ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCE

SCHOOL OF ALLIED HEALTH SCIENCES

DEPARTMENT OF MEDICAL LABORATORY SCIENCES

Evaluation of the diagnostic performance of Genotype MTBDR*plus* VER 2.0 Line Probe Assay for the detection of multidrug resistance TB (MDR-TB) in sputum samples referred to National TB Reference Laboratory, Ethiopian Public Health Institute.

By: Abyot Meaza Dasho

Advisor: Mr. Kassu Desta (MSc, PhD candidate; Assistant Professor)

A thesis submitted to Addis Ababa University, School of Allied Health Sciences, Department of Medical Laboratory Science in partial fulfillment of the Requirements for the Degree of Masters in Clinical Laboratory Science Specialty in Diagnostic and Public Health Microbiology.

September, 2015

Acknowledgment

I would like to express my deepest gratitude to my advisor Mr. Kassu Desta for guiding me while doing this thesis. I am also very grateful to acknowledge the unique and vital contribution of my coadvisor Abebaw Kebede, for his willingness to support this study and review the proposal and this final thesis. My thanks also goes to Mr. Zelalem Yaregal, coordinator of the National TB Reference Laboratory for his enormous support and all National TB Reference Laboratory team members who participated in the laboratory analysis of this study. Finally I would like to thank AAU, Department of Medical Laboratory Sciences and Ethiopian Public Health Institute, EPHI for supporting this study.

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List of Abbreviation

BSC – Biological Safety Cabinet

DOTS - Direct Observation Treatment Strategy

DNA - Deoxyribonucleic acid

DST - Drug Susceptibility Testing

EPHI - Ethiopian Public Health Institute

HIV - Human Immunodeficiency Virus

INH - Isoniazid

LPA - Line Probe Assay

LJ - Lowenstein - Jensen

MDR TB - Multi drug resistance Tuberculosis

MGIT - Mycobacterium Growth Indicator Tube

M. *tuberculosis-Mycobacterium tuberculosis*

MTBC **-** M*ycobacterium*. *tuberculosis* complex

MTBDR - *Mycobacterium tuberculosis* drug resistant

MOH - Ministry of Health

NPV - Negative Predictive Value

NTRL - National Tuberculosis Reference Laboratory

PPV - Positive Predictive Value

PMDT- Programmatic Management of Drug resistance Tuberculosis

RMP - Rifampicin

TB **–** Tuberculosis

WHO -World Health Organization

Abstract

Background: - Accurate and rapid detection of multi drug resistance Tuberculois(MDR-TB) is critical. Evaluating Genotype MTBDR*plus* VER 2.0 offer opportunity to scale up drug susceptibility testing (DST) capacity in Ethiopia.

Objective: - The aim of this study was to evaluate the diagnostic performance of Genotype MTBDR*plus* VER 2.0 for the detection of MDR-TB in sputum samples referred to National TB Reference Laboratory (NTRL) at Ethiopian Public Health Institute (EPHI).

Method and Design: - A cross sectional study was conducted from April to August, 2015 on presumptive MDR-TB patients. Analysis of 72 smear positive and 197 smear negative sputum samples was done with Genotype MTBDR*plus* VER 2.0 assay and compared with the reference, MGIT 960 culture and DST. Sensitivity, specificity, PPV and NPV of the assay was calculated, comparing the results with the reference method and results was interpreted based on 95% confidence interval, statistical significant was taken at p-value <0.05.

Result: - The sensitivity, specificity, PPV and NPV of Genotype MTBDRplus VER 2.0 assay were 96.4, 100, 100 and 96.9%, respectively for the detection of MDR-TB from direct smear positive sputum samples. Only 14(54%) samples had valid results with LPA among the 26 smear negative culture positive samples. The sensitivity and specificity of Genotype MTBDRplus VER 2.0 assay was 100% for the detection of MDR-TB among 14 direct smear negative and culture positive sputum samples. The most common mutations associated with RMP and INH resistance was S531L and S315TL, respectively. A single rare mutation (C15T/A16G) was also detected in this study.

Conclusion and Recommendation: - The diagnostic performance of Genotype MTBDR*plus* VER 2.0 assay in direct smear positive sputum sample was highly sensitive and specific for early detection of MDR-TB. However, the diagnostic performance of Genotype MTBDR*plus* VER 2.0 assay in direct smear negative sputum sample was low and showed high level of invalid results so it is unlikely to implement Genotype MTBDRplus VER 2.0 assay for the detection of MDR-TB in direct smear negative sample in our routine settings.

Key words:- performance, Genotype MTBDR*plus* VER 2.0, MDR-TB

1. Introduction

1.1 Background

Tuberculosis (TB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis (M. tuberculosis).* It typically affects the lungs (pulmonary TB) but can affect other sites as well (extrapulmonary TB). The disease is spread in the air when people who are sick with pulmonary TB expel bacteria, for example by coughing. In general, a relatively small proportion of people infected with *M. tuberculosis* will develop TB disease; however, the probability of developing TB is much higher among people infected with Human Immunodeficiency Virus (HIV). TB is also more common among men than women, and affects mostly adults in the economically productive age groups (1).

The first population-based national tuberculosis prevalence survey in Ethiopia which was done in 2010-2011 showed that the prevalence of smear-positive TB and bacteriologically confirmed TB were 108/100 000 (95%CI 73-143), and 277/100 000 (95%CI 208-347) respectively. The finding indicated that the actual TB prevalence in Ethiopia was much lower than the World Health Organization (WHO) estimates (2).

Globally in 2013, data from drug resistance surveys and continuous surveillance among notified TB cases suggest that 3.5% of newly diagnosed TB cases and 20.5% of those previously treated for TB had multidrug resistant TB (MDR-TB). The highest levels of MDR-TB are found in Eastern Europe and central Asia, where in some countries more than 20% of new TB cases and more than 50% of those previously treated for TB have MDR-TB (1).

The first countrywide anti-tuberculosis drug resistance survey in Ethiopia was carried out between 2003 and 2006. The survey reported the levels of MDR-TB in new and in previously treated patients, as 1.6% and 11.8% respectively (3).

The second round national anti-tuberculosis drug resistance surveillance in Ethiopia also done from November 2011 to June 2013. Multidrug resistant isolates were detected in 80 of the total 1651 samples from new and previously treated cases making the overall prevalence of MDR-TB

was 4.8%. The prevalence of MDR-TB among survey participants was 2.3% and 17.8% among new and previously treated cases, respectively. MDR TB prevalence among previously treated smear positive TB cases was almost 10 times higher than new smear positive TB cases (4).

Conventional drug susceptibility testing using solid medium such as Lowenstein - Jensen (LJ) is time consuming whereas liquid medium based methods such as Mycobacterium Growth Indicator Tube (MGIT) System 960 (BD Diagnostics, USA) are sensitive and faster but expensive. New tools such as LPA combined with greater resources for laboratory strengthening, offer opportunity to scale up DST capacity in Ethiopia. MTBDR*plus* VER 2.0 LPA is a rapid Deoxyribonucleic acid (DNA) strip based molecular technology which identify *M. tuberculosis* complex (MTBC) and detect its resistance to Rifampicin and/or Isoniazid from pulmonary clinical specimens or cultivated samples with average turnaround time of 2 days. Recently, WHO recommended the use of molecular line LPA for rapid screening of MDR-TB in low and middle income setting(5).

The Genotype Mycobacterium tuberculosis drug resistant(MTBDR) *plus* VER 2.0 is a qualitative in vitro test for the identification of the M. *tuberculosis* complex and its resistance to rifampicin (RMP) and /or isoniazid(INH) from pulmonary smear positive and negative clinical specimens and cultivated isolates. This molecular genetic assay is based on Deoxyribo Nucleic Acid (DNA) strip technology and it includes DNA extraction, master mix preparation and addition, multiplex amplification with biotinylated primers and detection with reverse hybridization. The identification of RMP resistance enabled by the detection of the most significant associated mutations of the *rpoB* gene. For the detection of INH resistance, the KatG gene and the promoter region of the *inhA* gene are examined (6).

1.2 Statement of the problem

TB remains a major global health problem, responsible for ill health among millions of people each year. TB ranks as the second leading cause of death from an infectious disease worldwide, after the HIV. The latest estimates included in this report are that there were 9.0 million new TB cases in 2013 and 1.5 million TB deaths (1.1 million among HIV-negative people and 0.4 million among HIV-positive people). Though most TB cases and deaths occur among men, the burden of disease among women is also high. In 2013, there were an estimated 3.3 million cases and 510 000 TB deaths among women, as well as an estimated 550 000 cases and 80 000 deaths among children. TB mortality is unacceptably high given that most deaths are preventable if people can access health care for a diagnosis and the correct treatment is provided. Short-course regimens of first-line drugs that can cure around 90% of cases have been available for decades (1, 7).

According to the 2011 Ministry of Health (MoH) report, TB is the eighth leading cause of hospital admissions and the third leading cause of hospital deaths in Ethiopia. In 1992, to prevent and limit the spread of the disease in Ethiopia, the government implemented direct observation treatment strategy (DOTS), the backbone of global TB control, whose objectives are the diagnosis of 70% of new smear positive TB cases and achieving 85% cure. TB is one of the diseases covered by the sixth Millennium Development Goals (2).

Ethiopia is one of among 22 high TB and 27 high MDR TB burden countries. There are a number of Presumptive MDR-TB living in the community undiagnosed. A total of 600 new MDR-TB patients has started the treatment (below the planned 900) in EFY 2005, reaching the cumulative number of 940 MDR-TB patients under treatment. In Ethiopia, currently there are 8 TB culture laboratories which serve the community for MDR-TB diagnosis using conventional and rapid molecular technology however the diagnostic performance of rapid molecular technology such as Line Probe Assay(LPA) for MDR-TB diagnosis demand performance evaluation(1,8)..

To date however, there is no published literature documenting the diagnostic performance of MTBDR*plus* VER 2.0 LPA assay in Ethiopia and also the performance of the test from smear negative sputum sample is not evaluated. Detection and interpretation variability of the assay

which is created among laboratories seen as a challenge for MDR TB diagnosis and treatment. In MTBDR*plus* test strip wild type 8 band seen as a challenge to interpret the result after detection due to strain diversity and detection capacity of the test and this may result in false rifampicin resistant that lead to unnecessary treatment.

One of the advantages of MTBDR*plus* VER 2.0 over VER 1.0 is detection of drug resistance TB from smear negative sputum sample however no evidence based documented literature and protocol in Ethiopia is available. So this study may highlights information on performance of the assay for the diagnosis of drug resistant TB from smear positive and negative sputum samples(6).

1.3 Significance of the study

- This study provide information about the diagnostic performance of Genotype MTBDR*plus* VER 2.0 LPA for the detection of drug resistance TB in smear positive and negative sputum samples in comparison with liquid culture based conventional technique.
- Evaluating new molecular tools such as Genotype MTBDR*plus* VER 2.0 offer opportunity to scale up DST capacity in Ethiopia.
- This study may resolve detection and interpretation variability which creates greater challenge in MDR TB diagnosis and treatment.

