COMPARISON OF XPERT MTB/RIF ULTRA AND LINE PROBE ASSAY FOR DETECTION OF RIFAMPICIN-RESISTANT MYCOBACTERIUM TUBERCULOSIS AT JIMMA UNIVERSITY MEDICAL CENTER, SOUTHWEST ETHIOPIA

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Jimma, Ethiopia

JIMMA UNIVERSITY

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Abstract

Background: *The new Xpert MTB/RIF Ultra was introduced to overcome imperfect sensitivity of first generation Xpert MTB/RIF in detection of tuberculosis (TB) and rifampicin-resistance. Given the rapid changes in the TB diagnostic arena, the added value of Xpert MTB/RIF Ultra for detection of RIF-resistant M. tuberculosis compared to VER 2.0 LPA and phenotypic drug susceptibility test (DST) is not well investigated.*

Objectives: *To compare diagnostic performance of Xpert MTB/RIF Ultra and MTBDRplus VER 2.0 line probe assay in detection of rifampicin-resistant M. tuberculosis in Jimma University Medical Center, Southwest Ethiopia.*

Methods: *A Hospital based cross-sectional study was conducted from June 2020 to October 2021 in Jimma, Southwest Ethiopia. A total of 275 pulmonary TB suspected patients were selected consecutively. Each study participants provided two cups of sputum samples. The first cup of sputum was subjected for Xpert MTB/RIF Ultra and the second sputum was processed for culture. Culture positive isolates were subjected for MGIT-DST and LPA. Sensitivity and specificity were determined to assess the performance characteristics of the Ultra and LPA.*

Results*: Of 275 paired-sputum samples tested, altogether 57 culture and Ultra positive samples were included. By MGIT-DST, 21 isolates were rifampicin-resistant and 36 were rifampicin-sensitive. There was one discrepant result, which was rifampicin-sensitive by Ultra but rifampicin-resistant by MGIT-DST. Against MGIT-DST as the gold standard, Ultra had a sensitivity and specificity of 95.5% and 100% respectively and showed excellent agreement with MGIT-DST (Kappa=0.96 (95%CI =88.8-99.8)). LPA correctly detected all rifampicin-resistant cases detected by MGIT-DST with 100% sensitivity and 100% specificity. We also found 96% overall agreement of two molecular methods (Ultra and LPA) for the detection of rifampicin-resistance.*

Conclusion: *Ultra has similar diagnostic performance with that of LPA for the detection of rifampicin-resistance. Considering its shorter turn-around time, relatively easier to perform and minimum biosafety requirement, the new Xpert MTB/RIF Ultra can be used for the detection of rifampicin-resistant and to screen for MDR-TB in Ethiopia.*

Key words*: Xpert MTB/RIF Ultra, LPA, sensitivity, specificity, Drug resistant TB*

Acknowledgement

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Abbreviations

DNA	Deoxyribo Nucleic Acid
DR-TB	Drug Resistance Tuberculosis
DST	Drug Susceptibility Test
HIV	Human Immune Deficiency Virus
INH	Isoniazid
JUMC	Jimma University Medical Center
LJ	Lowenson Jeensen
LPA	Line Probe Assay
MDR-TB	Multi Drug Resistance Tuberculosis
MGIT	Mycobacterial Growth Indicator Tube
MRC	Mycobacteriology Research Center
MTB	Mycobacterium Tuberculosis
NALC-NAOH	N-Acetyl-L-Cysteine sodium hydroxide
PCR	Polymerase Chain Reaction
RIF	Rifampicin
RRDR	Rifampicin Resistance Determining Region
RR-TB	Rifampicin Resistance Tuberculosis
SOPs	Standard Operational Procedures
SPSS	Statistical Package for Social Sciences
TB	Tuberculosis
WHO	World Health Organization

1. INTRODUTION

1.1. Background

Tuberculosis (TB) is an infectious disease caused by the *Mycobacterium tuberculosis* that largely affects the lungs. MTB is slender, curved rods aerobic, non-motile bacteria. It belongs to slow-growing intracellular bacilli of *the Mycobacteriaceae* family of the order of *Actinomycetales*. The main characteristic distinctive feature of this family is special staining properties due to their unique and complex cell wall. In this family, the genus Mycobacterium is classified into members seven very closely related mycobacterial species collectively called Mycobacterium tuberculosis complex, (1).

Though all the member of MTBC can cause TB in human, TB is mainly caused by MTB. Until the coronavirus (COVID-19) pandemic, TB was the leading cause of death from a single infectious agent, ranking above HIV/AIDS. Globally about 9.9 million (8.9–11.0 million) people developed the disease, which is equivalent to 127 cases (114–140) per 100 000 population, Moreover, about 1.5 million died from the disease including 214,000 deaths among people living with Human Immunodeficiency Virus (HIV). About 84% of TB deaths among HIV-negative people and 85% of the combined total of TB deaths in HIV-negative and HIV-positive people occurred in the WHO African and South-East Asia regions. (2).

The emergence of multidrug resistance TB (MDR-TB), TB with resistance to at least two main drugs isoniazid and rifampicin, and extensive drug-resistant TB (XDR-TB), TB caused by MDR TB tuberculosis strains which resistant to, any fluoroquinolones, and either bedaquiline or linezolid (or both); is a major public health threat worldwide, these are the main challenge to the global effort to fight TB (3). Globally 4.8 million peoples diagnosed with pulmonary Tb in 2020 and 59% were bacteriologically confirmed. Among these, 132 222 cases of MDR/RR-TB and 25 681 cases of pre-XDR-TB or XDR-TB were detected (2).

Ethiopia is among the 30 high TB and TB/HIV burden countries globally with an estimated TB incidence rate of 140/100,000 populations (157,000 persons annually); and 21,000 (19/100,000 population) TB deaths (4). The prevalence of RR-TB is 1.1% among new and 7.5% among previously treated TB cases, respectively and MDR prevalence was 1.03% among new and 6.52%

among previously treated TB patients according to the preliminary report of the 2019 national TB Drug Resistance Surveys (DRS) (5). According to second round survey the prevalence of MDR-TB was increased both in new (2.3%) and previously treated cases 17.8 (6). the overall prevalence of MDR-TB in all TB cases was estimated to be 1.4% (7).

One of the main challenges in detection of rifampicin resistant/MDR TB is long TAT of phenotypic DST(8). To overcome this, in 2008, the WHO endorsed the use of molecular line probe assays (LPA) for the detection MDR-TB in patients with smear positive or culture positive TB (9). LPAs had a shorter TAT (2-3 days) than Phenotypic DST but LPAs are more technically complex (designed for reference or regional laboratory settings). In order to minimize complexity and to improve TAT, in 2011, the WHO recommended replacement of LPA by the Xpert MTB/ RIF (Xpert) assay (Cepheid, Sunnyvale, USA), a molecular test that allows rapid diagnosis of *Mycobacterium tuberculosis* complex and simultaneous detection of resistance to rifampicin(10). However, Xpert MTB/RIF assay has low sensitivity in detection of smear-negative, pediatric and HIV-associated TB(11).

Since March 2017, the next-generation Xpert MTB/RIF Ultra assay was introduced to overcome the low sensitivity of Xpert MTB/RIF in smear-negative, pediatric and HIV-associated TB and to correct some of its limitations in the identification of rifampicin resistance(11). It has been suggested that, the sensitivity has been improved by: i) targeting the multi copy IS6110 and IS1081 genes for the detection of MTB, ii) applying or using more rapid thermal cycling with fully nested nucleic acid amplification, iii) using improved fluidics and enzymes, iv) using a larger DNA reaction chamber than the Xpert MTB/RIF . As a result, the assay able to detect traces of MTB DNA (a new semi-quantitative category with indeterminate RIF resistance) which were undetectable with the previous version of Xpert MTB/RIF which targeted only the Rifampicin Resistance-Determining Region (RRDR) of the MTB *rpoB* gene (12).

The threshold of detection of Xpert MTB/RIF Ultra is 15.6 bacterial colony-forming units per ml compared to 114 colony-forming units per ml of Xpert MTB/RIF. To improve the accuracy of rifampicin resistance detection, Xpert MTB/RIF Ultra incorporates melting temperature-based analysis instead of real-time PCR. Specifically, four probes identify rifampicin resistance mutations in the rifampicin resistance determining region of the *rpo*B gene by shifting the

melting temperature away from the wild type reference value. Xpert MTB/RIF Ultra performed similarly well as Xpert MTB/RIF in detection of rifampicin resistance (11).

Currently, the WHO recommends that all individuals presenting with symptoms or signs of TB should be diagnosed with Xpert MTB/RIF Ultra and that all individuals diagnosed with rifampicin resistant TB initiate an MDR-TB treatment regimen. Treatment should subsequently be optimized following confirmatory testing for rifampicin and INH resistance ,and second-line anti-TB drugs(13). Given the rapid changes in the TB diagnostic era, it is plausible to assess the added value of Xpert MTB/RIF Ultra for detection of rifampicin compared to LPA and phenotypic DST.

1.2. Statement of the problem

Over the last 6 years, efforts have been made to develop rapid diagnostic and drug susceptibility testing (DST) tools for TB. During this period, the WHO had issued 10 policy statements for improving diagnosis of TB and drug resistant TB. This includes the use of commercial and noncommercial DST methods and implementation of molecular methods such as the LPA (14).

In 2008, the WHO endorsed LPA for the detection of resistance to rifampicin and isoniazid in patients with smear positive or culture positive TB (9). LPA targets the *rpoB* gene, which consists of 81-bp hot-spot region from codons 507 to 533, called the rifampin resistance-determining region (RRDR) and *inhA* promoter (from -16 to -8 nucleotides upstream) and the *katG* (codon 315) region (15). Moreover, newer versions of the LPA technology have been developed since 2011, including (i) the GenoType MTBDR*plus* version 2 (referred to as GenoType MTBDR*plus* V2), and (ii) the Nipro NTM+MDRTB detection kit 2 (referred to as "Nipro", Tokyo, Japan). These newer LPAs aim to improve the sensitivity of Mycobacterium tuberculosis complex (MTBC) detection with simultaneous detection of resistance to rifampicin (RIF) and isoniazid (INH). The study demonstrated that there consistent performance among the three commercially available LPAs for detecting TB and resistance to RIF and INH(16). However, LPAs are more technically complex (designed for reference or regional laboratory settings) and take longer to perform the tests (14).

As an alternative, Xpert MTB/RIF was innovated in 2004 and indorsed by WHO in 2011. Xpert MTB/RIF is an automated PCR test using the GeneXpert platform. Xpert MTB/RIF is a single test that used for MTB and rifampicin resistance detection. In addition, it is rapid test with TAT of two hours. Moreover, it involve with minimal hands-on technical time because Xpert MTB/RIF follow closed sample processing ,PCR amplification and detection system in which all are integrated into a single self-enclosed test unit, the GeneXpert cartridge(10).

