

Addis Ababa University

College of Health Sciences, School of Allied Health Sciences

Department of Medical Laboratory Sciences



**Application of Home Validated Panel DNA Materials for Proficiency Testing
of WHO endorsed Molecular Assays: MTBDR*plus* and Xpert MTB/RIF
Assay**

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This is to certify that the Thesis prepared by Zekarias Dagne ,entitled: Application of Home validated Panel DNA Materials for Proficiency Testing of WHO endorsed Molecular Assays: MTBDR*plus* and Xpert MTB/RIF Assay submitted in partial fulfillment of the requirements for the degree of Master of laboratory science in Diagnostic and Public Health Microbiology track. Complies with the regulations of the University and meets the accepted standard with respect to originality and quality.

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Table of Content

List of Tables	VI
List of Figure.....	VII
Acronyms	VIII
Operational Definitions	X
1 INTRODUCTION	1
1.1 Background.....	1
1.2 Statement of the problem.....	3
1.3 Significance of the study	5
2 LITERATURE REVIEW	6
3 OBJECTIVES OF THE STUDY	9
3.1 General Objective	9
3.2 Specific Objective	9
4 METHOD AND MATERIAL.....	10
4.1 Study setting	10
4.2 Study design and period	11
4.3 Inclusion and Exclusion criteria.....	11
4.3.1 Inclusion Criteria.....	11
4.3.2 Exclusion Criteria.....	11
4.4 Variables of the study.....	11
4.4.1 Dependant variable.....	11
4.4.2 Independent Variables	12
4.5 Laboratory methods for Panel Preparation	12
4.5.1 Mycobacterium Tuberculosis Quality control strain source	12
4.5.2 Sub-Culturing and purity check up of the Isolates	12
4.5.3 MTB spiking	12
4.5.4 Extracted DNA panel Preparation from MTB isolate.....	13
4.5.5 Quality control	14
4.5.6 Panel validation.....	14
4.5.7 Panel Composition and Documentation	15
4.5.8 Transportation of PT panels.....	15

4.5.9	Evaluation of Performance	17
4.5.10	Proficiency test report.....	17
4.5.11	Data analysis and evaluation.....	17
4.5.12	Ethical Clearance.....	17
5	RESULTS.....	19
5.1	PT Validation Performance at EPHI,NTRL	19
5.2	Socio-demographic characteristics.....	21
5.3	Participating Laboratories.....	24
5.4	PT Performance using DNA Panel.....	25
5.4.1	MTBDR <i>plus</i> assay	25
5.4.2	Xpert MTB/RIF Assay	26
5.5	PT performance using spiked sputum	28
5.6	PT performance using extracted DNA	28
5.7	Overall performance indicators by drug and laboratory.....	29
6	DISCUSSION	31
7	LIMITATION OF THE STUDY	34
8	CONCLUSION AND RECOMMENDATION.....	35
	REFERENCE.....	36
9	ANNEX.....	40
9.1	Survey questionnaire (English version).....	40
9.2	Sop for MGIT culture inoculation and incubation	41
9.3	Sop for Preparation of Artificial Sputum	44
9.4	Sop for Extraction, Amplification and Hybridization of DNA for HAIN Probe Assays	47
9.5	LPA validation Result Technologist 1	56
9.6	LPA validation Result Technologist 2	57
9.7	Dispatch Form.....	58
9.8	Proficiency Test results form.....	59
9.9	Declaration	60

List of Tables

Table 1: Proficiency Test Panel strain composition validated at EPHI,NTRL and distributed to testing sites in 2015.....	16
Table 2: Performance Evaluation of NTRL,PT Validator and SRL Judicial Result in 2015.....	21
Table 3: Demographic characteristic of Personnel's working on TB Culture and DST Regional Reference Laboratories in Ethiopia (N=27) from July 2015 to January 2016.	22
Table 4: Overall Performance of RRL Professionals for susceptibility testing of M. tuberculosis.(N=13) from July 2015 to January 2016.....	23
Table 5 : Relationship between demographic characteristic with Passed ≥ 95 %) score on MTBc and drug susceptibility testing using LPA (N=13) from July 2015 to January 2016	24
Table 6: Proficiency performance of Participant Laboratories for MTBDR <i>Plus</i> and Xpert MTB/RIF in 2015 using DNA panels.	27
Table 7: Suitability performance of Spiked Sputum against Extracted DNA for panel testing of MTBDR <i>Plus</i> and Xpert MTB/RIF assays in 2015	29
Table 8: National TB Reference Laboratory Network DST proficiency testing Performance of seven laboratories for Isoniazid (% values).....	30
Table 9: National TB Reference Laboratory Network DST proficiency testing Performance of seven laboratories for Rifampicin (% values)	30

List of Figure

Figure 1: National TB Culture and DST Laboratory Network in Ethiopia, which are involved in the study.	10
Figure 2: Description of the 27 reaction zones on the GenoType® MTBDR <i>plus</i> strip	19
Figure 3: Description of the <i>rpoB</i> GENE RIF Resistance Determining Regions.....	20
Figure 4: Summary performance of all the participating laboratories (N=7) on LPA drug susceptibility testing in percentage	25

Acronyms

AFB	Acid Fast Bacilli
ATCC	American Type Culture Collection
CDR	Case detection rate
CLSI	Clinical Laboratory Standards Institute
DCS	Dried Culture Spot
DNA	Deoxyribonucleic acid
DOTS	Directly observed Treatment, Short course chemotherapy
DST	Drug susceptibility testing
EPHI	Ethiopian Public Health Institute
EQA	External Quality Assessment
FMoH	Federal ministry of health
HBCs	High TB burden countries
HIV	Human Immunodeficiency Virus
INH	Isoniazid
IUATLD	International Union against Tuberculosis and Lung Disease
LPA	Line Probe Assay
MC	Microscopy Center
MDR-TB	Multi drug Resistance Tuberculosis
NALC	N-acetyl-L cysteine
ND	Not done

NRL	National Reference Laboratory
NTP	National Tuberculosis control program
OSE	On-Site Evaluation
PCR	Polymerase chain reaction
PT	Proficiency Testing
PVR	Predictive value resistance
PVS	Predictive value susceptible
QA	Quality assurance
RMP	Rifampin
RRL	Regional Reference Laboratory
SERO	Scientific and Ethical Review Office
SNNPR	Southern nations, nationalities and peoples' region
SOP	Standard Operating Procedure
SRLN	Supranational reference laboratory network
RRDR	Rifampin Resistance Determining Region
TB	Tuberculosis
UN	United Nations
WHO	World Health Organization
WT	Wild type
XDR-TB	Extensively drug resistance Tuberculosis.
ZN	Ziehl-Neelsen

Operational Definitions

Agreement: It is a combination of sensitivity and specificity that describes the number of correct answers given or the amount of agreement between NTRL and the participant's answers, so both true resistant and true susceptible are counted toward this measurement.

Batch of samples: A batch of samples is defined as a group of samples that were processed, amplified and hybridized at the same time, under the same conditions, using the same PCR master mix, thermal cycler and hybridization solutions on the same hybridization platform.

Competency: is the skill of lab professionals for performing an accurate examination and reporting of GenoType® MTBDR*plus*Line Probe Assay and Xpert MTB/RIF assays

Efficiency: is the ability to correctly determine the proportion detected of the strains judicially classified as resistant and susceptible

External Quality Assessment (EQA): A process that allows participant laboratories to assess their capabilities by comparing their results with those in other laboratories in the network (RRLs and NRLs) through LPA panel testing and on-site evaluation of the laboratory.

Judicial Result: the results of the majority of SRLN participating laboratories, greater or equal to 85% concordance, considered the 'gold standard'.

Panel Testing: Sending PT strains (Half heat killed and Half DNA extracts) from the NRLs to the RRLs to check proficiency in GenoType® MTBDR*plus*Line Probe and Xpert MTB/RIF assays DST

Regional Reference Laboratory (RRL): Regional or intermediate level laboratory existing usually in the Regional headquarters. The RRL may exist as part of the state public health laboratory if no RRL is available in the Region.

Reproducibility: was defined as the proportion of pair of strains with an identical result among all pairs of results. If no result was available for one of the paired strains, the pair is excluded

Sensitivity: is the ability to correctly determine the proportion detected of the strains judicially classified as resistant.

Specificity: is the ability to correctly determine the proportion detected of the strains judicially classified as susceptible

Abstract

Background: Tuberculosis (TB) is a major public health problem in Ethiopia. Laboratory plays an important role in the diagnosis of TB and its treatment monitoring. Quality assured molecular drug susceptibility assay is an essential for rapid detection of MDR-TB cases and early treatment initiation Proficiency testing is one of the EQA methods in evaluating the quality service delivery however, it was not widely applied for molecular assays in Ethiopia mainly due to the inadequate capacity of preparing PT DNA panels' in-country.

Objective: The aim of this study was to assess Application of Homemade and validated panel DNA Materials for Proficiency Testing of the WHO endorsed Molecular Assays (GenoType® MTBDRplus and Xpert MTB/RIF assays) in TB Culture and DST laboratories, and in selected GeneXpert sites of Ethiopia.

Methods: A cross sectional study was conducted in five regional reference laboratories and two specialized hospital TB culture and DST laboratories which have molecular GenoType® MTBDRplus assay of Ethiopia, and in eight selected health facilities with Xpert MTB/RIF testing sites in Addis Ababa during the period between July 2015 and January 2016. PT DNA materials were validated at EPHI, NTRL and distributed to MTBDRplus and Xpert MTB/RIF testing facilities. All data from participating laboratories were captured and analyzed using *SPSS version 20*. A total of 194 (130 for MTBDRplus and 64 Xpert MTB/RIF) DNA panels were distributed to regional reference and hospital TB laboratories. Test results agreement between the participant laboratories and reference were estimated using *Kappa* statistics. The sensitivity, specificity, predictive value susceptible, predictive value resistance, accuracy and reproducibility of participant laboratories were calculated against the reference lab.

Results: 130 panel samples with a combination of 65 (50%) spiked sputum and 65 (50%) extracted DNA were tested in seven Regional Reference Laboratories which have MTBDRplus assay. Almost all, 129 (99.2 %) were correctly reported to the Isoniazid and Rifampicin susceptibility. Sixty nine (53.1%) reported isolates were resistance to both INH and RIF (MDR), 41 (31.5%) susceptible to both INH and RIF, and 19 (14.6%) INH susceptible but RIF resistance strains correctly reported with respect to the reference result. There was 100% agreement in detecting MDR and resistance to any of the two drugs (INH and RIF) ($\kappa = 1$) but the agreement was 97.8% for the detection of susceptible strains ($\kappa = 0.9$). Among 64 spiked sputum strains (32 *M. tuberculosis* DNA with no RIF resistance and 32 *M. tuberculosis* DNA with RIF resistance (*probe E* Mutant detected) distributed to GeneXpert sites, all of them were correctly reported. Overall agreement on detection of *M. tuberculosis* and RIF susceptibility was 100 % ($\kappa = 1.0$).

Conclusion: There was an excellent agreement between the participant laboratories and the reference laboratory for the detection *M. tuberculosis* and drug susceptibility using MTBDRplus and Xpert MTB/RIF assays. Therefore, we can apply home validated PT DNA panels as one of the EQA methods for WHO endorsed molecular assays MTBDRplus, whereas in the Xpert MTB/RIF assay a large scale validation is important to use as Panel test since, the sites we used for this study in Addis Ababa, are small compared to the general population in Ethiopian context.

1 INTRODUCTION

1.1 Background

Tuberculosis (TB) is a major global public health problem that affects millions of people around the globe, predominantly in low- and middle-income countries. It is also a persistent health threat in high-income countries, especially among the poorest, most vulnerable segments of the population [1]. In a globalized world, TB is a shared problem for all countries. Drug-resistant forms of TB is a particularly global issue that respects no borders, as further spread and amplification of drug resistance could undo all previous TB control efforts, rendering the disease yet again incurable [2]. The global threat of multidrug-resistant tuberculosis (MDR-TB); (strains of *Mycobacterium tuberculosis* resistant to at least rifampicin and isoniazid) to TB control, underscores the importance of prompt and rapid identification of such resistant *Mycobacterium tuberculosis* (MTB) strains. Isoniazid and Rifampicin are the key first-line anti-tuberculosis drugs, and resistance to these drugs i.e. MDR-TB, is likely result in treatment failure and poor clinical outcomes [3] TB strains resistant to the first-line drugs Isoniazid and Rifampin, now account for 5% of all TB cases globally.

