Evaluation of Xpert® MTB/RIF and Determine™ TB LAM assay for the diagnosis of extra-pulmonary tuberculosis at Jimma University Medical Center, Southwest Ethiopia

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A research thesis submitted to School of Medical Laboratory Science, Faculty of Health Science; Jimma University, in Partial Fulfillment for the Requirement of Masters of Sciences in Medical Microbiology

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Jimma, Ethiopia

JIMMA UNIVERSITY
INSTITUTE OF HEALTH
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APPROVAL SHEET

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As thesis research advisor, I hereby certify that I have read and evaluated this thesis prepared under my guidance by **Asnake Simieneh**, entitled: **"Evaluation of Xpert® MTB/RIF assay and Determine TB LAM test for the diagnosis of extra-pulmonary tuberculosis at Jimma University Medical Center, Southwest Ethiopia"**

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Signature **Date**

As members of the Examining Board of the Final M.Sc. Thesis Open Defense, we certify that we have read and evaluated the thesis prepared by **Asnake Simieneh** entitled: **"Evaluation of Xpert® MTB/RIF and Determine TB LAM assay for the diagnosis of extra-pulmonary tuberculosis at Jimma University Medical Center, Southwest Ethiopia"** and examined the candidate. We **recommend** that the thesis could be accepted as fulfilling the thesis requirement for the Degree of Masters of Science in Medical Microbiology.

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Abstract

Background: Ethiopia is one of the high burden countries for EPTB. Conventional techniques have low sensitivity for diagnosis of extra pulmonary tuberculosis (EPTB) due to its paucibacillary nature. Thus this study is aimed to evaluate the diagnostic performance of Xpert MTB/RIF and Urine-LAM assay for the diagnosis of EPTB.

Methods: An institution based cross-sectional study was conducted from April to October 2019. A total of 147 patients suspected for EPTB, including 23 HIV positive participants were enrolled. Socio demographic data were collected by a questionnaire. Extra-pulmonary samples were collected from all presumptive EPTB cases. From 126 study participants, urine sample were collected. All site-specific specimens were processed and tested by fluorescent microscopy, Xpert test and LJ culture whereas urine samples were tested by only Determine TB LAM test (Alere Inc, Waltham, USA). Clinical data's such as HIV status, clinician's decision to start anti TB treatment and treatment response were collected from patient's medical records. Sensitivity and specificity of Xpert and TB LAM tests were calculated by comparing them with culture alone and with composite reference standard (CRS), which comprises smear microscopy, culture and anti TB treatment response results.

Results: There were 37 overall EPTB cases (23 confirmed and 14 probable**).** The positivity rate of smear microscopy, Xpert and culture were 8.2%, 11% and 15.6% respectively. LAM test detects 13.5% of 126 participants. The sensitivity and specificity of Xpert test comparing to culture was 69.5% and 100% respectively; and its sensitivity and specificity were decreased to 43.2% and 100% respectively with the highest sensitivity for abscess (85.7%) and lower sensitivity for plural fluid (14.2%) with 100% specificity for all specimen types, compared to CRS. The sensitivity and specificity of LAM test comparing to culture was 35% and 91.3% respectively; and comparing to CRS it was 30.6% and 93.3% respectively with the highest sensitivity for HIV co-infected participants, 66.7%. Comparing with CRS the combination of Xpert and TB LAM test detected 61.1% of all TB participants and 83.3% of HIV co-infected TB cases.

Conclusions: Determine TB LAM test has low sensitivity for EPTB diagnosis but, the combination of TB LAM and Xpert test could improve sensitivity of EPTB diagnosis and could replace culture, which is time consuming and less use for early diagnosis and treatment of EPTB cases.

Keywords: EPTB, Lipoarabinomannan, Xpert MTB/RIF, Culture.

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Abbreviations and Acronyms

AIDS……Acquired immune deficiency syndrome

ATT…….Anti tuberculosis treatment

CRS……..Composite reference standard

EPTB……Extra pulmonary tuberculosis

HIV……...Human immunodeficiency virus

IGRA…....Interferon gamma release assay

JUMC…...Jimma University Medical Center

JUMRC….Jimma University Mycobacteriology research center

LAM…….Lipoarabinomannan

LJ…….Lowenstein-Jensen

MDR…...Multidrug-resistant

MTBc…..Mycobacterium tuberculosis complex

NPV ……Negative predictive value

NTM……Non tuberculosis mycobacterium

PPV……..Positive predictive value

TB……Tuberculosis

TST……..Tuberculin skin test

WHO……World health organization

Chapter 1: Introduction

1. Background

Tuberculosis (TB) is a contagious disease caused by a group of bacteria called Mycobacterium tuberculosis complex. The history of *Mycobacterium tuberculosis* started before three million years in east Africa however the modern strain of *M.tuberculosis* complex progenitor was originated around 20,000 years ago(1). Tubercle bacillus was identified as etiologic agent for TB by Robert Koch in 1882 (2).

TB affects all countries and all age groups but it is most common in Southeast Asia and sub-Saharan Africa. According to WHO 2018 report about 10 million people developed active disease, out of them 1.6 million people died. Risk of developing active tuberculosis diseases is higher in exposed infants than exposed children's (2-10 years) then the risk increases during adolescence and adult life. TB affects mainly adult's ≥15 years (90%), males (64%) and people living with HIV (9%) in 2017 (3-5). Every year 9-10 million peoples are diseased with tuberculosis and its incidence is decreased only by 2% year to year, which is an indication for the need of accurate and efficient diagnosis and diagnostic tools (6).

According to WHO Statistics for TB in 2016, Ethiopia was one of the 30 high TB burden countries, which accounts about 87% of all TB cases. Total TB incidence in Ethiopia was 182,000 out of these, 14,000 in HIV positive cases and there were around 26,000 and 4,000 deaths from TB among HIV negative and HIV positive patients respectively. However Ethiopia decreases TB mortality rate and incidence rate by 75% and 50% respectively in 2016(7).

Ethiopia is also one of the 14 high-burden countries for TB, TB/HIV and MDR-TB defined by World Health Organization for the period 2016–2020. In Ethiopia the prevalence of TB is highest among those aged 35–54 years and among men's with (male: female) ratios of 1.2 (8, 9). TB in Ethiopia is more prevalent among peoples who have a history of hospital admission, a TB positive household member, a house with less number of windows, smokers and peoples having low household income(10).

HIV/AIDS, diabetes mellitus, smoking, alcohol use, and the use of other drugs are the most common risk factors for active TB diseases and also contribute to poor tuberculosis treatment outcomes. Study shows that around 15% and 10% of TB cases worldwide have Diabetes mellitus and attributable to alcohol use respectively (11).

Although Ethiopia is one of the countries with better Anti-TB treatment success rates (>70%), it is reported that it has still high MDR-TB burden in 2018(8). A recent systematic review in Ethiopia shows heterogeneous distribution and prevalence rate of MDRTB report in the country and the overall prevalence of MDR-TB in all TB cases was estimated to be 1.4% from 1994–2014. The highest rate of MDR-TB recorded in Ethiopia was 46.3%, 42.9% and 38.3% in 2010, 2006 and 2005 respectively. Previous exposure to Ant-Tuberculosis Treatment was found as the most common risk factor for MDR-TB in Ethiopia (12).

M. tuberculosis is highly acid–alcohol–fast, slim, non-motile, aerobic non–spore-forming rods that are 0.2 to 0.6 × 1 to 10μm in size. It prefers to grow at body temperature and it requires enriched or complex media for primary growth. Even though its Growth is enhanced by 5 to 10% carbon dioxide it has very slow growth rate with a mean generation time of 12 to 24 hours. *M.tuberculosis* have dry, rough, buff-colored colonies about 3 to 6 weeks of incubation in Lowenstein–Jensen medium which contains homogenized egg with dyes to inhibit the growth of non-mycobacterial contaminants. *M. tuberculosis* cell wall is rich in lipids which make it resistant to drying, to many common disinfectants, and to different laboratory Staining technique. It is inactivated by UV light, heat and pasteurization techniques. *M. tuberculosis* cell wall structure is dominated by Mycolic acids and lipoarabinomannan (LAM) (3, 13).