Xpert MTB/RIF Ultra is currently the most sensitive tool for diagnosis of rifampicin resistant TB and is therefore recommended by WHO as the initial test for assessment of TB in all individuals with presumptive TB. WHO recommended that in patients who's Xpert MTB/RIF repeatedly detects rifampicin resistance, the recommended treatment regimen for MDR-TB should be

initiated and optimized following confirmatory testing for rifampicin resistance and additional DST to test resistance to isoniazid and second line anti-TB agents. Line probe assays can play an adjunctive role in the current TB diagnostic landscape. One potential adjunctive role is confirmation of rifampicin resistance detected by Xpert MTB/RIF Ultra. Confirmation of rifampicin resistance is important to avoid unnecessary treatment (17). Another potential role of line probe assays is the detection of INH resistance in patients diagnosed with rifampicin sensitive TB who respond poorly to standard first-line treatment. Multiple studies have shown poor treatment outcomes for INH mono-resistant cases when treated with the standardized TB treatment regimen (18,19).

Another alternative for confirmation of rifampicin resistance detected by an initial Xpert MTB/RIF Ultra assay is culture-based phenotypic DST. The advantage of a phenotypic culture-based DST is its ability to use the same culture to subsequently test for other first and second line drugs. Clear disadvantages remain, including slower turn-around time, the technical infrastructure needs of a centralized laboratory, and frequent contamination of liquid cultures (20). Confirmatory testing for rifampicin resistance in groups with a low prevalence of MDR-TB shown by Xpert MTB/RIF using other genotypic testing or conventional DST techniques may be unreliable and can produce discordant results which requires solution by DNA sequencing (17).

The use of Xpert MTB/RIF Ultra assay is expanding at different TB diagnosis setting for rapid diagnosis and RIFs resistance detection of *M. tuberculosis*. Currently Ethiopia also includes Xpert MTB/RIF Ultra assay in national Tb diagnostic algorithm. However, at least to our knowledge, there was no study on test performance of Xpert MTB/RIF Ultra compared to line probe assays and phenotypic drug susceptibility assays for detection of drug-resistant *M. tuberculosis*. therefore, this study aimed to compare Xpert MTB/RIF Ultra and line probe assay and phenotypic DST in detection of RIF-resistant mycobacterium tuberculosis in Jimma University medical center southwest Ethiopia.

1.3. Significance of the study

This study will provide information on diagnostic accuracy of Xpert Ultra for detecting rifampicin resistant *M. tuberculosis*. The information will be used by national TB control program, to evaluate the diagnostic yield and performance of Xpert MTP/RIF Ultra. It can also provide information on the role using LPA for Xpert MTB/RIF Ultra rifampicin resistance cases. Furthermore, it will use as baseline for users such as health professionals, researchers, experts and policy makers.

2. LITRATURE REVIEW

Over the last 6 years, efforts have been made to develop rapid diagnostic and drug susceptibility testing (DST) tools for TB. During this period, the WHO had issued 10 policy statements for improving diagnosis of TB and drug resistant TB (15).

Main concerns about detection of rifampicin resistant/MDR TB is related to limit of long turnaround time (TAT) of phenotypic DST (9). To overcome this, in 2008, the WHO endorsed the use of molecular line probe assays (LPA) for the detection of resistance to rifampicin and isoniazid in patients with smear positive or culture positive TB (10). LPAs are more technically complex (designed for reference or regional laboratory settings) and take longer to perform than the Xpert MTB/RIF assay. In 2011, the WHO recommended replacement of smear microscopy by the Xpert MTB/ RIF (Xpert) assay (Cepheid, Sunnyvale, USA), a molecular test that allows rapid diagnosis of Mycobacterium tuberculosis complex and simultaneous detection of resistance to rifampicin (11). Xpert MTB/RIF assay has low sensitivity in detection of smear-negative, pediatric and HIV-associated TB (12).

Since March 2017, the next-generation Xpert MTB/RIF Ultra assay was introduced to overcome the low sensitivity of Xpert MTB/RIF in smear-negative, pediatric and HIV-associated TB and to correct some of its limitations in the identification of rifampicin resistance (12). It has been suggested that, the sensitivity has been improved by: i) targeting the multi copy IS6110 and IS1081 genes for the detection of MTB, ii) more rapid thermal cycling with fully nested nucleic acid amplification, iii) improved fluidics and enzymes, iv) a larger DNA reaction chamber than the Xpert MTB/RIF . As a result, the assay can detect traces of MTB DNA (a new semi-quantitative category with indeterminate rifampicin resistance) which were undetectable with the previous version of Xpert MTB/RIF which targeted only the Rifampicin Resistance-Determining Region (RRDR) of the MTB rpoB gene (13).

Different studies were carried out to evaluate the effectiveness of Xpert MTB/RIF Ultra assay for detection of MTBC and rifampicin resistance in specimens. Mostly the performance of Xpert MTB/RIF Ultra assay has been evaluated against existing reference standards, Xpert MTB/RIF, and culture, for TB testing and phenotypic DST for Rifampicin resistance testing.

2.1. Xpert MTB/RIF ultra and Xpert MTB/RIF

A retrospective study conducted in Bologna (Italy) on improvement of *Mycobacterium tuberculosis* detection by Xpert MTB/RIF Ultra: A head-to head comparison on Xpert MTB/RIF samples. Xpert MTB/RIF Ultra showed a 100% agreement with phenotypic susceptibility test for detection of rifampicin resistance from 19 Xpert MTB/RIF negative sputum samples (21). This study didn't compare Ultra with LPA for detection of rifampicin resistance; however the current study includes the comparison of Xpert MTB/RIF Ultra, LPA and Culture.

A study conducted in Barcelona (Spain) showed that 75.9% sensitivity (95% confidence interval [CI], 66.6 to 83.4%) and 100% specificity of Xpert MTB/RIF Ultra for detection of TB from 108 smear negative and culture positive extra pulmonary samples(22). Another study conducted in Montpellier University Hospital (France), showed that Xpert MTB/RIF Ultra had highest sensitivity 100% compared to Xpert MTB/RIF 42% ($P < 0.001$) for detection of MTB (23). A study conducted in Switzerland, on Added Value of Xpert MTB/RIF Ultra for Diagnosis of Pulmonary Tuberculosis in a Low-Prevalence Setting showed that Xpert Ultra had higher sensitivity 95.7% (45/47), and 82.9% (39/47) Xpert MTB/RIF respectively. For detection of rifampicin resistance both shows the same sensitivity and specificity result(24).

A prospective cohort study conducted in Beijing China on Xpert MTB/RIF Ultra improved the diagnosis of paucibacillary tuberculosis. Showed that the Sensitivity of detection of RIF resistance was slightly higher for Xpert MTB/RIF Ultra than Xpert MTB/RIF, 100% (16/16) and 93.75%, (15/16) respectively, $P = 0.310$ and both have 100 % specificity in comparison to phenotypic DST (25). A prospective, multicenter, diagnostic accuracy study conducted on Xpert MTB/RIF Ultra for detection of Mycobacterium tuberculosis and rifampicin resistance: showed that both Xpert MTB/RIF and Xpert MTB/RIF Ultra have similarity in detection of rifampicin resistance. Out of 684 culture positive sputum samples Xpert MTB/RIF Ultra detects 588 (86%) and 580 (85%) for Xpert MTB/RIF (26).

A review on Xpert Ultra versus Xpert MTB/RIF for pulmonary tuberculosis and rifampicin resistance in adults with presumptive pulmonary tuberculosis, on nine studies (3500) participants Xpert Ultra showed pooled sensitivity and specificity of 90.9% (86.2 to 94.7) and 95.6% (93.0 to 97.4) (7 studies, 2834 participants) respectively whereas Xpert MTB/RIF showed 4.7% (78.6

to 89.9) and 98.4% (97.0 to 99.3) (7 studies, 2835 participants) pooled sensitivity and specificity respectively for detection of pulmonary *Mycobacterium Tuberculosis*. For detection or RIF resistance both test methods has similar sensitivity and specificity(27).

A comparative study conducted in Johannesburg, South Africa on Performance of Xpert MTB/RIF, Xpert Ultra, and Abbott RealTime MTB for Diagnosis of Pulmonary Tuberculosis in a High-HIV-Burden Setting showed that sensitivity of the Ultra of 89.3% (95% confidence interval [CI], 78.1 to 96) was higher than that of the Xpert MTB/RIF (82.1% [95% CI, 69.6 to 91.1]; P0.13) but Xpert MTB/RIF shows higher specificity (100% [95% CI, 98 to 100) than Xpert Ultra (96.7% [95% CI, 92.9 to 98.8]; P0.007) the study did not compare for detection of rifampicin resistance(28).

2.2. Genotype MTBDRplus LPA and Xpert MTB/RIF

According to a double blinded prospective study conducted in India on Comparison of Xpert MTB/RIF with Line Probe Assay for Detection of Rifampin-Monoresistant *Mycobacterium tuberculosis:* Seventy-two (25.8%) samples showed multidrug resistance, 62 (22.2%) showed rifampicin monoresistance, 29 (10.3%) showed isoniazid monoresistance, and 116 (41.5%) were pan-susceptible. Of 62 LPA rifampicin monoresistance, 38 (61.4%) showed rifampin resistance, while 21 (33.8%) were found susceptible and of the 116 pan-susceptible samples, only 83 were available for Xpert MTB/RIF testing; 4 (5.1%) were rifampin resistant, 74 (94.8%) were susceptible by Xpert MTB/RIF assay. From the 25 discrepant samples, the LPA results showed 100% and Xpert MTB/RIF only 64.4% agreement with culture (MGIT960)(29).

A study conducted in Bangladesh on Comparison of Xpert MTB/RIF Assay and GenoType MTBDRplus DNA Probes for Detection of Mutations Associated with Rifampicin Resistance in Mycobacterium tuberculosis. The overall 92.4% agreement of Xpert MTB/RIF and LPA for detection of rifampicin resistance with LJ-DST from 92 sputum samples(20).

A comparative crossectional study conducted in German Nepal Tuberculosis Project showed that the overall sensitivity, specificity, positive predictive value and negative predictive value of Xpert MTB/RIF 98.6%, 100%, 100% and 93.8% respectively and the result showed that Xpert MTB/RIF assay was found to be highly sensitive, specific and comparable to gold standard conventional DST method for the diagnosis of MDR-TB(30).

A study conducted in Georgia on Comparison of the Xpert MTB/RIF and MTBDR*plus* 2 Assays, the Xpert MTB/RIF and LPA showed a good sensitivity (87% and 83% respectively), and both 99% specificity in detection of rifampicin resistance. Both showed an excellent agreement with culture based DST for detection of rifampicin resistance Xpert (k=0.89) and LPA (0.88)(31).

A study conducted in National Health Laboratory Service (NHLS) TB referral laboratory, Cape Town, South Africa, sowed that both Xpert MTB/RIF and LPA have a 100% sensitivity and specificity of detecting rifampicin resistance(32). Another prospective study conducted in Algeria showed that 100% Sensitivity, specificity, positive predictive value and negative predictive value of Xpert MTB/RIF to detect RIF resistance in comparison to conventional phenotypic drug susceptibility technique(33).