Laboratory confirmation of TB and drug resistance is key to ensuring that individuals with TB signs and symptoms are correctly diagnosed and treated. Definitive diagnosis of drug-resistant TB requires that *Mycobacterium tuberculosis* bacteria be detected and resistance to anti-TB drugs determined. This can be done by isolating the bacteria by culture, identifying it as belonging to the *M. tuberculosis* complex (MTBc), and conducting drug susceptibility testing (DST) using solid or liquid media or by performing a WHO endorsed molecular test to detect TB DNA and mutations associated with resistance [4].

Conventional phenotypic methods using solid media remain more common, relatively inexpensive and highly standardized for testing susceptibility to many drugs, but have long turnaround time. Compared with solid culture methods, liquid culture methods significantly reduce the turnaround time for results and are around 10% more sensitive. The mean time to detect drug resistance on egg based LJ media is around 3–4 months [5]. Even using the more modern broth-based liquid culture system culture and drug susceptibility results from sputum specimens still takes several weeks [6]. However, newly developed molecular based methods

have advantages over conventional phenotypic methods in terms of both accuracy and turnaround time [7].

The WHO recommends using rapid molecular tests *MTBDRplus* and Xpert MTB/RIF to diagnose TB and MDR-TB in developing and high-burden countries [8]. Molecular tests dramatically shorten time to diagnosis from months to days (*MTBDRplus*) or hours (Xpert MTB/RIF). Both tests are based on PCR amplification of the beta subunit of mycobacterial DNA-dependent RNA polymerase (*rpoB*) followed by detection of mutations in its 81bp Rifampin Resistance Determining Region . Such mutations are found in 95- 98% of all (MTB) strains resistant to Rifampin (RIF) [9]. Furthermore, most isolates resistant to RIF are also resistant to Isoniazid (INH) therefore the presence of *rpoB* mutations can be used as a surrogate marker for MDR-TB [10].

Although Xpert MTB/RIF has dramatically shorten the time required for patients to receive an accurate diagnosis of tuberculosis and Rifampicin resistance, there are some challenge include: a stable uninterruptable electrical supply, ambient operating temperature of the instrument which should not exceed 30 °C, annual calibration, and additional DST methods may be needed to investigate resistance to other first- line and second-line drugs [11]. *Mycobacterium tuberculosis* arises from spontaneous chromosomal mutations at low frequency. Clinical drug-resistant TB largely occurs as a result of man-made selection during disease treatment of these genetic alterations through erratic drug supply, suboptimal physician prescription and poor patient adherence. The relationship between drug resistance in *M. tuberculosis* strains and their virulence/transmissibility needs to be further investigated. Understanding the mechanisms of drug resistance in *M. tuberculosis* would enable the development of rapid molecular diagnostic tools and furnish possible insights into new drug development for the treatment of TB. [12]

Laboratory services have an important role in improving the delivery of health care and reducing the prevalence of TB, particularly drug resistant TB. Quality control and proficiency testing are fundamental tools that ensure accuracy in laboratories by comparing quality to other laboratories, evaluating the general performance level of the laboratory's service, and detecting laboratory facilities with an acceptable levels of proficiency [13].

1.2 Statement of the problem

Extensively drug-resistant (XDR) TB has been reported from more than 58 countries and is estimated to occur in up to 10% of MDR-TB patients. Treatment of MDR-TB remains challenging and complex, and treatment success is considerably lower than drug susceptible TB. Sub-Saharan Africa is especially burdened with drug-resistant TB [14]. In 2013, 58% of the 4.9 million pulmonary TB patients notified globally were bacteriologically confirmed via a WHO recommended test [15].

Globally, an estimated 3.3% of new TB cases and 20% of previously treated cases have MDR-TB. In 2014, an estimated 190 000 people died of MDR-TB. The number of cases detected (123 000) worldwide represented just 41% of this global estimated 300 000, and only 26% of the 480 000 incident cases of MDR-TB estimated to have occurred in 2014. Extensively drug-resistant TB (XDR-TB) had been reported by 105 countries by 2015. An estimated 9.7% of people with MDR-TB have XDR-TB. The funding required for a full response to the global TB epidemic in low- and middle-income countries is estimated at US\$ 8 billion per year in 2015, excluding research and development [16].

Ethiopia among the 27 high MDR-TB burden countries, the prevalence of MDR-TB increased from a base line rate of 1.6% among new smear positive TB cases in 2005 to current level of 2.3% in 2014; similarly the rate has increased from 11.8% to 17.8% among previously treated smear positive TB cases. In the 2014 TB Global report :TB incidence rate is 224/100.000 population TB prevalence rate was at 211/100.000 population and the country is funding more than 50 million USD from 2015-2017 [17].

The first nationwide anti-TB drug resistance surveillance has been carried out during the period of 2003 - 2005 ,on the basis of the findings of the surveillance, the country started to introduce the programmatic management of multidrug resistant tuberculosis (MDR-TB) on pilot since 2010. In addition to the National TB reference laboratory in Addis Ababa, about seven TB culture labs are providing TB culture and DST services at major regional labs (Adama, Hawassa, Harar, Bahir Dar, Jimma and Mekele)and specialized Hospitals (St.Peter Hospital) [18].

According to 2013/14 Ethiopian Ministry of health report, a total of 116,633 TB cases (all forms)

were reported with a TB case notification rate of 133/100,000 populations. Out of the reported 116,633 cases, 34.0% were smear positive pulmonary TB, 34.8% were smear negative pulmonary TB, and 31.2% were extra pulmonary TB. The TB case detection rate was 53.7%, which is below the target set for the year (81.0%), the prevalence of smear-positive TB among persons aged 15 years and above was 108/100,000, whereas the prevalence of bacteriologically confirmed TB in the same age group was 277/100,000 [19]. By the second round anti-tuberculosis drug resistance surveillance in Ethiopia, the prevalence of multi-drug resistance TB among newly diagnosed and previously treated cases was 2.3 % and 17.8%, respectively. WHO estimates that about 2100 MDR-TB occur in Ethiopia among notified TB patients annually, Majority of the estimated MDR-TB cases could be undiagnosed due to poor access to diagnostic services[19].

There are limited opportunities for EQA TB culture and drug susceptibility test in resource-poor countries, although the WHO SRL network does have a DST proficiency testing program for its member laboratories. Difficulties in expanding this service globally involve labor-intensive preparatory work and the high cost and strict packaging requirements for shipping drug-resistant isolates of TB. To overcome this lack of available EQA, performance indicators were recently implemented for mycobacterial culture as a means of internal quality assessment.[20]

To ensure that results of drug susceptibility testing are reliable and internationally comparable, the WHO and The IUATLD established a network of TB supranational reference laboratories network (SRLN) in 1994. The first purpose of this network was to standardize *M. tuberculosis* drug susceptibility testing throughout the world. Currently the supranational network provides proficiency testing of drug susceptibility testing results for national laboratories engaged in anti-tuberculosis drug resistance surveillance[21].

The proficiency testing by the SRLN involves regular distribution to national reference laboratories of panels of coded *M. tuberculosis* strains with predefined drug-resistance profiles. The test results of the reference laboratory are compared with the coded SRL results in blinded fashion and specific performance indicators (sensitivity, specificity, reproducibility) calculated for each drug and for the reference laboratory as a whole [22]. Accuracy of the laboratory diagnosis is therefore crucial and a comprehensive laboratory quality assurance program must be in place to ensure the accuracy, reliability and reproducibility of DST results [23] Currently, inter laboratory proficiency testing is available for Phenotypic Culture and DST but, for

molecular tests (MTBDR*plus* and Xpert MTB/RIF) assays is limited. PT in molecular diagnostics is further complicated by scarcity of established programs, as well as the complexity of the tests. As CLSI defined participant laboratories benefit because external quality assessment serve as an early warning system, identifying systematic problems, provides objectives evidence of laboratory quality, serve as indicator for focusing improvement efforts, identifying training needs, provide a source for continuing education and provides a source of materials for practice[24]. Although proficiency testing (PT) is one of the EQA methods in evaluating the quality service delivery, it was not widely applied for molecular assays in Ethiopia. This is mainly due to the inadequate capacity of preparing PT DNA panels' in-country.

1.3 Significance of the study

- To provide information about the ability of NTRL to prepare PT material for TB molecular diagnostics.
- Provide information about the performance of professionals who are working at Regional TB culture Laboratories in the country
- To provide tangible information for different stakeholders about the performance of MTBDR*plus* and Xpert MTB/RIF) assay
- Provide information which is used to improve the performance of TB Laboratories based on identified gaps.

2 LITERATURE REVIEW

In a recent study done in South Africa on A Pilot Evaluation of External Quality Assessment conducted Using Dried Culture Spot (DCS) Material revealed well performance of GenoType MTBDR*plus* Version 2 while recommending increased bacterial concentration for use with version 1. The study demonstrates suitability of DCS program not only for the Xpert MTB/RIF assay but also for the MTBDR*plus* assay version 2 and its ability to incorporate measurements for the entire testing process (DNA extraction through result reporting), as opposed to just amplification and hybridization steps on DNA prepared EQA materials. The study also showed the added advantage of the DCS technology to transport inactivated tuberculosis-positive specimens at room temperature, thereby reducing the overall program costs. [25]

A LPA EQA programme can help to ensure the quality of and promote improvement in TB laboratory performance. A filter-paper-based *Mycobacterium tuberculosis* DNA samples external quality assurance study conducted at five laboratories in four countries on line-probe assay for detection of anti-tuberculosis drug resistance demonstrated inter- and intra-laboratory reproducibility of 97% and 96% respectively. Most of the participating laboratories reproduced their blinded duplicates correctly and with similar banding intensities. In the study differences between participating laboratories were prominently observed when comparisons were made between laboratories [26].

A 2008 meta-analysis found that the GenoType MTBDR *plus* assay had a pooled sensitivity of 98% for detecting Rifampicin resistance and 89% for detecting Isoniazid resistance and specificity of 99% for Rifampicin and Isoniazid. Testing can be performed on culture isolates or acid fast bacilli (AFB) positive sputum specimens, and can provide results within 8 hours, making this a promising tool to accelerate the diagnosis of MDR-TB cases, and hence improve management of MDR-TB cases [27]

Performance of First Drug Susceptibility testing of *Mycobacterium tuberculosis* conducted in Saudi Arabia among 1904 isolates found 44 inconsistencies. The majority of MDR-TB cases reported from referral Laboratories lacked to reproduce the same results at different site. Of the 44 inconsistent results, 16 showed false resistance to isoniazid and rifampicin and 14 demonstrated false susceptibility to the same drugs.

The network of supranational tuberculosis reference laboratories (SRLs) was conducted a research to evaluate the annual SRL Rounds 6–14 for nine rounds (1999–2007) of proficiency testing for first-line drug susceptibility testing (DST) in 2011, Panels consisted of 20–30 cultures (including 10 pairs of duplicate strains), aiming at 50% resistance prevalence with a variety of profiles in the 27 SRLs participating laboratories. Of 600 strains, 10% were excluded from evaluation. The average SRL sensitivity and specificity varied between rounds, without attaining significance or trends. Both sensitivity and specificity remained at >95% for isoniazid (INH), rifampicin (RMP) and streptomycin and at >80% for ethambutol. The 16 SRLs participating in all rounds performed consistently better and concluded the rounds succeeded in comparing the proficiency of laboratories, and should be further promoted for DST quality assessment [28].