A LAM is an immunogenic lipopolysaccharide which is found in mycobacterial cell walls and has similar function with O-antigenic lipopolysaccharides present in other bacteria. It is released from metabolically active or degenerating bacterial cells and present mainly in people with active TB disease and has shown only low cross-reactivity with non-tuberculosis mycobacterial infections (3, 14).

LAM plays significant role in mycobacterial cell wall integrity and the pathogenesis of tuberculosis. A study by Fukuda et al. reports that a structural defect in LAM of *M. tuberculosis* resulted in loss of acid fastness, susceptible to macrophage killing and different antibiotics and failed to infect mice; which strongly suggests loss of cell wall integrity and pathogenicity(15).

LAM is excreted and detected in urine during advanced immunodeficiency such as HIV co infection; which results in systemic dissemination of *M. tuberculosis* and high mycobacterial burden due to podocyte dysfunction and change in glomerular filtration rate that leads higher antigen concentration of lipoarabinomannan in urine (16, 17). Another study indicates the other possible way for lam excretion in urine; it is due to renal involvement of patients with disseminated TB in patients living with HIV and advanced immunodeficiency (18).

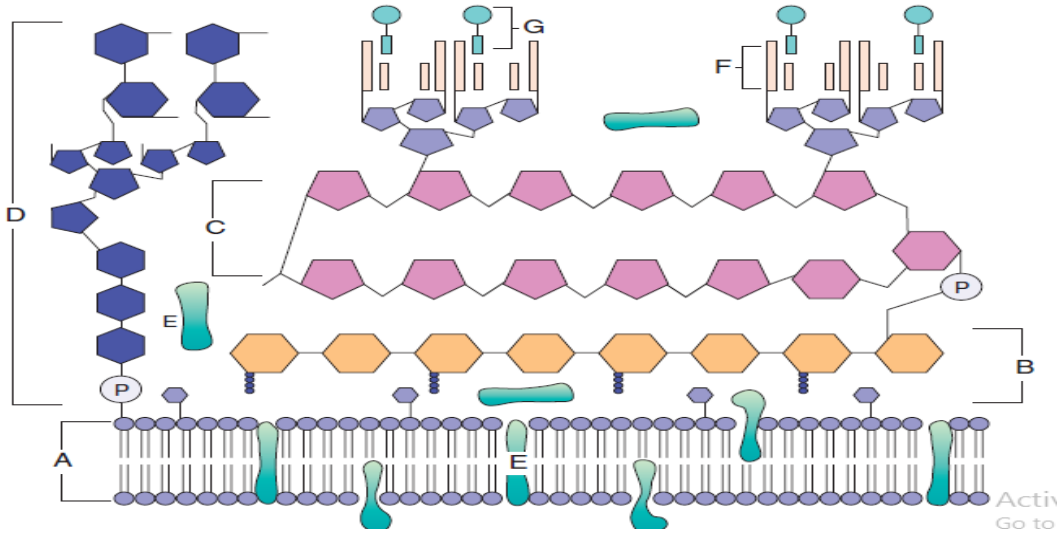


Figure 1: mycobacterium cell wall composition which includes (A) plasma membrane, (B) peptidoglycans, (C) arabinogalactan, (D) mannose-capped lipoarabinomannan, (E) plasma-associated and cell wall–associated proteins, (F) mycolic acids, and (G) glycolipid.
(source from : Murray P, Pfaller MA, Rosenthal K. Medical Microbiology. 7th Edition ed. philadelphia 2013. 1023 p)

M. tuberculosis which is an intracellular pathogen transmitted through aerosol spread of TB bacilli. After exposure, it enters the respiratory airways and tubercle bacilli penetrate to the alveoli and engulfed by alveolar macrophages, which results in cell necrosis, apoptosis or survival of the infected macrophages. Survival of infected macrophages enables the bacilli to persist and proliferate before the adaptive immune response is activated(3, 13).

After antigen presentation in lymph nodes, CD4+ T cells are activated and produce IFN-γ which stimulates formation of the phago-lysosome in infected macrophages, resulted in uncomfortable acidic environment for MTB. Another cytokine TNF-α is produced by activated macrophages, dendritic cells and T cells plays main role in granuloma formation and macrophage induction.

Granuloma which is an aggregation of immune cells and debris prevents further spread of the bacteria. Alveolar macrophages, epithelioid cells, and Langhans giant cells with intracellular mycobacteria form the central core of a necrotic mass that is surrounded by dendritic cells, neutrophils, B cells, T cells, natural killer (NK) cells and fibroblasts(3, 19, 20).

Tuberculosis is classified as pulmonary and extra pulmonary TB diseases. Pulmonary TB is the most common active TB infection of the lung, which is characterized by the presence of persistent cough, fever, night sweats and weight loss (21). EPTB is less common types of TB, which is due to lymphohematogenous dissemination of tuberculosis from the primary site of infection in the lung during immunosuppression. EPTB is characterized by fever, weight loss, night sweats, anorexia, weakness, wasting, hepatomegaly, lymphadenopathy and splenomegaly (22).

There are different TB diagnostic tests such as smear microscopy, which is developed more than 100 years ago but still it is used for diagnosis of active TB especially for pulmonary tuberculosis. Rapid molecular test method such as Xpert MTB/RIF tests which is used for diagnosis of active TB and for detection of rifampicin-resistance. Culture-based method is the current gold standard tests for TB diagnosis; however it is Complex, expensive, time-consuming and requires more developed laboratory capacity and can take up to 8 weeks to provide results. There are also new points of care tests for active tuberculosis diagnosis such as Lateral flow urine lipoarabinomannan assay which is Urine-based test for MTB cell wall component mainly for the Diagnosis of TB in HIV+ patients with low CD4 counts. There are also tests for the diagnosis of latent TB infection such as tuberculin skin test (TST) and interferon gamma release assay (IGRAs) (4, 6, 23).

2. Statement of problem

Tuberculosis (TB) is a major public health concern worldwide. Globally, TB causes a morbidity of an estimated 10 million people in 2018. EPTB accounts about 15% of 10 million notified TB cases globally. It also accounts 15%, 17% and 24% all TB cases in Africa, south East Asia and Eastern Mediterranean respectively. Ethiopia has high EPTB rates, about 31% of all notified TB cases (24).

Laboratory diagnosis of EPTB is critical, since clinical diagnosis alone may lead to diagnostic delays, misdiagnosis and result in resistant strains and increased mortality. Although there are different laboratory diagnostic techniques for the diagnosis of tuberculosis so far, the diagnosis of EPTB is difficult for many different reasons such as variable and non-specific presentations of the disease, pauci-bacillary nature or low bacillary load, non-uniform distribution of bacilli and difficulty in obtaining appropriate and adequate samples, and poor performance of conventional microbiological techniques during EPTB and disseminated TB diagnosis (23).

There is a need of rapid point of care and non-invasive tests for the diagnosis of extra pulmonary tuberculosis. Determine TB LAM Ag assay is a urine based test for the diagnosis of tuberculosis. Determine TB LAM test is recommended for the diagnosis of TB in HIV positive adult and children patients with signs and symptoms of TB (25).

There are different studies done on Determine TB LAM Ag and Xpert MTB/RIF Assay (18, 25-27). But most of the studies are done on only HIV positive and pulmonary TB cases. Thus the objective of this study was to evaluate the diagnostic performance of Determine TB LAM Ag and Xpert MTB/RIF Assay for extra pulmonary tuberculosis.

3. Significance of the study

The findings of this study would be an input to strength WHOs evidence of policy recommendation on Determine TB LAM test and contribute towards Improving active EPTB case detection and patient management. Provide information on the sensitivity and specificity of LAM and Xpert test for policy makers and national TB control programs.

Chapter 2: Literature review

Tuberculosis is a global health threat but it is treatable and curable, even in people living with HIV but its diagnosis is challenging. TB diagnoses in resource constrained settings are mainly through sputum smear microscopy which is rapid, cheap, simple to use and also used for grading of disease. Besides its advantage sputum smear microscopy, has poor performance or low sensitivity for diagnosing active tuberculosis especially in children's, peoples living with HIV(PLHIV) and in TB patients without lung involvements, it cannot differentiate tuberculosis from non-tuberculosis mycobacteria and also it is unable to detect drug-resistance (28-30).