A study conducted in Kenya on Comparison of GeneXpert and line probe assay for detection of *Mycobacterium tuberculosis* and rifampicin-mono resistance, showed that LPA haves a better performance with sensitivity, specificity, positive predictive value, and negative predictive value of 98.4, 66.0, 65.4 and 98.4% respectively than Xpert MTB/RIF which had a sensitivity, specificity, positive predictive value, and negative predictive value of 78.5, 64.9, 59.4 and 82.2% respectively(34). According to a study conducted in Southwest Ethiopia, Xpert MTB/RIF detects RIF resistance with a 100% sensitivity and 97.8% specificity and a positive-predictive value of 95.7% in comparison to LPA from TB positive sputum samples(35).

2.3. Xpert MTB/RIF Ultra and Genotype MTBDRplus LPA

A comparative evaluation study on new GneXpert Utra and other diagnostic assays for detecting Tuberculosis in pulmonary and extra pulmonary specimens at Pretoria, South Africa showed that Xpert MTB/RIF Ultra had high sensitivity 88 % than LPA (80%) for pulmonary samples. In terms of specificity Xpert MTB/RIF Ultra also had higher 58.57% compared to LPA 11.11% on pulmonary samples for detection of MTB. Xpert MTB/RIF Ultra detects more RIF resistance than LPA (36). This study compares only for detection of *M. tuberculosis*, our study compares Xpert MTB/RIF Ultra and MTBDRplus VER 2.0 LPA for detection of rifampicin resistance.

All of the above findings in the literature, which were conducted at different times and locations, evaluated Xpert MTB/RIF Ultra assay either with Xpert MTB/RIF or GenoType MTBDRplus and phenotypic DST for detection of *M. tuberculosis* on direct clinical specimens, culture isolates, or both, Furthermore, to my knowledge, no study has been undertaken in this study site compare Xpert MTB/RIF Ultra and GenoType MTBDRplus for detection of rifampicin resistant *M. tuberculosis*. As a result, the goal of this research is to compare performance of Xpert MTB/RIF Ultra and GenoType MTBDRplus for detection of rifampicin resistant *M. tuberculosis.*

3. OBJECTIVE OF THE STUDY

3.1. General objective

- To compare performance of Xpert MTB/RIF ultra and line probe assay for detection of rifampicin resistant *M. tuberculosis* in Jimma University Medical Center (JUMC), Southwest Ethiopia.

3.2. Specific objective

- To determine the diagnostic performance of Xpert MTB/RIF Ultra compared to phenotypic DST in detecting rifampicin resistant M. tuberculosis.
- To determine the diagnostic performance of Xpert MTB/RIF Ultra compared to MTBDR*plus* LPA in detecting rifampicin resistant M. tuberculosis.
- To evaluate the role of LPA for the detection of drug resistance in the era of Xpert MTB/RIF ultra.

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4. MATERIALS AND METHODS

4.1. Study setting

The study was conducted at Jimma University Medical Center 350 km in the southwest of Addis Ababa. Jimma University Medical Center is a referral hospital and provides services for about15 million populations in catchment area. It provides service for approximately 15, 000 inpatients, 160, 000 outpatient attendants, and 11, 000 emergency cases annually.

Laboratory investigations for this study were carried out at Mycobacteriology Research Center of Jimma University (JU-MRC). The JU-MRC was established as part of a collaborative research project between Jimma University and a consortium of Flemish Universities from Belgium in November 2010. The center activities are mainly focused on research, training and service in the field of Mycobacteriology. The research component of the center is mainly on basic and applied research which ranges from optimization of detection methods to molecular level research. The center is also open for training on Mycobacteriology techniques up on request by academic departments. It is also involved in the provision of service to the patients as a part of a national Mycobacteriology laboratory network. Conventional bright field microscopy, fluorescent microscopy, culture to detect MTB using solid media (L-J and Middlebrooke media) and liquid media (BACTEC MGIT 960 TB detection system); DST on solid media, liquid media and line probe assay (Geno Type MTBDRplus assay), Xpert MTB/RIF and Xpert MTB/RIF Ultra are the currently available technologies at the center (37).

4.2. Study design and study period

An institution based cross-sectional study was carried out from June 2020 to October 2021.

4.3. Source population

All presumptive pulmonary TB patients who visit JUMC during the study period were considered as source population.

4.4. Study population

All presumptive pulmonary TB patients who visit JUMC and had bacteriological confirmed *M. tuberculosis* with Xpert MTB/RIF Ultra, MTBDR*plus* LPA and Phenotypic DST during the study period were considered as study population.

4.5. Inclusion and exclusion criteria

4.5.1. Inclusion Criteria

- Presumptive Pulmonary TB cases
- Participants with age >18 years

4.5.2. Exclusion criteria

- Critically Ill patients that can't provide sample

4.6. Sample size determination

4.6.1. Sample size

The sample size required for this study was calculated using Conner's formula for estimating sample size for evaluating sensitivity and specificity in matched group study (38).

$$N= \frac{[SLF*(\psi)^{1/2} + PF * (\psi-\delta^2)^{1/2}]^2}{\delta^2}$$

Where:-

N= Number of individuals to be participated in the research

P_1= Sensitivity of LPA = 96%(39)

P_2= Sensitivity of Xpert Ultra = 88%(11)

SLF = Significance level factor at $\alpha = 0.05$=1.645

PF= Power factor at β=80% =0.84

Ψ= the probability of disagreement between the techniques = $P_1 (1- P_2) + P_2 (1- P_1)$

$\delta = P_2 - P_1$= (-0.08)

$$N= \frac{[1.645*(0.15)^{1/2} + 0.84*(0.18-(-0.08)^2)]^2}{(-0.08)^2}$$

N= 144 sample size

The calculated sample size was 144 we increase the sample size to increase our findings accuracy and making final size of 275.

4.6.2. Sampling technique

Sampling technique

Non probability, consecutive sampling technique was used.

4.6.2.1. Data collection procedure

A written informed consent was obtained from 275 patients with sign and symptoms of TB at JMC. After informed consent, all participants were interviewed using structured questionnaires to gather information on socio-demographic characteristics (age, sex, clinical related TB illness (duration of cough, weight loss, duration of fever, duration of night sweats, duration of shortness of birth, presence and duration of chest pain). HIV status was taken from MRN.

The patient were requested to submit two cups of sputum samples each of 5 mL, one for same day Xpert MTB/RIF Ultra assay and one for solid and liquid culture at JU-MRC. From culture positive isolates phenotypic DST were performed and first line LPA also tested after DNA was extracted from culture positive samples. All laboratory procedures involving clinical specimens were carried out in Biological safety cabinet (BSC class II) at the MRC.

4.7. Laboratory tests

4.7.1. Xpert MTB/RIF Ultra assay

Sputum sample was tested by Xpert MTB/RIF Ultra as per the protocol. Briefly, samples were treated with a sodium hydroxide and isopropanol-containing sample reagent (with 2:1 sample reagent ratio. The solution was incubated at room temperature for 15 min. About 2ml of the liquefied sample was transferred to the sample chamber of the Xpert MTB/RIF Ultra cartridge using sterile pipette. Then the lid was firmly closed and inserted into Gene Xpert instrument to start the test within the time frame specified in the package insert (13).

The Xpert MTB/RIF Ultra test result was interpreted as MTB detected, MTB not detected and trace detected. In case of trace result, a new sputum samples was requested for a second Xpert MTB/RIF Ultra test. For second test positive (trace or higher) the test was considered as positive. Trace result was considered as bacteriological confirmation of TB in HIV-positive patients and HIV-negative TB cases without a history of TB. Rifampicin resistance results were reported as susceptible, resistance and indeterminate (40).

4.7.2. Mycobacterial culture and identification

4.7.2.1. Sputum sample processing

The second cup of sputum from each participant was used for mycobacterial culture. All sputum specimens were processed by N-acetyl-L-cysteine -Sodium Hydroxide (NALC-NaOH) decontamination method. Briefly, equal volumes of clinical specimens were mixed with NALC-NaOH with a final concentration of 1% NaOH in a sealable 50mL falcon tube, then vortexed and incubated at room temperature (15-20°C) for 15 minutes. Phosphate buffered saline (PBS, pH 6.8) was added to the 50mL mark and centrifuged at 3,000 x g for 15 minutes at 4°C. The supernatant was decanted into and sterile PBS was added to the pellet to a volume of 1-2 ml, and re-suspended (41).

4.7.2.2. Ziehl–Nielsen Smear microscopy

Direct smears were prepared from the thick purulent portion of sputum specimens, which were then air-dried, heat-fixed. The slides were stained with carbolfuschin for 5 minutes and decolorized by 3% acid alcohol for 3 minutes. Finally the slides stained with methylene blue one minute. The smears were examined and the positive results were graded according to WHO guidelines after being scanned with an oil immersion objective (42).

4.7.2.3. Inoculation and incubation of L-J and MGIT culture

After L-J tubes were labeled with study ID, 200µl of sputum pellet was inoculated on the LJ medium with evenly spreading over entire surface of the medium. Tubes were left with loosen cap for 2-3 days at 37°C in a horizontal position, after which the cap was tightened securely and incubated in an upright position for 6 weeks and inspected for rough, tough and buff colony formation at weekly intervals. Positive growth was confirmed by smear microscopy using Ziehl-Neelsen staining (43).

For liquid culture, the Mycobacterial Growth Indicator Tube (MGIT) method was used according to manufacturer`s instructions. Tubes were inspected for contamination or damage and each tube were labeled with study ID using a permanent marker. The antibiotic supplement (PANTA) was prepared and reconstituted with 15mL MGIT growth supplement. To each MGIT tube, 0.8mL of the PANTA with growth supplement mixture and 0.5mL of well-mixed sputum sediment were added using a sterile graduated transfer pipette. The closed tubes then placed in the BACTEC 960 instrument until signaling "positive" or no growth was detected after 42 days (41).

4.7.2.4. Identification of MTBC using SD Bioline TB Ag MPT64 rapid test

Identification was done for 63 culture positives (MGIT or Lowenstein Jensen's) samples using SD Bioline TB Ag MPT64 Rapid test, an immunechromatographic method which can detect MPT64 antigens produced by *M. tuberculosis* complex. From 63 samples 61 were positive for MPT64 antigens (44).

4.7.3. First Line MIGT DST (Phenotypic DST)

Drug susceptibility test was performed for 57 culture positive samples using a MIGT DST protocol. Briefly a set test assay that consists of a Growth Control tube and one tube for each drug were prepared. About 1% Growth control was prepared by adding 0.1ml of 1-2 days MGIT *M. tuberculosis* positive in to 10ml of sterile saline (1:100 dilution) then, 0.5 ml were transferred to MGIT tube. A volume of 100µl known concentration of (1.0 mg/ml for rifampicin and 0.1mg/ml for isoniazid) drug was added to a MGIT tube after that 0.5ml of sample were added and growth was compared with a drug free control of the same specimen. The result was interpreted as susceptible, if the growth was inhibited and fluorescence was suppressed in the drug-containing tube; meanwhile, the drug-free control grow and show increasing fluorescence. Resistant, if there is growth and its corresponding increase in fluorescence in both the drug-containing and the drug-free tube. The MGIT 960 system monitors these growth patterns and will automatically interprets results as susceptible or resistant (44).

