid (H)	☐	☐	Amikacin	☐	☐
Streptomycin (S)	☐	☐	Capreomycin	☐	☐

Rifampicin (R)	☐	☐	Cycloserine	☐	☐
Ethambutol	☐	☐	Ethionomide	☐	☐
Pyrazinamide (Z)	☐	☐	Kanamycin	☐	☐
			Oxloxacin	☐	☐

DNA P-amino Salicylic acid ☐ ☐

rpoB

rpoB

katG

InhA

Identification test Human ☐ Bovine ☐ Typical ☐

Name……Signature……Designation

APPENDIX III: Automated BACTEC 960 SYSTEM instrument loaded with samples

APPENDIX IV: Line probe assay mtbdrplus protocol

1. PROCEDURES

Workflow- uses the 3 room strategy

In order to protect unamplified samples from contamination from amplified DNA, key steps/assay of this technique must be performed in distinct and physically separated work areas.

Reagent preparation room (PCR 1): This area is used for storing and setting up master mixes and aliquoting reagents. Nucleic acids, specimens, and amplified products are forbidden in this room.

DNA extraction Area (BSL 3): This area is used for performing DNA extraction from live culture. A BSC and BSL3 is used to work with infected TB samples.

Amplification Area (PCR 2): This area is used for setting up the reactions for PCR.

Post-amplification Area (PCR 3): This area is used for post-PCR analysis or procedures.

DNA EXTRACTION

Mechanical extraction

Bacteria colonies from solid culture are collected with an inoculating loop and re-suspended in 300ul distilled water.

1 ml of bacteria suspension from a positive MGIT is aliquoted into a labeled 1.5 ml eppendorf tube and pelleted by centrifuging at 10,000g for 15 minutes in a microfuge with an aerosol tight rotor for liquid media.

Discard the supernatant and add pipette 100ul of distilled water.

Re-suspend bacterial pellet by vortexing for 10 seconds in both solid and liquid cultures in a class II bio-safety cabinet.

Heat inactivate bacteria suspensions for 20 min in a water bath at 95 degree centigrade

Incubate for 15 minutes in ultrasonic bath

Spin down for 5 minutes at 14000g and use 5ul of the supernatant for PCR

Chemical extraction

Collect specimen to a 1.5ml cryo-vial tube which is pre-labeled with the lab number. 500µl of decontaminated specimen and 1ml of liquid culture isolate.

The samples are centrifuged at 10,000g for 15 minutes in a micro-centrifuge with an aerosol tight rotorDiscard the supernatant, pipette 100µl of lysis buffer (A-LYS) and re-suspend the pellet by vortexing for 5 seconds.

Incubate the samples in a heating block at 95◦c for 5 minutes

Pipette 100µl of neutralization buffer (A-NB) and add to the sample

Vortex briefly for 5 seconds to completely mix the mixture

Centrifuge the tubes at 14,000g speed for 5 minutes.

Collect the supernatant in a separate 1.5ml tube which is pre-labeled with the lab no.

AMPLIFICATION

Remove AM-A and AM-B from the -20◦c freezer and thaw them carefully

Pipette AM-A and AM-B in a room free of contaminating DNA (PCR 1).

Prepare PCR tubes according to number of tests to be performed and allow for 2 controls for each batch.

Prepare 45ul of the amplification mix for each sample as follows:

Per sample

10ul AM-A

35ul of AM-B

5ul DNA solution is added at the PCR 2

AM- A and AM-B can be prepared and stored in aliquots depending on the work load. These is to avoid freeze and thaw

AMPLIFICATION PROFILE

Transfer the PCR tubes to the thermal cycler and perform PCR using the following amplification profile

15sec in 95degree centigrade 1 cycle
30sec at 95 degree centigrade
2min at 65 degree centigrade 20 cycles
25sec 95 degree centigrade
40 sec 50 degree centigrade
40 sec 70 degree centigrade 30 cycles
8 minutes 70 degree centigrade 1 cycle
Amplification products are stored at +8 to -20◦c

HYBRIDIZATION

TWINCUBATOR

Note: use the appropriate kit/strips for the appropriate test

Dispense 20ul of denaturizing solution (blue) in a corner of each well to be used

Add to the solution 20ul of the amplified sample , pipette up and down to mix well and incubate at room temperature for 5 minutes

Carefully add 1ml of pre warmed hybridization buffer (green) to each well. Shake gently to homogenize.