Culture method is a gold standard for active TB diagnosis, which is more sensitive than other diagnostic tests with detection limit about 10-100 bacilli/ml of clinical sample. Unlike smear microscopy culture method is used for species identification; provide viable bacteria colony and single isolates for drug susceptibility testing. However its complexity, Slow turnaround time, high cost , bio-safety need and also bacterial killing during decontamination process may inhibit its applicability (23, 28).

Xpert MTB/RIF test is a nucleic acid amplification test which is endorsed by WHO in 2010 for the diagnosis of pulmonary TB and rifampicin resistance in adults and children and in 2014 for the diagnosis of extra pulmonary tuberculosis(31). According to different studies, it has different advantages when it is compared to conventional TB diagnostic techniques such as short turn-around-time (~2 h), highly sensitive and specific in smear positive patients, has good sensitivity and specificity in extra pulmonary specimens (32). It can also provide rapid DST results, its technique is not prone to cross-contamination and it requires minimal biosafety facilities but infrastructure requirements, more expensive in comparison to smear microscopy, limited sensitivity in smear-negative patients may limit its applicability especially in resource constrained settings and developing countries (6, 33).

There are several antibody based serological tests for the diagnosis of tuberculosis. Antibody detection test are simple, inexpensive and ideal for point-of-care diagnosis. This test may be used for screening of a high risk population, early diagnosis and treatment of TB cases and also it could be able to provide significant evidences along with smear microscopy and culture for diagnosis of EPTB. Currently available antibody detection test for tuberculosis diagnosis are not reproducible and the results vary in different settings. Antibody detection

test provide false results due to non-tuberculosis mycobacterium (NTMs) and during HIV co infection. Due to those limitations, WHO recommends not to use antibody detection test for tuberculosis diagnosis(23).

Detecting mycobacterial antigens in various body fluids may provide direct evidence of tuberculosis. BCG, non-protein cell wall antigen, antigen 5, 14kDa, antigen A60, 45/47 kDa antigen, lipoarabinomannan (LAM), haemoglycolipid-lipid antigen and cord factor (trehalose-6, 6_dimycolate) are some of the antigens which can be detected in patients body during TB infection with a minimum concentration of 3-20 ng/m L (23).
A Systematic Review and Meta-Analysis on Antigen Detection test shows a variable sensitivity from 0% to 100% and specificity ranges from 62% to 100% of this test. This variable sensitivity of antigen detection test limits the applicability of the test for TB diagnosis(34).

Alere Determine TB LAM Ag strip test is an immunochromatographic point of care test for the qualitative detection of lipoarabinomannan (LAM) antigen of Mycobacteria in urine specimen. TB LAM test provides result within 25 minutes (26, 35). Determine TB LAM test is recommended for the diagnosis of TB in HIV positive adult and children patients with signs and symptoms of TB (25). According to some studies, Determine TB LAM test shows a promising result for the diagnosis of smear-negative TB patients and disseminated TB patient, especially those with advanced immunosuppression and HIV-infection (36, 37).

Including point of care Determine TB LAM test in TB diagnostic algorithms could improve TB diagnosis especially in HIV co-infected TB patients and this is supported by a study in Kenya. The study in Kenya shows, adding TB LAM test increases the yield of diagnostic algorithm by 36.6% from (47.4 to 84%), 19.9% from (62.2 to 82.1%) and 13.4% from (74.4 to 87.8%) when they used clinical sign and x ray, clinical sign and microscopy and clinical sign and Xpert test as diagnostic algorithm, respectively (26).

The performance of Determine TB LAM test for diagnosis of TB in HIV negative individuals is low but it might be used as an alternative TB diagnostic tool in patients who are co-infected with HIV and patients with advanced immunosuppression. A study in Ethiopia, on Determine TB LAM test shows a sensitivity and specificity of 37.1% and 97.7% respectively, for TB patients regardless of their HIV status. But the sensitivity and specificity of Determine TB LAM test increased to 55.6% and 100% respectively, in HIV infected TB patients. The literature also shows the association between performance of TB LAM test and level of immunosuppression of the HIV-infected TB patients (36).

EPTB which results from lymphohematogenous dissemination of M. tuberculosis bacteria is a highly lethal form of TB (38), as a result it needs highly sensitive, fast and point of care tests. Thus the aim of this study is to evaluate the diagnostic performance of Xpert MTB/RIF assay and Determine TB LAM test for extra pulmonary tuberculosis.

Chapter 3: Objectives of the study

3.1 General objective

- To evaluate the diagnostic performance of Xpert MTB/RIF and Determine TB LAM Assays for extra pulmonary tuberculosis at Jimma University Medical Center

3.2 Specific Objectives

- To determine the diagnostic performance of Xpert MTB/RIF Assay for diagnosing EPTB.
- To determine the diagnostic performance of Determine TB-LAM Assay for diagnosing EPTB.
- To determine the yield of Xpert MTB/RIF and Determine TB-LAM Assays used in combination for the diagnosis of EPTB.

Chapter 4: Materials and Methods

4.1 Study area and period

The study was conducted at Jimma University Medical Center, South west Ethiopia from April to October 2019. Jimma zone is located in Oromia Regional State in Southwest Ethiopia. Geographically, it is located in Jimma city, 352 km southwest of Addis Ababa. JUMC has 500-inpatient beds and more than 15 million catchment populations. Every quarter of the year approximately 600-800 TB cases are treated.

Laboratory investigations for this study were carried out at Mycobacteriology Research Center of Jimma University (JUMRC), South west Ethiopia. The JUMRC 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 is mainly focused on basic 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 M. tuberculosis and drug susceptibility testing (DST), line probe assay (GenoTypeMTBDRplus assay) and Xpert MTB/RIF are the currently available technologies at the center.

4.2 Study design

The study design that we used in our study was an institution based cross-sectional study at Jimma University Medical Center, South west Ethiopia.

4.3 Source population

All EPTB patients who are attending to Jimma University medical center

4.4 Study population

All patients with signs and symptoms suggestive of EPTB, who were attending to Jimma University medical center during the study period, were included.

4.5 Inclusion and exclusion criteria

Inclusion criteria

- ✓ The amount of specimen was 3ml for body fluids and pus, 2.5 ml in case of CSF

Exclusion criteria

- ✓ Patients with pulmonary and extra pulmonary involvement
- ✓ Incomplete record
- ✓ Patients who received anti-tuberculosis drugs
- ✓ Severely ill patients, who were unable to give samples

4.6 Sample size

The sample size for this study was calculated based on the formula for sample size estimation of diagnostic test studies in health sciences (40). This formula requires the following assumptions; the expected sensitivity/specificity values of the new diagnostic test, the minimum acceptable lower confidence limit and the estimated prevalence of disease or condition under study. Based on sensitivity (previous study) the following formula is used to calculate the sample size.

$$N(Sen.) = \frac{Z^2 \times [Sen.\ (1-Sen.)]}{L^2 \times Prevalence}$$

$$N = \frac{(1.96)^2 \times [0.65x\ (1-0.65)]}{(0.1)^2 \times 0.54}$$

N =160

Where N =required sample size

Sen. = sensitivity= 65 % (39)

Z=CI normal distribution value i.e. for 95%, z=1.96

P=Prevalence of disease in the test population= 54% (32)

L= absolute precision desired on either side (half width of the confidence interval) of sensitivity (10%) = 0.1

4.7 Sampling Technique

Consecutive non probability sampling technique was applied.

4.8 Variable

Dependent variable

- ✓ Detection of TB from the collected sample

Independent variables

- ✓ Sex
- ✓ Age
- ✓ Residency
- ✓ HIV status
- ✓ HIV WHO staging
- ✓ Type of EPTB sample

4.9 Participant recruitment and data collection

From all patients visiting JUMC during the study period, those patients with sign and symptom of EPTB and able to give appropriate specimen (N=147) were enrolled. Once consent and assent was obtained demographic and clinical data were collected.