4.7.4. First Line LPA

DNA Extraction was done for 57 culture positive samples using by Genolyse DNA extraction method. Extracted DNA was amplified to facilitate detection of mycobacterial drug resistance (45).

Amplification and Detection: Following amplification, labeled PCR products was hybridized with specific oligonucleotide probes immobilized on a strip. Captured labeled hybrids were detected by colorimetric development, enabling detection of the presence of *M. tuberculosis* complex, as well as the presence of wild-type and mutation probes for resistance. If a mutation is present in one of the target regions, the amplicon will not hybridize with the relevant probe. Mutations are therefore detected by lack of binding to wild-type probes, as well as by binding to specific probes for the most commonly occurring mutations (45).

4.8. Data analysis procedures

Double data entry was performed on EpiData V3.1 and exported to SPSS version 21 for analysis. Descriptive statistics were used to present participant's characteristics and the data was presented in figure and graph. Sensitivity, Specificity, Positive Predictive value (PPV), and Negative Predictive value (NPV) of Xpert MTB/RIF Ultra was determined by taking culture as a gold standard. The level of agreement among the different diagnostic tools was assessed by Cohen's Kappa statistics. Kappa values were interpreted as poor, slight, fair, moderate, substantial and almost perfect agreed if the calculated values are 0-0.20, 0.21-0.40, 0.41-0.60, 0.61-0.80 and 0.81-1.00 respectively. The precision of the estimates was reported using 95% confidence interval (CI).

4.9. Data quality assurance

All samples were collected, packaged, and transported following the laboratory's standard operating procedure. All patient data were double-checked for consistency and completeness regularly. The performance of the culture media was tested by inoculating H37Rv and incubating it at 37 0C (known susceptible MTB). The sterility of sample processing reagents was checked by inoculating all reagents in a separate Blood heart infusion, (BHI). Start and end control were included in every run of sample inoculation. Positive and negative control were included in molecular assay.

4.10. Ethical consideration

Ethical approval was obtained from the Ethics Review Board of Jimma University, Ethiopia; Permission was obtained from hospital managers/medical director. The purpose of the study was explained to the participant and written consent was obtained. All the information's were kept confidential.

4.11. Dissemination plan

This study will be submitted to Jimma University, Faculty of Health Science, School of Medical Laboratory Science, and Mycobacteriology Research Center. So it can serve as a reference in the library. Summery will be also submitted to Jimma University Medical center. Additional effort will also be made to present on conferences to reach the medical/scientific community and publish the article on reputable Journals after the final reports.

5. Results

5.1. Socio-demographic and Clinical characteristics

In this study, a total of 275 presumptive TB cases were enrolled. The mean age the participant was 40.12 years SD ± 15.8 years. Highest number of participants 146/275 (53.1%) were female. Majority of 229/275 (88.3%) of the patients had no TB contact history. Among the 243 (88.4%) patients with documented HIV status, 25.2% were HIV positive **(Table 1)**.

Table 1 Socio-demographic and Clinical characteristics of study participants at JMC, Jimma, Ethiopia, 2021

	Frequency n=275	Percent (%)
Socio-demographic Characteristics		
Age		
18-40	163	59.3
>40	112	40.7
Sex		
Female	146	53.1
Male	129	46.9
Clinical Characteristics		
TB contact history		
Yes	46	16.7
No	229	83.3
Pleuratic chest pain		
Yes	187	68
No	88	32
Weight loss		
Yes	161	58.5
No	114	41.5
Duration of fever		

1-20 Days	235	85.5
21-40 Days	37	13.5
> 40 Days	3	1.1
Night sweating		
Yes	90	32.7
No	185	67.3
Cough duration		
> 14 Days	27	9.8
< 14 Days	248	90.2
HIV Status		
Positive	53	19.3
Negative	157	57.1
Unknown	65	23.6

5.2. Performance of Xpert MTB/RIF Ultra

From 275 sputum samples run in Xpert MTB/RIF Ultra, 19.6% (54/275) were positive, 1.5% (4/275) Trace call and 78.9% (217/275) were negative for MTBC. Out of the positives, 37% (20/58) were rifampicin resistant. Of 275 samples processed for culture on LJ and/or MGIT, 22.2% (61/275) showed growth, 73.8% (203/275) no growth, 3.3% (9/275) contaminated and 0.7% (2/275) had presumptive NTM result **(Figure 1)**. From Xpert MTB/RIF Ultra positive samples, 1.9% (1/54) were negative for culture and from Xpert MTB/RIF Ultra negatives 1.8% (4/217) were culture positive **(Table 2)**.

Table 2 Xpert MTB/RIF ultra and culture results for detection of MTB, at JUMC, Jimma, Ethiopia, 2021

		Culture Results n (%)				
		Positive	**Negative**	**Presumptive NTM**	**Contamination**	**Total**
	MTB	53	1	0	0	54
	Detected	98.1%	1.9%	0%	0%	100%
Xpert Ultra						
Result n (%)	MTB Not	4	202	2	9	217
	Detected	1.8%	93.1%	0.9%	4.1%	100%
	Trace	4	0	0	0	4
	Detected	100%	0%	0%	0%	100%
Total		61	203	2	9	275
		22.2%	73.8%	0.7%	3.3%	100%

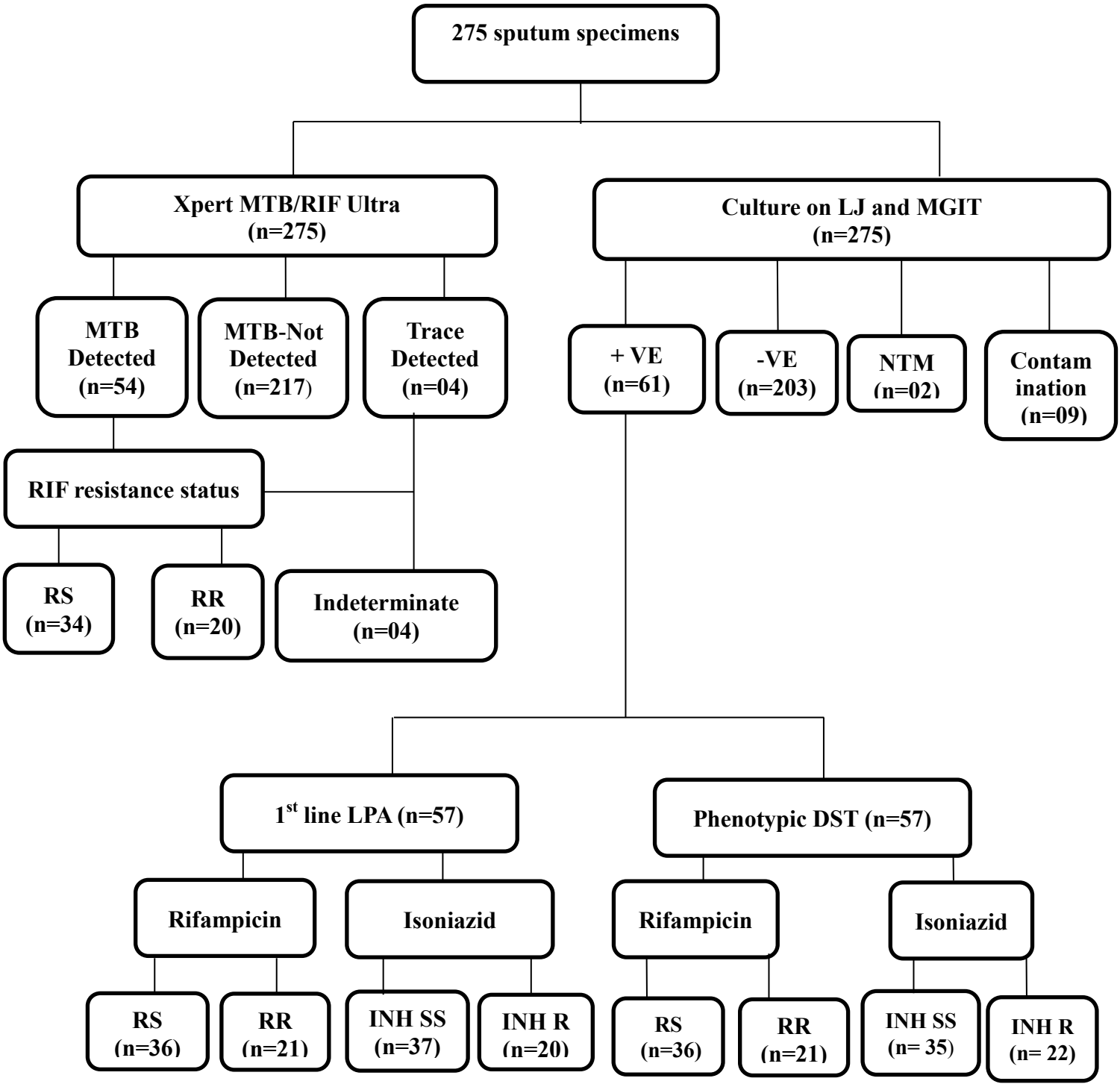

Figure 1 flow chart showing lab procedure and result at JMC, Jimma, Ethiopia, 2021

Key: - RR (rifampicin resistant), RS (Rifampicin sensitive), +VE (culture positive), -VE (culture negative) INH R (isoniazid resistance), INH SS (isoniazid sensitive)

Out of 275 sputum samples processed for AFB microscopy, 3.64% (10/275) were positive. All of them were (100%) were also positive by Xpert MTB/RIF Ultra and Xpert MTB/RIF Ultra detected additional four positive from smear negatives (**Table 3**).

Table 3 Frequency distributions for AFB smear result of participants with positive MTB detection pattern of Xpert MTB/RIF ultra at JUMC, Jimma, Ethiopia, 2021.

		Xpert MTB/RIF Ultra			
		Detected	**Trace detected**	**Not detected**	**Total**
Sputum smear (AFB)	Positive	10 (18.5%)	0 (0%)	0(0%)	10 (3.64%)
	Negative	44 (81.5%)	4 (100%)	217 (100%)	265(96.4%)
Total		54 (100%)	4 (100%)	217 (100%)	275 (100%)

5.3. Performance of MTBDRplus VER 2.0 LPA

First line Genotype MTBDR*plus* VER 2.0 LPA result was available for 57 smear and/or culture-positive samples. One Xpert MTB/RIF Ultra-positive sample was not subjected for MTBDRplus LPA due to the fact that this sample was smear and culture-negative. Out of 57 samples processed 61.4% (35/57), were Sensitive for both INH and RIF and 33.3% (19/57) were Resistance for both Rifampicin and Isoniazid. Two samples were mono resistant to RIF and one mono resistant for INH **(Table 4)**.