2. Literature Review

2.1 Genotype MTBDR*plus* LPA and TB Culture-DST method

A multi-site validation in India of the Line Probe Assay for the rapid diagnosis of MDR-TB and Smear positive sputum specimens from 320 patients subjected to LPA and results compared against those from conventional LJ culture and drug susceptibility testing. Significantly higher proportions of interpretable results were observed with LPA compared to LJ. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of LPA were 96, 99, 99 and 95%, respectively and LPA proved highly accurate in the rapid detection of RMP resistance and potentially enable earlier commencement of the appropriate drug therapy, leading to some reduction of transmission of drug-resistant. The most common mutation associated with RMP was S531 in *rpoB* gene (9).

In the study done in USA Genotype MTBDR plus was compared to gold standard MGIT liquid culture. MTBDRPlus had a sensitivity of 81.0% and a specificity of 100%, with sensitivities of 44.1% in AFB smear-negative versus 94.6% in AFB smear-positive specimens. For specimens that were positive for M. tubeculosis by MTBDR*plus*, the sensitivity and specificity for RMP resistance were 91.7 and 96.6% respectively and for INH they were 70.6 and 99.1% (10).

A study done in Brazil evaluated Genotype MTBDR*plus* assay directly on sputum of patients who had treatment failure or relapse in a routine outpatient setting and compared with conventional DST and gene sequencing. The sensitivity and specificity of the MTBDR*plus* assay were lower than those in other studies, in which the sensitivity of the MTBDR*plus* assay for detecting RMP and INH resistance ranged from 92% to 99% and from 73% to 92%,respectively . They observed that the MTBDR*plus* assay is more sensitive for detecting RMP resistance than INH resistance (82.0% versus 60.0% using the conventional DST) (11).

The commercially available line probe assay MTBDR*plus* 2.0 was evaluated for its ability to detect MTBC and mutations conferring resistance to RMP and INH directly in smear-negative and smear-positive pulmonary clinical specimens under routine laboratory conditions in Moldova, a high-incidence country for tuberculosis (TB). Two hundred fiftyseven (73.9%) smear negative and 81(23.3%) smear positive specimens done with the new method and compared with culture and clinical data as the reference standard. Overall sensitivity and specificity were 87.6

and 99.2%, respectively. One hundred four of the 257 smear-negative samples turnedout to be culture positive, and 20 were MTBC culture negative but were positive based on clinical symptoms. The combined sensitivity and specificity in the subgroup of smear negative samples were calculated to be 79.8 and 99.2%, respectively. MTBDR*plus* 2.0 detected RMP and INH resistance with sensitivity and specificity of 94.3 and 96.0%, respectively (12).

The study done in India evaluated the utility of the line probe assay for the early diagnosis of drug-resistant pulmonary tuberculosis as compared to the 'Gold standard' 1% proportion method. A total of 687 patients suspected of pulmonary tuberculosis were screened. One hundred smear positive samples (95 sputum and 5 bronchial aspirates) were included in the study and the sensitivity for the detection of RMP, INH, and MDR-TB was 98.1, 92.1, and 95%, respectively, with a specificity of 97.8% for RPM and 98.33% for MDR-TB detection. It also had the additional advantage of allowing a study of mutation patterns. High performance characteristics and a short turnaround time makes LPA an excellent diagnostic tool, for an early and accurate diagnosis, in a high MDR-TB prevalent region (13).

A study was validated GenoType MTBDR*plus* assay for detection of MDR-TB in a public health laboratory in Thailand and 50 stored isolates and 164 stored AFB-positive sputum specimens were tested using both the MGIT DST and the GenoType MTBDR*plus* assay. The GenoType MTBDR*plus* assay had a sensitivity of 95.3%, 100%, and 94.4% for INH resistance, RMP resistance, and MDR-TB, respectively (14).

A study in Uganda assessed the performance of a commercial LPA (Genotype MTBDR*plus*) for rapid detection of RMP and INH resistance directly on smear-positive sputum specimens from 118 previously treated TB patients. Results were compared with MGIT 960 liquid culture and DST. Sensitivity, specificity, positive and negative predictive values were 100.0%, 96.1%, 83.3% and 100.0% for detection of RMP resistance; 80.8%, 100.0%, 100.0% and 93.0% for detection of INH resistance; and 92.3%, 96.2%, 80.0% and 98.7% for detection of MDR compared with conventional results. The most common mutation associated with RMP and INH was S531L and S315TL, respectively (15).

A cross sectional study conducted to assess the efficacy of LPA MTBDR*plus* as rapid diagnostic test for early detection of DRTB compared to conventional susceptibility methods in Pakistan. In

comparison to the agar proportion method, the detection rate and specificity of resistance using MTBDR*plus* was 92.5% and 98.2% for RMP, and 76.3% and 100% for INH. The study showed that MTBDR*plus* had a high detection rate for RMP resistance. However, additional probes need to be included in the assay to improve the detection of INH-resistant mycobacterium tuberculosis strains in Pakistan (16).

A study in India, the performance of the Genotype MTBDR*plus* assay was evaluated using MGIT 960 as gold standard. A total of 120 smear positive sputum specimens received from 120 patients during a period of five months were processed by LPA MTBDR *plus* assay and MGIT 960 as gold standard. The sensitivity, specificity, positive and negative predictive values for detection of RMP resistance by Genotype MTBDR*plus* was found to be 97.6, 94.4, 97.6 and 94.4 percent respectively. Overall concordance of RIF and INH result between Genotype MTBDR*plus* and MGIT 960 was found to be 96.6 and 84.7 per cent, respectively (5).

Another study done in Germany evaluated the new GenoType MTBDR*plus* assay (Hain Lifescience GmbH, Nehren, Germany). In total, 106 RMPr/ INHr, 10 RMPs/INHr, and 80 RMPs/INHs *Mycobacterium tuberculosis* complex (MTBC) strains were comparatively analyzed with the new and the old MTBDR assays. In comparison to conventional drug susceptibility testing, both assays were able to identify RMP resistance correctly in 74 of 75 strains (98.7%) and 30 of 31 specimens (96.8%). The misidentification of RMP resistance was obtained for two strains containing *rpoB* P533L mutations. Compared to the old MTBDR assay, the new GenoType MTBDR*plus* assay enhanced the rate of detection of INH resistance from 66 (88.0%) to 69 (92.0%) among the 75 INH-resistant strains and 36 (87.8%) to 37 (90.2%) among the 41 specimens containing INH-resistant strains. Thus, the new GenoType MTBDR*plus* assay represents a reliable and upgraded tool for the detection of INH and RMP resistance in strains or directly from smear-positive specimens (17).

A study carried out a systematic review and used meta-analysis methods for diagnostic accuracy of GenoType MTBDR assays. 14 comparisons for RMP and 15 comparisons for INH were identified in 10 articles that used GenoType MTBDR assays. The pooled sensitivity (98.1%, 95% confidence interval (CI) 95.9–99.1) and specificity (98.7%, 95% CI 97.3–99.4) estimates for RMP resistance were very high and consistent across all subgroups, assay versions and

specimen types. The accuracy for INH was variable, with lower sensitivity (84.3%, 95% CI 76.6–89.8) and more inconsistent than specificity (99.5%, 95% CI 97.5–99.9). GenoType MTBDR assays demonstrate excellent accuracy for RMP resistance, even when used on clinical specimens. While specificity is excellent for INH, sensitivity estimates were modest and variable. Together with data from demonstration projects, the meta-analysis provides evidence for policy making and clinical practice (18).

2.2 Genotype MTBDR*plus* LPA and Xpert MTB/RIF method

Study in South Africa that evaluated the diagnostic performance of GenoType MTBDR*plus* VER 2.0 assay with the Xpert MTB/RIF on smear-positive and smear-negative patient specimens who submitted to a high-throughput diagnostic laboratory. A total of 282 consecutive specimens were subjected to the two new molecular assays, and their performance characteristics were assessed relative to the routine diagnostic standard. Both assays showed similar diagnostic performance characteristics. The sensitivities of the GenoType MTBDR*plus* VER 2.0 and Xpert MTB/RIF assays for the detection of culture-positive *M. tuberculosis* were 73.1% and 71.2%, respectively, while the specificities of both assays were 100%. Both assays were able to diagnose the presence of *M. tuberculosis* in 57 to 58% of smear-negative cases, suggesting that the performance characteristics were dependent on bacillary load. The detection of *M. tuberculosis* in culture-negative specimens confirmed that molecular assays should not be used for treatment monitoring. The sensitivity and specificity for RMP resistance detection were 100% in both assays; however, the GenoType MTBDR*plus* VER 2.0 assay provided additional information on INH susceptibility (19).

3. Hypothesis

The diagnostic performance of Genotype MTBDR*plus* VER 2.0 is high (>95%) and moderate (>80%) for the detection of drug resistant TB in smear positive and negative sputum samples respectively.

4. Objectives

4.1 General Objective

- To evaluate the diagnostic performance of Genotype MTBDR*plus* VER 2.0 Line Probe Assay for the detection of MDR-TB by comparing with conventional liquid culture based DST in smear positive and negative sputum samples of presumptive MDR-TB patients.

4.2 Specific Objectives

- To determine the sensitivity, specificity, PPV and NPV of Genotype MTBDR*plus* VER 2.0 Line Probe Assay for the detection of MDR-TB in direct smear positive sputum sample.
- To determine the sensitivity, specificity, PPV and NPV of Genotype MTBDR*plus* VER 2.0 Line Probe Assay for detection of *M. tuberculosis* and MDR-TB from direct smear negative sputum sample.
- To determine the frequency of mutations associated with RMP and INH drug resistant TB.

5. Materials and Methods

5.1 Study design

A cross sectional study was conducted on presumptive MDR-TB patients referred to the NTRL, EPHI. The Genotype MTBDR*plus* VER 2.0 assay was compared with the reference standard method, conventional liquid culture based DST.

5.2 Study area and study period

The study was conducted at NTRL, EPHI from April to August 2015.

5.3 Study population

All patients who referred to the NTRL and submitted their sputum sample for laboratory diagnosis of drug resistance TB in the study period were the source of population. Those presumptive MDR-TB and smear positive and culture positive isolates were the study population of the first objective. Presumptive MDR-TB and smear negative were the study population for the second objective.

5.4 Sample Size Calculation and Sampling Technique

Presumptive MDR-TB sputum samples were collected based on non probability convenience sampling technique until it reach the total sample size (20).

$$\mathbf{N= (z\alpha/2)^2 p\ (1-P)/d^2}$$

N = Number of individuals to be participated in the research,

z = standard normal distribution curve value for 95% confidence level, zα/2=1.96

d=margin of error taken as 5%

P= sensitivity of the assay, 97 %(0.97)

N= 194, Sample size

Contingency, for culture (10%) and contamination (10%) = 38 making a total sample size of 232

A total of 274 samples collected for the study to increase the sample size for smear negative culture positive category.