1. Study Participants' demographic and clinical information were collected using a pre-tested questionnaire by face to face interview.
2. As part of routine clinical practice fluids such as cerebrospinal fluid, pleural fluid, pericardial fluid, peritoneal fluid, and pus from cold abscess were collected according to standard operating procedure by physicians in different wards. Each sample was divided in to two and transferred in to two different falcon tubes. The first sample was processed for Xpert MTB/RIF test in JUMC Microbiology laboratory and the second sample was transported to Jimma University Mycobacteriology research center where smear microscopy and LJ culture were done.
3. All suspected EPTB patients were requested to submit urine specimen (10-30ml) for the research purpose. Urine specimen was collected and transported to the Mycobacteriology research center where TB-LAM test were done. The collected sample was centrifuged at 10,000g for 5minutes. Supernatant was used for Determine TB LAM assay (Annex 2).
4. Participants' medical records were reviewed for HIV status, WHO staging for HIV, clinicians decision to initiate ATT and treatment outcome. In addition, records of non TB participants were reviewed for their diagnosis (clinician's final diagnosis).

4.10 Laboratory processing and testing of specimens

Fluid specimens such as (cerebrospinal fluid, pleural fluid, pericardial fluid, peritoneal fluid, and synovial fluid) were processed and tested by Xpert MTB/RIF test, smear microscopy and culture whereas urine specimens were tested by Determine TB LAM test.

4.10.1 Lowenstein Jensen (LJ)

Culture was done using Lowenstein Jensen (LJ). Abscess or pus and blood-stained specimens were decontaminated by the standard *N*-acetyl-L-cysteine and sodium hydroxide (NALC/NaOH) method with a final NaOH concentration of 1% (41). An equal volume of standard NALC/NaOH solution was added to the specimen and incubated for 20 minutes. After centrifugation (15 minutes at 3000g), the sediment was re suspended in 1.0 to 1.5 ml of sterile phosphate buffer (pH 6.8). Specimens expected to be sterile (such as cerebrospinal fluid (CSF), pleural fluid, and peritoneal fluid) were directly centrifuged to concentrate the samples. Tubes were inoculated with 0.2 mL (2–4 drops) of the processed specimens (42). For positive LJ tubes, a smear was prepared to detect acid-fast bacilli, and MTBC was confirmed by a p-nitro benzoic acid test.

4.10.2 Para-nitrobenzoic acid inhibition (PNB) test

PNB test was performed in our laboratory to differentiate MTBc from NTM. LJ media containing PNB drug was prepared according to JUMRC-SOP manual. Colonies from the surface of the LJ medium was scraped and emulsified on tubes containing glass beads and 2 ml of sterile water. The content of the tube was Homogenized and allowed to stand for 5min to allow larger clumps to settle. Then 1 ml of the supernatant suspension was transferred to another tube and the turbidity is adjusted to McFarland 1standard by adding distilled water. For every sample one PNB containing and one PNB free-control media was inoculated with bacterial suspension and incubated at 37^0c for 28 days. The results are interpreted as, if No growth was seen on PNB media it is MTBc, if growth was seen on PNB media it is NTM. But there should be a growth on drug free LJ media to be valid (43).

4.10.3 Fluorescent Smear microscopy

After processing of the specimens, smears were prepared from all samples other than urine and examined at Mycobacteriology Research Center of Jimma University (JU-MRC) for the presence of AFB. All smears were stained by the Auramine-phenol method according to standard operating procedures and examined with fluorescent LED Microscope (42).

4.10.4 Xpert MTB/RIF test

The Xpert MTB/RIF assay was performed as previously described by Helb et al. and WHO (44, 45), Briefly sample reagent was added in a 2:1 ratio to patient specimen. The mixture was vortexes and incubated at room temperature for 15 minutes. Two ml of the reagent sample mixture was transferred to an Xpert cartridge using a Pasteur pipette, the cartridge was loaded onto Xpert machine and results was automatically generated after1 hour and 50 minute. Xpert MTB/RIF assay detects both presence of MTB and RIF Resistance. If the result is MTB not detected; it means it is negative for MTB. MTB detected and RIF Resistance not detected means it is positive for MTB and sensitive for rifampicin. MTB detected and RIF Resistance detected means it is positive for MTB and it is resistant for rifampicin. MTB detected and RIF Resistance indeterminate means it is positive for MTB and the test could not accurately determine if the bacteria are resistant to RIF; during RIF Resistance indeterminate result susceptibility testing to first-line TB drugs should be performed. For 'Invalid', 'No Result' or 'Error' results, the sample was reprocessed and retested.

4.10.5 Urine-LAM test

Immediately after collection, urine specimen was stored at -20^{o}c. While we got the LAM strip test kit, the frozen specimen was de-frozen and centrifuged at 10,000g for 5 minutes. About 60 μL of supernatant was applied to the sample pad (White pad marked by the arrow symbol). After 25 minutes two trained laboratory technologists who were blinded to the culture and Xpert results read Determine TB LAM test and results was recorded. Positive tests were graded on a scale that ranged from grade 1 to 4. Results were stable for up to 35 minutes after sample application and it should not be interpreted beyond 35 minutes(25). For Determine TB LAM test all cases greater than or equal to grade 1+ was considered as LAM test positive.

4.11 Diagnostic classification for analysis

Based on clinical and laboratory findings, study participants were categorized as follows: (i) Confirmed TB: defined as a positive culture of MTBC and /or smear positive; (ii) Probable TB: culture negative but clinical improvement after ATT; (iii) Non TB: patients for whom no microbiological (smear-negative and culture-negative) evidence of TB, and/or for whom an alternative diagnosis is available.

Since TB culture has high sensitivity and specificity it is used as a gold standard for diagnosis of pulmonary tuberculosis, however its sensitivity is limited in EPTB cases because of the pauci-bacillary load of the diseases. Due to the above reason we have used composite reference standard (CRS), as a standard or a reference to calculate the sensitivity and specificity of new diagnostic tests. CRS in this study comprises of smear microscopy, LJ culture and clinical improvement after ATT initiation. Any patient that was positive for any one component of the CRS was considered as 'TB' cases.

4.12 Quality control

We checked out the completeness, accuracy and clarity of our data to ensure data quality.

Training was given to data collectors on how to approach study subjects and on how to use the questionnaire. Quality checking was done daily, during and after data collection; amendments were made before the next data collection.

Internal quality control procedure was implemented for all laboratory procedures. A known positive and a known negative control specimen were included with each run and each batch of laboratory procedure. We used known $H_{37}RV$ strain to check whether culture media supports growth. Known specimen was used to check Xpert MTB/RIF test quality. Quality control line in Determine LAM test kit was used to check validity of LAM test. All laboratory procedures were carried out as per the approved protocol/ SOPs.

4.13 Data management

Data were entered through Epidata version 3.1 and analyzed using SPSS software package version 20. Descriptive analysis such as frequency, mean and proportion was calculated. The sensitivity, specificity, PPV and NPV of Determine TB LAM test and Xpert MTB/RIF test was calculated in comparison with culture alone and with CRS, which comprises smear microscopy, culture and clinical improvement after ATT. Any patient that was positive for any one component of the CRS was considered 'TB'. ROC and AUC are also calculated to evaluate the discriminatory ability of Determine TB LAM test and Xpert MTB/RIF test. Cohen's kappa coefficient was calculated to assess the agreement between the two readers. Overlapping 95% CI data were considered as showing no significant difference between test results.

4.14 Ethical clearance

Ethical clearance was obtained from Jimma University institution of health science, institutional review board (IRB) before we conduct this study. The study participants were informed to voluntarily participating in the study. Also informed consent was obtained from each study participant, and assent form from parents or guardians of participants whose age is less than 15 years, with full right to refuse to participate during the study at any time. Confidentiality was maintained by coding participant's identity starting from data collection to analysis.

4.15 Dissemination plan

The results of this study will be presented to Jimma University institute of health and school of medical laboratory sciences. It will also be disseminated to Jimma University medical center, Jimma town health office and finally, an attempt will be made to publish the findings of this study in reputable open access international journals.