Table 4 Genotype MTBDRplus VER 2.0 LPA for detection RIF and INH resistance MTB at JUMC, Jimma, Ethiopia, 2021

LPA result		Frequency	Percent
RIF	Sensitive	36	63.2
	Resistance	21	36.8
Total		57	100
INH	Sensitive	37	64.9
	Resistance	20	35.1
Total		57	100
RIF and INH	Sensitive	35	61.4
	Resistance	19	33.3
Total		54	94.7

5.4. Comparison of results of Xpert MTB/RIF ultra and Line probe assay

The two molecular methods were compared for detection of rifampicin resistance. Fifty seven MTBC culture and Xpert MTB/RIF Ultra positive samples were used for comparison. Of 57 positive samples, Xpert MTB/RIF Ultra detected 57.9% (33/57) and LPA detected 56.1% (32/57) samples as RIF sensitive. One sample had discordant result of rifampicin resistant which was RIF sensitive by Xpert MTB/RIF Ultra and resistant with MTBDRplus VER 2.0 LPA (**Table 5**).

Four Trace call samples detected by Xpert MTB/RIF Ultra had rifampicin indeterminate results and LPA detected all as rifampicin sensitive. Xpert MTB/RIF Ultra had 95.5%, (95%CI, 76.18-99.88), 100%, (95%CI, 90.26-100), 100% and 97.3% (95%CI, 84.17-99.29) of sensitivity, specificity, negative and positive predictive value respectively and also showed almost perfect agreement compared to MTBDRplus VER 2.0 LPA (Kappa= 0.96 (95% CI, 88.8-99.88) using phenotypic DST as a gold standard (**Table 5**).

Table 5 Performance of Xpert MTB/RIF Ultra and MTBDRplus VER 2.0 LPA for detection of RIF resistant MTB at Jimma Ethiopia, 2021

	MTBDRplus VER 2.0 LPA			Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)	K(95% CI)
	RIF resistance (%)	RIF sensitive (%)	Total					
Xpert MTB/RIF Ultra								
RIF resistance	20 (95.2)	0	20(35.1)	95(76.5-99.8)	100(90.26-100)	100	97.3(84.1-99.29)	0.96(88.8-99.88
RIF susceptible	1 (5)	32 (88.9)	33(57.9)					
RIF indeterminate	0	4 (11.1)	4 (7)					
Total	21 (100)	36 (100)	57(100)					

5.5. Performance of Xpert MTB/RIF Ultra and MTBDRplus VER 2.0 LPA compared to phenotypic drug susceptibility test for RIF resistance

Phenotypic DST of 57 samples against INH and RIF was done using BACTEC MGIT 960 indirect proportion method. Of 57 samples, 57.9% (33/57) were sensitive and 33.3% (19/57) resistance for both RIF and INH. Two samples (3.5%) were INH mono sensitive and three samples (5.3%) had INH mono resistance result **(Table 6)**.

Table 6 Phenotypic DST for detection RMP and INH resistance MTB at JUMC, Jimma, Ethiopia, 2021

Phenotypic DST		Frequency	Percent
RIF	Sensitive	36	63.2
	Resistance	21	36.8
Total		57	100
INH	Sensitive	35	61.4
	Resistance	22	38.6
Total		57	100
RIF and INH	Sensitive	33	57.9
	Resistance	19	33.3
Total		54	91.2

Thirty three samples were identified RIF sensitive by Xpert MTB/RIF Ultra, whereas 97.0% (32/33) were sensitive by phenotypic DST. All the RIF resistance by Xpert MTB/RIF were RIF resistant by DST. Four samples detected trace MTB and RIF indeterminate with Xpert MTB/RIF Ultra, 100% (4/4) were RIF sensitive with phenotypic DST (Table 7). By considering trace results as confirmed positive and RIF sensitive, Xpert MTB/RIF Ultra had 95.5%, 100%, 100%, and 97.3% of sensitivity (95%CI, 76.18-99.88), specificity (95%CI, 90.26-100), positive predictive (95%CI,100%) and negative predictive (95%CI, 84.17-99.29) value respectively. There was almost perfect agreement between Xpert MTB/RIF Ultra and phenotypic DST (Kappa= 0.96 (95% CI, 88.8-100)) in detection of rifampicin resistance (**Table 7**).

MTBDR*plus* LPA correctly detected all rifampicin resistant cases detected by phenotypic DST with 100% sensitivity (95%CI, 83.89-100), 100% specificity (95%CI,99.28-100), 100% positive (95%CI,100) and 100% negative (95%CI,100) predictive values, with perfect agreement (Kappa =1.00, (95%CI, 100)) (**Table 7**).

Table 7 Performance of Xpert MTB/RIF Ultra and MTBDRplus VER2.0 LPA compared with phenotypic DST for detection of RIF resistant MTB at Jimma Ethiopia, 2021

	Phenotypic DST			Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)	K(95% CI)
Xpret MTB/RIF Ultra	RR (%)	RS (%)	Total					
Rifampicin resistance	20 (95.2)	0 (0)	20(35)	95.5(76.9-99.8)	100 (90.26-100)	100	97.3(84.17-99.29	0.96(88.8-100
Rifampicin susceptible	1(4.8)	36(100)	37(65)					
Total	21(100)	36(100)	57(100)					
MTBDRplus								
Rifampicin resistance	21(100)	0(0)	21(37)	100(83.89-100)	100(99.28-100)	100	100	1(100)
Rifampicin susceptible	0(0)	36(100)	36(63)					
Total	**21(100)**	**36(100)**	**57(100)**					

Among 57 samples, 64.9% (37/57) were sensitive for INH by MTBDRplus VER2.0 LPA., MTBDRplus VER2.0 LPA had 90.91 sensitivity (95%CI, 70.84-98.88), 100%, specificity 100% (95%CI, 90.00-100), PPV (95%CI,100) and 94.59% NPV (95%CI, 82.36-98.50). There was almost perfect agreement between LPA and phenotypic DST in detection of INH resistance (Kappa=0.93, 95%CI, 82.2-100) (**Table 8**).

Table 8 Performance of MTBDRplus VER2.0 LPA compared with phenotypic DST for detection of INH resistant MTB at Jimma Ethiopia

	Phenotypic DST			Sensitivity(%) (95% CI)	Specificity(%) (95% CI)	PPV (%) (95% CI)	NPV(%)(95% CI)	K(95% CI)
	INHR (%)	INHS (%)	Total					
MTBDRplus								
Isoniazid resistance	20(90.9)	0(0)	20(35.1)	90.91(70.84-98.88)	100(90.00-100)	100	94.59(82.36-98.50)	0.93(88.2-100
Isoniazid susceptible	2(9.1)	35	37(64.9)					
Total	**22(100)**	**35**	**57(100)**					

6. Discussion

Drug-resistant tuberculosis is a major challenge to global tuberculosis control efforts. A drug-resistant pattern evaluates the effectiveness of a country's current tuberculosis control program. Early detection of drug resistance *M. tuberculosis* is critical for determining the best treatment regimen composition. Only by rapidly identifying drug-resistant tuberculosis cases and treating them with a combination of effective drugs can drug-resistant tuberculosis be prevented. The availability of low-cost, effective diagnostic tests that may be employed in resource-constrained settings is a critical gap in the fight against DR-TB(46). Nucleic acid-based diagnostics have provided a unique approach for developing very efficient point-of-care tests. Among these Xpert MTB/RIF, Xpert MTB/RIF Ultra and MTBDRplus VER 2.0 LPA have been widely used to diagnose DR-TB and bring significant improvement in time-to-treatment of MDR-TB.

Main concerns about detection of rifampicin resistant/MDR TB is related to limit of long turnaround time (TAT), of phenotypic DST compared to molecular testes such as Xpert MTB/RIF Ultra and MTBDRplus VER 2.0 LPA. Compared to MTBDRplus VER 2.0 LPA, Xpert MTB/RIF Ultra is an automated PCR test with short TAT and minimal hands on contact but, has a limitation of detecting INH and also rifampicin result of MTB trace rifampicin indeterminate result. MTBDRplus VER 2.0 LPA has an advantage of detection of both INH and rifampicin but it has a long TAT, more technical and also had long hand contact compared to Xpert MTB/RIF Ultra (9).

In our study sensitivity and specificity of Xpert MTB/RIF Ultra was 95.5%, (95%CI, 76.18-99.88) and 100%, (95%CI, 90.26-100) respectively using phenotypic DST as a gold standard. Our finding showed similar result with previous studies done on comparing with other diagnostic tests. A recent meta-analysis of 9 studies found that Xpert MTB/RIF Ultra could detect rifampicin resistance with pooled sensitivity 94.9% (88.9 to 97.9) and 99.1% (97.7 to 99.8) specificity (27). A review of 95 study conducted on comparison with Xpert MTB/RIF, showed 95% (90% to 98%) and 98% (97% to 99%) sensitivity and specificity respectively (47). Another comparative study reported sensitivity and specificity of Xpert MTB/RIF Ultra 92.7% (80.1, 98.5) 98% (92.8, 99.9) respectively (12).

The main distinguishing factor between Xpert MTB/RIF Ultra and MTBDRplus VER 2.0 LPA is that MTBDRplus VER 2.0 LPA assay can detect INH resistance. The present study showed that, among 33 rifampicin susceptible cases determined by Xpert MTB/RIF Ultra, 32 were not discordant with the MTBDRplus VER 2.0 LPA test (i.e. the Xpert MTB/RIF Ultra result and the MTBDRplus VER 2.0 LPA results were concordant), while 1 (2.9%) case was found to be discordant (i.e. rifampicin susceptible on Xpert MTB/RIF Ultra but resistance on MTBDRplus VER 2.0 LPA testing). The discordance may be due to the presence of hetroresistance Xpert MTB/RIF Ultra failed to detect on samples that contains 5% or less mutant DNA population (12).

Xpert MTB/RIF Ultra has been developed to overcome limitation of Xpert MTB/RIF, to address sensitivity, Xpert MTB/RIF Ultra uses two different multi-copy amplification targets (IS6110 and IS1081) and has larger PCR reaction chamber (50µl) As a result, the assay can detect traces of MTB DNA (a new semi-quantitative category with indeterminate RIF resistance)(48). In the current study 1.5% (4/275) MTB Trace call and rifampicin indeterminate with Xpert MTB/RIF Ultra. MTBDRplus VER 2.0 LPA and phenotypic DST detected all trace MTB and rifampicin resistance as rifampicin and isoniazid sensitive. Our study supports WHO technical group recommendation of culture and drug susceptibility testing for rifampicin resistance be performed to confirm or exclude resistance (11). 5.7% (2/35) of rifampicin resistance by Xpert MTB/RIF Ultra had INH mono sensitive and 10% (2/20) Xpert MTB/RIF Ultra RIF sensitive samples showed INH mono resistance with MTBDRplusVER 2.0 LPA.

MTBDRplus VER 2.0 LPA and Phenotypic DST had 100% (57/57) concordant result in detection of RIF resistance with 100% sensitivity, specificity, positive and negative predictive value respectively. In comparison with other studies, the present study showed comparable result in a study conducted in Germany, Georgia, South Africa, Algeria, Kenya, and Ethiopia with 100%, 83%, 100%, 100%, 90% and 100% sensitivity and 100%, 99%, 100%, 100%, 99.1% and . 97.8% specificity respectively. MTBDRplus VER 2.0 LPA showed less sensitivity 90.1% in detection of INH resistance in comparison with Phenotypic DST.