5.5 Inclusion and Exclusion criteria

5.5.1 Inclusion criteria

Presumptive MDR-TB who were >15 years of age with consent were included in the study. Smear positive presumptive MDR-TB were included for the first objective of the study and smear negative presumptive MDR-TB were included for second objective of the study.

5.5.2 Exclusion criteria

TB patients who were taking 1st line anti-TB drugs, extraplumunary TB cases and study participants without consent were excluded from the study.

5.6 Specimen collection, storage and transportation

Prior to collection of specimen, eligible study participants were signed on consent form and basic demographic data and clinical information concerning the previous history of TB checked and obtained from the request form. Based on the criteria for Presumptive MDR-TB and Programmatic Management of Drug resistant Tuberculosis (PMDT) (21) who satisfied the study inclusion criteria were asked to provide one morning 5ml sputum sample in a sterile screw cap in which universal disposable container provided for the routine diagnosis. Specimens that did not fulfill the standard quality, volume, time of delivery and storage were rejected based on the rejection criteria annexed in this document.

The sputum samples were kept in a refrigerator at a maximum temperature of +4^{0}c until the specimens were processed for culture within two days of delivery.

5.7 Laboratory Investigation

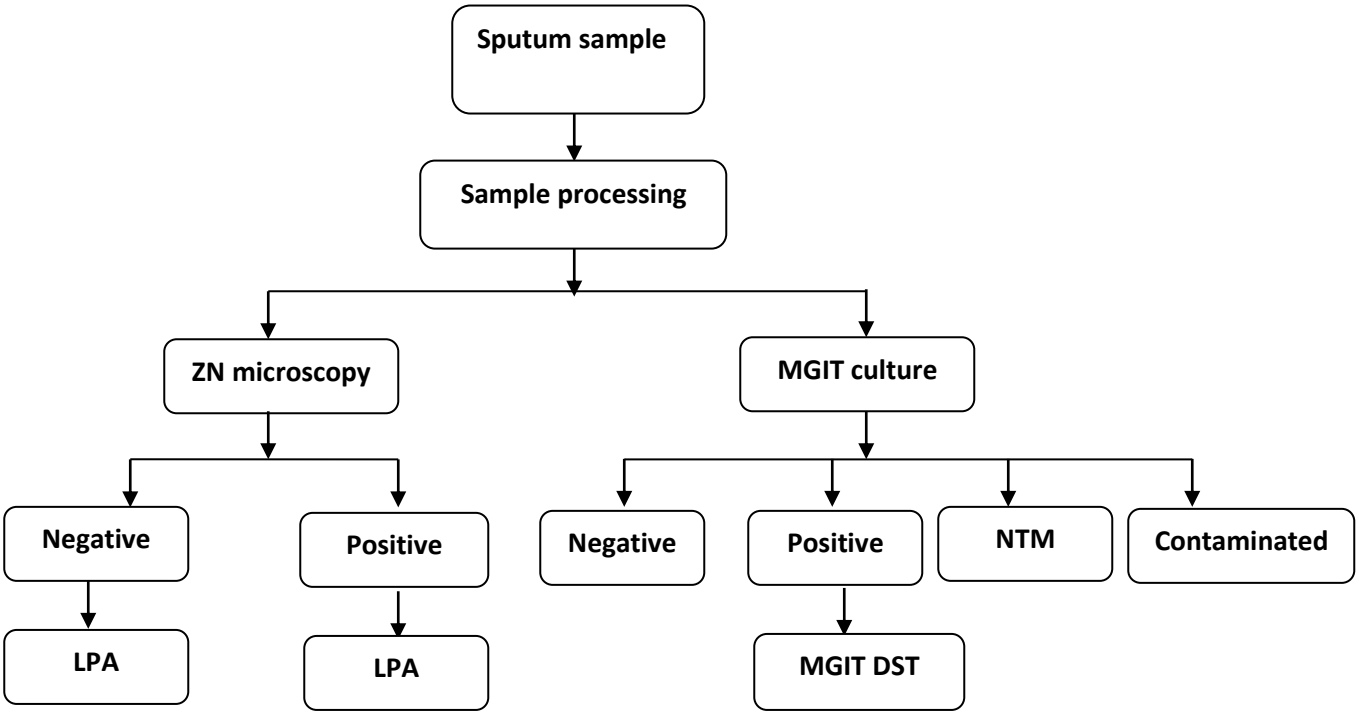


Figure 5.7 flow chart for laboratory investigation of LPA and MGIT culture and DST

5.7.1 Sample processing and inoculation to MGIT

Prior to culture and LPA, sputum sample processed for digestion, decontamination and concentration. Equal volume of sputum and reagents (NaoH-Nacitrate with NALC) added to 50ml falcon tube up to 10ml inside biological safety cabinet (BSC)and mixed with vortex for 20 seconds incubated for 15 minutes and at the min time mixed by inverting 2-3 times. After addition of 35ml of Phosphate buffer solution the mixture centrifuged for 15minutes at 3000g in 4^0c safety centrifuge. Smear preparation was done from the sediment with sterile loop for AFB microscopy. Then the sediment was neutralized and resuspended in 1ml phosphate buffer solution and inoculated to Liquid media (Middle brook 7H9 broth base) in which PANTA and Supplement were added for the growth of the bacilli. The inoculated liquid media loaded to MGIT 96o machine for incubation and growth of the culture. The machine can detect fluorescence in a liquid culture medium, enriched with oxygen, to indicate the presence of bacteria. As bacteria grow in the culture, the oxygen is utilized, causing it to be fluorescent when

placed under UV light. For quality control process start and end control sterile distilled water were processed as a sample and results expected to be negative (22, 23).

5.7.2 Smear Microscopy (concentrated)

Smears which were prepared from the sediment were stained with Zeihl Nelson and stained smears were read with light microscopy. Smear positive sediments were directly transferred to LPA. Smear negative sediment sample also transferred to LPA and the result would be interpretable when the culture growth was confirmed with MGIT or Xpert MTB/RIF if the culture was contaminated. (22).

5.7.3 Identification

To differentiate whether the growth is due to contamination with other microorganisms or true *M. tuberculosis* complex growth, inoculation to blood agar and ZN staining technique were performed because MGIT shows only microbial growth. If there is growth in the blood culture and no AFB is seen in the sample taken from MGIT culture, the result in MGIT is due to contamination. But, if there is no growth in blood culture and AFB is seen in ZN staining technique the result in MGIT shows true infection. Finally, it is necessary to confirm the infection whether it is by *M. tuberculosis* complex or other non tuberculosis mycobacterium species. To do this rapid TB anigen(SD Bioline) was done. SD Bioline rapid TB antigen test is an immunochromatography strip speciation test that can detect a TB-specific antigen (MPT64) from positive liquid or solid cultures to confirm the presence of organisms belonging to M. tuberculosis complex once growth is observed in MGIT It provides results within 15 minutes and is highly sensitive and specific (98.6% and 97.9%, respectively). Known positive and negative control strain were tested for Rapid TB antigen per new batch (23, 24).

5.7.4 Line Probe Assay

The Genotype line probe tests are molecular assays based on DNA strip technology and allow for detection TB and resistance to RMP and INH. The MTBDR*plus* detects resistances to two most important anti-tuberculosis drugs RMP and INH either direct from smear positive specimens processed and concentrated for culture or indirect from culture isolates. DNA was extracted from the appropriate sample using chemical A and B inside BSC. After final centrifugation the supernatant was taken as DNA extract. Master mix was prepared in clean room to prevent contamination of molecular laboratory. In addition room 5 µl of DNA extracts

was added to the corresponding PCR tubes, 5 µl of DNA extract from H37Rv quality control strain to the positive control tubes and 5 µl of distilled water the negative control tube. After addition, the mixture with polymerase chain reaction (PCR) tube placed in to PCR machine for amplification. After completion of PCR process, the amplicon was detected with series of procedures by adding different reagents to the strip. The strips were formed color bands after addition of the final substrate reagent. Finally the result was interpreted based on deletion of wild type and addition of mutant band following the protocol (6).

5.7.5 MGIT DST

Drug susceptibility testing can be performed based on the same principle with MGIT culture. Two MGIT tubes were inoculated with the test culture. A known concentration of a test drug was added to one of the MGIT tubes, and growth was compared with the MGIT tube without the drug (growth control). If the test drug was active against the isolated mycobacterium, it would inhibit the growth and thus there would be suppression of fluorescence, while the growth control would grow uninhibited and would have increasing fluorescence. Growth was monitored by the BACTEC 960 instrument which automatically interprets results as susceptible or resistant. One H37RV sensitivity strain was run per batch of DST set for quality control purpose (23).

5.8 Quality Control

All the laboratory procedures have been tested for specific internal quality control described above and performed by trained TB Laboratory expert. The discrepant results were resolved by repeating the tests and using Xpert MTB/RIF assay.

In addition to these, samples for proficiency testing requested from internationally known TB culture laboratory and tested in the laboratory that scored 100% for DST (LPA and MGIT DST).

5.9 Study Variables

The dependent variable is the performance (sensitivity, specificity, PPV and NPV) and the independent variables are sex and age and previous treatment history of TB.

5.10 Ethical Clearance

The study was approved by Department Research Ethical Review Committee, Addis Ababa University. Support letter obtained from the Department. Written consent was gained from the

study participant. Results were reported to the clinician for free and the data would used for this project only.

5.11 Data Processing and Statistical Analysis

Statistical analysis was carried out using SPSS version 20 software packages. Sensitivity specificity, PPV and NPV of the MTBDR*plus* VER 2.0 assay was calculated, comparing the results with the reference method and results was interpreted based on 95% confidence interval, statistical significant was taken at p-value <0.05.

5.12 Operational definition

Presumptive MDR-TB is any person (whether adult or child) who belongs to any of the MDRTB high risk groups, such as: retreatment cases, new TB cases that have contacts of confirmed MDR TB cases or non-converter of Category 1, and people living with HIV with signs and symptoms of TB (21).

MDR-TB is a resistant form of TB caused by an organism that is resistant to at least RMP and INH, the two most potent TB drugs (21).

6. Results

6.1 Sociodemographic Characteristics

A total of 274 subjects were included in this study. Among these 169(61.7%) were male and the average age is 37 years. Subjects with previous TB treatment history 133(48.5%) were new case and 128(46.7%) were retreatment (Table 6.1).