Chapter 5: Result

5.1 Demographic and clinical data

A total of 147 presumptive EPTB patients, who visited the hospital during the study period were included. Participants had a median age of 35(IQR) (22–45) years; 82 (56%) were males and 23(15.6%) were HIV infected. The majority, 84(57.1%) of the participants were from rural areas (Table 2). Regarding clinical signs and symptoms, about 124(85%) and 122(83%) of the study participants had Tiredness and loss of appetite respectively. Weight loss, night sweating, fever, cough, shortness of breathing, abdominal distention, headache, diarrhea, chest pain and vomiting were also observed in 106(72%), 100(68%), 84(57%), 72(49%), 50(34%), 20(13.6%), 16(11%), 15(10%), 12(8%) and 9(6%) of the study participants respectively. About 23 participants were positive for HIV and 22 (95.7%) of them were classified in WHO stage IV and 1(4.3%) of them were stage III. Out of the total HIV infected patients about 3(13%) were not started anti-retroviral treatment during data collection.

All participants provided sufficient volume of extra pulmonary specimens including 28 CSF, 49 peritoneal fluid, 45 pleural fluids, 19 abscesses, 2 pericardial and 4 other fluids (2 gastric aspirate and 2 synovial fluids). In addition all study participants were requested to provide urine specimen but only 126(86%) of study participants provided urine. Of the total 147 patients, there were 37 EPTB cases in which (23 culture confirmed TB cases and 14 ‘probable TB’ cases with clinical improvement after clinician’s decision to start ATT) and 110 patients were classified as ‘non TB’ cases because no bacteriological or clinical evidence for TB is observed.

Table 1: Socio demographic and clinical characteristics of presumptive EPTB cases visited Jimma University medical center from April to October 2019(N= 147).

Characteristics		All,(N=147)	Confirmed TB(n=23)	Probable TB (n=14)	Non TB (n=110)
Sex	Male	82(55.8%)	10(43.5%)	11(78.6%)	61(55.5%)
	Female	65(44.2%)	13(56.5%)	3(21.4%)	49(44.5%)
Residence	Urban	63(42.9%)	12(52.255)	6(42.9%)	45(40.9%)
	Rural	84(57.1%)	11(47.8%)	8(57.1%)	65(59.1%)
Age(years)	0-15	24(16.3%)	4(17.4%)	2(14.3%)	18(16.4%)
	16–30	35(23.8%)	10(43.5%)	3(21.4%)	22(20%)
	31–45	52(35.3%)	6(26.1%)	4(28.6%)	42(38.2%)
	>45	36(24.5%)	3(13.0%)	5(35.7%)	28(25.5%)
Types of specimen	peritoneal fluid	49(33.3%)	2(8.7%)	6(42.9%)	41(37.3%)
	pleural fluid	45(30.6%)	4(17.4%)	3(21.4%)	38(34.5%)
	cerebrospinal fluid	28(19%)	3(13%)	3(21.4%)	22(20%)
	Pus(abscess)	19(12.9%)	13(56.5%)	1(7.1%)	5(4.5%)
	pericardial fluid	2(1.4%)	0	0	2(1.8%)
	Others*	4(2.7%)	1(4.3%)	1(7.1%)	2(1.8%)
HIV status	Positive	23(15.6%)	4(17.4%)	2(14.3%)	17(15.5%)
	Negative	111(75.5%)	16(69.6%)	12(85.7%)	83(75.5%)
	Unknown	13(8.8%)	3(13%)	0	10(9.1%)

*****Others include 2 gastric aspirates and 2 synovial fluid specimens

5.2 The different forms of EPTB

A total of 37 different forms of extra pulmonary tuberculosis (23 culture confirmed and 14 clinically treated probable TB) were identified (Figure 2).

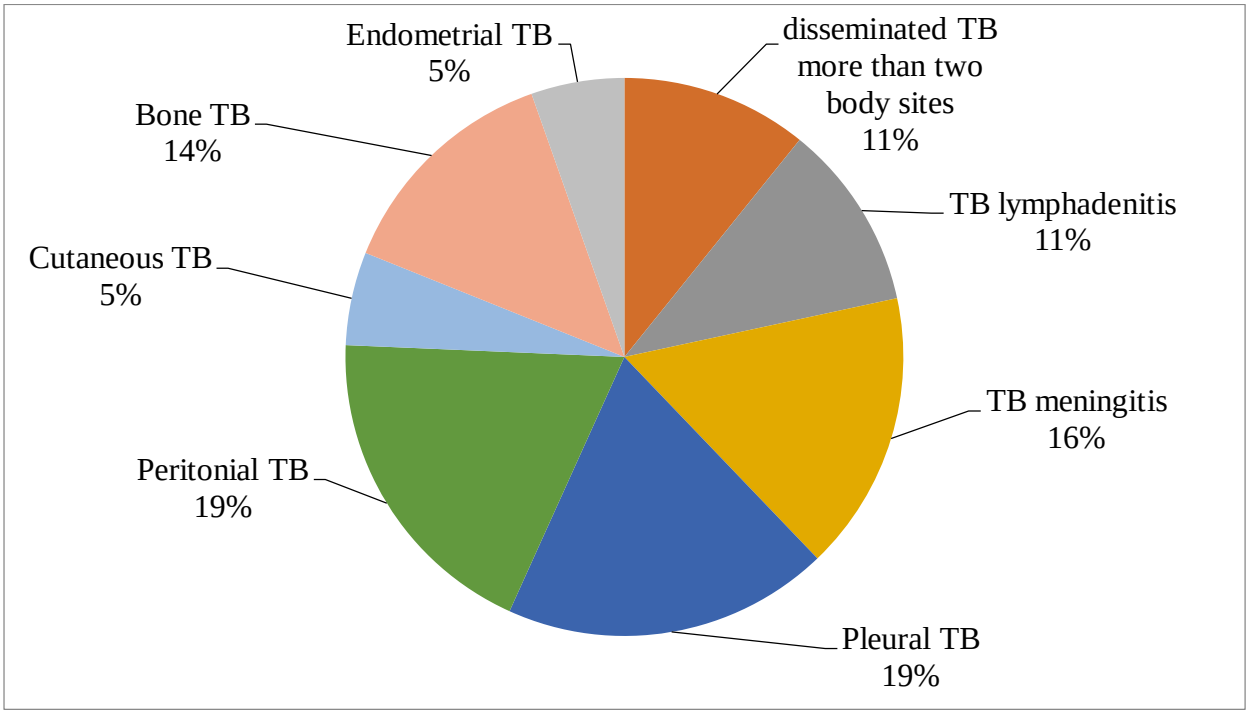


Figure 2: Types of identified forms of extra pulmonary tuberculosis (37 overall EPTB cases) at JUMC from April to October 2019.