Add marked strips to respective wells using tweezers

Place tray on the twincubator and incubate for 30 minutes at 45° centigrade.

Aspirate hybridization buffer completely

Wash with 1ml stringent buffer (Red) and incubate for 15 minutes at 45° centigrade in shaking twincubator

Work at room temperature from this step. Remove stringent wash buffer completely.

Wash each strip with 1ml rinse buffer for 1 minute.

Add 1ml of diluted conjugate to each strip and incubate for 30minutes on the shaking twincubator

Remove solution and wash each strip twice for 1 minute in rinse solution and once in 1ml water.

Add 1ml diluted substrate to each strip and incubate for 5-10 minutes protected from light without shaking.

Stop reaction by rinsing twice with distilled water

Remove strips from the tray and dry them on absorbent paper.

GT BLOT 48

Before using the instrument for analyzing samples the following series of instrument checks should be carried out:

Ensure the power is switched OFF.

Ensure the waste tubes are routed into a suitable empty container.

One of the waste tubes is gravity fed. Ensure the waste container is below the level of the machine and the waste tubes are not trapped under feet.

Switch power ON (back left of the machine) and ensure that the instrument initializes properly.

The initialization sequence of events is as follows:

Place all tubing's in a container filled with distilled/de-ionized water.

Follow the keypad commands, and ensure the water flows though the appropriate dispensing needles under the arm when washing is in progress.

"BeeBlot press start"

Using either the left or right arrow select "Washing – Assay 11" then press START three times:

"Cleaning cycle A" Press START

After cleaning cycle A is complete press START

"Cleaning cycle B" Press START

While the cleaning cycle is in progress, prepare dilutions of the conjugate and substrate solutions in dilutions of 1:100. After cleaning cycle B is complete switch the power to the instrument OFF.

Dry tubing's with a dry cloth.

Switch off the instrument and close the lid prior to cleaning. On a weekly basis clean the insert for the tray inside the instrument carefully using 70% ethanol and cotton tipped applicator stick to remove any residues from between the wells. Don't use bleach inside the instrument.

Annual maintenance is done by HAIN lifescience company. Place all solutions in their respective places (color match and insert the conduction tubes (e.g. the HYB solution is green, and the slot is marked with a green dot). Check that there is sufficient solution in each bottle for the number of strips being loaded into the tray.

Note: stock solution should be returned in the fridge at temperatures of (2-8)℃

Switch the power to the instrument ON."BeeBlot start" Press START.

By pressing the right arrow key select "Genotype A assay 01" three times.

It is important to locate the temperature sensor in the tray before the assay starts. Lift the temperature sensor up carefully and put into the tray. Make sure that the sensor is properly positioned down before starting the assay.

Reagent of HYB and STR should be pre- heated for 15 minutes before the start of the run.

While the pre-heat is in progress, dispense 20µl of denaturation "DEN" solution in a corner of each of the wells in the tray. One tip can be used, but should be changed in cases of possible contamination.

Take the LPA strips out of the tube using tweezers and label with a pencil (or Hainmarker pen) on the coloured side mark the strips and always use gloves when handling strips.

Add 20µl of amplified sample to the DEN solution, pipette up and down to mix and incubate at room temperature for 5 minutes. Take great care not to contaminate the neighboring wells during addition of amplified samples. Use a fresh pipette tip for each addition.

Include a positive control of ATCC H37rv prepared from culture and a negative control from molecular water.

After the pre-heat is complete, press START and select the number of wells to be used using the left or right arrow key. The choice must be an even number between 2 and 48. Press START.

Position sensor and press START.

Begin assay – press START

Close lid – press START

After the priming sequence is completed the instrument will dispense pre-warmed hybridization reagent (HYB) into the tray and it will dispense water into the wells that are not used, in order to gain optimal temperature:

Check to ensure that all the wells were filled correctly.

The strips must be completely covered by the solution and the coated side must be facing upwards (identifiable by the mark near the lower end).