Table 6.1 Sociodemographic characteristics of the study participant at NTRL, from April to August 2015

Age and treatment history	Male(n=169)	Female(n=105)
Age		
Mean	37	37
Range	Min:15, Max:70	Min:15, Max:70
History		
New	81	53
Retreatment	81	47
MDR-contact	1	0
Uncertain	6	5

6.2 performance of LPA from smear positive sample

Seventy-two samples were smear positive and culture positive. Results of LPA were compared with results of MGIT first line DST. The sensitivity, specificity, PPV and NPV of Genotype MTBDR*plus* VER 2.0 LPA were 88.2, 89.5, 88.2 and 89.5%, respectively for the detection of RMP resistance directly from smear positive sputum sample. The sensitivity, specificity, PPV and NPV of Genotype MTBDR*plus* VER 2.0 LPA were 91.7, 97.2, 97.1 and 92.1%, respectively for the detection of INH resistance directly from smear positive sputum sample. The sensitivity, specificity, PPV and NPV of Genotype MTBDR plus VER 2.0 LPA were 96.4, 100, 100 and 96.9%, respectively for the detection of MDR-TB directly from smear positive sputum sample (Table 6.2).

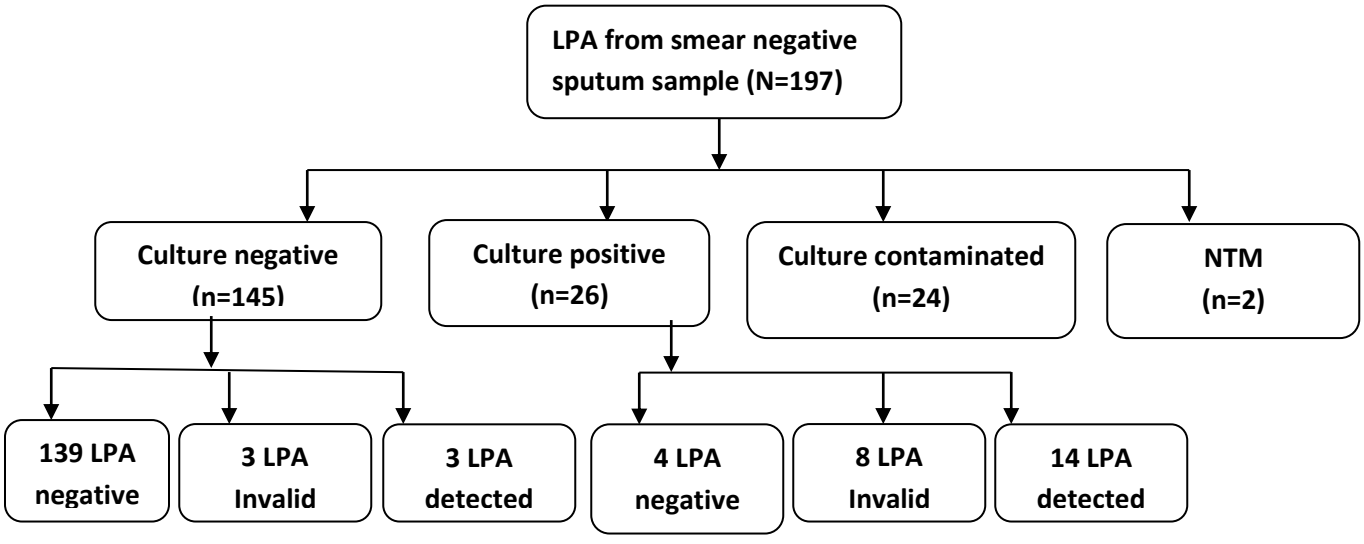
Table 6.2 Performance of Genotype MTBDR*plus* VER 2.0 LPA for detection RMP and INH resistance and MDR-TB in smear positive direct sample, 2015

Detection of drug Resistance	Sensitivity% (95% CI)	Specificity% (95% CI)	PPV% (95% CI)	NPV% (95% CI)	Kappa	p-value
RMP	88.2(72.6-96.7)	89.5(75.2-97.1)	88.2(72.6-96.7)	89.5(75.2-97.1)	0.78	<0.001
INH	91.7(77.5-98.3)	97.2(85.5-99.9)	97.1(84.7-99.9)	92.1(78.6-98.3)	0.89	<0.001
MDR-TB (RMP&INH)	96.4(81.7-99.9)	100(88.8-100)	100(87.2-100)	96.9(83.8-99.9)	0.97	<0.001

*RMP- Rifampicin, INH-Isoniazid, MDR-TB-Multidrug resistant Tuberculosis, CI-Confidence Interval

6.3 Performance of LPA from smear negative direct sputum sample

A total of 197 smear negative sputum samples were processed for direct LPA and from these 145(73.6%) were culture negative and 26(13.2%) were culture positive. Among 145 smear negative and culture negative samples, LPA results were negative in 139(96%), Invalid in 3(2%) and falsely detected in 3(2%) of the sample (Figure 6.3).



*LPA-Line Probe Assay, NTM-Nontubreculos Mycobacterium

Figure 6.3 flow chart for LPA from smear negative direct sputum sample

The sensitivity of Genotype MTBDR*plus* VER 2.0 LPA found to be 77.8% for the detection of *M. tuberculosis* from direct smear negative sputum sample (Table 5.3(1)).

Table 6.3(1) Performance characteristics of Genotype MTBDR *plus* VER 2.0 LPA for detection *M. tuberculosis* in smear negative sample, 2015

Sensitivity% (95% CI)	Specificity% (95% CI)	PPV% (95% CI)	NPV% (95% CI)	Kappa (95% CI)	P-value
77.8(52.4-93.6)	97.9(94-99.6)	82.4(56.6-96.2)	97.2(93-99.2)	0.78	<0.001

*CI-Confidence Interval

Among the 26 smear negative and culture positive sample the LPA had valid results in 14 of the sample for the detection of RMP and INH resistance. Low specificity and PPV was found for the detection of RMP resistance directly from smear negative and culture positive sputum sample. The sensitivity of Genotype MTBDR plus VER 2.0 LPA was low, 60 %(3/5) for the detection of INH resistance directly from smear negative and culture positive sputum sample. The sensitivity, specificity, PPV and NPV of Genotype MTBDR*plus* VER 2.0 LPA were 100(2/2), 100(5/5), 100(2/2) and 100(5/5)% respectively for the detection of MDR-TB directly from smear negative and culture positive sputum sample (Table 6.3(2)).

Table 6.3(2) Performance characteristics of Genotype MTBDR*plus* VER 2.0 LPA for detection RMP and INH resistance and MDR-TB in smear negative and culture positive direct sample, 2015

Detection of drug Resistance	Sensitivity% (95% CI)	Specificity% (95% CI)	PPV% (95% CI)	NPV% (95% CI)	Kappa	P-vale
RMP	100(29.2-100)	63.6(30.8-89.1)	42.9(9.9-81.6)	100(59-100)	0.43	<0.192
INH	60(14.7-94.7)	100(66.4-100)	100(29.2-100)	81.8(48.2-97.7)	0.66	<0.028
MDR-TB (RMP&INH)	100(15.8-100)	100(47.8-100)	100(15.8-100)	100(47.8-100)	1	<0.048

*RMP- Rifampicin, INH-Isoniazid, MDR-TB-Multidrug resistant Tuberculosis, CI-Confidence Interval

6.4 Mutations associated with RMP and INH drug resistant TB

Frequency of mutations associated with RMP and INH drug resistant TB analysed among 35 results of Genotype MTBDR*plus* VER 2.0 and concordant with MGIT DST.

Twenty seven (77.1%) were missing of wild type 8 (530-533) and mutation S531L in *rpoB* gene and this was the most frequent mutation associated with RMP resistance. On the other hand 28 (80%) were missing wild type (315) and mutation S315TL in *KatG* gene and this was the most frequent mutation associated with INH resistance. A single rare mutation (C15T/A16G) in *inhA* gene was detected in this study (Table 6.4).

Table 6.4 Mutations associated with RMP and INH drug resistant TB among concordant Resistant result with Genotype MTBDR*plus VER* 2.0 and MGIT DST NTRL, 2015

RMP resistance		INH resistance				
rpoB gene		*KatG* gene		*inhA* gene		
WT1-8 missing	Mutant	WT missing	Mutant	WT1-2 missing	Mutant	Frequency
530-533(WT8)	S531L	315	S315TL	-	-	23
530-533(WT8)	S531L	315	-	-	-	3
526-529(WT7)	H526Y	315	S315TL	-	-	1
530-533(WT8)	S531L	315	-	-	-	1
526-529(WT7)	H526Y	315	S315TL	-15,-16(WT1) -8(WT2)	C15T A16G	1
*[1]510-513(WT2) 516-519(WT4) 522-526(WT6) 526-529(WT7)	-					1
*[1] 505-509(WT1) 513-517(WT3) 526-529(WT7) 530-533(WT8)	-					1
		*[2]315	S315TL	-	-	3
		*[2]315	-	-15,-16(WT1)	-	1

*[1]- Mutations associated with RMP mono resistance, *[2]- Mutations associated with INH mono resistance

7. Discussion

In this study the performance of Genotype MTBDR*plus* VER 2.0 LPA was evaluated both from direct smear positive and negative sputum sample comparing with liquid based MGIT culture and DST for the detection of RMP resistance, INH resistance and MDR-TB.

The diagnostic performance of Genotype MTBDR*plus* VER 2.0 LPA was high for the detection of MDR-TB (sensitivity-96.4, specificity-100%) in smear positive sputum sample. Similar studies have reported high sensitivity and specificity for the detection of MDR-TB in smear positive sputum sample in Uganda by Albert *et al*(13), India by Maduri *et al*(11), Thailand by Anek *et al* (12) and Moldova by Crudu *et al*(10). Only one sample (1.4%) detected as falsely susceptible for detection of MDR-TB and the same result was also detected from Xpert MTB/RIF assay. This could be explained by the fact that, about 5% and 10% to 25% of resistant strains are thought to have mutations outside *rpoB* and, katG and *inhA* loci respectively (9).

In this study relatively high sensitivity and specificity was observed in smear positive sample for the detection of RMP resistance (sensitivity-88.2 and specificity-89.5%) and INH resistance (sensitivity-91.7 and specificity-97.2%). Higher sensitivity and specificity reported for the detection of both RMP and INH resistance in study done in India by Raizada *et al* (7) and Germany by Hillemann *et al* (16). The reason for higher sensitivity and specificity could be due to large sample size used in the study by Raizada *et al*(7) and gene sequencing used as reference standard to characterize the genotype of resistance mutations in the study by Hillemann *et al*(16). A systematic review and meta-analysis of 14 comparisons reviewed by Ling *et al*(17) were also identified very high and consistent pooled sensitivity and specificity for the detection of RMP and INH resistance in smear positive sample. On other hand lower sensitivity reported in Brazil by Maschmann *et al* (9) and in India by Singhal *et al* (15) for INH. This could be explained by the fact that, it is well known that about 10% to 25% of INH resistant strains are thought to have mutations outside *katG* and *inhA* loci (9).

From the total of 72 smear positive sputum sample four (5.6%) and four (5.6%) samples reported as falsely susceptible and falsely resistant respectively for the detection of RMP resistance in smear positive samples. Four samples that has been falsely susceptible might be due to inexistence of the probes in the *rpoB* gene region of Genotype MTBDR*plus* VER 2.0 LPA to detect the resistance of RMP and the same result was detected from Xpert MTB/RIF assay

suggesting additional probes need to be included in the molecular assay to improve the detection of RMP-resistant *Mycobacterium tuberculosis* strains. Four samples that have been falsely resistant might be due to low bacillary load in the sample that might decreased the target DNA and resulted in absence of *rpoB* wild types and the other possible reason could be silent mutation detected by molecular techniques that do not significantly alter the phenotype of the organism in which they occur.