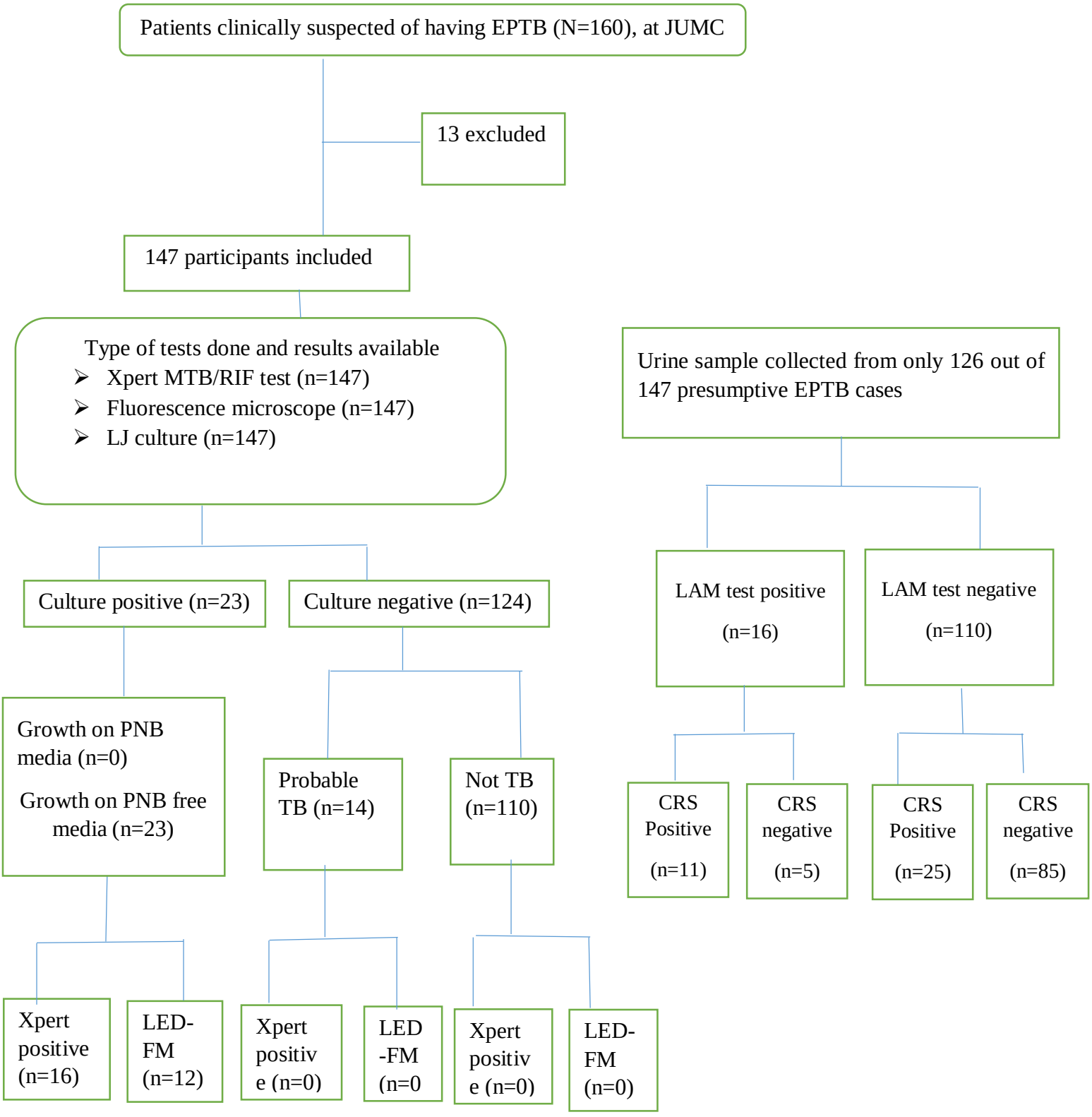


Figure 3: flow chart which shows overall patient flows, diagnostic test result and diagnostic classifications. CSF, cerebrospinal fluid; conta*, contamination; EPTB, extra pulmonary tuberculosis; LAM, Lipoarabinomannan; LJ, Lowenstein-Jensen; PNB, p-nitro benzoic acid

5.3 Detection/positivity rate of tests

There were overall 37 EPTB cases, of which about 23(15.6%), 16(11%) and 12(8.2%) of 147 site-specific specimens were positive by culture, Xpert MTB/RIF test and fluorescent microscopy respectively. There were 3 (2%) Xpert MTB/RIF error result and 2 (1.4%) culture contaminations but all of them were re-processed, re-tested and provide negative results. All Xpert MTB/RIF test positive cases, were RIF sensitive. Determine TB LAM test was positive for 16(12.7%) of 126 urine specimens. A total of 42 participants including (23 culture positive and 19 culture negative cases with clinician's decision) started ATT. After two weeks all culture positive and 14 culture negative cases had good response to ATT. Out of 5 cases that were culture negative, but started ATT through clinicians decision, 3 cases were died and 2 cases were not improved within two weeks follow up. About 37(25.2%) of the 147 participants were positive by CRS.

5.4 Diagnostic performance of Determine TB LAM, Xpert MTB/RIF test and Fluorescence microscopy

The accuracy of Determine TB LAM test and Xpert MTB/RIF test to diagnose EPTB regardless of patients' HIV status was calculated (Table 3). Using grade 1 cut off point the results of the two independent readers of TB LAM test were concordant in 124 of 126 tests which gives an agreement of 98.4 % and kappa statistics was 95.6%(95% CI, 92.5%-98.7%).

Out of 126 participants 16(12.7%) were positive for Determine TB LAM test (≥ grade 1). From the LAM positive participants, 8 of them were culture positive whereas the remaining 8 was culture negative. About 4 of 8 LAM positive but culture negative cases had a result of grade 2 and above. Medical records of those 8 LAM positive, but culture negative cases was reviewed and 4 patients were started ATT, through clinicians decision; after two weeks follow up 3 patients improved and 1 patient was died.

Adding Determine TB LAM test to Xpert MTB/RIF test increases the sensitivity of Xpert MTB/RIF test from 69.5% to 82.6 comparing to culture reference standard and from 43.2% to 61.1% comparing to CRS reference standard respectively, however its specificity is decreased from 100% to 91.3% and 93.3% as compared to culture and CRS respectively.

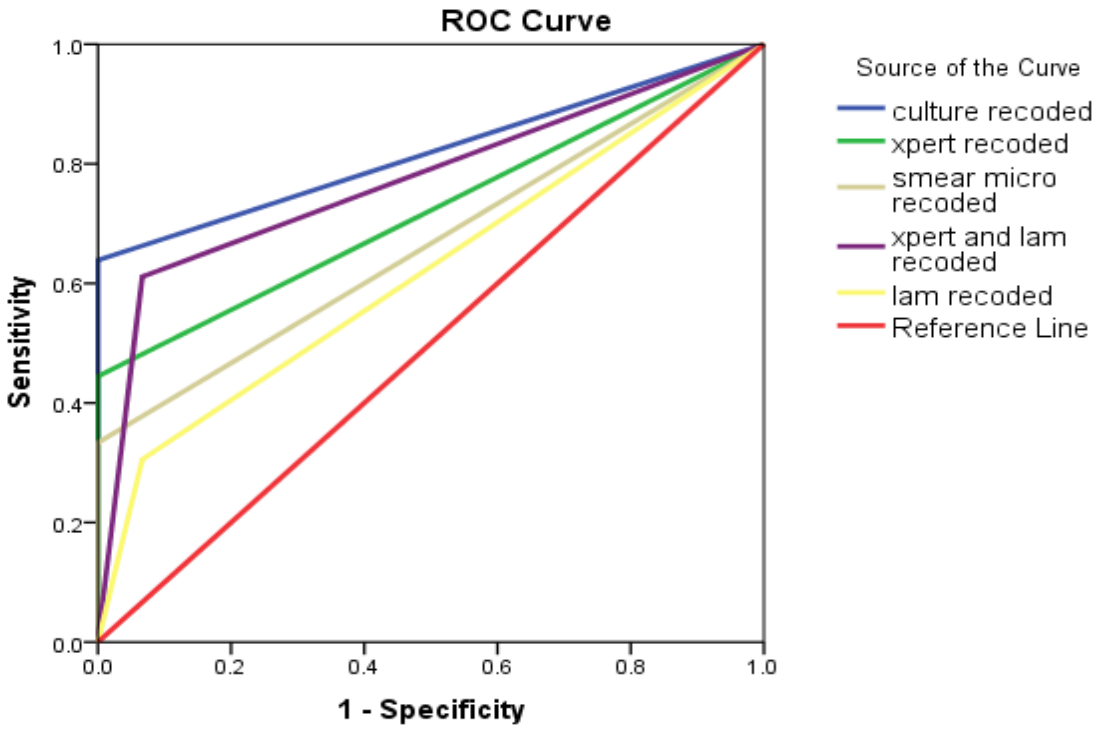
TB detection capability of Fluorescence microscopy was calculated. Fluorescence microscopy detected, 52.5% (12/23) of culture positive cases with sensitivity, specificity, PPV and NPV of 52.5%, 100%, 100% and 91.8% respectively. However its sensitivity, specificity, PPV and NPV is changed in to 32.4%, 100%, 100% and 81.5% respectively, when it is compared with CRS. Comparing to CRS, the sensitivity of LED-FM was 57.1%, 33.3% and 28.6% for abscess, CSF and pleural fluid respectively, with 100% specificity for all sample types.