Using tweezers (or sterile pipette tip), turn over any strips that may have turned over during immersion. Clean tweezers using bleach to avoid cross contamination.

Close the door and press START.

The tray should start to rock and the green LED will come ON. Check that the temperature starts to rise.

Once the temperature of 45ºC is attained, hybridization will start and continues for 20 minutes incubation.

According to the assay program, the instrument will aspirate and add the necessary reagents at the correct time interval and incubate reagents at the appropriate temperature throughout the test method.

After reaction has stopped ASPIRATE tray? Will be displayed. Press START.

After aspiration is complete open the door and remove the tray and put it on the bench. Take out the tubing's from the reagent containers and place them in the container filled with 1% bleach solution and run the washing assay twice and repeat the washing cycle twice with de-ionized water.

Dry tubing's and places them in an empty container. Close the reagent containers and store at (2-8)ºC.

To remove the tray, remove the two tray clamps at the bottom of the tray, lift sensor and slide tray outwards.

Interpretation of Results on the Genoscan

Use forceps to transfer strips to the Geno Type MTBDR*plus* or MTBDRsl Results Sheet provided with the kit or downloadable from Hain Life science website.

Be careful to follow instructions on the pdf file which can be downloaded from the website to ensure that the correct size sheet is printed (otherwise the size of the strip on the form is smaller than the actual size and the bands will not line up).

Read results by lining strips up to code provided with kit.

In order for results to be valid, CC (conjugate control) and AC (amplification control) bands must appear for every sample. The presence of TUB band indicates that *M. tuberculosis* complex is present in the sample. (For positive samples the AC band may be very weak or absent, but as long as TUB band is present the result can be evaluated. For negative samples, however, the AC band MUST be present or the result cannot be evaluated).

A mutation in the relevant gene (and resistance to the relevant drug) is signified by either an absent wild type band and/or the presence of a mutant band for each gene cluster.

The *rpoB, katG* and *inhA* each have a control band which must be present for *MTBDRIplus* and gyrA, gyrB, rrs, eis control band must be present in MTBDR*sl* in order to interpret the results.

rpoB predicts RIF resistance, *katG* predicts high level INH resistance, *inhA* predicts low level INH resistance.

Both the gyrA and gyrB genes are examined for detection of resistance to FLQ (e.g., ofloxacin or moxifloxacin).

The rrs gene is examined for detection of cross-resistance to AG/CP antibiotics such as kanamycin (KAN) and amikacin (AMK), both AG, or capreomycin (CAP) and viomycin (VIO)

The eis gene is examined for detection of a low-level KAN-resistance

In order for a batch of results to be valid, the negative control strip must have a CC and AC band present, but no other bands must be visible.

If a positive result is obtained with the negative control, the results of the whole batch must be repeated and measures taken to remove amplicon contamination from all rooms and equipment.
If a negative result is obtained with the positive control, the results of the whole batch must be repeated and do a root cause analysis.
Refer to the product insert for more details of interpretation of banding patterns and troubleshooting.
Record the results on the report form and store forms in a file.

Cleaning of GT Blot trays

Soak the trays thoroughly using diluted bleach solution in the sink. Remove excess liquid and then spray with 70% ethanol and wipe wells thoroughly to remove residues (using cotton tipped applicator stick).
After cleaning, wash well using distilled water to ensure all bleach is removed. Bleach residues not removed may affect the colour development of the strips.
Discard the water and wash a second time with distilled water.
Pat remaining liquid on a fresh paper towel and leave to air dry.
The tray can be reused once dry.
Keep several trays available, so that they can be alternated. Trays can be reused for quite some time, but check during cleaning that there are no holes in the bottom of the black trays.
Discard trays once a hole appears.

Cleaning the GT Blot 48 machine

Clean the outside of the instrument on a monthly basis using a moist lint free cloth. Switch off the instrument and close the lid prior to cleaning.
On a weekly basis clean the insert for the tray inside the instrument carefully using 70% ethanol and cotton tipped applicator stick to remove any residues from between the wells. Do not use bleach inside the instrument.

QUALITY CONTROL

Positive control

Prepare positive control by removing a cryovial containing live ATCC 27294 H37RV M. Tuberculosis from the -80 freezer,

Place the vial on a bench in BSL3 to thaw

Label a MGIT 960 medium tube as H37Rv and date.