In Taiwan clinical mycobacteriology laboratory was conducted a research to evaluate the impact of external quality assessment (EQA) on the quality of drug susceptibility testing (DST) in 2007–2011. Panels consisting of 20–30 *Mycobacterium tuberculosis* strains were used. Efficiency of 95% in detecting resistance to both isoniazid (INH) and rifampicin (RMP), and of 90% to ethambutol (EMB) and streptomycin (SM) was used to define a competent laboratory. The proportion of laboratories that fulfilled the competency criteria for all first-line drugs was 16.7% in 2007, increasing to 85.7% in 2008, 86.1% in 2009, 82.4% in 2010, and to 96.8% in 2011. The mean efficiency in detecting resistance to INH and RMP reached >99% during 2008–2011 (INH and RMP), and for EMB it increased from 82.0% in 2007 to 92.2% in 2008 and 99.5% in 2011, while that for resistance to SM increased from 82.0% in 2007 to 98.1% in 2008 and 99.5% in 2011. Preparations of inoculums for DST and detection of EMB resistance were the main reasons for non-competence. The EQA programme was effective in improving the competency of clinical laboratories in performing DST for tuberculosis [29].

In a similar study conducted by Quality assurance for the World Health Organization (WHO)/International Union Against Tuberculosis and Lung Disease (The Union) global tuberculosis (TB) drug resistance surveillance programme to monitor the quality of drug susceptibility testing (DST) in different countries. From 2002–2003 and 2005–2006, 20 *Mycobacterium tuberculosis* strains were sent by the WHO/ Union Supranational Reference Laboratory of Rome to TB reference laboratories in Albania, Bahrain, Kosovo, Mozambique, Oman, Qatar and Turkey for external quality control (EQC). In 2002–2003, the specificity, sensitivity, efficiency, reproducibility and predictive values for resistance/ susceptibility were

90% for streptomycin (SM), isoniazid (INH) and ethambutol (EMB). In 2005–2006, all statistical values were 96% for SM, INH, rifampicin and EMB. The study concluded that EQC improved the quality of *M. tuberculosis* DST in the participating countries [30]

In the study conducted South African, Johannesburg to assess the implementation of Xpert MTB/RIF quality requirement a pilot program using dried culture spots (DCSs) of inactivated *Mycobacterium tuberculosis* were described of 274 DCS results received, 2.19% generated instrument error related to sample volume (too little volume/too viscous/fluid transfer failed) testing, such as errors 5006 and 5007; the remainder yielded 100% correct *Mycobacterium tuberculosis* detection. The probe A cycle threshold (CT) variability of three DCS batches was <3.47. The study of longer-term DCS stability was ongoing [31].

In a study conducted in India to assess proficiency assessment system enable LPA scale-up, proficiency testing procedures and implemented after initial LPA training, each project site collected remnant sputum specimens from 50 smear-positive patients for anonymous LPA testing. Results were assessed for negative control validity, successful amplification and internal and external reproducibility. Any site failing to achieve the pre-defined proficiency benchmarks was reviewed for their practices and subsequently were reassessed, after undertaking the necessary corrective action. As a result Six laboratories underwent LPA proficiency assessment and this mechanism revealed several procedural weaknesses, including errors in deoxyribonucleic acid (DNA) extraction practices, reagent contamination, and inter-observer variability in interpretation. LPA proficiency assessment facilitated successful implementation of LPA in India. Based on these results, the described proficiency testing mechanism was accepted by National Laboratory Committee of India's Revised National TB Control Programme, and was being implemented at all LPA laboratories under country's laboratory scale-up plan [32].

3 OBJECTIVES OF THE STUDY

3.1 General Objective

- To assess the application of home validated panel DNA materials for Proficiency testing of WHO endorsed molecular assays (GenoType® MTBDR*plus* and Xpert MTB/RIF assays) in TB Culture and DST laboratories, and selected GenXpert sites of Ethiopia.

3.2 Specific Objective

- To assess the capacity of NTRL to prepare PT materials for molecular assays.
- To assess the agreement of participant laboratories against the reference PT results for detection of *M. tuberculosis* and drug susceptibility.
- To compare the suitability of MTB Spiked material with Extracted DNA as panel samples for proficiency testing of molecular assays.
- To determine the sensitivity, specificity, predictive value resistance, predictive value susceptible and efficiency of participant Laboratories

HYPOTHESIS

We hypothesize that home validated PT materials are suitable and comfortable to use for Molecular GenoType® MTBDR*plus* Line Probe Assay (LPA) and Xpert MTB/RIF proficiency testing of RR TB-Culture and DST laboratories, and assume performance of at least $\geq 95\%$.

4 METHOD AND MATERIAL

4.1 Study setting

This study was conducted at five regions reference and two specialized hospitals (see Fig. 1), all of them have the capacity to perform TB culture, identification and drug susceptibility test using molecular GenoType® MTBDR*plus*Line Probe Assay (LPA), equipped with required minimum safety standards to carry the test such as serviced Biosafety cabinets, mycobacterial aerosol containment work practices, and separate rooms for LPA DST workflows. In addition Seven government hospitals and one health center with Xpert MTB/RIF testing sites in Addis Ababa were also included for Xpert MTB/RIF proficiency testing.

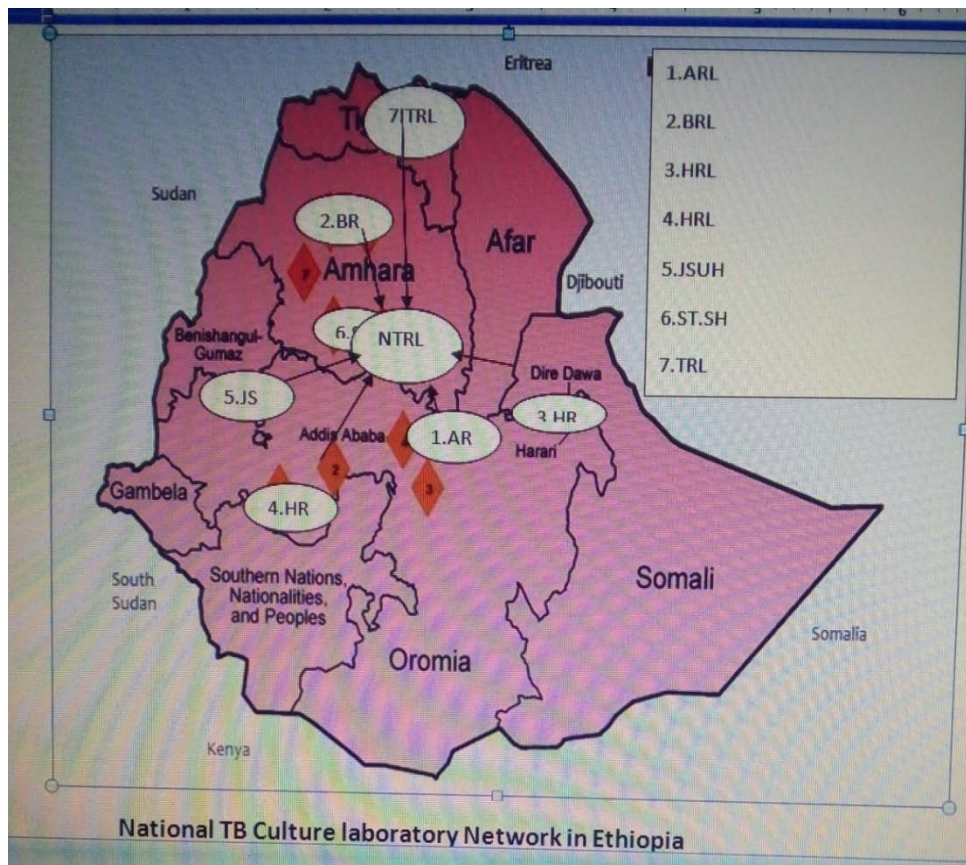


Figure 1: National TB Culture and DST Laboratory Network in Ethiopia, which are involved in the study.

1. **ARL:** Adama Regional TB Culture Laboratory(Oromia Regional State)
2. **BRL:** Bahir Dar Regional TB Culture Laboratory(Amhara Regional state)
3. **HRL:** Harar Regional TB Culture Laboratory (Harrari Administrative State)
4. **HRL:** Hawassa Regional TB Culture Laboratory(SNNPR)
5. **JSUH:** Jimma University specialized Hospital (Jimma)
6. **ST.SH:** St. Peter TB Specialized Hospital (Addis Ababa)
7. **TRL:** Tigray Regional TB Culture Laboratory (Mekele)

4.2 Study design and period

A cross sectional study was conducted in five regional and two specialized hospitals which can perform TB culture and DST laboratories with molecular GenoType® MTBDR*plus* assay of Ethiopia, and seven hospitals and one health center with Xpert MTB/RIF testing sites in Addis Ababa during the period between July 2015 and January 2016. 194 PT DNA materials were prepared and validated at NTRL of EPHI and distributed to MTBDR*plus* and Xpert MTB/RIF facilities, with a designed questionnaire for the information of the lab workers in MTBDR*plus* sites.(Annex 9.1) All 194 PT materials were provided for two rounds in three months interval, feedback was given following result submission and data analysis.

4.3 Inclusion and Exclusion criteria

4.3.1 Inclusion Criteria

RRL, Government hospital laboratories and selected health centers providing DST service with molecular MTBDR*plus* or Xpert MTB/RIF assays.

4.3.2 Exclusion Criteria

Facilities not volunteer to participate in the study are excluded from the study.

4.4 Variables of the study

4.4.1 Dependant variable

- Performance ability of MTBDR*plus* testing sites and lab professionals.

4.4.2 Independent Variables

- Educational background,
- Experience In TB Culture and DST Laboratory.
- Training on LPA Diagnostic technique.

4.5 Laboratory methods for Panel Preparation

4.5.1 Mycobacterium Tuberculosis Quality control strain source

Panel of strains with good concordance (more than 80% for each drug) from previous rounds of supranational tuberculosis laboratories (SRL) for drug susceptibility testing of *Mycobacterium tuberculosis* was used. These strains that have a known resistant pattern for Rifampicin and Isoniazid anti-TB drugs through molecular typing in Antwerp supranational reference Laboratories (SRL) were used for the preparation of LPA and Xpert Panels.

4.5.2 Sub-Culturing and purity check up of the Isolates

Selected isolates were grown in triplicate in MGIT culture for multiplication according to the manufacturer standard operating procedure. One portion was subjected to prepare MTB spiked artificial sputum and the second for direct preparation of DNA from MTB isolates, while the third tube was stored at -20°C as a backup.(Annex-9.2)

4.5.3 MTB spiking

Artificial sputum was prepared from methylcellulose and egg albumin which has similar characteristics with natural sputum help to monitor cross contamination. These artificial specimens were submitted blindly to LPA processing team and had negative results for both microscopy and culture. This artificial sputum mixed was with 3-5 days positive liquid culture or 0.5 McFarland MTBc Positive solid culture isolate and heat killed.(Annex-9.3)

Transfer the contents of the MGIT cultures into a labeled 15ml falcon tube or 2ml Cryovial for large volume and small volume preparation respectively ,heat kill the strains and non spiked artificial sputum in a water bath set at 95⁰c for 15ml falcon tube and 105⁰c for 2ml Cryovial for 10 minutes. Heat kill the known grown strains of MTB from MGIT culture (10ml) in duplicate

including the blinded non-spiked artificial sputum (10ml) prepared to monitor cross contamination and personnel integrity at 105°C for 10 minutes on a heating block. The strains are then quality checked. When QC has passed, the strains are spiked into artificial sputum in the ratio of 1:2 respectively. Spiked strains are then stored at -20°C freezer. LPA is then performed as per Manufacturer SOP to determine the banding patterns of each strain (Annex 9.4)

4.5.4 Extracted DNA panel Preparation from MTB isolate.

The LPA diagnostic technique is based on the PCR amplification of specific regions of DNA. DNA must therefore be extracted from the specimen under investigation in order to make a genotypic diagnosis of the drug susceptibility pattern. The extraction of the DNA from the positive MTB culture is done after partial cell lysis during a heat-killing step. An initial centrifugation step concentrates the bacilli at the bottom of the micro centrifuge tube to form the bacterial containing “pellet”. The pellet should be resuspended in molecular grade water, to ensure that the suspension of extracted DNA is free of impurities that might inhibit the PCR reaction. The tubercle bacilli tend to clump so the resuspended solution should be mixed well so that it appears slightly opaque (milky). This will ensure that the dispersed bacilli will be killed during the heat-inactivation step and a higher yield of DNA obtained.