In our study Xpert MTB/RIF Ultra showed almost perfect agreement with MTBDRplus VER 2.LPA and phenotypic DST [Kappa= 0.96 (95% CI, 88.8-100)] and non-inferior in detection of rifampicin resistance. Therefore we recommended that Xpert MTB/RIF Ultra is can be used for initial diagnosis with confirmation of rifampicin and isoniazid resistance MTBDRplus VER 2.LPA and phenotypic DST.

7. Conclusion and Recommendation

7.1.Conclusion

Ultra has similar diagnostic performance with that of LPA for the detection of rifampicin-resistance. Considering its shorter turn-around time, relatively easier to perform and minimum biosafety requirement, the new Xpert MTB/RIF Ultra can be used for the detection of rifampicin-resistant and to screen for MDR-TB in Ethiopia.

7.2. Recommendation

Xpert MTB/RIF Ultra has a number of other advantages over MTBDRplus VER 2.LPA. Xpert MTB/RIF Ultra is relatively easier to perform, bio safety requirement is minimum, less hands-on time ensures lower probability of human errors and turn-around time is faster. Considering these facts and the similar performance of Xpert MTB/RIF Ultra found in our study, we recommend that the national programs may consider using Xpert MTB/RIF Ultra to screen for MDR TB. As Xpert MTB/RIF Ultra is being rolled out in different countries, there is scope of further research work should be conducted to inform the rest of world about the feasibility of wide scale roll out of Xpert MTB/RIF Ultra.

7.3.Limitation of Study

- LPA was done from Culture Isolates instead of smear positive samples

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9. ANNEXES

9.1. Annex I：Information sheet English version

Dear sir /Madam_____

My name is Dawit Abera I am currently studying in Jimma University, faculty of health science and undertaking a master's degree (Msc) in Medical Microbiology. You are invited to participate in the study. This study compare Xpert MTB/RIF ultra with line probe assay for detection of drug-resistant tuberculosis in Jimma University Medical Center . The aim of this study will be to compare Xpert MTB/RIF ultra with line probe assay and culture for detection of RIF resistant *Mycobacterium tuberculosis*.

a. **Duration**: the duration of this study deepened on the availability of study subjects.
b. **Procedure to be carried out**: the procedure is easy and simple; first you will be asked few questions and then you are asked to provide two sputum samples.
c. **Expected benefits**: The information gained from you and other study participants will help to consider prevention strategy for tuberculosis infection that remains a major public health problem in Ethiopia, if you are positive for TB/MDR-TB appropriate medical care will be provided to you.
d. **Confidentiality:** we respect your privacy and confidentiality. Any information that identifies you will not be shared with anyone else outside the study team. If a research article or publication comes from this study, you will not be identified by name. The information we collect from you as part of the study will be kept in locked file cabinet, or be protected by a password on the computer only accessible to personnel involved in the study.
e. **Voluntary participation and withdrawal from the study:** The participation is completely voluntary and you have the right not to participate in the study. You can stop participating in study at any time after giving your consent .This decision will not affect in any way yours current or future medical care in the health facility.

Contact information: if you have any question about the study you can contact the investigator

Dawit Abera: +251911884642/+251937930336

Thank you for your assistance.

9.2. Annex II ፡ Information sheet Amharic version

ውድ ጌታ / እመቤት _____

ስሜ አቶ ዳዊት አበራ ይባላል በአሁኑ ጊዜ በጂማ ዩኒቨርሲቲ ፤ በጤና ሳይንስ ፋኩልቲ ውስጥ ሁለተኛ ድግሪዬን (ኤም.ኤስ.ሲ.) በ በሕክምና ማይክሮባዮሎጂ እያጠናሁ እገኛለሁ፡፡ እርስዎም በጥናቱ ላይ እንዲሳተፉ ተጋብዘዋል ፡፡ ይህ ጥናት በጅማ ዩንቨርስቲ ሜዲካል ሴንተር ውስጥ የ ጂን ኤክስፐርት አልተራን ከ ላይን ፕሮብ አሴይ ጋር መድኒት የተላመደን የሳንባ ነቀርሳን በሽታን ለመለየት መቻልን የሚያነፃፅር ይሆናል፡፡

ሀ. ጥናቱ የሚፈጀው ጊዜ : የዚህ ጥናት ቆይታ ከ6-12 ወራት ፡፡

ለ. መከናወን ያለበት ሂደት: መጀመሪያ ጥቂት ጥያቄዎች ይጠየቃሉ ከዚያም ሁለት አክታ ናሙናዎችን እንዲያቀርቡ ይጠየቃሉ፡፡

ሐ. የሚጠበቁ ጥቅሞች: ከእርስዎ እና ከሌሎች የጥናት ተሳታፊዎች የተገኘው መረጃ የሳንባ ነቀርሳ በሽታን በተገቢው መንገድ ለመከላከል የሚያግዝ ዘዴ ለማግኘት ይረዳል፡፡ መድሃኒትን የተላመደ የሳንባ ነቀርሳ ከተገኘብዎት ተገቢው ህክምና እንዲያገኙ ይደረጋል፡፡

መ. ምስጢራዊነት: ግላዊነትዎን እና ምስጢራዊነትዎን እናከብራለን ፡፡ እርስዎን የሚለይ ማንኛውም መረጃ ከጥናቱ ቡድን ውጭ ለማንም አይጋራም ፡፡ የእንደ አንድ የጥናቱ አካል እኛ ከእርስዎ የምንሰበስበው መረጃ በተቆለፈ ፋይል ካቢኔ ውስጥ ይቀመጣል ወይም በጥናቱ ለተሳተፉ ሠራተኞች ብቻ በሚገኝ ኮምፒተር ላይ በይለፍ ቃል የተጠበቀ ይሆናል ፡፡

ሠ በፈቃደኝነት መሳተፍ እና ከጥናቱ መውጣት: ተሳትፎው ሙሉ በሙሉ በፈቃደኝነት ሲሆን በጥናቱ ውስጥ ላለመሳተፍ መብት አለዎት ፡፡ ፍቃድዎን ከሰጡ በኋላ በማንኛውም ጊዜ በጥናት ላይ መሳተፍዎን ማቆም ይችላሉ ፡፡

አድራሻ: - ስለ ጥናቱ ማንኛውም ጥያቄ ካልዎት ተመራማሪውን ማነጋገር ይችላሉ

ዳዊት አበራ: +251911884642/+251937930336

ለተሳትፎዎ እናመሰግናለን፡፡

9.3.Annex III：English version of consent form

I_____ I have been requested to participate in this study which involves collection of two sputum samples from me and in which I will answer few question. The purpose of this study and sample collection procedure has been explained for me .I have also read the information sheet (or it has been read to me); I have asked some questions and clarification has been given to me .I have given my consent on behalf of myself to participate in study and I hereby confirm my agreement with my signature.

Signature _____ Date_____

Thank you for your participation in this important study.

N.B: If you want to request additional information about the study, you can contact me by

+25191188462/+251937930336/davideyou34@gmail.com

9.4. Annex IV: Amharic Version of Consent Form

<u>ለጥናቱ ተሳታፊዎች የተዘጋጀ የፍቃደነት መግለጫ ቅፅ (ኮንሰንት)</u>

እኔ…….. ሁለት የአክታ ናሙና መሰጠት እንዲሁም ጥቂት ጥያዎች መመለስ በሚያካትተው በዚህ ጥናት እንድሳተፍ ጥያቄ ቀርቦልኛል።ጥናቱ ስለሚሰጠውም ጥቅም እንዲሁም የናሙና አሰጣጥ ሂደቱ ላይ ገለጻ ተደርጎልኛል።የመረጃ መሰጫ ቅፅ አንብቤ / ተነቦልኝ ተረድቻለሁ። የነበሩኝ ቀጥቂት ጥያቄዎች በሚገባ ተብራርቶልኛል። ስለዚህ በጥናቱ ለመሳተፍ ፍቃደኛ መሆኔን በፊርማዬ አረጋግጣለሁ።

ፊርማ---------- ቀን------/------/------

በዚህ ጠቃሚ ጥናት ስለተባበራችሁ አመሰግናለሁ።

ስለጥናቱ ተጨማሪ መረጃ ማግኘት ከፈለጉ በ +25191188462/+251937930336/davideyou34@gmail.com

ማግኘት ይችላሉ ።

9.5. Annex V: Afan Oromo Version of Information sheet

Odeefannoo hirmaattotaaf

Ani Daawwit Abarraa kanan jedhamu yeroo ammaa Yuunivarsiitii Jimmaa Muummee Saayinsii Fayyaatti barataa digirii 2ffaa Medical Microbiology ti.Qorannoo kanatti akka hirmaattuuf afeeramteetta. Kaayyoon qo'annoon kanaa Giddugala Yaalaa Yuuniversiitii Jimmaatti XPERT MTB/RIF ultra with conventionaL line probe assay fi culture for detection of drug-resistant tuberculosis wal madaalchisuudha.

a. **Turtii Qo'annoo:** turtiin qo'annoo kanaa argama hirmaattota qorannoorratti hundaa'ee ji'a 2-3kan turu ta'a
b. **Adeemsa hojii**: adeemsichi hojichaa salphaa fi gabaabaadha; jalqaba gaaffii waa'ee keessan muraasa gaafatamtu kana booda akkee lama akka kennitaniif gaafatamtu.
c. **Bu'aa eegamu**: Odeeffannoon isinii fi hirmaattota biroorraa argannu ittisa dhibee daanyoo sombaa akka iyyattiitti rakkoo hamaa ta'e kana, yoo dhibee daranyoo sombaa kan qabaattan ta'e yaala akka argattan goona

d. **Icitummaa;** Iccita dhuunfaa keessanii ni kabajna; ragaa dhuunfaa keessaniis icitaan ni qabna. Dhimmi dhuunfaa keessanii marti beekumsa keessaniin ala hin saaxilamu. Ragaan isin irraa funaanamu marti maqaa keessaniin osoo hin ta'in lakkoofsa icitaa addaan galmeeffama. Ragaan isinirraa funaanamu marti hamma seeraan deeggamarumutti icitaan ni qabama. Ragaan isinirraa funaanamus saanduqa keessatti itti cufamee, jecha dabiitiin koompittara keessatti cufamee dhimma armaan olitti ibsame qofaaf oola.

e. **Mirga hirmaattotaa fi hirmaannaa addaan kutuu;** Hirmaannaan keessan fedhii keessan qofarratti waan hundaa'uuf diduuf mirga guutuu qabdu. Erga jalqabdaniis yeroo barbaaddanitti addaan kutuu ni dandessu. Murtoon keessan garuu tajaajila argachuu qabdan mara irratti dhiibbaaa hin fidu. Qorattoonni qo'annoo kanaa yeroo barbaadanitti akka isin hirmaannaa keessan addaan kuttan godhuu ni danda'u. Kunis kan ta'u yoo isin fedhii dhabdan fi adeemsa qoranichaa hordofuu diddan ta'a.Gaaffii waa'ee qorannoo kanaa qabdan mara gaafachuu ni dandeessu. Nutis hanga beekumsi keenya nuuf heeyyametti isiniif debisuu ni yaalla.