In a clean BSC pipette 800µl of OADC supplement to the MGIT tube.

Add 0.5ml of thawed organism to the MGIT tube and invert the tube to mix.

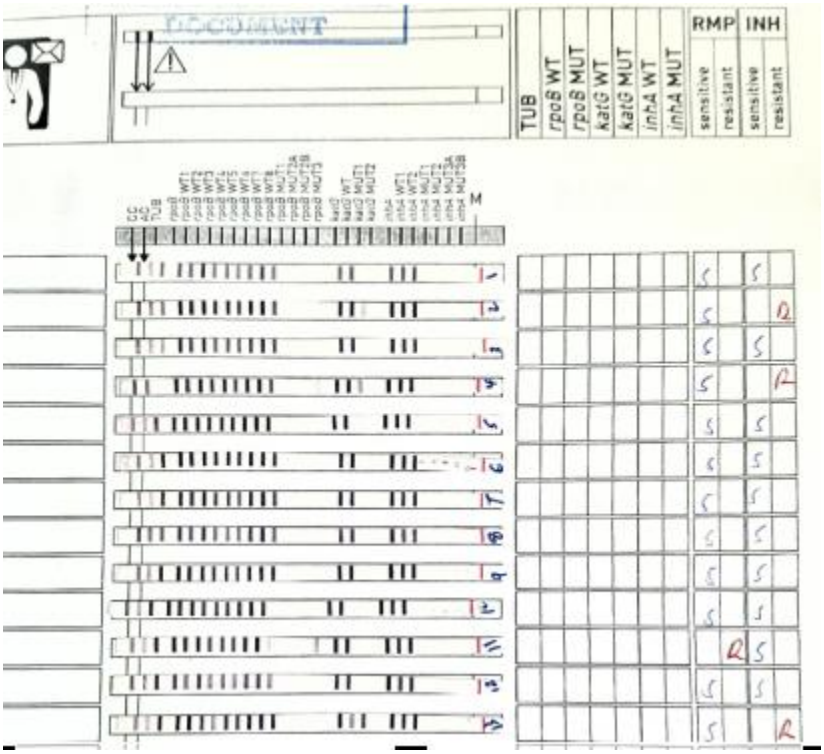
Incubate the inoculated MGIT in a BACTEC machine.

When the tube is flagged positive by the machine remove it and aliquote the organisms in 1 ml parts in the cryovials.

Use 1ml cryovial with every batch of HAIN samples and process them as normal patient samples.

Negative control –use 1ml of molecular water as the negative control and process as the normal sample.

APPENDIX V: Mounted strips showing resistance patterns



SS is sensitive to both rifampicin and isoniazid, RS is resistant to rifampicin but sensitive to isoniazid and SR refers to sensitive to rifampicin but resistant to isoniazid**.**

APPENDIX VI: Genexpert manufacturer's instructions

GeneXpert IV System

GeneXpert MTB/RIF assay is a rapid diagnosis test of Tuberculosis (TB) and drug resistance. It is revolutionizing TB control with aids in prompt diagnosis and treatment (selection of appropriate TB regimen).

GeneXpert MTB/RIF assay is a **nucleic acid amplification (NAA) test** which simultaneously **detects DNA of *Mycobacterium tuberculosis* complex (MTBC) and resistance to rifampin (RIF) (i.e. mutation of the rpoB gene)** in less than 2 hours. In comparison, standard cultures can take 2 to 6 weeks for MTBC to grow and conventional drug resistance tests can add 3 more weeks.

This system integrates and automates sample processing, nucleic acid amplification, and detection of the target sequences.

The primers in the XpertMTB/RIF assay **amplify a portion of the *rpoB* gene** containing the 81 base pair "core" region. The probes are **able to differentiate** between the conserved wild-type sequence and mutations in the core region that are associated with rifampicin resistance.

The Centers for Disease Control and Prevention (CDC) recommends that NAA testing be performed on at least one respiratory specimen from patients who have a moderate or high suspicion of having pulmonary TB.