The heat generated by the heating-block, hot air oven or water-bath is meant to kill the bacilli and partially lyses the cells, thereby rendering the solution non-infectious. The solution can be now be transferred out of the BSL-3 laboratory for performance of the rest of the assay. The powerful ultrasonic shockwaves created by the sonicator disrupt the cell walls of the tubercle bacilli, causing further cell lysis, and releasing the DNA and other cell debris into the molecular grade water. The final centrifugation step, at maximum speed, is needed to separate the impure cell debris (containing the cell wall, proteins and other macromolecules) from the DNA. The heavier debris will form the pellet and the lighter DNA (free from impurities) will be suspended in the supernatant (molecular grade water). The extracted DNA was pure and stored in the -80°C freezer.

4.5.5 Quality control

Heat killed MTB isolates were inoculated in MGIT tubes according to the SOP and then checked for MTB growth, the batch of panels culture were negative, then batch were passed proceeding to ascertain banding patterns.

4.5.6 Panel validation

Validation is the process of verifying each batch of LPA PT strain prior to sending test panels to participant laboratories. Extracted DNA from both artificial spiked sputum or MTB isolates were subjected to LPA analysis and Xpert MTB/RIF testing following SOP and the results clearly recorded as PT panels. The known resistance or sensitivity pattern of each strain with LPA band patterns and Mutant probes with Xpert MTB/RIF were recorded and blinded (known only to PT panel's coordinator and EQA supervisor). The same but blinded strains were duplicated and randomized, then given to the LPA laboratory technologist and in charge of Xpert MTB/RIF workstation for analysis.

LPA panels (each comprising 10 vials; 5 spiked sputum and 5 extracted DNA) were initially tested blindly with different individuals in EPHI NTRL, according to the manufacturer procedure in order to validate competency for the LPA, positive and negative QC samples were performed on a routine basis for each batch of specimens. Each batch of specimens tested with the LPA had a positive band for "extraction positive control" (ATCC strain H37Rv), checks the extraction procedure and also verified that the LPA Panel was capable of detecting the M. tuberculosis complex, as well as all the WT (wild type) regions associated with a pan-susceptible strain of M. tuberculosis. and "extraction negative control", (molecular grade water) monitors for contamination throughout the entire LPA procedure (DNA extraction, master mix preparation, template addition, and hybridization) verified that no contaminating nucleic acids or amplicons had been introduced. Each strain on the batch were verified for the presence of the two internal controls on the strip shown in (Annex 9.5 and 9.6) : the Conjugate Control(CC) (line no. 1), which verified efficiency of the conjugate binding to the biotinylated primers and; efficiency of the substrate reaction with the conjugate to detect the positive bands, and the Amplification Control(AC) (line no. 2), band indicated that the DNA extraction and PCR procedures were carried out successfully. the strip were interpreted as a valid test when the CC AC, TUB, *rpoB*,

katG and *inhA* locus controls and their respective bands were present, using the intensity of the AC band as the baseline against which all other bands on the strip were interpreted. Xpert MTB/RIF panels (each comprising 5 spiked and 5 extracted DNA) were initially tested blindly with different individuals in EPHI NTRL, according to the manufacturer procedure in order to validate competency for the Xpert MTB/RIF assays. Results from the two laboratory technologists from the two methods were compared against the judicial results. The documented result interpretation is reviewed by the LPA and Xpert MTB/RIF section reviewer and successfully processed spiked artificial MTB sputum samples or directly extracted DNA and internalized LPA and Xpert MTB/RIF results were automatically qualified as EQA samples for participant laboratories.

4.5.7 Panel Composition and Documentation

A total of 194 home validated panel DNA materials for proficiency testing were prepared. Each laboratory participating in PT were receive 20 panel strains in two rounds (Five spiked sputum and Five DNA extracts to each round) for MTBDR *Plus*(LPA) and 10 (five spiked sputum on the first round and five DNA extracts on the second round) were prepared to Xpert MTB/RIF sites, since the Xpert MTB/RIF machine is with four module only four result is involved in the analysis. (Table 1) Each labeled randomly and identification numbers (which differs from laboratory to laboratory) entered in the EXCEL worksheet. The unique identification number for each strain were used for sending results back to PT provider using the result form that was sent together with the PT panel.

4.5.8 Transportation of PT panels

4.5.8.1 Liquid samples

These were products derived from living organisms that were prepared and distributed in accordance with the requirements of national governmental authorities which may have special licensing requirements. The PT strains for purposes of genotypic techniques performance monitoring and evaluations liquid material had sufficient amount of detectable nucleic acids which was heat killed nonviable organism and no possibility of causing TB infection, were aliquoted 1.0 ml into each Cryovial and tighten the lead, triple packaged, then ready following

the instructions that accompany the PT samples instruction stated the exact date by which the participants must return results to the PT provider.(Annex 9.7)

Table 1 Proficiency Test Panel strain composition validated at EPHI,NTRL and distributed to testing sites in 2015

Types of Panel	No. of Vials to be tested	Vials strain	Susceptibility band pattern
MTBDRplus	10		
	3	<i>M.tuberculosis</i> RIFs, INHs	Positive, RIF s, INHs No missed WT and MUT band present , identified by <i>rpoB</i> gene No missed WT and MUT band present ,identified by KatG gene
	1	<i>M.tuberculosis</i> RIFr, INHs	Positive, RIFr INHs; Rifampin resistance was identified by an <i>rpo B</i> wild-type 7 (WT7) missing band and no mutant band present, Isoniazid susceptible.
	6	<i>M.tuberculosis</i> RIFr, INHr	Positive, RIFr, INHr 1/6 Rifampin resistance was identified by an <i>rpoB</i> wild-type 7 (WT7) missing band and no mutant band present , Isoniazid resistance was identified by KatG wild-type (WT) missing band and a mutant 1(MUT1)band present . 5/6 Rifampin resistance was identified by an <i>rpoB</i> wild-type 8 (WT8) missing band and mutant (MUT3)band present , Isoniazid resistance was identified by KatG wild-type (WT) missing band and a mutant 1(MUT1)band present.
Xpert MTB/RIF	5		
		MTB Detected	1- MTB Detected (Medium) RIF resistant Detected (probe E missed)
			1-MTB Detected (Medium) RIF resistant NOT Detected
			1-MTB Detected (Medium) RIF resistant NOT Detected
			1-MTB Detected (Medium) RIF resistant Detected (probe E missed)
			1-MTB Detected (Medium) RIF resistant NOT Detected

4.5.9 Evaluation of Performance

The performance of participants with regard to the overall performance against prior expectations, taking measurement uncertainties into account; variation within and between participants. Possible sources of error (with reference) and suggestions for improving performance; advice and educational feedback to participants as part of the continual improvement procedures of participants any other suggestions, recommendations or general comments; and conclusions were included in the feedback. (Annex 9.8)

4.5.10 Proficiency test report

Clear and comprehensive Proficiency test reports, include data covering the results of each strains tested together with an indication of the total performance of individual participants, and comments or recommendations, based on the outcomes of the proficiency testing round were made available to participants within two weeks.

4.5.11 Data analysis and evaluation

Results received from participants were cross-checked, data cleaned and entered to excel sheet and transported to Statistical Package for Social Sciences (SPSS) 20.0 for analysis. Data processing and statistical analysis performed by using the SPSS software (Windows version 20.0). The percentages of different types of errors were calculated for MTB DR Plus and Xpert MTB/RIF assay Participant laboratories. The sensitivity, specificity, predictive value Susceptible (PVS), predictive value Resistance (PVR), accuracy and reproducibility were calculated. The agreement of reading between the participant laboratories and the reference laboratory result were assessed using *kappa* statistics. Missing results because of failure to test were recorded as “ND” and excluded from all evaluations. The pass mark of a laboratory for any tested drug was based on efficiency (accuracy of performance) which is $\geq 95\%$ and above for Isoniazid and Rifampicin drugs.

4.5.12 Ethical Clearance

The study was conducted after approved by Department Research Ethical Review Committee (DREC) Addis Ababa University. Information about the study was provided to all participant

involved in the study and to all concerned bodies found at all studies area. All participants were assured about the confidentiality, protection and anonymity of the data.

5 RESULTS

5.1 PT Validation Performance at EPHI,NTRL

Performance of EPHI, NTRL results against SRL judicial result for Rifampicin and Isoniazid anti TB drugs : a total of 60 (40 for MTBDRplus and 20 for Xpert MTB/RIF) DNA panels were validated for the type of missed probes in Xper MTB/RIF assay and banding patterns detected on a strip of MTBDR*Plus* following hybridization with PCR products amplified from target DNA in a specimen.

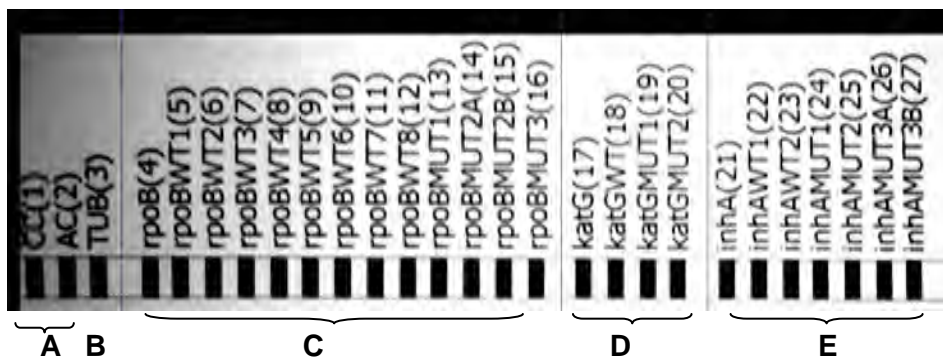


Figure 2: Description of the 27 reaction zones on the GenoType® MTBDR*plus* strip

Banding pattern of strains for susceptibility to *M. tuberculosis*. (A) The internal control reaction zones 1 (CC), 2 (AC) are present indicating that the DNA extraction, amplification and hybridization steps were successful. (B) Reaction zone 3 (TUB) is present, thus identifying the specimen as positive for *M. tuberculosis* complex. (C) The *rpoB* locus control (reaction zone 4) and all the *rpoB* wild-type probes (zones 5 to 12) and mutant probes (zones 13-16) are indicating susceptibility to RMP. (D) The *katG* locus control (zone 17) and *katG* wild-type probe (zone 18), mutant probe (zones 19&20) as well as (E) the *inhA* locus control (zone 21) and its associated wild-type probes (zones 22 and 23), mutant probes (zones 25-27) shows that the same strain also exhibits susceptibility to INH.

Out of 40 PT panels validated for MTBDR*Plus*, 24 (60%) were MDR strains: Rifampin resistance was identified by both an *rpoB* wild-type 8 (WT8) missing band and mutant (MUT3) band present and type 7 (WT7) missing band and no mutant band missing. Isoniazid resistance was identified by *KatG* wild-type (WT) missing band and a mutant 1 (MUT1) band

present , 12(30%) of them were susceptible to both INH and RIF: No missed WT and no hybridized MUT band present ,identified by *rpo B* gene and KatG , and 4 (10%) resistance to any of the two drugs: Rifampin resistance was identified by an *rpo B* wild-type 7 (WT7) missing band and no mutant band appeared, Isoniazid susceptible ,no missed wild type and hybridized mutant band observed.

Xpert MTB/RIF assay is based on RT-PCR amplification of *rpoB* gene Rifampicin resistance determining regions. 95% of all resistance to rifampicin are due to mutations in the *rpoB* gene and 5% due to mutations outside the gene > 90% of mutations in the *rpoB* gene are located in the 81 base pairs (codon 507–533) of the Rifampicin Resistance Determining Region 5 probes bind to wild type (do not bind to mutant sequence) . Rifampicin resistance can be used as a surrogate marker for MDR-TB, as more than 90% of RIF-resistant isolates are also resistant to INH, another potent first-line anti-TB drug .