Table 2: 2x2 table for LAM and Xpert MTB/RIF Assay comparing with Culture and CRS reference standard

	Culture				CRS		
LAM Assay		Positive	Negative	Total	Positive	Negative	Total
	Positive	8	8	16	11	5	16
	Negative	15	95	110	25	85	110
	Total	23	103	126	36	90	126
Xpert Assay	Positive	16	0	16	16	0	16
	Negative	7	124	131	21	110	131
	Total	23	124	147	37	110	147
Xpert and/or LAM	Positive	19	8	27	22	5	27
	Negative	4	95	99	14	85	99
	Total	23	103	126	36	90	126

Table 3: Diagnostic performance of Determine TB-LAM and Xpert MTB/RIF test

Diagnostic Accuracy	Culture as a reference standard		
	Determine TB LAM test	Xpert MTB/RIF test	Determine TB LAM and Xpert MTB/RIF test
Sensitivity (95%CI)	34.9% (95%CI, 34.1%-35.5%)	69.5%(95%CI,68.9-7.0)	82.6% (95%CI, 82.1%-83.1%)
Specificity (95%CI)	91.3% (95%CI, 91%-91.4%)	100%	91.3% (95%CI, 91.1%-91.4%)
PPV (95%CI)	47% (95%CI, 46.3%-47.8%)	100%	67.9% (95%CI, 67.3%-68.4%)
NPV (95%CI)	86.2% (95%CI, 86%-86.4%)	94.6%(95%CI,94.5-94.7)	95.2 %(95%CI, 95%-95.4%)
	CRS as a reference standard		
	Determine TB LAM test	Xpert MTB/RIF test	Determine TB LAM and Xpert MTB/RIF test
Sensitivity (95%CI)	30.6% (95%CI, 30%-31%)	43.2% (95%CI, 42.7-43.7)	61.1% (95%CI, 60.6%-61.6%)
Specificity (95%CI)	93.3% (95%CI, 93.2%-93.5%)	100%	93.3% (95%CI, 93.2%-93.5%)
PPV (95%CI)	64.7% (95%CI, 63.9%-65.4%)	100%	78.6% (95%CI, 78%-79.1%)
NPV (95%CI)	77.1% (95%CI, 76.8%-77.3%)	83.9% (95%CI ,83.7-84)	85.7% (95%CI, 85.5%-85.9%)



Diagonal segments are produced by ties.

Footnote: culture recoded means *culture test*, xpert recoded means *xpert mtb/rif test*, smear micro recorded means *smear microscopy*, xpert and lam recoded means *combination of xpert and lam test* and lam recorded means lam test

Area Under the Curve

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
culture recoded	.819	.051	.000	.720	.919
Xpert recoded	.722	.058	.000	.610	.835
smear micro recoded	.667	.059	.004	.550	.783
Xpert and lam recoded	.772	.053	.000	.669	.876
lam recoded	.619	.059	.037	.504	.735

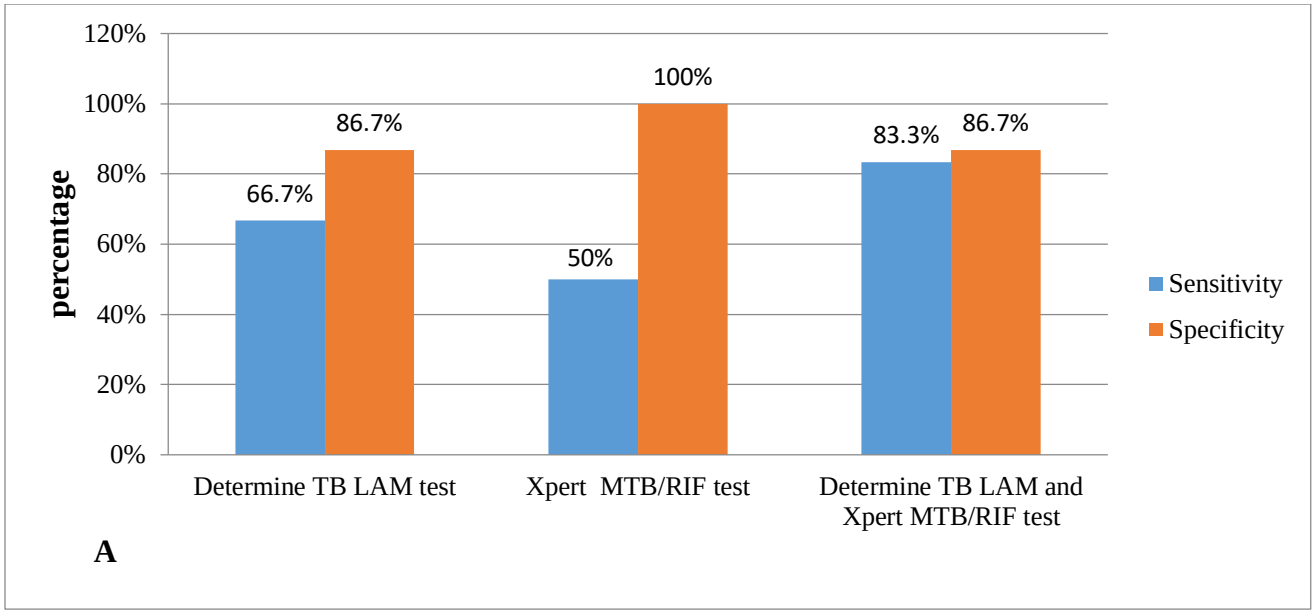
Null hypothesis: true area = 0.5
Alternative hypothesis: true area >0.5

In this study all the tests has AUC >0.5 with statistically significant at ($p<0.05$). As a result we rejected the null hypothesis and all tests has a significant discriminatory ability of EPTB.

Figure 4: ROC analysis for culture, smear microscopy, Determine TB LAM test, Xpert MTB/RIF test and the combination of LAM and Xpert test against CRS.

5.5 Diagnostic performance of Determine TB LAM and Xpert MTB/RIF test among HIV-positive and HIV negative patients

The strength of Determine TB LAM and Xpert MTB/RIF test to diagnose TB in HIV co-infected participants were calculated (figure 5). Out of the total HIV positive TB suspected patients (n=23), about 2(8.7%), 3(13%), 4(17.4%), 4(17.4%) and 5(21.7%) were diagnosed as TB positives by Smear microscopy, Xpert MTB/RIF test, Determine TB LAM test, culture and combination of LAM and Xpert tests respectively. There is a significant difference in sensitivity of Determine TB LAM test in HIV positive and HIV negative participants, which was 66.7%(95%CI, 65.4%-67.8%) and 26%(95%CI, 25.4%-26.5%), respectively.



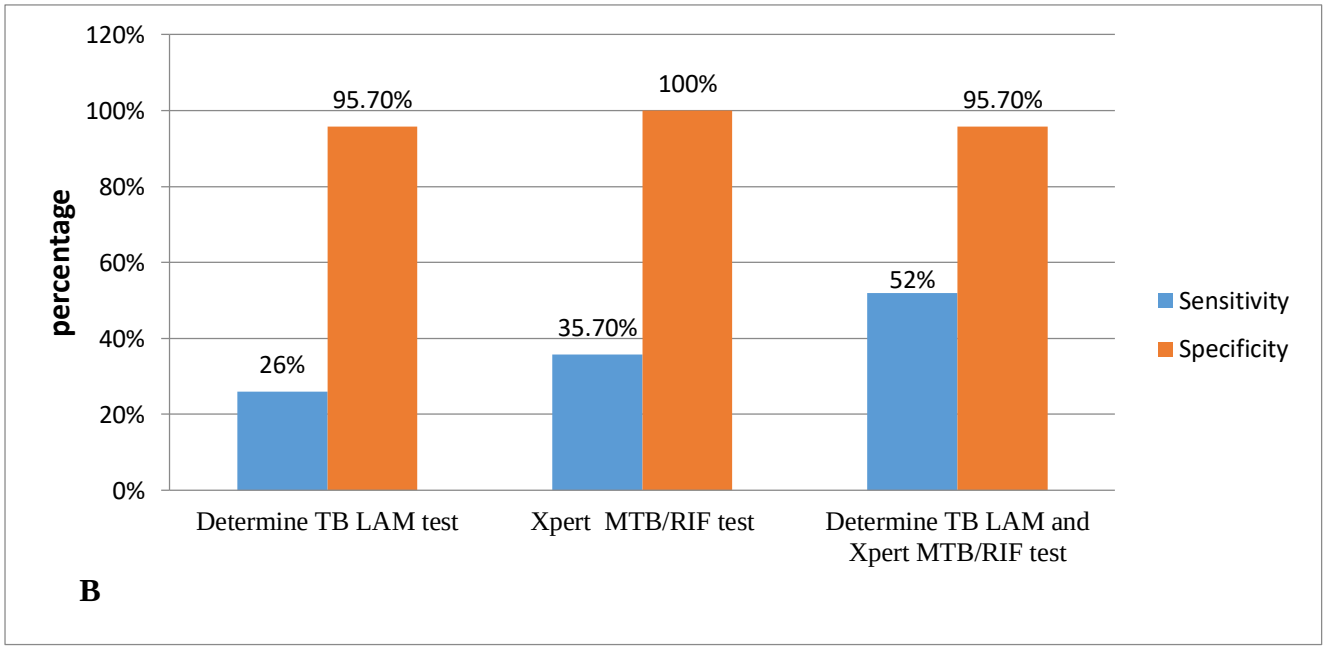


Figure 5: performances of Determine TB LAM, Xpert MTB/RIF test and combination of TB LAM and Xpert MTB/RIF test comparing to CRS among HIV positive (A) and HIV negative (B) TB cases