Materials/System requirement:

GeneXpert Cartridge

1. **GeneXpert System**
   - equipped with GX2.1 software/computer/printer/barcode wand-reader and operator manual (Cepheid Inc, Sunnyvale, USA).
   - It is available in a one, two, four, or 16-module configuration
2. **GeneXpert Cartridge**:
   - Single-use disposable XpertMTB/RIF cartridges
   - Sample extraction, amplification and detection are all carried out within this self-contained cartridge.
3. **Class II biological safety cabinet (BSC)**
4. **Sample reagent** (provided in Xpert MTB/RIF kit), 8ml volume pack per each cartridge. The sample reagent solution is clear, but may range from colorless to golden yellow.
5. **Permanent marker** pens.
6. **Sterile (individually packed) disposable transfer pipettes**– with single mark for minimum volume of sample transfer to cartridge (provided in Xpert MTB/RIF kit).
7. **Sterile screw-capped specimen collection containers/cups**.
8. **Discard containers** for pipettes and sputum containers.

Basic Procedure:

1. Collect sputum sample from the patient with suspected TB.
2. The sputum is mixed with the reagent that is provided with the assay, and a cartridge containing this mixture is placed in the GeneXpert machine.
3. All processing from this point on is fully automated.

Advantages of the Xpert MTB/RIF Assay

1. Time efficient methods for detecting *Mycobacterium tuberculosis* bacteria and mutations isoniazid (INH) resistance.

2. Availability of quick test results leads to improved patient management and outcomes, and preventing unnecessary use of resources (avoiding unnecessary treatment, respiratory isolation).
3. Fully automated system; minimal technical training is required to run the test.
4. Prompt (quick) identification of multidrug-resistant TB (MDR TB)* cases as resistance to RIF, in most instances, co-exists with resistance to INH. Rapid diagnosis of rifampin (RIF) resistance potentially allows TB patients to start on effective treatment much sooner than waiting for results from other types of drug susceptibility testing. If rifampin resistance is detected, confirmation of resistance can be done by **DNA sequencing**.

*MDR TB is TB that is resistant to both isoniazid (INH) and Rifampicin (RIF).

Interpretation of GeneXpert results:

- **Detected**: Mycobacteria have a high probability of resistance to RIF; should be confirmed by additional testing. If RIF resistance is confirmed, rapid molecular testing for drug resistance to both first-line and second-line drugs should be performed so that an effective treatment regimen can be selected.
- **Not detected**: Mycobacteria are probably susceptible to RIF; All tests that are positive for MTBC should have growth-based susceptibility testing to first-line TB drugs.
- **Indeterminate:** the test could not accurately determine if the bacteria are resistant to RIF. Growth-based susceptibility testing to first-line TB drugs should be performed.

APPENDIX VII: Ethical approval.

amref health africa

Amref Health Africa in Kenya

REF: AMREF – ESRC P256/2016 18th October 2016

Mr. Stephen Aricha
National Public Health Laboratories
Tel: +254725200812
E-mail: arichasteve@gmail.com

Dear Stephen,

RESEARCH PROTOCOL: COMPARATIVE EVALUATION OF GENE XPERT AND LINE PROBE ASSAY WITH CULTURE FOR DETECTION OF RIFAMPIN-MONO RESISTANT *MYCOBACTERIUM TUBERCULOSIS* AT THE NTRL TUBERCULOSIS LABORATORY IN KENYA.

Thank you for submitting your research protocol to the Amref Ethics and Scientific Review Committee (ESRC).

This is to inform you that the ESRC has approved your protocol. The approval period is from 18th October 2016 to 18th October 2017 and is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by Amref ESRC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the ESRC immediately.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to Amref ESRC immediately.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period (attach a comprehensive progress report to support the renewal).
f) Clearance for export of biological specimen or any form of data must be obtained from Amref ESRC, NACOSTI and Ministry of Health for each batch of shipment/export
g) Submission of an executive summary report within 90 days upon completion of the study. This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

Please do not hesitate to contact the ESRC Secretariat (esrc.kenya@amref.org) for any clarification or query.

Yours sincerely,

for

Prof. Mohamed Karama
Chair, Amref ESRC

CC: Dr. George Kimathi, WASH Programme Manager, Amref Kenya and Vice Chair Amref ESRC
Samuel Muhula, Monitoring & Evaluation and Research Manager, Amref Kenya