***rpoB* GENE 81 bp RIF RESISTANCE DETERMINING REGION**

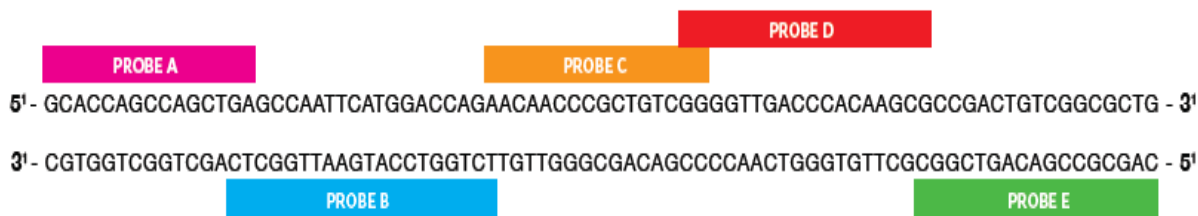


Figure 3: Description of the *rpoB* GENE RIF Resistance Determining Regions.

Among 20 PT panels validated for Xpert MTB/RIF, 12 (60%) were MTB detected ,Rifampin resistance was identified by *probe E* Mutation detected, 8 (40%) were MTB detected, Rifampicin resistance was not detected (all the five probes from A-E were present) were correctly identified with reference to the judicial result. There was 100% agreement in detecting Rifampicin resistance with a specified resistance by scanning the Rifampicin resistance determining region DNA for associated (*probe E*) mutations. (kappa = 1). Concordance with the judicial results and level of agreement between the two Technologist against Judicial results were described (Table 2).

Table 2: Performance Evaluation of NTRL,PT Validator and SRL Judicial Result in 2015

	SRL Judicial result		NTRL, validator.1 result		NTRL, validator.2 result	
	INH	RIF	INH	RIF	INH	RIF
Total correct results	10	10	20	20	20	20
True resistant	6	7	12	14	12	14
False resistant	0	0	0	0	0	0
True susceptible	4	3	8	6	8	6
False susceptible	0	0	0	0	0	0
Sensitivity	100%	100%	100%	100%	100%	100%
Specificity	100%	100%	100%	100%	100%	100%
Predictive value resistance	100%	100%	100%	100%	100%	100%
Predictive value susceptible	100%	100%	100%	100%	100%	100%
Accuracy	100%	100%	100%	100%	100%	100%
Reproducibility	100%	100%	100%	100%	100%	100%

5.2 Socio-demographic characteristics

From a total of 27 laboratory professionals who were working at TB culture and susceptibility sites, 13 (52%) were participated in the study. Based on the data, 21 (77.7%) of the participants were from regional reference TB culture laboratories and 6 (22.3%) from specialized hospital TB culture laboratories, 22 (81.5%) participants had BSc, and 5 (18.5%) were MSc holders. The mean age of the participants was 27 years and 23 (85.1%) were male. More than half (55.5%) of the participants had at least 2-5 years experience in TB culture, identification and susceptibility test reporting using a *MTBDRPlus*. Half of the participants examined one round, ten PT samples.

Table 3: Demographic characteristic of Personnel's working on TB Culture and DST Regional Reference Laboratories in Ethiopia (N=27) from July 2015 to January 2016.

Variables	Number	Percent	Cumulative Percent
Age			
20-30	12	44.4	44.4
31-40	15	55.6	100.0
Sex			
Male	23	85.1	85.1
Female	4	14.9	100.0
Educational Status			
Degree	22	81.5	81.5
MSc and Above	5	18.5	100.0
Experience			
<2 Years	2	7.4	7.4
2-5 Years	15	55.5	51.6
> 5 Years	10	37.1	100.0
Type of Facility			
Regional Reference Laboratory	23	15.0	15.0
Specialized Hospital TB Culture Laboratory	4	20.5	35.5
Trained for LPA			
Yes	22	81.5	81.5
No	5	18.5	100.0
Currently working in TB Lab			
Yes	22	81.5	81.5
No	5	18.5	100.0

Majorities (92.6 %) of the participants have been working in TB lab for two and more years and well experienced, specifically trained with different methodologies of TB culture and identification, drug susceptibility techniques including LPA, in each round only one participant

in charge of LPA work station were participated. However 14 (51.8%) of the total professionals were not included in this PT evaluation because of limitation of resource that high cost is required even to conduct a single round of PT. Among 13 professionals who participated in the PT 12(93%), 13(100%), 13 (100%) were competent and correctly reported for susceptible strains for INH and RIF anti TB drugs , either of the two(INH or RIF) anti TB drugs and MDR-TB strains (resistance both for INH and RIF) respectively (Table 4).

Table 4 Overall Performance of RRL Professionals for susceptibility testing of M. tuberculosis.(N=13) from July 2015 to January 2016.

Performance Classification	Frequency	Percent	Cumulative Percent
Percent agreement of susceptible strains			
95-100 %	12	92.8	92.8
90-94 %	0	0	92.8
80-89%	1	7.2	100.0
<80 %	0	0	100.0
Percent agreement of Mono resistance strains			
95-100 %	13	100.0	100.0
90-94 %	0	0	100.0
80-89 %	0	0	100.0
<80%	0	0	100.0
Percent agreement of MDR strains			
95-100%	13	100.0	100.0
90-94 %	0	0	100.0
80-89 %	0	0	100.0
<80 %	0	0	100.0

Of the 130PT panels 110 (84.6%), 20 (15.4%) PT isolates for LPA were performed by BSc Degree and MSc holders respectively. From a total of 13 participants 12 (92.3%) were scored > 95 % in the first round while 13 (100%) were scored > 95 % in the second round. The relationship between participants performance with educational statutes, MTBDR*Plus* Training and TB Lab Work Experience among participants with >5years, 2-5 years and < years work experience showed a significance association (P=0.001).

Table 5 : Relationship between demographic characteristic with Passed (≥ 95 %) score on MTBc and drug susceptibility testing using LPA (N=13) from July 2015 to January 2016

Variables	Greater or Equal to 95%(Passed)	Chi-Square	Degree of freedom	p-value
Performance Vs Educational background	12	9.488	4	0.001
Performance Vs MTBDR <i>Plus</i> Training	13	5.99	2	0.000
Performance Vs TB Lab Work Experience	13	9.44	4	0.000

5.3 Participating Laboratories

On August 2015, seven TB Culture and DST testing laboratories were participated in the performance evaluation program and received first round PT panels for LPA. Six laboratories reported results: with response rates of (75%) out of six laboratories only half of them respond to the expected turnaround time of ten working days. One laboratory, initially it was functional but, dropped out due to renovations of the PCR rooms. On December, 2015 the same group of laboratories, Participated in the second round program for PT. All the seven TB culture and DST testing laboratories reported result: with a response rate 100 % and the expected turnaround time, 15 days from PT delivery of 71.4 %. Overall 6 (86%) of the laboratories were participated on both rounds and 1 (14%) participated only once. response rate across laboratories ranged from 75% to 100 % and turnaround time were ranged from 50 % to 71.4%. From July 2015 through

January 2016, the same home validated DNA PT strains were tested for Xpert MTB/RIF assay in Addis Ababa with eight testing sites: All 8 (100%), and half (50%) facilities were respond results within the expected turnaround time, of 5 days on the first and second round respectively.

5.4 PT Performance using DNA Panel

5.4.1 MTBDRplus assay

In both PT rounds between July 2015 to January 2016 a total of 130 panels (equal proportion of spiked sputum and extracted DNA), were tested in seven Regional Reference Laboratories using MTBDRplus assay. Almost all, 129 (99.2 %) were correctly reported for the Isoniazid and Rifampicin susceptibility, 69 (53.1%) were resistance to both INH and RIF (MDR), (Rifampicin resistance was identified by the *rpoB* (WT8) missing band and mutant MUT3 and where *rpoB* (WT7) missing band ,no mutant band by the LPA, which correspond to the Xpert MTB/RIF *Probe E* mutant). 41 (31.5%) susceptible to both INH and RIF (no deleted wild type and hybridized mutant band seen), and 19 (14.6%) were INH susceptible but RIF resistance strains correctly reported in comparing with the reference result. There was 100% agreement between NTRL and participating sites in detecting MDR and resistance to any of the two drugs (INH and RIF) ($\kappa = 1$) but the agreement was 97.8% for the detection of susceptible strains ($\kappa = 0.9$), where 1(0.8%) were reported Rifampicin susceptible, which was having *rpoB* (WT8) missing band and mutant MUT3 bands, by a single participant lab on the first round as false susceptible which were resistance both for Rifampicin and Isoniazid.

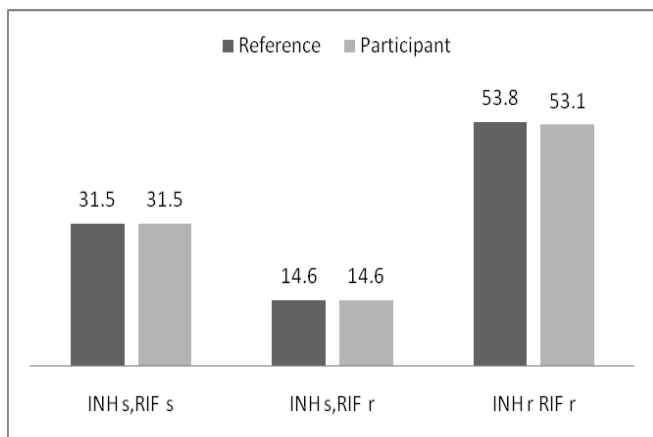


Figure 4: Summary performance of all the participating laboratories (N=7) on LPA drug susceptibility testing in percentage

The Predictive value resistance for INH and RIF were both 100% ,while the predictive value susceptible for INH and RIF were 100% and 94.4% respectively. The accuracy of the lab detecting strains for INH and RIF were 100% and 98.3% respectively, while the reproducibility were both 100%. Overall agreement on detection of Susceptible strains was 97.8% (Kappa = 0.9) which is almost 'perfect agreement' while on identification of MDR strains and resistance either of the two drugs (INH and RIF) was 100% (kappa = 1) which is a perfect Agreement .There was no false resistance report observed while, false susceptible 1(0.8%) case was reported which is an indication of loosing MDR strains.

5.4.2 Xpert MTB/RIF Assay

Among 64 home validated PT strains (32 *M. tuberculosis* DNA with no RIF resistance and 32 *M. tuberculosis* DNA with RIF resistance (*probe E* Mutant detected)), distributed to eight health facilities who have Xpert MTB/RIF sites all of them were correctly reported. The Predictive value resistance and the predictive value susceptible for RIF were both 100%. The accuracy of the lab detecting MTB and RIF susceptibility were 100%. Overall agreement on detection of *M.Tuberculosis* and RIF susceptibility was 100 % (Kappa =1.0) which is a perfect Agreement. There was no false susceptible or false resistance result report observed which is an indication of the participants lab and technology capacity to detected MTB and Rifampicin susceptibility specially proxy indicators for MDR-TB.(Table 6)

Table 6: Proficiency performance of Participant Laboratories for MTBDR*Plus* and Xpert MTB/RIF in 2015 using DNA panels.

Facilities		MTBDR <i>Plus</i>		Total	Xpert MTB/RIF		Total
		Strains Passed	Strains Failed		Strains Passed	Strains Failed	
Lab 01	Count	20	0	20	8	0	8
	% of Total	15.4%	0.0%	15.4%	12.5%	0.0%	12.5%
Lab 02	Count	20	0	20	8	0	8
	% of Total	15.4%	0.0%	15.4%	12.5%	0.0%	12.5%
Lab 03	Count	19	1	20	8	0	8
	% of Total	14.6%	0.8%	15.4%	12.5%	0.0%	12.5%
Lab 04	Count	20	0	20	8	0	8
	% of Total	15.4%	0.0%	15.4%	12.5%	0.0%	12.5%
Lab 05	Count	10	0	10	8	0	8
	% of Total	7.7%	0.0%	7.7%	12.5%	0.0%	12.5%
Lab 06	Count	20	0	20	8	0	8
	% of Total	15.4%	0.0%	15.4%	12.5%	0.0%	12.5%
Lab 07	Count	20	0	20	8	0	8
	% of Total	15.4%	0.0%	15.4%	12.5%	0.0%	12.5%
Lab 08	Count	--	--	0	8	0	8
	% of Total	--	--	0	12.5%	0.0%	12.5%
Total	Count	129	1	130	64	0.0%	64
	% of Total	99.2%	0.8%	100.0%	100.0%	0.0%	100.0%

5.5 PT performance using spiked sputum

A total 97 spiked sputum PT isolates (65 for MTBDR*Plus* and 32 for Xpert MTB/RIF) distributed for validation. 96 (99.2%) were correctly reported for MTB detection and susceptibility testing to Isoniazid and Rifampicin anti TB drugs: 37 (58.4%) resistance to both INH and RIF (MDR): Isoniazid resistance was identified by KatG wild-type (WT) missing band and a mutant (MUT1) band appeared, Rifampicin resistance was identified by the rpoB (WT8) missing band and mutant MUT3 present by the LPA, which correspond to the Xpert MTB/RIF *Probe E* mutant. 21 (30.7%) were susceptible no missed WT and hybridized MUT band present, identified by rpoB and KatG genes to RIF and INH respectively, 7 (10.7%) INH susceptible but RIF resistance was detected by rpoB (WT7) missing band, no hybridized mutant band by the MTBDR*Plus*, while 16 (50%) RIF resistance which correspond to the Xpert MTB/RIF *Probe E* mutant, 16 (50%) RIF resistance not detected strains correctly reported with respect to the reference result. There was 100% agreement in detecting MDR and resistance to any of the two drugs (INH and RIF) ($\kappa = 1$) but the agreement was 98.2% for the detection of susceptible strains ($\kappa = 0.9$). 1 (1.0%) were reported Rifampicin susceptible, which was having rpoB (WT8) missing band and mutant MUT3 bands.