Wal-qunnamtii

Gaaffii kamuu yoo qabaattaniif, yaadni ifa hin taane yoo jiraate fi shakkii waa’ee qo'annoo yoo qabdan teessoo armaan gadiin na quunnamuu ni dandeessu.

Dawit Abera: +251911884642/+251937930336

Turtii keessaniif galatoomaa

9.6. Annex VI: Afan Oromo Version of Consent Form

Unka Waliigaltee Afaan oromoo

Ani maqaan koo_____qorannoo kana, kan saamuuda haakkee yeroo lamaa fi gaaffii afanii muraasa narra fuudhan keesssattti akkan hirmaadhuuf gaafatameera. Kaayyoon qorannoo kanaa fi haalli saamuda itti fuudhan naaf ibsameera. Akkasumas waraqaa odeeffannoo dubbifadheera(ykn naaf dubbifameera); gaaffii ifaa naaf hin taane tokko tokkos akka naaf ibsaniif gaafadhee jarris naaf ibsaniiru. Kanumafis fedhakootiin qorannoo kana kessatii hirmaachuukoo mallattoo kootin nan mirkanessa.

Mallattoo_____Guyyaa_____

Galatoomaa!

Yoo gaaffii ykn odeffannoo dabalataa barbaddan;

+25191188462/+251937930336/davideyou34@gmail.com

9.7. Annex VII: Data collection tool, English Version

Patient card no _____ study identification no _____

s.no	Questions	Answer
1.	Age	
2.	Sex	1. Male 2. Female
3.	TB contact History of?	1. Yes 2. No
4.	Date of sputum Xpert MTB/RIF Ultra test performed?	
5.	Xpert MTB/RIF Ultra results?	1. Positive 2. Negative
6.	Date of sputum culture performed?	
7.	Sputum culture results?	1. Positive 2. Negative
TB symptoms		
8.	Duration of cough (week)	
9.	Duration of fever (day)	
10.	Chest pain?	1. Yes 2. No
11.	Duration of chest pain (day)	
12.	Presence of night sweet	1. Yes 2. No
13.	Weight loss?	1. Yes 2. No
14.	Loss of appetite?	1. Yes 2. No
Chest X-ray pathology/findings		
15.	TB related abnormality on CXR?	1. Yes 2. No
16.	HIV status	1. Positive 2. Negative 2. 3. Unknown

9.8. Annex VIII : Data collection tool, Afan Oromo Version

_____ _____

	Gaaffii	**Deebiikee**
1	Umrii	
2	Saala	1. Dhiira 2. Dubara
3	Seenaa daranyoo sombaa qabduu?	1. Eenyee 2. Lakki
4	Dhukkubbii hamaa qabduu?	1. Eeyyee 2. lakki
5	Guyyyaa Xpert MTB/RIF Ultra isiniif hojjetame	
	Bu'aa qorannoo Xpert MTB/RIF Ultra	1.Positiiva 2. Negatiiva
7	Guyyaa sputum cuture'n hojjetme	
8	Bu'aa qorannoo Sputum culture	1. Positiiva 2. Negatiiva
Mallattoo TB		
09	Turtii qufaa (torbee)	
10	Turtii qaama gubaa (guyyaa)	
11	Waraansa laphee?	1. Eeyyee 2. Lakki
12	Turtii waraansa laphee	
	Dafqa halkanii	1. Eeyyee 2. Lakki
14	Hir'ina qaamaa?	1. Eeyyee 2. Lakki
15	Fedhii nyaataa dhabuu?	1. Eeyyee 2. Lakki
Bu'aa qorannoo raajii laphee		
16	Rakkina raajii laphee dhukkuba TB waliin wal fakkaatu	2. Eeyyee 2. Lakki
17	Dhibee HIV	3. Positiiva 2. Negativa 1. Hin beeku

9.9. Annex IX ፡ Data collection tool, Amharic Version

ካርድ ቁጥር---------- የጥናት መለያ ቁጥር ----------

ተ.ቁ	ጥያቄ	መልስ
1	ዕድሜ	
2	ፆታ	1. ወንድ 2. ሴት
3	ከዚህ በፊት በ ተቢ ታመው ያውቃሉ?	1. አዎ 2. አይደለም
4	ኤክስፐርት ኤምቲቢ/ሪፍ አልትራ የተሰራበት ቀን?	
5	ኤክስፐርት ኤምቲቢ/ሪፍ አልትራ ውጤት?	1. ፖዘቲቭ 2. ነጌቲቭ
6	ካልቸር የተሰራበት ቀን?	
7	የካልቸር ውጤት?	1. ፖዘቲቭ 2. ነጌቲቭ
	የቲቢ ምልክቶች	
8	ሳል ከጀመሮት ምን ያህል ጊዜ ይሆናል	
9	ትኩሳት ለምን ያህል ግዜ ቀየቦት	
10	ደራትዎ ላይ የህመም ስሜት አለ ?	1. አዎ 2. አይደለም
11	የደረት ህመም ስሜት የቆየበት ቀን	
12	ለሊት ማላብ ስሜት አለዎት	1. አዎ 2. አይደለም
13	የክብደት መቀነስ አለ?	1. አዎ 2. አይደለም
14	የምግብ ፍላጎት መቀነስ አለ?	1. አዎ 2. አይደለም
	የደረት ራጅ ውጤት	
15	በ ራጅ ላይ ከ ተቢ ጋር የተያየዘ የጤና መቃወስ ይታያል?	1. አዎ 2. አይደለም
16	የ ኤችአይቪ ውጤት	➢ ፖዘቲቭ 2. ነጌቲቭ 3. አይታወቅም

9.10. Annex X: Formats for recording laboratory results

Xpert MTB/RIF result collection sheet

No.	Sample ID	Sample collection date	Sample processing date	Xpert MTB result	Xpert RIF result	remark

Xpert Ultra result collection sheet

No.	Sample ID	Sample collection date	Sample processing date	Xpert MTB result	Xpert RIF result	remark

Culture result collection sheet

S.no	Sample ID	Sample processing date	Primary Media	Growth detection								Media smear	Report date	Remark
				Weeks										
				1	2	3	4	5	6	7	8			
			LJ											
			MGIT											
			LJ											
			MGIT											
			LJ											
			MGIT											
			LJ											
			MGIT											
			LJ											
			MGIT											
			LJ											
			MGIT											
			LJ											
			MGIT											
			LJ											

Xpert MTB/ RIF Ultra Assay procedure

2. Check the sample integrity and visually inspect and estimate the volume of specimen in the collection container.
3. Open the lid of the collection container and pour Sample Reagent buffer (supplied in kit) to the specimen in a 2:1 (SR buffer: specimen) volume ratio.
4. Close the lid of the container tightly and vortex for 15 seconds.
5. Allow the mixed specimen to stand for 15 minutes at room temperature
6. Shake the container once during the 15 minute incubation i.e. after 10 minutes.
7. At the end of 15 minutes if the sample is still viscous, shake the sample again and leave it for another 5 min until it is properly liquefied.
8. Remove an Xpert Ultra cartridge from its wrapper, taking care NOT TO TOUCH the back of the cartridge. Once the foil wrapper is removed, the specimen must be added to the cartridge within 30 minutes.
9. Label the side of the Xpert Ultra cartridge with the sample ID. Do not write or place sticker over cartridge barcode.
10. Open the lid of the cartridge.
11. Open the lid of the collection container containing the processed specimen
12. Open a sealed sterile pipette (supplied in kit) without touching the tip.
13. Use the pipette to aspirate >2ml of the specimen (just above 2ml mark on pipette) and slowly dispense it into the open port of the Xpert Ultra cartridge. Do not add less than 2ml of mixed specimen to the cartridge
14. Close the cartridge lid firmly.
15. Dispose the specimen collection container, pipette and leftover SR buffer into a suitable waste bin.
16. Take the Xpert cartridge to the bench with the GeneXpert instrument. NOTE: the test must be started within 30min of adding the specimen to the cartridge.
17. Load the cartridge with the specimen to the GeneXpert machine.

Result Interpretation:

1. **MTB Not Detected** – MTB target DNA is not detected hence the test is negative for TB
2. **MTB Detected RIF Resistance not Detected**- MTB present is not resistant to Rifampicin
3. **Trace call-** lowest bacillary burden for MTB detection.
4. **MTB Detected RIF Resistance Detected** - MTB present is resistant to Rifampicin
5. **Invalid** – Repeat test
6. **Error** – Repeat test
7. **No Result** – Repeat test

Culture and Identification testing procedure

Pre-Specimen processing preparation:

2. Label the sample and request form with culture number
3. Check all the material required for sample processing is in place
4. Arrange sample, and required material in the biological safety cabinet

Step	Acton
1	1. Use the TB processing checklist to collect supplies, reagents, and specimens Label the specimen and LJ media (with lab code, date inoculated) 2. Disinfect BSC and perform daily maintenance 3. Set up BSC for specimen processing with absorbent liner, disinfectant, and waste containers
2	1. Sort specimens into batches using sterile water blanks as the first and last negative processing controls 2. Batch size was based on the centrifuge load including negative processing controls

Preparation for process using NALC-NaOH

Sputum sample

Step	Action
1	Check the volume of the sputum (at least 2 ml, not more than 5 ml) (if the volume of the sample was above 5ml, transferred 5ml of the purulent part to another falcon tube and proceed this allocate for the subsequent steps)
2	Add equal volumes of NALC-NaOH solution. Use aliquots of NALC-NAOH(1 vial of NALC-NAOH per one specimen)
3	Tighten cap of container and vortex slowly
4	Shake intermittently to aid homogenization and decontamination
5	Invert each bottle to ensure that NALC-NaOH solution contacts all the sides and inner Portion of caps.

6	Keep at 20°C – 25°C for 15 min for decontamination
7	Fill the tube with phosphate buffer up to 45 ml mark on the tube. Use aliquots of phosphate buffer(1 vial of PBS per one specimen)
8	Mix-well or vortex
9	Centrifuge at 3,000 ×g for 15 minutes
10	Carefully pour off the supernatant into a discard container containing 5% sodium Hypochlorite or other germicide. Make sure the final concentration of bleach is 1% after Pouring off the supernatant.
11	Re suspend the deposit with 2ml PBS.

Inoculation

MGIT Media

Step	**Action**
1	Mark each MGIT tube with laboratory number.
2	Using repeater pipette Add 0.8 ml of the PANTA solution to each MGIT tube just prior to inoculation.
3	with disposable Pasteur pipette, Add 0.5 ml of a well-mixed processed and/or direct specimen to the appropriately labeled MGIT tube
4	Tightly recap the tube and mix by inverting the tube several times.
5	Wipe tubes and caps with a Mycobactericidal disinfectant.
6	Leave inoculated MGIT tubes at room temperature for 30 min.
7	Scan the inoculated MGIT tube and load into the slot identified by the MGIT 960
8	Check MGIT 960 daily for indicator lights flagging positive and negative cultures
9	Incubate MGIT tubes until the instrument flags them as positive (red flag) or negative (green flag)
10	Positive and negative tubes will be issued by pull the respective drawer and press

	"positive or negative button as needed", the machine displayed by the indicator light changing to green at the exact location of the tube in the instrument drawer
11	Remove the tube and scan
12	Continue MGIT culture work up.