From the total of 72 smear positive sputum samples three (4.2%) and one(1.4%) samples reported as false susceptible and false resistant, respectively for the detection of INH resistance in smear positive samples. Three samples that have been falsely susceptible might be due to inexistence of the probes in the *katG* and *inhA* gene region of Genotype MTBDR*plus* VER 2.0 LPA to detect the resistance of INH suggesting additional probes need to be included in the katG and/or inhA gene region to improve the detection of INH-resistant *Mycobacterium tuberculosis* strains. one sample that has been falsely resistant might be due to low bacillary load in the sample that might decreased the target DNA and resulted in absence of *katG* wild type and the other possible reason could be silent mutation.

The performance of LPA from smear negative sample also evaluated for detection of *M. tuberculosis* and the resistance of RMP and INH and result showed low detection and similar report observed in USA by Luetkemeyer *et al* (8) that evaluated LPA for the detection of MTB and resistance to RMP and INH found very low sensitivity (44.1%) for AFB smear negative specimen however relatively high sensitivity (79.8%) reported in Brazil by Maschmann *et al* (9). This might be due to appropriate selection of study population that used sputum of patients who had treatment failure or relapse in a routine outpatient setting in the study. In addition, in the study done in South Africa by Barnard *et al* (19) to evaluate LPA with Xpert MTB/RIF from smear positive and negative sample reported that the diagnostic performance of the GenoType MTBDR*plus* VER 2 LPA was equivalent to that of the Xpert MTB/RIF assay. This could be that retreatment cases were selected given their high risk of associated drug resistance and both assays were molecular techniques and share the same principle of testing that detect mutations conferring in *rpoB* gene region for the detection of RMP resistance.

From a total of 197 smear negative sputum samples that have been referred for MDR-TB diagnosis, 139(70.1%) interpreted as true negative, 14(7.1%) samples detected and interpreted

as true positive, this might be due to very low or no bacilli found in the sample that resulted no DNA extracted and no detection identified suggesting that MDR-TB diagnosis with LPA from smear negative sputum sample unlikely to be implemented as routine diagnosis due to inappropriate selection of presumptive drug resistant TB patients. Among 145 smear negative and culture negative sample 139 found to be LPA negative and from 139 LPA negative, 2 samples were MTB detected in Xpert MTB/RIF and this finding contradict with the study done by Barnard et al. From 145 smear negative and culture negative sample LPA detected falsely drug resistant in 3(2 RMP monoresistant and 1 MDR-TB) however no positive result found from the Xpert MTB/RIF and MGIT culture in 3 of the samples. This might be due to other bacteria or contamination mixed with the sputum samples and could be detected in the *rpoB* and *katG* gene region of the probe. High number of LPA negative turnout result was also observed and this could due to over diagnosis MDR-TB that did not follow appropriate selection and screening of presumptive MDR-TB.

Among the 26 smear negative culture positive sample LPA detected valid result in 14(54%), negative in 4(15.4) and invalid in 8(30.6%) samples and this suggests that detection of *M. tuberculosis* and its resistance to RMP and/or INH prone high level of invalid results. High sensitivity (3/3) and low specificity (7/11) found in RMP resistance detection, low sensitivity (3/5) and high specificity (9/9) observed in INH resistance detection and high sensitivity (2/2) and specificity(5/5) found in MDR-TB detection directly from smear negative and culture positive sputum sample. The possible reasons for low sensitivity and specificity could be very low or no bacillary load in the sample for extraction of DNA, the probes to detect from smear negative sample might not be improved compared to VER 1 or other bacteria might easily contaminate the detection and made false detection in the absence of AFB and target DNA in the sample. In this study high sensitivity and specificity observed in smear negative culture positive sample for the detection of MDR-TB however there were few number of valid results (2 for sensitivity and 5 for specificity) for comparison from other studies. In addition, high level of invalid and false negative results observed.

Missing of wild type 8(530-533) and mutation S531L was the most common mutation associated with *rpoB* gene and missing wild type(315) and mutation S315TL was the most common mutation associated with *KatG* gene. A single rare mutation (C15T/A16G) in *inhA* gene

was detected in this study. The most common mutation associated with RMP by Raizada *et al* (7) was the same as this study. This finding was also common with findings in study done in Uganda by Albert *et al* (13).

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8. Strength and Limitation

The Genotype MTBDR*plus* VER 2.0 was compared with liquid culture based MGIT 960 instrument which has high performance and gold standard method for both culture and DST.

Genotype MTBDR VER 1 LPA was not included in the study as the company had stopped producing the product. If the version 1 would have been available this might have helped to compare the two products in our setting.

Gene sequencing was not available for troubleshooting discordant results between LPA and MGIT DST.

The clinical diagnosis was not controlled and there was high number of smear negative and culture negative results.

9. Conclusion

The diagnostic performance of Genotype MTBDR*plus* VER 2.0 LPA in direct smear positive sputum sample was high for the detection of MDR-TB. It is highly sensitive and specific for early detection of MDR-TB.

In this study relatively high sensitivity and specificity observed for the detection of RMP and INH resistance in smear positive sample.

The diagnostic performance of Genotype MTBDR*plus* VER 2.0 LPA in direct smear negative sputum sample was low. Only 14(54%) samples had valid results with LPA among the 26 smear negative culture positive samples. The remaining 8(30.6%) and 4(15.4%) were invalid and negative with LPA, respectively. The sensitivity and specificity of Genotype MTBDR*plus* VER 2.0 LPA was 100% for the detection of MDR-TB among 14 direct smear negative and culture positive sputum samples.

10. Recommendations

- The diagnostic performance of Genotype MTBDR*plus* VER 2.0 LPA in direct smear positive sputum sample found to be high and offer opportunity to scale up DST capacity in Ethiopia however, additional probes for resistance determining region need to be included to identify other unrecognized mutations.
- Additional large scale study that compare molecular and phenotypic methods with controlled clinical diagnosis and that use gene sequencing as reference standard should be done.
- The diagnostic performance of Genotype MTBDR*plus* VER 2.0 in direct smear negative sputum sample found to be low so it is unlikely to implement MTBDR*plus* VER 2.0 for the detection of MDR-TB in direct smear negative sample in our routine setting.
- Presumptive MDR-TB patients should be selected and screened appropriately based on PMDT criteria for laboratory diagnosis of MDR-TB to decrease unnecessary diagnosis and negative turnout result.

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12. Annexes

Annex I- Clinical Form

A. Identification of the patient

Name of Health facility:_____ Facility Code:_____

Patient's Full Name: _____ Patient Reg. No. (Card No.):_____

Unique survey ID number of the patient:_____

(Facility code + Patient Sequential No. for study)

---------- CUT HERE ----------

Name of Health facility:_____ Facility Code:_____

Unique survey ID number of the patient:_____

(Facility code + Patient Sequential No. for Survey)

Date registered (Ethiopian Calendar): _____Patient's Age: _____ Years

(Day/Month/Year)

Patient's Sex: Male ☐ Female☐

B. History given by the patient

Have you previously received treatment for TB? No☐ Yes

What was the outcome of the most recent treatment?

Cured & treatment completed ☐ Defaulted☐ Failed☐ Relapse☐

Chronic☐ Transferred out☐

Relapse not distinguishable/default not distinguishable☐ Unknown☐

According to your evaluation, indicate patient classification:

New☐ Retreatment☐

Responsible Officer Name:_____

Annex II. Laboratory Result form

Culture and DST result form

Name of Health Facility _____ Healthy facility Code: _____

Patient Reg. No_____

Unique ID of the patient: _____

(Health facility code+ Patient Sequential No. for Study)

Date registered _____

Sex: Male _____ Female _____ Age: _____ Years

Concentrated smear result---------- ☐ZN

Identification

_____ ***M. tuberculosis complex***

_____ NTM

_____ Negative

_____ Contaminated

Susceptibility of *M. tuberculosis*

MGIT DST		LPA	
_____	Isoniazid	_____	Isoniazid
_____	Rifampicin	_____	Rifampicin
_____	Ethambutol		
_____	Streptomycin		

Date of reporting _____Reported by----------

Annex III. Consent form

Good Morning/Afternoon, My name is _____, and I am collecting data for a study which assess the performance of MTBDR *plus* VER 2.0 Line Probe Assay. The main purpose of this study is to evaluate the diagnostic performance of MTBDR*plus* VER 2.0 Line Probe Assay. This study may resolve detection and interpretation variability which creates greater challenge in MDR TB diagnosis and treatment. The study also offers opportunity to scale up DST capacity in Ethiopia.

If you agree to take part in the study, we will do additional TB laboratory tests with your leftover sample. You are free to choose to be part of the study.

There will not be any risk for you if you choose to be part of the study. Every person who is part of this study will not receive any incentives. There may not be direct benefit to you as a participant. If you decide to participate in the study, your sputum sample will be sent to the central laboratory for culture and DST testing and your result will be communicated to your physician. This will give your physician better understanding about your illness and help him/her to link you to the right TB treatment. All the information in your test result will be kept strictly confidential. We will keep the records in a safe place and only investigator/s will be allowed to look at them. Your name and other personal identifier/s will not appear if we present this study or publish the results. If you have any questions please contact Abyot Meaza at 0911468388

Do you agree to participate and willing to spare some time to answer only some questions?

Y / N

Participant's statement:

The above study has been explained to me and I agree to take part.

Participant's name: _____

Signature _____Date_____

IV. Consent form (Amharic Version)

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Annex V. Protocol for Laboratory Investigations

1. Protocol for Sample Processing Using NALC-NaOH

Principle	Specimens for tubercle bacilli isolation do usually contain associated flora which has to be eliminated before inoculation of the specimen onto culture media. Culture examination detects fewer bacilli than microscopy and increases the number of tuberculosis cases found, in the range of 20-50%, subject to the local incidence. Culture methods provide definitive diagnosis by establishing the viability and identity of the organisms and enable the detection of drug resistance. The present procedure applies to the use of solid or liquid media for culture purposes.