5.6 PT performance using extracted DNA

Of all 97 extracted DNA panels (65 for MTBDR*Plus* and 32 for Xpert MTB/RIF) distributed for validation: All 97 (100.0%) were correctly reported for MTB detection and susceptibility testing. 36 (55.3%) resistance to both INH and RIF (MDR): Isoniazid resistance was identified by KatG wild-type (WT) missing band and a mutant (MUT1) band appeared, Rifampicin resistance was identified by the rpoB (WT8) missing band and mutant MUT3 present by the LPA, which correspond to the Xpert MTB/RIF *Probe E* mutant. 21 (32.3%) were susceptible no missed WT and hybridized MUT band present, identified by rpoB and KatG genes to RIF and INH respectively, 8 (12.3%) INH susceptible but RIF resistance was detected by rpoB (WT7) missing band, no hybridized mutant band by the MTBDR*Plus*, whereas 16 (50%) RIF resistance which correspond to the Xpert MTB/RIF *Probe E* mutant, 16 (50%) RIF resistance not detected strains correctly reported with respect to the reference result. There was 100% agreement in detecting MDR, resistance to any of the two drugs (INH and RIF) and susceptible strains ($\kappa = 1$) for both MTBDR*Plus* and Xpert MTB/RIF methods.

Table 7: Suitability performance of Spiked Sputum against Extracted DNA for panel testing of MTBDR*Plus* and Xpert MTB/RIF assays in 2015

			Proficiency Performance		Total
			Proficiency Passed	Proficiency Failed	
Isolate type	Spiked Sputum	Count (%) within Proficiency Performance	96 (98.9%)	1 1.1%	97 100.0%
	Extracted DNA	Count % within Proficiency Performance	97 100.0%	0 0.0%	97 100.0%
Total		Count % within Proficiency Performance	193 99.4%	1 0.6%	194 100.0%

5.7 Overall performance indicators by drug and laboratory

Table 8 and 9 shows the overall efficiency or accuracy of PT performance in the 7 regional laboratories participating in the two rounds of the proficiency testing.

According to the reference results for Isoniazid, agreement values of 95% or higher were obtained in all six test parameters in the two rounds average values for the 7 laboratories were: 100% for sensitivity, specificity , predictive value resistance, predictive value susceptible, and accuracy, as well as reproducibility. (Table 8)

Table 8: National TB Reference Laboratory Network DST proficiency testing Performance of seven laboratories for Isoniazid (% values)

ISONIAZID						
	No. of labs with results in the range of					Average score
	100%	95-99%	90-94%	80-89%	<80%	
SENSITIVITY	7	0	0	0	0	100.0%
SPECIFICITY	7	0	0	0	0	100.0%
PREDICTIVE VALUE RESISTANT	7	0	0	0	0	100.0%
PREDICTIVE VALUE SUSCEPTIBLE	7	0	0	0	0	100.0%
ACCURACY	7	0	0	0	0	100.0%
REPRODUCIBILITY	7	0	0	0	0	100.0%

According to the reference results for rifampicin, agreement values of 95% or higher were obtained in all six test parameters in the two rounds of testing, except for predictive value susceptible (94.4%), the two round average values for the 7 laboratories were: sensitivity (97.6%), accuracy (98.3%), 100 % for specificity, predictive value resistance and reproducibility (Table 9).

Table 9: National TB Reference Laboratory Network DST proficiency testing Performance of seven laboratories for Rifampicin (% values)

RIFAMPICIN						
	No. of labs with results in the range of					Average score
	100%	95-99%	90-94%	80-89%	<80%	
SENSITIVITY	6	0	0	1	0	97.6%
SPECIFICITY	7	0	0	0	0	100.0%
PREDICTIVE VALUE RESISTANT	7	0	0	0	0	100.0%
PREDICTIVE VALUE SUSCEPTIBLE	6	0	0	0	1	94.4%
ACCURACY	6	0	1	0	0	98.3%
REPRODUCIBILITY	7	0	0	0	0	100.0%

6 DISCUSSION

While molecular methods have considerable advantages for scaling up programmatic management of drug resistant TB by offering rapid results and potential for high throughput, the care of patients with tuberculosis (TB) starts with a quality assured diagnosis [16]. In the context of the global drive to scale up diagnostic and treatment services for MDR-TB, rapid molecular tests such as LPA will play an increasingly important role in early detection and start of treatment. With the expanding availability of these low cost rapid tests for the detection of Multidrug resistance TB both in public and private sector the quality of test results being provided routinely needs to be ensured. Easy to implement standard quality assurance procedures are crucial to ensure that laboratories are proficient in laboratory procedures and are providing reliable results to providers, so that patients with MDR-TB are accurately detected and those Without MDR TB are not subject to unnecessary expensive and potentially toxic treatment.

In the current study which was conducted to assess the performance of RRL using Genotype MTBDR*plus* version 2, LPA using home validated PT material was conducted and an agreement between participant lab and National Reference Laboratories in the detection of M. tuberculosis was 94.4.% we should also in agreement with the study conducted in Johannesburg ,South Africa which was 96%. An overall agreement(kappa = 0.9) our study finding on detection of M. tuberculosis between the reference lab and the sites ,which is classified as ‘almost perfect agreement’ based on kappa index [,33], and it was similar to the study conducted in, South Africa (Kappa = 0.9).

Overall, the sensitivity ,specificity ,predictive value susceptible, predictive value resistance and accuracy was all 100% in the National TB Reference laboratory validation result which is similar to the result of the research laboratory in Johannesburg, South Africa, which was 100%. An overall agreement of (kappa = 1.0) which is perfect. Professionals in detecting susceptible and resistance for INH and RIF were 100 % and 96.7%, respectively. Both the sensitivity and specificity of this study were higher even though, no similar studies conducted in our country this study finding showed that our Regional Reference TB culture and DST laboratories quality to diagnose MDR-TB is very good and plays a significant role to control nationwide transmission of TB by reducing delay in treatment and prolonged hospitalization, which is the

priorities of the national TB control program. This is not simply achieved rather frequent capacity building through, recruiting the right professionals, training, experiencing, and a close site supervision of the NTRL.

In a similar study conducted in India after initial LPA training, each project site collected remnant sputum specimens from 50 smear-positive patients for anonymous LPA testing. A proficiency assessment mechanism for LPA testing was developed and piloted in the 6 laboratories. These laboratories were already providing MDR-TB diagnostic services using LJ C&DST. However, these reference laboratories had no prior experience in the implementation of LPA. As a result Only 2 of the 6 laboratories achieved proficiency benchmarks on the first attempt. Three of the six laboratories were successful in achieving the proficiency benchmarks on the 2nd or 3rd attempt after retraining . However, in the present study six of the eight laboratories that began proficiency assessment successfully achieved proficiency benchmark in the first round and seven of the seven laboratories were achieved proficiency benchmarks on the second round successfully because the laboratories were gone through different training and experience in the implementation of LPA [34].

In the study conducted South African, Johannesburg to assess the implementation of Xpert MTB/RIF quality requirement a pilot program using dried culture spots (DCSs) of inactivated *Mycobacterium tuberculosis* were described of 274 DCS results received, 2.19% generated errors; the remainder yielded 100% correct *Mycobacterium tuberculosis* detection. The probe A cycle threshold variability of three DCS batches was <3.47. In the present study even though, liquid inactivated *Mycobacterium tuberculosis* strains were applied of the 64 results received, 100% yielded correct *M. tuberculosis* positivity and RIF sensitivity with probe E mutated resistance showed an excellent agreement to use as an option for PT material for molecular tests [31,35].

The performance of reporting DST results of rifampicin and isoniazid from Saudi Arabia were assessed in 2009-10. An automated liquid culture-based DST and a molecular mutation detection technique were used. Performance of first-line drug susceptibility testing of 1904 clinical isolates showed 44 inconsistent results. The majority of the cases reported as MDR-TB from the referral laboratories could not reproduce the same results at a different site (Mycobacteriology Research Section). Of the 44 cases, 16 (36.3%) showed false resistance to isoniazid and rifampicin and on the other hand, 14 (31.8%) cases showed false susceptibility to the same drugs. An urgent

reevaluation and training were needed for the staff involved in the TB-DST. Even though the discrepancies are common in low grade around the globe, false reporting of MDR-TB cases has serious clinical implications. In this study though, the method used are different and the number of PT isolates are not that much comparable with the Saud Arabia, in the present study accuracy of the lab detecting strains for INH and RIF were 100% and 98.3% respectively, There was no false resistance report observed while, false susceptible 1(0.8%) case was reported which is an indication of loosing MDR strains, which needs further root cause analysis to identify the source of error the discrepancy from the obtained result compared to the Saud Arabia indicate an implication of good performance consequence on better patient management rely on the ability and experience of professionals [36]

In summary ,this study showed that the current home validated DNA material is able to assess the proficiency of a laboratory in conducting DST which contribute to better diagnosis of drug-resistant TB and in turn lead to more effective control of TB in the country so, it should therefore continue to be promoted as one of the available tools for DST quality assessment.

7 LIMITATION OF THE STUDY

This performance assessment MTBDRplus version 2 and Xpert MTB/RIF assay through proficiency testing using home validate PT material doesn't take into account all professionals working in the Laboratories only those who assigned in each work stations were included. This mechanism assesses the proficiency of the laboratory as a unit, and assigned personnel, the other aspect may be of a major concern where there are many technologists. The number of participants in Xpert MTB/RIF assay were very small compared to the general population.

In this study culture positive spiked sputa and Extracted DNA were assessed but, processed specimens sediment which is positive for smear ,were not included to know the correlation between LPA results and smear grading .

8 CONCLUSION AND RECOMMENDATION

In this study we should conclude that the current home validated genotypic PT materials were suitable, able to assess the proficiency of a laboratory in conducting MTBDR*Plus* drug susceptibility test for MDR-TB in Ethiopia. Whereas in Xpert MTB/RIF assay even though, the selected sites in Addis Ababa, are small, compared to the general population, the finding in this study is very promising. This study provides a preliminary demonstration through the use of inactivated *M. tuberculosis* material that an EQA program can be safely provided.

We recommend from these results that the performance of PT DST will be maintained at a high level and can even be significantly improved, if a large scale assessment conducted in addition to this with other possible more safe and less cost alternations available like DCS in parallel. However, proficiency testing does not necessarily reflect routine performance and it should always be used in conjunction with internal quality control and, if possible, also re-testing of routine samples at National reference laboratory. All laboratories conducting DST should be encouraged to participate in annual proficiency testing for all methods. National tuberculosis programmes should also provide both financial and technical support for DST quality assurance systems.

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International Journal of Mycobacteriology 2 2013; 14 – 17

9 ANNEX

9.1 Survey questionnaire (English version)

Questionnaire for “Performance Evaluation of health professionals for *MTBDRPlus* working at Regional Reference TB culture and DST Laboratories in Ethiopia, 2015.