LJ Media

Step	**Action**
1	Mark each LJ tube with laboratory number
2	Decant excess water from the media
3	Inoculate 2 to 3 drops of sediment and/or direct preparation in to two LJ media
4	Wipe tubes and caps with a Mycobactericidal disinfectant.
5	Loose the cap slightly and put the LJ tube in slant position facing upward in the incubator
6	Make sure the fluid cover the surface
7	Keep in a slant position for one week in the incubator (35 – 37 ^{O}C temperature)
8	Check LJ two times a week for any contamination and fast grower Mycobacterium /NTM
9	Read LJ tubes weekly until a positive growth obtained
10	If the LJ tube have growth and enough (>50 matured colony) for subsequent work , quantify the colony
11	If the LJ tube have growth and not enough for subsequent work write "P" on the work sheet and wait 1 to 3 week till enough growth obtained
12	Positive tube will be kept in the incubator till (maximum of 1 week) the subsequent work is done
13	If the LJ tube have no growth in 8th week issued as negative

First line LPA procedure

DNA extraction from liquid culture isolates

Step	Action
1	Using a sterile disposable Pasteur pipette, transfer 1000µl of each thoroughly mixed liquid culture sample to labeled 1.5ml screw cap tube
2	Load the 1.5ml screw cap tubes in a micro-centrifuge with aerosol-tight rotor.
3	Centrifuge for 15 minutes at 10,000 RCF or 10263RPM
4	Unload tubes from the microcentrifuge and carefully carry the tubes to the BSC
5	Discard supernatant from each tube by use of a 1000µl adjustable pipette
6	Resuspend each pellet in 100µl Lysis Buffer (A-LYS)
7	Mix the contents of each tube by use of a sterile tip followed by thorough vortexing for at least 15 to 20 seconds
8	Incubate the tubes for 5 minutes at 95 °C in a thermoblock
9	Add 100 µl Neutralisation Buffer (A-NB) and vortex the sample for 5 seconds
10	Load the tubes into a microcentrifuge and spin for 5 minutes at 13,000 x g
11	Carefully carry the tubes to the BSC. Uncap tubes one at a time, and transfer 100µl of DNA-containing supernatant to a sterile 1.5ml screw cap tube

DNA extraction from solid culture isolates

Step	Action
1	Pipette 100µl lysis buffer (A-LYS) into sufficient number of 1.5ml screw cap tubes (1 tube per culture)
2	Use 1µl sterile disposable inoculation loop to collect bacteria from solid media with sufficient growth
3	Inoculate the bacteria into the lysis buffer. Break the clumps by aid of the inoculation loop
4	For 15-20 seconds thoroughly vortex to adequately mix
5	Incubate the tubes for 5 minutes at 95 °C in a thermoblock
6	Add 100 µl Neutralisation Buffer (A-NB) and vortex the sample for 5 seconds

7	Load the tubes into a microcentrifuge and spin for 5 minutes at 13,000 x g
8	Carefully carry the tubes to the BSC. Uncap tubes one at a time, and transfer 100µl of DNA-containing supernatant to a sterile 1.5ml screw cap tube

Reagent Preparation and Master Mix procedures:

Step	Action
1	Thoroughly apply freshly prepared 0.5% bleach solution to all surfaces of PCR hood and worktop. Allow 15 minutes contact time release UV for 15-20 minutes
2	Wipe with damp cotton wool
3	Apply 70% ethanol to all the surfaces PCR hood and worktop
4	Thaw the two amplification mixes, A and B (AM-A and AM-B) at room temperature.
5	Gently invert to mix the contents of each tube, AM-A and AM-B
6	Carefully remove sufficient PCR tubes from stock pack, place in a PCR tube rack in the PCR hood. Ensure that the lids are closed.
7	Determine the number of samples to be amplified, and positive and negative control samples plus one contingency
8	Label PCR tubes accordingly.
9	Prepare a master mix containing AM-A and AM-B, 10:35 ratio respectively in a sterile screw cap tube (1.5ml) according to the number of samples and controls
10	Mix the master mix gently
11	Pipette 45 µl of the master mix to each labeled PCR tube
12	Ensure all PCR tubes are tightly closed. Close the PCR tube rack
13	Place the stock reagents AM-A and AM-B back in the freezer at -20 °C
14	Wipe pipettes, PCR hood surfaces and worktop with 0.5% bleach followed by 70% ethanol

DNA addition

Step	Action
1	Thoroughly apply freshly prepared 0.5% bleach solution to all surfaces of PCR hood or worktop. Allow 15 minutes contact time
2	Wipe with damp cotton wool
3	Apply 70% ethanol to all the surfaces PCR hood
4	Assemble PCR tubes containing 45µl master-mix reagent
5	Add 5µl of DNA to corresponding mastermix PCR tubes in the PCR hood. NB:- No DNA should be added to the reagent control
6	Check that all PCR tubes are tightly closed
7	Wipe pipette, PCR hood surfaces with 0.5% bleach followed by 70% ethanol

Amplification

Step	Action
1	Thoroughly apply freshly prepared 0.5% bleach solution to work top surfaces.
2	Wipe with damp cotton wool
3	Apply 70% ethanol to all work top surfaces
4	For first use, set up the thermal cycler to the correct amplification profiles according to the instruction manual (see SOP on Use and Maintenance of Thermal Cycler).
5	If PCR tubes have bubbles at base, remove by swinging arm with tubes in hand in arc.
6	Transfer PCR tubes to middle section of thermal cycler. Avoid placing tubes at the edges as this can result in evaporation of the contents during heating
7	Check program parameters and follow menu options to start appropriate program
8	Run the specific program ,Ver.2-cul for samples from solid or liquid culture, Ver.2-dirsample for decontaminated smear positive sediments
9	After the cycles are complete, proceed to the detection stage. If detection cannot be performed on same day, store PCR tubes with amplicons at 4°C for a maximum of 7days

Detection

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

	described for HYB removal.
16	Add 1 ml rinse solution (RIN) to each well. Press right arrow key to rinse the strips for 1 minute. When alarm goes off, press right arrow key. Completely remove RIN.
17	Add 1 ml of diluted Conjugate per well. Press right arrow to incubate at 37°C for 30 minutes on Twincubator
18	When alarm goes off, press right arrow to stop
19	Completely aspirate CON-D solution using Pasteur pipette
20	Add 1ml RIN per well. Press right arrow and incubate for 1 minute on Twincubator
21	When alarm goes off, press right arrow key to stop. Remove RIN completely and repeat Step 20
22	Remove RIN completely and wash with 1 ml sterile distilled water per well on TwinCubator. Press right arrow key and incubate for 1 minute on TwinCubator
23	When alarm goes off, press right arrow key to stop. Remove water and add 1 ml of diluted substrate per well.
24	Place on Twincubator under aluminum foil for a maximum of 10 minutes. Look for color reaction to indicate reaction completion after 4-5 minutes. If color reaction is too weak, replace the foil and reincubate for several more minutes, up to a maximum of 10 minutes
25	Wash twice for 1 minute with distilled water. Remove distilled water after each wash
26	Use forceps to transfer membrane strips to an absorbent paper and allow to air dry
27	Soak trays in 0.5% bleach for 15 minutes and rinse with distilled water
28	Clean pipettes, instruments and work area with freshly diluted 0.5% bleach, followed by 70% alcohol
29	Switch off the Twincubator after use

Interpretation of LPA strip results

Step	Action
1	Use forceps to transfer strips to the GenoType MTBDRplus Results Sheet provided with the kit
2	Align the bands Conjugate Control (CC) and Amplification Control (AC) on each strip with the respective lines on the sheet
3	Attach the strips to the results sheet using clear adhesive tape
4	Determine the band positivity and positions on each strip using the reference reading chart of the kit and mark the results on the worksheet
5	In order for a batch of results to be valid, the negative control strip must have a CC and AC band present, but no other bands must be visible.
6	If a positive result is obtained with the negative control, the whole batch must be repeated and measures taken to remove amplicon contamination from all rooms and equipment.
7	In order for patient results to be valid, CC (conjugate control) and AC (amplification control) bands must appear for every sample. The presence of TUB band indicates that M.tuberculosis complex is present in the sample
8	If CC is negative the conjugation or substrate reaction was unsuccessful either due to error in the procedure or due to problems with the reagent
9	If AC is positive, errors during extraction and amplification set-up and presence of amplification inhibitors in the specimen can be excluded
10	Signal of AC can be weak or even absent while results for other bands (*TUB, rpoB, katG and inhA* locus controls) may be positive. This might be due to competitive reactions between AC and TUB, *ropB, katG, inhA* during amplification. In this case, the strip can be evaluated.
11	A weak or missing AC band with negative test result for TUB, *rpoB, katG and inhA* locus controls may indicate potential mistakes during extraction and amplification set-up, or presence of amplification inhibitors. In this case, the test results are invalid
12	*rpoB* predicts RIF resistance, *katG* predicts high level INH resistance, *inhA* predicts low level INH resistance

13	The *rpoB, katG and inhA* each have a control band which must be present in order to interpret the results. Locus Control zones *(rpoB, katG, inhA)* detect a gene region specific for their respective genes. If the locus control zones are negative, then their respective mutation- specific positive bands cannot be considered for evaluation
14	A mutation in the relevant gene (and resistance to the relevant drug) is signified by either an absent wild type band and/or the presence of a mutant band for each gene cluster.
15	For results to be valid the bands (except CC) must be of intensity approximately equal to or greater than the intensity of the AC band.
16	If the TUB zone is negative, the tested bacteria does not belong to M. tuberculosis complex; therefore, presence or absence of any other bands (except CC and AC) cannot be considered for evaluation
17	When all wild type probes of a gene stain are positive and there is no detectable mutation within the examined regions, the tested strain may be considered sensitive for the respective antibiotic
18	In case of mutation, the respective amplicon cannot bind to the corresponding wild type capture probe on the strip due to the mismatch
19	The absence of a signal for at least one of the wild type probes may predict resistance to the respective antibiotic *indirectly*
20	Positive hybridization signal with a mutation-specific capture probe (for common mutations only!) may predict resistance to the respective antibiotic *directly*
21	Presence of rare mutations that do not have mutation-specific capture probes may only be indicated by the lack of hybridization with one or more wild type probes

9.11. Annex XI: Declaration Sheet

I, the undersigned, MSc Medical Microbiology student declares that this thesis is my original work in partial fulfillment of the requirement for the degree of master science in Medical Microbiology. Where others work has been used, it has been carefully acknowledged and referenced in accordance with the requirements.

Name of principal investigator

Dawit Abera Signature _____ Date_____

Approved by advisors;

Dr. Mulualem Tadesse (PhD, Assistant Professor) Signature _____ Date_____

Mr. Kedir Abdella (MSc, Assistant Professor) Signature _____ Date_____

Mr. Wakjira Kebede (MSc, Assistant Professor) Signature _____ Date_____

Prof. Gemeda Abebe (Professor of Molecular Medical Microbiology) Signature _____ Date_____

Approved by Assessors

_____ Signature _____Date_____

_____ Signature _____Date_____