Step	Action
1.	Transfer the sputum (at least 2 ml, not more than 5 ml) into a centrifuge tube
2.	Add equal volumes of NALC-NaOH solution. Use aliquots of NALC-NAOH(1 vial of NALC-NAOH per one specimen)
3.	Tighten cap of container and vortex slowly
4.	Shake intermittently to aid homogenization and decontamination
5.	Invert each bottle to ensure that NALC-NaOH solution contacts all the sides and inner portion of caps.
6.	Keep at 20°C – 25°C for 15 min for decontamination
7.	Fill the tube with phosphate buffer up to 45 ml mark on the tube. . Use aliquots of phosphate buffer(1 vial of PBS per one specimen)
8.	Mix-well or vortex
9.	Centrifuge at 3,000 ×g for 15 minutes
10.	Carefully pour off the supernatant into a discard container containing 5% sodium hypochlorite. Make sure the final concentration of bleach is 1% after pouring off the supernatant.
11.	Prepare smear on slide labelled with culture number for microscope

12.	Re suspend the deposit with 2ml PBS.

2. Protocol for BACTEC MGIT **Culture 960 System**

Principle

The MGIT (Mycobacteria Growth Indicator Tube) consists of liquid broth medium that is known to yield better recovery and faster growth of mycobacteria. The MGIT contains 7.0 ml of modified Middlebrook 7H9 broth base. This medium is terminally sterilized by autoclaving. An enrichment, MGIT OADC (Oleic acid, Albumin, Dextrose and Catalase) or MGIT 960 Growth Supplement, is added to make the medium complete. This Growth Supplement is essential for growth of many mycobacteria, especially those belonging to *M. tuberculosis* complex. Addition of the MGIT PANTA is necessary to suppress contamination. In addition to Middlebrook 7H9 liquid media, the MGIT tube contains an oxygen-quenched fluorochrome, tris 4, 7-diphenyl-1, 10-phenonthroline ruthenium chloride pentahydrate, embedded in silicone at the bottom of the tube. During bacterial growth within the tube, the free oxygen is utilized and is replaced with carbon dioxide. With depletion of free oxygen, the fluorochrome is no longer inhibited, resulting in fluorescence within the MGIT tube when visualized under UV light. The intensity of fluorescence is directly proportional to the extent of oxygen depletion.

Procedure for inoculation

1.	Reconstitute MGIT PANTA with 15.0 ml MGIT growth supplement
2.	Mix until completely dissolved. Add 0.8 ml of this enrichment to each MGIT tube
3.	Label MGIT tubes with specimen number.
4.	Unscrew the cap and aseptically add 0.8 ml of MGIT growth supplement/PANTA to each MGIT tube
5.	Using a sterile pipette or a transfer pipette, add up to 0.5 ml of a well mixed processed/concentrated specimen to the appropriately labeled MGIT tube
6.	Immediately recap the tube tightly and mix by inverting the tube several times

7.	Load the MGIT tube in to MGIT 960 machine scanning with the barcode reader

2. Protocol for MGIT Culture positive Work up

2.1Blood agar inoculation and reading

Step	Action
1.	Take the positive tube from the incubator
2.	Inoculation in BA plate
3.	Incubate blood agar at 37ºC for 48 hours, checking for growth of contaminants at 18-24 and 48 hours.
4.	Record growth / no growth on BA plate in culture reading worksheet

2.2 Smear of positive MGIT cultures

Step	Action
1.	Mix the broth
2.	Using a sterile pipette remove a small aliquot
3.	Place 1 drops of fixative and 1 drop of broth on the slide and spread it on a small area (2 x 1 cm).
4.	Let the smear air-dry.
5.	Heat-fix the smear by passing it over a flame a 2-5 times
6.	Stain the smear with Ziehl-Neelsen method
7.	Place a drop of oil on the stained and completely dried smear

8.	Screen under a low power objective to locate stained bacteria
9.	Switch to oil immersion objective lens for detail observation
10.	Record results in culture reading worksheet
11.	If the smear is negative for AFB and the broth is clear and no growth on BA, re-incubate the tube for further monitoring
12.	After 3 days, visually inspect MGIT tube for growth and repeat AFB smear as above
13.	If AFB smear remains negative, contamination is not found on the blood agar plate, and the LJ slants are not positive by 8 weeks, the culture is considered to be negative.

3. Protocol for Identification of MTBC using SD Biline Rapid TB Antigen

Principle

The SD Bioline TB Ag MPT64 Rapid test is an immunochromatographic method which can detect MPT64 antigens produced by *M. tuberculosis* complex from AFB positive MGIT or Lowenstein Jensen's media. The test cassette consists of a sample pad, a gold conjugate pad, a nitrocellulose membrane and an absorbent pad. Mouse monoclonal anti-MPT64 antibodies are immobilized on the nitrocellulose membrane as the capture material (Test Line). Another antibodies conjugated with colloidal gold particles are used for antigen capture and detection in a sandwich type assay. The test device has a letter of T and C as "Test Line" and "Control Line" on the surface of the case. Both the "Test Line" and "Control Line" in result window are not visible before applying any sample. The "Control Line" is used for procedural control. Control line should always appear if the test procedure is performed properly and the test reagents of the control line are working. As the test sample applied in the sample well flow laterally through the membrane, the antibody-colloidal gold conjugate binds to the MPT64 antigen in the sample. This complex then moves across a chromatographic carrier, and is then captured by a second fixed antibody located in the middle of the test cartridge. This reaction produces a red to purple colour band which indicates a positive

result for *M. tuberculosis* complex. In the absence of MPT64, there is no line in the test band region.

Test procedure

Step	Action
1	Vortex the tightly capped broth culture for 20 seconds to ensure that it is well mixed
2	Remove the test cartridge from the foil pouch and place it on the BSC work area
3	Avoid touching the specimen placing area on the cartridge with hands
4	Label each cartridge with the sample identification number
,5	Place 100µL of the prepared bacterial culture on the specimen placing area of the test cartridge. Pipette tips should be changed between samples
6	Examine the reading area of the test plate after 15 minutes
7	A colour band will appear at the left section of the result window to show that the test is working properly. This band is the Control Line
8	The right section of the result window indicates the test results. If another colour band appears at the right section of the result window, this band is the Test Line
9	The formation of a purple to red line on the reading areas labeled [T] and [C] of the cartridge indicates a POSITIVE result
10	The formation of a purple to red line on the reading area labeled [C] of the cartridge but not [T] indicates a NEGATIVE result
11	If no line is observed on the reading area [C], technical errors or product damage has occurred. In this case, the test should be considered invalid and repeated using a new cartridge

4. Protocol for MGIT Drug Susceptibility Test

Principle

Two MGIT tubes are inoculated with the test culture. A known concentration of a test drug is added to one of the MGIT tubes, and growth is compared with the MGIT tube without the drug (growth control). If the test drug is active against the isolated mycobacteria, it will inhibit the growth and thus there will be suppression of fluorescence, while the growth control will grow uninhibited and will have increasing fluorescence. Growth is monitored by the BACTEC 960 instrument which automatically interprets results as susceptible or resistant. Drug susceptibility testing using BACTEC MGIT 960 (MGIT 960) is the fully automatic system for detection of mycobacterial growth and drug susceptibility testing of *M. tuberculosis.*

4.1 Preparation of inoculums from MGIT Tube

Step	Action
1.	Label each MGIT tube with relevant drug and laboratory number and date
2.	Add 0.8 ml MGIT SIRE Supplement to each SIRE tube and the SIRE growth control tube.
3.	Add 0.1 ml (100 µl) of the appropriate reconstituted drug solutions into each of the corresponding labeled BACTEC MGIT 960 tubes.
4.	The day a MGIT tube is positive by the instrument is considered **Day 0**
5.	The tube should be kept incubated for at least one more day (**Day 1**)
6.	A positive tube may be used up to and including the fifth day (**Day 5**) after it becomes instrument positive.
7.	Label each MGIT tube with relevant drug and laboratory number and date

4.1.1 For Day 1 or Day 2 growth

Step	Action
1.	If growth in a tube is of Day 1 or Day 2, mix well to break up clumps (vortex).
2.	Leave the tube undisturbed for about 15-20 minutes to allow large clumps to settle.
3.	Use the supernatant undiluted for inoculation of the drug set.

4.1.2 For Day 3, 4, or 5 growth

Step	Action
1.	If growth is on Day 3, 4, or 5, mix well to break up the clumps.
2.	Let the large clumps settle for 15-20 minutes.
3.	Dilute 1.0 ml of the positive broth in 4.0 ml of sterile saline (1:5 dilutions).
4.	Use this well–mixed, diluted culture for inoculation of the drug set.

4.2 Inoculation of Positive MGIT Tube Specimen

4.2.1 SIRE Growth Control Tube

Step	Action
1.	Using sterile pipettes dilute 0.1 ml of MGIT inoculum prepared in 10 ml sterile saline and mix well.
2.	Add 0.8 ml MGIT SIRE Supplement to each SIRE tube
3.	Inoculate 0.5 ml of this suspension into the growth control-labeled tube
4.	Immediately recap the tube tightly and mix by inverting the tube several times.

4.2.2 Drug-Containing Tubes

Step	Action
1	Inoculate each labeled, drug-containing tube with 0.5 ml of the MGIT specimen prepared step 3 in 4.1.1 or step 4 in 4.1.2.
2	Immediately recap the tube tightly and mix by inverting the tube several times.
3	Wipe all tubes and caps with a mycobactericidal disinfectant.

4.3 Incubation

Step	Action
1.	Enter the inoculated set of DST specimens into the BACTEC 960 instrument using the AST set entry feature.
2.	Be sure that the tubes are loaded according to the order specified for the AST set entry feature
3.	Be sure that the caps are tightly closed
4.	Open the desired MGIT 960 drawer and press the “tube enter” key.
5.	The barcode scanner will light up.
6.	Scan the inoculated MGIT tube and load into the slot identified by the MGIT 960.
7.	Incubate MGIT tubes until the instrument flags them as positive
8.	Check MGIT 960 daily for indicator lights flagging positive
9.	Positive tubes will be displayed by the indicator light changing to green at the exact location of the tube in the instrument drawer.

5. Protocol for MTBDRplus VER 2.0 Line probe Assay

5.1 DNA extraction using Genolyse chemical method

Principle	DNA extraction is a procedure whereby DNA is fetched from bacterial cells or fragments of bacterial cells to be used for molecular biology analysis. With the Genolyse chemical method test, this implies that: the bacterial cells in the decontaminated patient sample or culture samples are chemically broken to expose the DNA by using a lyses buffer.