Questionnaire Number: _____ Code of Health Facility: _____

Interviewee Code : _____ Date of Report: _____

Socio-demographic information

- 1) Age (in years) _____
- 2) Sex
 - a) Male b) Female
- 3) Educational status
 - a) Diploma
 - b) Degree
 - c) MSc and above
 - d) Other Specify-----
- 4) Work Experience in TB culture Laboratory
 - a) ≤ 1 year
 - b) 1-5 Years
 - c) ≥ 5 Years
- 5) Types of Health Facility
 - a) RRL
 - b) Specialized Hospital TB Lab
 - c) Other Specify-----
- 6) Are you full time employee
 - a) Yes b) No
- 7) Have you taken any training on TB culture or DST
 - a) Yes b) No

9.2 Sop for MGIT culture inoculation and incubation

Equipment and materials

- MGIT960 instrument
- BSC cabinet, class II, certified
- Racks 50 ml Falcon tubes
- Racks for MGIT 7 ml tubes
- Adjustable automated pipettes, 1 ml or repeat pipettor
- Sterile filtered tips, 1 ml or tips for the repeat pipettor
- Sterile serological pipettes, 10 or 25 ml
- Sterile Pasteur pipettes, graduated, 1 or 3 ml
- Sharps container
- Biohazard bags, autoclavable
- Permanent marker
- Glass slides
- Pencil or diamond pen

Reagents and solutions

- MGIT 7 ml tubes
- MGIT growth supplement
- PANTA
- Disinfectants (tuberculocidal)
- Phosphate buffer, 0.01 M, pH 6.8

Detailed instructions for use

Reconstitution of PANTA

- Label the PANTA bottle with the date and initials
- Bring the growth supplement tubes to room temperature before start
- Aseptically open bottles of supplement and PANTA
- Add 15 ml of growth supplement to each of PANTA bottles
- Close the bottle and mix until PANTA powder dissolved

Preparation of MGIT 7 ml tubes

- Label the tubes with the laboratory number, the date of inoculation and initials
- Add 2 tubes for positive and negative controls
- Place positive control tube at the last before the last position and the negative control tube at the last position
- Prepare the MGIT 7 ml before inoculation of specimens
- Slightly loosen the caps of the tubes
- Add 0.8 ml of reconstituted PANTA to each of the tubes
- Close the tubes tight immediately
- In case of leftover of the reconstituted PANTA keep it at 2 – 8⁰C and use within 5 days; do not freeze
- Add reconstituted PANTA to MGIT tubes right before inoculation, do not store prepared tubes

Inoculation of MGIT 7 ml tubes

- Work in BSC
- Open tubes for one specimen at a time
- Use separate pipette for each specimen
- Add 2 ml of phosphate buffer to the specimen sediment and mix well
- With the same pipette inoculate 0.5 ml of specimen to each tube and prepare smear (refer to SOP “Specimen processing for culture” for smear preparation)
- Close the tube tight immediately and mix well by inverting several times
- Let the tubes stand at room temperature for 30 min

Incubation of inoculated MGIT 7 ml tubes

- Perform daily maintenance of the MGIT960 instrument
- Enter the tubes into the instrument (SOP “Use and maintenance of MGIT960 instrument”)
- Incubate the tubes in the MGIT960 until it flags them positive or negative (in 42 days)

Reading, interpretation, recording and reporting

- Prepare worksheets prior start of work to include list of specimens, quality controls, date of culture set up, initials and lot numbers of MGIT 7 ml tubes, growth supplement and PANTA
- Record maintenance of MGIT960 instrument and BSC in the relevant log sheets

Quality control

- Use quality controls with every batch of specimens
- Use sterile phosphate buffer for negative control and suspension of *M. tuberculosis* quality control (QC) strain (equal for McFarland standard 0.5; SOP “Preparation of McFarland standard”), diluted 1:500 as positive control; process the controls the same way and at the same time as specimens
- Interpret result for negative control as acceptable if no growth detected
- Interpret results of positive control as acceptable if time-to-detected is within a specified time for testing. Establish baseline time-to-detected by collecting data from several tests/runs
- Open one tube at a time and work with one specimen at a time
- Maintain MGIT960 instrument and BSC before work

Waste management and other safety precautions

- Dispose of used materials as per the laboratory SOPs and the national regulations
- Disinfect and decontaminate surfaces according to the laboratory procedures
- Make sure the BSC reading are within the accepted range before start of the procedure
- Use personal protective equipment for culture/DST laboratories while performing this procedure
- Air dry smear inside BSC; do not flame wet smears
- Transfer infectious materials only on a laboratory trolley

9.3 Sop for Preparation of Artificial Sputum

The purpose of this Standard Operating Procedure (SOP) is to describe the method of preparing artificial specimens to help monitor cross contamination. These artificial specimens will be submitted blindly to the sample processing team and should have negative results for both microscopy and culture.

If positive results are received as an outcome of these control artificial sputum, this demonstrates cross contamination occurred and correctional measures should be implemented to this effect.

This SOP is applicable to all laboratory employees of the National Tuberculosis Reference Laboratory, NTRL responsible for the preparation of the artificial sputum.

Abbreviations, definitions and terms

- MGIT Mycobacteria Growth Indicator Tube
- NTRL National Tuberculosis Reference Laboratory
- NTLP National Tuberculosis and Leprosy Control Programme
- CPC Cetylpridinium chloride
- LJ Löwenstein Jensen
- NALC N acetyl L cysteine
- NaOH Sodium Hydroxide
- N.A. Not applicable
- QC Quality Control
- SOP Standard Operating Procedure

Safety and environment

- For safety measures use Personal Protective clothing and equipment (gloves, laboratory coat and lamina flow hood) must be used when performing this procedure.
- In case of biohazard incident/Accident, refer to SOP P012 “Safety Manual”

Reagents and Materials

- 50 ml sterile conical tube
- Balance sensitive to 0.1g
- Lamina flow hood
- Disinfectant (e.g. phenol, Lysol)
- Incubator
- Autoclave
- Methyl cellulosulphate powder
- Blood agar plate
- At least 3 eggs
- Sterile distilled water.

Procedure

- i. Weigh 5.0g of methylcellulose
- ii. Add to 500ml sterile distilled water
- iii. Mix to dissolve by using a Bunsen flame to facilitate the dissolving process.
- iv. Emulsify the 3 eggs/50mls of egg albumen-**do not include the egg yoke.**
- v. Add emulsified egg to the mixture when methylcellulose is dissolved
- vi. Autoclave for 20 minutes at 121°C
- vii. Leave to cool and inoculate on Blood Agar.
- viii. Aliquote about 5ml in sterile falcon tubes ready for use and store at 2-8°C in a refrigerator.

Quality control

Inoculate the prepared media onto one blood agar plate-there should be no growth after 24-48 hours.

Prepared by.....

Date.....

ARTIFICIAL SPUTUM PREPARATION WORKSHEET

Item	Amount	Amount
Methylcellulose	5g	10g
Egg albumen used	50ml	100ml
Distilled water	500ml	1000ml

Quality control

Is there growth on the blood agar plate after 24-48 hours

Yes/No.....

If there is growth on the blood agar plate, then discard the entire batch of the prepared artificial sputum, fill out an occurrence management form and then repeat the process with a new batch of eggs.

Prepared artificial sputum is stored at 2-8C for 1 month

Tech/date..... Reviewed by/date.....

9.4 Sop for Extraction, Amplification and Hybridization of DNA for HAIN Probe Assays

Equipment and materials

- Bio-safety cabinet, Class II, certified
- Vortex and mini-vortex
- Micro centrifuge, at least 10 000 g, refrigerated, with bio-safety rotor
- Mini centrifuge for spin down
- Heat block, at least 95⁰C
- Hot oven, at least 95⁰C
- Ultra sonic bath
- Thermal cyclers, specifications
- Water bath, at least 45⁰C
- TwinCubator or GT-BLOT 48
- Automated pipettes, 5 µl, 10 µl, 50 µl, 200 µl and 1000 µl
- Sterile filtered tips for the automated pipettes, 5 µl, 10 µl, 50 µl, 200 µl and 1000 µl
- Sterile Pasteur pipettes 1 or 3 ml
- Sterile disposable plastic loops
- Timer
- Sterile, Eppendorf tubes, 1.5 or 2.0 ml
- PCR tubes, to fit the thermal cycler
- Hybridization trays
- Forceps
- Cellophane tape
- Paper towels
- Frosted slides
- Pencil, permanent markers, marker for strips
- Personal protective equipment
- Soft cloth

Reagents and solutions

- Line probe assay kits
- Sterile molecular grade, DNase-, RNase-, pyrogen-free water

- Sodium hypochlorite, 1%, freshly prepared
- Alcohol, 70%

Detailed instructions for use

Preparation of processed for culture specimens for decontamination (heat-kill)

- Work in a dedicated room/area (refer to SOP “Prevention of contamination in line probe assay laboratory”)
- Handle infectious materials in BSC
- Transfer 0.5 ml of sediment suspended in phosphate buffer into sterile 1.5 – 2.0 ml conical Eppendorf tubes properly labelled with the laboratory number and date
- Open one tube and one specimen at a time to prevent cross contamination
- Load the rotor inside BSC
- Gently wipe the tubes with 1% solution of sodium hypochlorite before removal from BSC and placing into the rotor
- Place the tube into the rotor in such a position so that to make location of the pellet position easily notable
- Secure the rotor lid
- Centrifuge the tubes at 10 000 g for 15 min
- Remove the tubes inside BSC in 2-5 min after the centrifugation stopped
- Let the tubes stand for 1-2 min in a rack before you proceed for decanting
- Gently aspirate and discard the supernatant using automated pipette and 1.0 ml sterile filtered tips
- Use new tip for each sample
- If the pellet is disrupted sediment it down again
- After all samples were decanted add 100 µl of molecular grade water, vigorously pipette the pellet and then vortex for 1 min to ensure homogeneity of the suspension to facilitate the heat-kill

Preparation of liquid cultures isolates for decontamination

- Work in a dedicated room/area (refer to SOP “Prevention of contamination in line probe assay laboratory”)
- Work in BSC
- To prepare liquid culture isolates vortex MGIT tubes for 2 min to break the clumps
- Transfer 1.0 ml of culture to a sterile conical Eppendorf tube 1.5 – 2.0 ml
- Vortex the tubes again for 1 min to make sure the clumps completely broken up

- Refer to the section 4.5.1 for the next steps

Preparation of solid culture isolates for decontamination

- Work in a dedicated room/area (refer to SOP “Prevention of contamination in line probe assay laboratory”)
- Work in BSC
- Re-suspend representative colonies in 300 µl of molecular grade water using sterile loops
- Make sure to avoid pick up of Lowenstein-Jensen media to prevent inhibition of PCR reaction
- Vortex the tubes for 1 min break the clumps
- Refer to the section 4.5.1 for the next steps

Decontamination (heat kill)

- Work in a dedicated room/area (refer to SOP “Prevention of contamination in line probe assay laboratory”)
- Inactivate live bacilli in the suspensions by heating in pre-warmed heat block or hot oven at 95⁰C for 20 min
- Check the temperature right before placing the tubes
- If you use a hot oven for decontamination keep an appropriate rack inside to make sure it is always pre-warmed

Extraction of DNA by mechanical method

- Work in a dedicated room/area (refer to SOP “Prevention of contamination in line probe assay laboratory”)
- De-gas the distilled water in the sonicator for 15 min
- Check the sonicator’s function as follows: draw a medium thick pencil line on the frosted end of a slide; submerge the end into the de-gassed water; the line should disappear in 1-2 min
- Place the tubes into a floating rack to keep the tubes upright
- Disrupt the bacilli by ultrasound sonification for 15 min
- After sonification is complete place the tubes on a paper towel to remove water from outside
- Centrifuge the tubes at 13 000 g for 5 min
- Transfer the supernatants into fresh labeled tubes
- Store the supernatants at +4⁰C to -20⁰C

- Discard the water from the sonicator and clean with 1% sodium hypochlorite followed by 70% alcohol