5.1.1 DNA extraction from decontaminated sediments

Step	Action
1	Log specimen numbers onto a DNA extraction worksheet and record date and initials
2	Work on no more than 10 sediments and 2 controls at a time
3	Assemble sediments for the batch in the BSC
4	Vortex thoroughly for 15-20 seconds each of the 50ml conical tubes containing the sediments
5	Using a sterile disposable Pasteur pipette, transfer 500µl of each decontaminated sample to labelled 1.5ml screw cap tube
6	Load the 1.5ml screw cap tubes in a microcentrifuge with aerosol-tight rotor.
7	Centrifuge for 15 minutes at 10,000 x g
8	Unload tubes from the microcentrifuge and carefully carry the tubes to the BSC
9	Discard supernatant from each tube by use of a 1000µl adjustable pipette
10	Resuspend each pellet in 100µl Lysis Buffer (A-LYS)
11	Mix the contents of each tube by use of a sterile tip followed by thorough vortexing for at least 15 to 20 seconds

12	Incubate the tubes for 5 minutes at 95 °C in a thermoblock
13	Add 100 µl Neutralisation Buffer (A-NB) and vortex the sample for 5 seconds
14	Load the tubes into a microcentrifuge and spin for 5 minutes at 13,000 x g
15	Carefully carry the tubes to the BSC. Uncap tubes one at a time, and transfer 100µl of DNA-containing supernatant to a sterile 1.5ml screw cap tube
16	Store DNA at 2-8 °C for not more than 7 days. For longer storage, keep at -20 °C

5.1.2 DNA extraction from liquid culture isolates

Step	Action
1	Using a sterile disposable Pasteur pipette, transfer 1000µl of each thoroughly mixed liquid culture sample to labelled 1.5ml screw cap tube
2	Proceed similarly to the procedure for **decontaminated sediments**, from step 6 to 16

5.2 Mastermix reagent preparation

Principle

All reagents needed for amplification are included in the Amplification Mixes A and B and are optimized for the PCR step of MTBDR*plus* test. The AM-A contains Taq polymerase, PCR buffer and nucleotides. The nucleotides acts as DNA precursors (the four deoxynucleoside triphosphates, dATP, dCTP, dGTP and dTTP) which will be used as building blocks during the elongation of the single stranded DNA. DNA polymerase (Hot Start *Taq*) is required to elongate the DNA molecule by facilitating the incorporation of the free nucleotides onto the end of the primer, according to the complementary base on the single stranded target DNA. The AM-B

contains biotinylated primers for the amplification of specific regions of the mycobacterial chromosome. The Mg^{2+} in the salts forms soluble complexes with the free nucleotides allowing for the DNA polymerase to recognise them as substrates during the amplification procedure.

Procedure

Step	Action
1	Thoroughly apply freshly prepared 0.5% bleach solution to all surfaces of PCR hood and worktop. Allow 15 minutes contact time
2	Wipe with damp cotton wool
3	Apply 70% ethanol to all the surfaces PCR hood and worktop
4	Thaw the two amplification mixes, A and B (AM-A and AM-B) at room temperature.
5	Gently invert to mix the contents of each tube, AM-A and AM-B
6	Carefully remove sufficient PCR tubes from stock pack, place in a PCR tube rack in the PCR hood. Ensure that the lids are closed.
7	Determine the number of samples to be amplified plus control samples.
8	Label PCR tubes
9	Prepare a master mix containing AM-A and AM-B in a sterile screw cap tube (1.5ml) according to the number of samples and controls
10	Mix the master mix gently by light vortexing for 10-15 seconds
11	Briefly spin down the master mix in a mini spin centrifuge (5 seconds)
12	Pipette 45 µl of the master mix to each labelled PCR tube

13	Ensure all PCR tubes are tightly closed. Close the PCR tube rack
14	Place the stock reagents AM-A and AM-B back in the freezer at -20 °C
15	Wipe pipettes, PCR hood surfaces and worktop with 0.5% bleach followed by 70% ethanol

5.3 DNA addition, amplification and detection

Principle

All reagents needed for amplification are included in the master mix and are optimized for the PCR step of MTBDR*plus* test. The AM-A contains Taq polymerase, PCR buffer and nucleotides. The nucleotides acts as DNA precursors (the four deoxynucleoside triphosphates, dATP, dCTP, dGTP and dTTP) which will be used as building blocks during the elongation of the single stranded DNA. DNA polymerase (Hot Start *Taq*) is required to elongate the DNA molecule by facilitating the incorporation of the free nucleotides onto the end of the primer, according to the complementary base on the single stranded target DNA. The AM-B contains biotinylated primers for the amplification of specific regions of the mycobacterial chromosome. The Mg^{2+} in the salts forms soluble complexes with the free nucleotides allowing for the DNA polymerase to recognise them as substrates during the amplification procedure.

The membrane strips used in the hybridization or detection step are pre-coated with specific probes complementary to the amplified nucleic acids. After chemical denaturing, the single amplicons bind to the probes. Highly specific binding of complementary DNA strands is ensured by stringent conditions which result from the combination of buffer composition and a certain temperature. Thus, the probes reliably discriminates several sequence variations in the gene regions examined. The streptavidin-conjugated alkaline phosphatase binds to the amplicons' biotin via the streptavidin moiety. Finally, the alkaline phosphatise transforms an added substrate into a dye which becomes visible on the membrane strips as a coloured precipitate.

5.3.1 DNA Addition

Step	Action
1	Thoroughly apply freshly prepared 0.5% bleach solution to all surfaces of PCR hood or worktop. Allow 15 minutes contact time
2	Wipe with damp cotton wool
3	Apply 70% ethanol to all the surfaces PCR hood
4	Obtain DNA samples from the refrigerator or freezer and allow to reach room temperature
5	Assemble PCR tubes containing 45µl master-mix reagent
6	Prepare a DNA addition worksheet
7	Add 5µl of DNA to corresponding PCR tubes in the PCR hood. NB- No DNA should be added to the reagent control
8	Check that all PCR tubes are tightly closed
9	Wipe pipette, PCR hood surfaces with 0.5% bleach followed by 70% ethanol

5.3.2 Amplification

Step	Action
1	Thoroughly apply freshly prepared 0.5% bleach solution to work top surfaces.
2	Wipe with damp cotton wool
3	Apply 70% ethanol to all work top surfaces
4	For first use, set up the thermal cycler to the correct amplification profiles according to the instruction manual (see SOP on Use and Maintenance of

	Thermal Cycler).
5	If PCR tubes have bubbles at base, remove by swinging arm with tubes in hand in arc.
6	Transfer PCR tubes to middle section of thermal cycler. Avoid placing tubes at the edges as this can result in evaporation of the contents during heating
7	Check program parameters and follow menu options to start appropriate program
8	Run the specific program • **Ver.2-cul** for samples from solid or liquid culture, • **Ver.2-dirsample** for decontaminated smear positive sediments
9	After the cycles are complete, proceed to the detection stage. If detection cannot be performed on same day, store PCR tubes with amplicons at 4°C for a maximum of 7 days

5.3.3 Detection

Step	Action
1	Pre-warm HYB and STR solutions (green and red) to 45°C in water bath (15 minutes total). Pre-warm RIN (rinse solution) and sterile distilled water to room temperature
2	Pre-warm TwinCubator to 45°C
3	Remove DNA strips from tube (shake strips down to end of tube then remove carefully holding the end of the strip with forceps) and mark them with provided pen according to detection worksheet.
4	Pipette 20µl DEN (denaturing solution) to one end of each well of a clean tray

	to be used
5	Add 20µl of corresponding amplified DNA sample to the denaturing solution in each well, and mix well by pipetting up and down 5 times
6	Incubate for 5 minutes at room temperature
7	Carefully add 1 ml HYB (hybridization solution) to each well and in the opposite end to the DEN/DNA mixture. Use single tip for each well. Do not splash mixture or contaminate neighbouring well
8	Gently tilt to shake and homogenize solution. Do not splash mixtures
9	Add each labelled strip to each well with coloured marker facing up. If strips turn over, re-position them with a fresh pipette tip. Strips must be completely covered by hybridization solution
10	Place tray on TwinCubator and press "START" to incubate for 30 minutes at 45°C. From this point, press right arrow on TwinCubator once to advance steps in protocol.
11	When alarm goes off, press right arrow key to stop
12	Remove HYB carefully by pipetting with individual sterile pipette tip or sterile disposable Pasteur pipette into a small plastic discard container containing undiluted bleach solution. Change tips or Pasteur pipettes between wells
13	Wipe off condensation that forms on TwinCubator lid before every incubation step.
14	Add 1 ml STR (stringent buffer) per well and incubate for 15 minutes in TwinCubator at 45°C. Press right arrow key to start.
15	When alarm goes off, press right arrow key. Completely remove STR as previously described for HYB removal.

16	Add 1 ml rinse solution (RIN) to each well. Press right arrow key to rinse the strips for 1 minute. When alarm goes off, press right arrow key. Completely remove RIN.
17	Add 1 ml of diluted Conjugate per well. Press right arrow to incubate at 37°C for 30 minutes on TwinCubator
18	When alarm goes off, press right arrow to stop
19	Completely aspirate CON-D solution using Pasteur pipette
20	Add 1ml RIN per well. Press right arrow and incubate for 1 minute on TwinCubator
21	When alarm goes off, press right arrow key to stop. Remove RIN completely and repeat Step 20
22	Remove RIN completely and wash with 1 ml sterile distilled water per well on TwinCubator. Press right arrow key and incubate for 1 minute on TwinCubator
23	When alarm goes off, press right arrow key to stop. Remove water and add 1 ml of diluted substrate per well.
24	Place on TwinCubator under aluminum foil for a maximum of 10 minutes. Look for colour reaction to indicate reaction completion after 4-5 minutes. If colour reaction is too weak, replace the foil and re-incubate for several more minutes, up to a maximum of 10 minutes
25	Wash twice for 1 minute with distilled water. Remove distilled water after each wash
26	Use forceps to transfer membrane strips to an absorbent paper and allow to air dry
27	Soak trays in 0.5% bleach for 15 minutes and rinse with distilled water

28	Clean pipettes, instruments and work area with freshly diluted 0.5% bleach, followed by 70% alcohol
29	Switch off the TwinCubator after use

5.4 Interpretation of LPA strip results

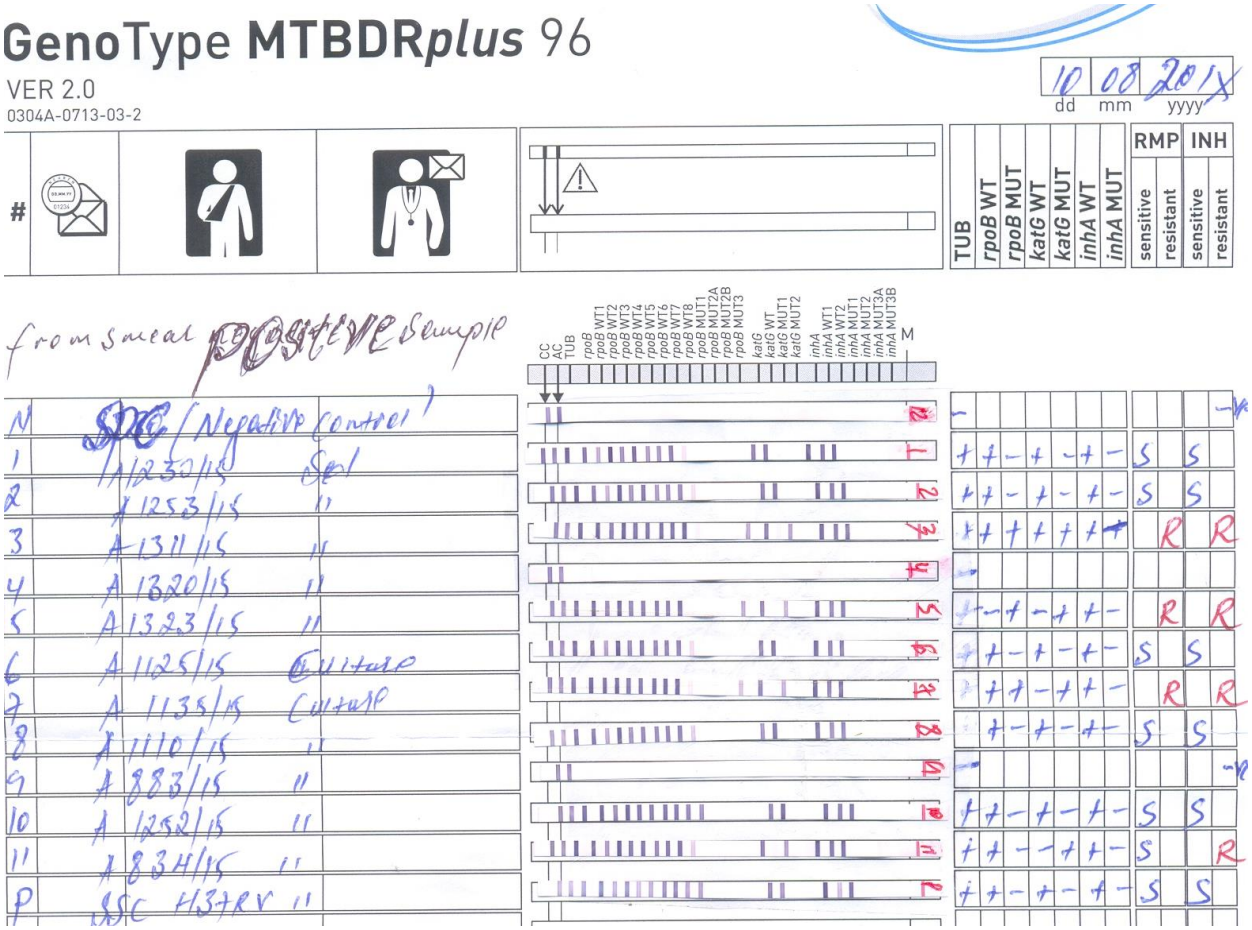
Step	Action
1	Use forceps to transfer strips to the GenoType MTBDR*plus* Results Sheet provided with the kit
2	Align the bands Conjugate Control (CC) and Amplification Control (AC) on each strip with the respective lines on the sheet
3	Attach the strips to the results sheet using clear adhesive tape
4	Determine the band positivity and positions on each strip using the reference reading chart of the kit and mark the results on the worksheet
5	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.
6	If a positive result is obtained with the negative control, the whole batch must be repeated and measures taken to remove amplicon contamination from all rooms and equipment.
7	In order for patient 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
8	If CC is negative the conjugation or substrate reaction was unsuccessful either due to error in the procedure or due to problems with the reagent
9	If AC is positive, errors during extraction and amplification set-up and presence of

	amplification inhibitors in the specimen can be excluded
10	Signal of AC can be weak or even absent while results for other bands (TUB, rpoB, katG and inhA locus controls) may be positive. This might be due to competitive reactions between AC and TUB, ropB, katG, inhA during amplification. In this case, the strip can be evaluated.
11	A weak or missing AC band with negative test result for TUB, rpoB, katG and inhA locus controls may indicate potential mistakes during extraction and amplification set-up, or presence of amplification inhibitors. In this case, the test results are invalid
12	rpoB predicts RIF resistance, katG predicts high level INH resistance, inhA predicts low level INH resistance
13	The rpoB, katG and inhA each have a control band which must be present in order to interpret the results. Locus Control zones (rpoB, katG, inhA) detect a gene region specific for their respective genes. If the locus control zones are negative, then their respective mutation- specific positive bands cannot be considered for evaluation
14	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.
15	For results to be valid the bands (except CC) must be of intensity approximately equal to or greater than the intensity of the AC band.
16	If the TUB zone is negative, the tested bacteria does not belong to M. tuberculosis complex; therefore, presence or absence of any other bands (except CC and AC) cannot be considered for evaluation
17	When all wild type probes of a gene stain are positive and there is no detectable mutation within the examined regions, the tested strain may be considered sensitive for the respective antibiotic
18	In case of mutation, the respective amplicon cannot bind to the corresponding wild type capture probe on the strip due to the mismatch

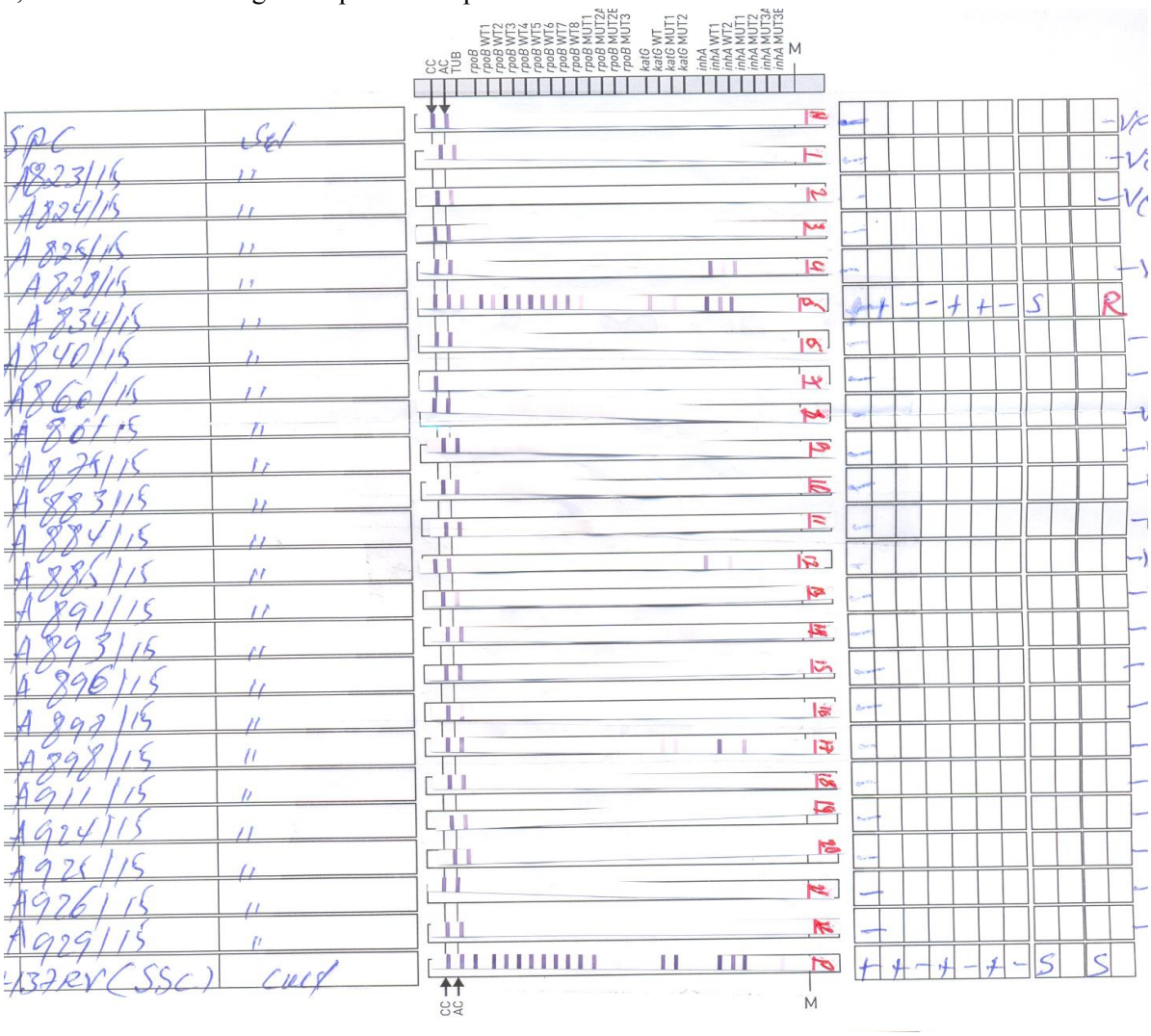
19	The absence of a signal for at least one of the wild type probes may predict resistance to the respective antibiotic indirectly
20	Positive hybridization signal with a mutation-specific capture probe (for common mutations only!) may predict resistance to the respective antibiotic directly
21	Presence of rare mutations that do not have mutation-specific capture probes may only be indicated by the lack of hybridization with one or more wild type probes

Annex VI. Results of LPA strips

i, Results of smear positive sputum samples



ii, Results of smear negative sputum samples



Annex VII. Specimen rejection criteria log

EPHI Pt ID or LIS ACCESSION NO	NO. OF SPECIMENS	PROBLEM	ACTION TAKEN
		1 2 3 4 5 6 7 8 9 10 11 12	A B C D E F G H
		1 2 3 4 5 6 7 8 9 10 11 12	A B C D E F G H
		1 2 3 4 5 6 7 8 9 10 11 12	A B C D E F G H
		1 2 3 4 5 6 7 8 9 10 11 12	A B C D E F G H
		1 2 3 4 5 6 7 8 9 10 11 12	A B C D E F G H
		1 2 3 4 5 6 7 8 9 10 11 12	A B C D E F G H
		1 2 3 4 5 6 7 8 9 10 11 12	A B C D E F G H

PROBLEMS

1. UNLABELLED SPECIMEN
2. UNLABELLED FORM
3. NO SPECIMEN RECEIVED
4. NO FORM RECEIVED
5. NO TEST ON FORM
6. NO LOCATION ON FORM
7. INSUFFICIENT
8. RECEIVED BROKEN
9. WRONG TUBE/SPECIMEN & FORM DIFFER
10. NAME ON SPECIMEN & FORM DIFFER
11. RECEIVED EMPTY
12. SPECIMEN LEAKED IN TRANSIT

ACTION TAKEN

- A PHONED <5MIN TO RESOLVE
- B PHONED >5MIN TO RESOLVE
- C PHONED TO Dr.
- D PASSED ON TO LABORATORY
- E MADE OUT FORM
- F HELD AT RECEPTION FOR 1 WEEK
- G DISCARDED IMMEDIATELY
- H OTHER

NAME: _____ **DATE:** _____

ASSURANCE OF PRINCIPAL INVESTIGATOR

I the undersigned accomplished all responsibilities for the scientific and ethical conduct of the research project and provided timely progress report to my advisors and got the necessary advice and approval in the course of the research. I also communicated timely to my advisors and all stakeholders involved in the study.

Name of the student: Abyot Meaza Dasho

Signature: _____

Date:_____

Approval of Advisors

Name: Mr. Kassu Desta

Signature: _____

Date: _____

Name: Mr. Abebaw kebede

Signature: _____

Date: _____

Approval of Examiners

Internal examiner

Name: Mrs. Mulu Hussien

Signature: _____

Date: _____

External Examiner

Name: Mr. Ahmed Esmael

Signature: _____

Date: _____