Preparation of master mix

- Work in a dedicated room/area (refer to SOP “Prevention of contamination in line probe assay laboratory”)
- Clean the room/area as per SOP “Prevention of contamination in PCR laboratory” before and after the procedure
- Label PCR tubes with laboratory numbers quality control identifications
- Label the last 3 tubes as follows: PC (positive control), EC (extraction control) and MC (master mix control)
- Estimate amount of reagents to be used based on the number of samples and quality controls to be tested plus one or two to account for loss on pipettes tips
- Remove the needed amount of reagents from fridge/freezer and thoroughly thaw and mix
- Spin down the drops from the walls and the caps if necessary
- Keep the DNA polymerase and the reagents on the bench in a mini-cooler during the procedure
- Combine ingredients of the PCR reaction in the order indicated in the Table 1 in an Eppendorf tube of an appropriate size
- Open one reagent tube at a time only to prevent contamination and close the reagent tube as soon it is added
- Visually observe the addition of DNA polymerase the tube to make sure it is added
- Gently vortex the tube and spin down for 10 – 15 sec after all reagents added
- Distribute the master mix to labeled with laboratory numbers PCR tubes by 45 µl
- Cover the lid of the PCR rack with the master mix tubes and take the rack to the room/area for addition of DNA templates
- If the tubes are not used the same day keep them at – 20°C

Table 1 Preparation of master PCR mix

Ingredient	Reaction volumes (µl)				
	1x	2x	8x	24x	24x+2x
PNM mix	35	70	280	840	910

Buffer	5	10	40	120	130
MgCl ₂	2	4	16	48	52
Water	3	6	24	72	78
DNA polymerase	0.2	0.4	1.6	4.8	5.2

Addition of DNA template

- Work in a dedicated room/area (refer to SOP “Prevention of contamination in line probe assay laboratory”)
- Clean the room/area as per SOP “Prevention of contamination in PCR laboratory” before and after the procedure
- If master mix tubes were kept at – 20⁰C thoroughly thaw
- Add 5 µl of DNA extracts to the corresponding PCR tubes, 5 µl of DNA extract from H37Rv quality control strain to the PC tubes and 5 µl of distilled water the negative control tubes
- Open one tube at a time to prevent contamination and cross-contamination of PCR reaction
- Use a new tip for each sample
- Discard and replace tip in case it touched irrelevant surfaces
- Discard the tips into 1% sodium hypochlorite solution
- Right before addition of samples to master mix tubes to gently agitate the DNA template extract to bring negatively charged DNA stacked to the wall into the solution
- After addition of template gently mix the solution by pipetting up and down
- Open one PCR tube at a time and immediately close after addition of template to prevent contamination and cross contamination
- Spin the tubes for 10 -15 sec to bring down the drops
- After all samples added place the tubes into PCR machine if it is in the same room
- If PCR machine is in another location close the lid of the PCR rack with the prepared PCR tubes and take it to the area

Amplification

- Work in a dedicated room/area (refer to SOP “Prevention of contamination in line probe assay laboratory”)

- Clean the room/area as per SOP “Prevention of contamination in PCR laboratory” before, after placing the tubes into the PCR machine and after removal of the tubes from the instrument
- Place PCR tube into the machine evenly distributing the tubes over the block
- Use separate PCR protocols for direct testing of processed specimens and for culture isolates (for the protocols refer to the Table 2)
- Avoid using the isolates protocol for direct testing as the number of cycles is smaller culture isolates is smaller and this can lead to insufficient amount of PCR product and subsequently to false negative results
- Avoid using the direct specimen protocol for testing of isolates as the amount of template in the DNA extract from isolates is much higher and the reagents will be depleted before number of copies of the DNA product reaches the needed level. This also can lead to false negative results
- After completion of the protocols relocate the tubes into a PCR rack, cover the lid and take the tubes to the hybridization room/area

Table 2 Amplification protocols for line probe assays by type of samples

Step	Processed specimens			Culture isolates		
	t ⁰ C	Duration	No of cycles	t ⁰ C	Duration	No of cycles
Initial denaturation	95	15 min	1	95	15 min	1
Denaturation	95	30 sec	10	95	30 sec	10
Elongation	58	2 min		58	2 min	
Denaturation	95	25 sec	30	95	25 sec	20
Annealing	53	40 sec		53	40 sec	
Elongation	70	40 sec		70	40 sec	
Final extension	70	8 min	1	70	8 min	1

Hybridization

- Work in a dedicated room/area (refer to SOP “Prevention of contamination in line probe assay laboratory”)

- Clean the room/area as per SOP “Prevention of contamination in PCR laboratory” before and after the procedure

Preparation to hybridization

- Pre-warm hybridization buffer (HYB) and stringent wash solution (STR) to 45⁰C to dissolve all crystals
- Bring to the room temperature rinse solution (RIN) and distilled water
- Estimate amount of the working solutions of conjugate and substrate needed as the total number of well plus 1
- Freshly dilute the concentrates 1:100 with the relevant diluting solutions (CON-C with CON-D and SUB-C with SUB-D)
- Keep the solutions protected from light (wrapped in foil or placed in a drawer) until use

Denaturation

- Put 20 µl of denaturation solution (DEN) in each well of the hybridization tray; add 20 µl of DNA PCR products in each tray and mix by pipetting up and down. Avoid bubbling and spillage to prevent cross-contamination
- Incubate at room temperature for 1 min (at the bench).
- Add 1ml of pre-warmed hybridization solution (HYB) to each well containing DEN + amplified DNA.
- Mix the solution by gentle tilting the mixture up and down

Incubation of strips with the denatured amplicon

- Perform hybridization either in TweenCubator or in GT-Blot 48 (the GT-Blot will perform the described steps automatically; for use and maintenance of the instruments refer to the relevant SOPs)
- Label the strips according to the samples labeling, using DNA strip marker and forceps for clean strips
- Using forceps for clean strips place the labeled strips to the corresponding wells
- Incubate for 20 min. at 45⁰C in the Twincubator or in GT-BLOT 48
- After completion of hybridization remove the hybridization solution from each well using individual pipette/tip

- Discard pipettes/tips into 1% sodium hypochlorite solution

Washing

- Use individual pipettes/tips for each solution and well. Use filtered tips if an automated pipette is used
- Discard pipettes/tips into 1% sodium hypochlorite
- Add 1 ml of STR (fixing and washing solution) to each well of the tray
- Incubate for 10 min at 45⁰C
- Remove the STR completely from each well
- Add 1 ml of RIN (rinsing solution) to each well of the tray and incubate for 1 min at room temperature
- Remove the whole quantity of RIN

Conjugation

- Add 1 ml of the diluted 1:100 conjugate incubate for 20 minutes at 37⁰C
- Aspirate the conjugate completely.
- Add 1ml of RIN (rinsing solution) to each well of the tray and incubate for 1 min. at room temperature
- Aspirate the whole quantity of RIN completely, and then rinse once with water for 1 min

Detection

- Add 1 ml of the diluted 1:100 substrate. Dilution to each tray and incubate for 2-10 minutes at room temperature Aspirate the substrate completely after the band are sufficiently developed
- Stop the reaction by rinsing twice with water for 1 minute in each rinse

Transfer of strips to the score sheet

- Remove the developed strips from the tray and air partially dry DNA strips on absorbent paper protected from light. Remaining water in the strips will prevent the strips from sticking to the cellophane tape.
- Using forceps dedicated to handling of hybridized strips and cellophane tape attach the strips to the score sheet

- Make sure that the conjugate control (CC), amplification control (AC) and TUB bands on a test strip aligned with the respective lines on the core sheet
- Let the strips on the score sheet completely air dry protected from the light

Reading, interpretation, recording and reporting

- Prepare worksheets prior to work separate for the direct specimens and for culture isolates
- Record cleaning and maintenance of equipment in the relevant log sheets
- Report failure of equipment in the equipment failure log/register

Quality control

- Strictly follow the procedure
- Use quality controls with every batch of tested samples
- Use quality control strain of *Mycobacterium tuberculosis* H37Rv as a positive control
- Use phosphate buffer used for dilution of specimens sediments and solution used for DNA extract from culture isolates as the extraction negative control
- Use sterile distilled water as the negative master mix control
- Place quality control tubes in the last 3 positions of the testing batch in the following order: positive control, the extraction negative control and the master mix control
- Perform the procedures in dedicated areas only
- Perform cleaning of the PCR facilities and maintenance of equipment according to the relevant SOPs
- For detailed quality control measures refer to the SOP "Procedure for quality control of line probe assays"

Waste management and other safety precautions

- Discard disposable pipettes/tips into 1% sodium hypochlorite
- Discard the sodium hypochlorite and other waste according to the laboratory procedure for waste management and the national regulations
- Do not autoclave sodium hypochlorite solutions as it released chlorine gas during heating what may cause explosion and corrosion of autoclave
- Follow the laboratory safety procedures

9.5 LPA validation Result Technologist 1



GenoType MTBDRplus 96
 ER 2.0
 IQ4A-0713-03-2

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6
2011
dd mm yyyy






TUB	rpoB WT	rpoB MUT	KatG WT	KatG MUT	inhA WT	inhA MUT	RMP	INH
	sensitive	resistant	sensitive	resistant	sensitive	resistant		


NC	Ref Control (Molecular grade H ₂ O)	1	2	3	4	5	6	7	8	9	10	PC	pos Control (H37Rv)						
		7321	3380	6586	9047	1217	9508	1704	7585	9173	6339								

01000058

 HYB 30 min
 
 STR 15 min
 
 SUB 10 min


Jepain

9.6 LPA validation Result Technologist 2

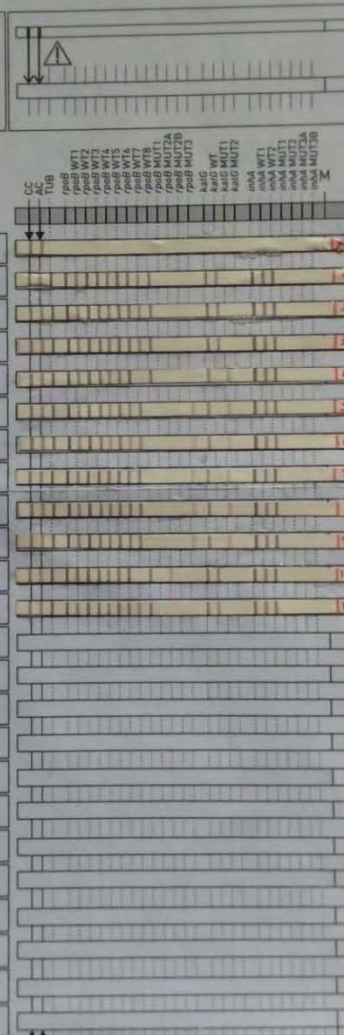


GenoType MTBDRplus 96
 VER 2.0
 0304A-0311-03-1

23 06 2015
 dd mm yyyy


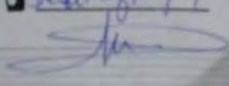
#	Icon 1	Icon 2	Diagram	TUB	rpoB-WT	rpoB-MUT	katG-WT	katG-MUT	inhA-WT	inhA-MUT	RMP	INH
											sensitive	resistant
											resistant	sensitive
											resistant	resistant

Wc	Neg Control (Molecular quality)
1	7321
2	3350
3	6586
4	7047
5	1217
6	9508
7	1704
8	9585
9	9173
10	6339
PC	H37RV



1	-	-	-	-	S	S
2	+	+	+	+	S	S
3	+	+	+	+	S	S
4	+	-	-	+	R	R
5	+	+	+	+	R	R
6	+	+	+	+	R	R
7	+	+	+	+	R	R
8	+	+	+	+	R	R
9	+	+	+	+	R	R
10	+	+	+	+	R	S
PC	+	+	+	+	S	S

LOT 0100058 HYB 30 min STR 15 min SUB 10 min


Technologist A


9.9 Declaration

I ,the undersigned, was conducted this research by adhering with all responsibilities for the scientific and ethical conduct of the research . I was provided a timely progress report to my advisors and got the necessary advise and approval from them in the course of the research .I was communicated timely to my advisors and all stakeholders involved in the study including source of funding for this research.

Principal Investigator : Zekarias Dagne

Signature: _____ Date : _____

Advisor : Kassu Desta (MSc, Assistant. Professor)

Signature: _____ Date : _____

Co- advisor : Walelign Dessie (MSc)

Signature: _____ Date : _____