5.6 Diagnostic performance of Xpert MTB/RIF test across different clinical samples

The diagnostic accuracy of Xpert MTB/RIF test among non-respiratory specimens were calculated. Comparing to CRS reference standard, the sensitivity of Xpert MTB/RIF test were 85.7%, 33.3% and 14.2 for Abscess, CSF and Pleural fluid, respectively (Table 4).

Table 4: performance of Xpert MTB/RIF test among different non respiratory samples

		Xpert MTB/RIF test		CRS		Xpert MTB/RIF test			
Sample type	total	positives	negatives	positives	negatives	Sensitivity (95%CI)	Specificity (95%CI)	PPV	NPV
Abscess	19	12	7	14	5	85.7%	100%	100%	71.4%
CSF	28	2	26	6	22	33.3%	100%	100%	84.6%
Pleural fluid	45	1	44	7	38	14.2%	100%	100%	86.4%
Other*	4	1	3	2	2	-	-	-	-

Note: other* includes (2 gastric aspirate and 2 synovial fluid). All peritoneal fluid and pericardial fluid specimens were negative for Xpert MTB/RIF test.

Chapter 6: Discussion

Our study shows that about 25.2% of study participants had EPTB. This high proportion of EPTB cases is comparable to a report from WHO 2019 for Ethiopia that reports 31% of EPTB cases (8). This high proportion of EPTB might be due to the high HIV prevalence in the country (46).

Sensitive and rapid diagnostic tests will increase the number of diagnosed patients; facilitate early treatment, contributing potentially to decreased disease transmission, reduced case fatality and prevention of adverse effect of the disease, especially in the highly lethal form of tuberculosis (as disseminated tuberculosis). Conventional techniques (smear microscopy and culture) have limited sensitivity for the diagnosis of EPTB, due to the paucibacillary load of the diseases. Mycobacterium tuberculosis needs about 6-8 weeks to grow on LJ medium, which limits the applicability of culture. It is difficult to obtain appropriate extra pulmonary specimen and there is a need for invasive procedures. Incorporating tests like Determine TB LAM test which uses urine sample for tuberculosis diagnosis is attractive than the other EPTB diagnostic tests which needs invasive procedures for sample collection(4, 14).

Xpert MTB/RIF test and Determine TB LAM test was evaluated in HIV positive and HIV negative EPTB patients. In our study the overall sensitivity and specificity of Xpert MTB/RIF test, comparing to LJ culture reference was 69.5% and 100% respectively, but the sensitivity was reduced from 69.5% to 43.2% when comparing to CRS reference, whereas the specificity was the same.

Culture is the gold standard for the diagnosis of pulmonary TB; however it may miss cases of EPTB due to the paucibacillary nature of the disease(23). As a result when Determine TB LAM and Xpert MTB/RIF test is evaluated against culture alone, it leads to underestimating the specificity of Determine TB LAM and Xpert MTB/RIF test since culture may overestimate false positive EPTB cases(cases identified as positive by Xpert MTB/RIF and Determine TB LAM test but negative by culture). Thus using CRS reclassifies false positive cases to true positives and improves specificity. The other limitation of using culture alone as a reference is overestimation of the sensitivity of Xpert MTB/RIF and Determine TB LAM test since culture may miss true positive cases of paucibacillary diseases (32, 47). So, Sensitivity overestimation could be decreased and corrected by using CRS which comprises anti-TB treatment response data in addition to culture.

.The positivity rate of Xpert MTB/RIF test for non-respiratory specimens in this study was 10.8% (16/147), which is comparable to studies done in India by Pravin et.al, who reports overall 9.4% positivity of Xpert MTB/RIF test for body fluids(30).

The finding of the present study shows that the sensitivity of Xpert MTB/RIF test was less than the sensitivity of culture, however it can improve and fasten or decrease the turnaround time (TAT) for the diagnosis of tuberculosis especially when a rapid diagnosis of TB and early treatment initiation is significantly necessary (like in the case of disseminated TB). The detection time for MTB with Xpert MTB/RIF test and LJ culture test was less than two hour and 6-8 weeks respectively in our study.

In addition we found variable sensitivity of Xpert MTB/RIF test among different non-respiratory sample types. Sensitivity of Xpert test was 85.7 % for abscess, 33.3% for CSF and 14.2 % for pleural fluid, with 100% specificity for all specimen types. This finding indicates that high sensitivity of Xpert test in abscess sample and lower sensitivity in CSF and pleural fluid specimen for EPTB diagnosis; which is consistent with findings of previous studies (32, 48, 49).

Our finding also shows that high specificity and low sensitivity of Xpert MTB/RIF test on body fluids, which is also reported by previous studies (32, 48), and it might be due to the paucibacillary nature of the diseases. Other studies suggested Xpert MTB/RIF Ultra over Xpert MTB/RIF assay with an overall improvement of 45.6% for the diagnosis of respiratory and non-respiratory smear-negative cases and paucibacillary diseases(50).

The most striking result to emerge from the data is that the high specificity and positive predictive value of Xpert MTB/RIF test, which provides significant evidences for the clinicians to rule in extra pulmonary tuberculosis and to treat patients as early as possible following a positive Xpert MTB/RIF result. The overall low sensitivity of Xpert MTB/RIF test (43.2%) as compared to CRS shows that negative Xpert MTB/RIF result does not exclude or rule out EPTB diagnosis especially from body fluid specimens such as pleural and peritoneal fluids. As a result where available, other diagnostic tools and approaches such as Xpert MTB/RIF Ultra test should be considered (50, 51).

In this study the overall sensitivity and specificity of Determine TB LAM test comparing to culture reference to diagnose EPTB regardless of HIV status was 35% and 91.3% respectively, which is comparable to findings reported by Sahle et al (36). Comparing to CRS Sensitivity was decreased to 30.6% while specificity was improved to 93.3%. This low sensitivity of Determine TB lam test indicates that negative LAM test result does not used to rule out extra pulmonary tuberculosis. In addition Determine TB LAM test alone should not be used to diagnose extra pulmonary tuberculosis. However it has high sensitivity for HIV infected EPTB patients.

The sensitivity and specificity of Determine TB LAM test to detect TB in HIV-co infected patients comparing to CRS were 66.7% and 86.7%, respectively. The sensitivity of the present study shows similarity to some other study findings, reported by Hurega et al and Juma et al, 65.4% and 65.52%, respectively (26, 39). The conventional diagnostic tool; Smear microscopy is still used to diagnose TB including HIV co infected patients in resource constrained settings however its limited sensitivity shows there is a need for additional sensitive and point of care tests. In our study the sensitivity of Determine TB LAM test in HIV positive patients was much higher than in HIV negative patients. In this study Determine TB LAM test detected TB in additional 2(33%) and 1(17%) of HIV infected patients who were missed by smear microscopy and Xpert MTB RIF test respectively. This suggests that Determine TB LAM test could address the challenges for diagnosing TB in HIV co infected patients through smear microscopy and Xpert MTB RIF test. The high sensitivity of Determine TB LAM test in HIV positive patients than HIV negative patients might be due to podocyte dysfunction that results change in glomerular filtration which is most of the time secondary to HIV disease that leads higher antigen concentration of lipoarabinomannan in urine (16). Although not performed in our study, different studies shows a significant association between the performances of Determine TB LAM test and level of immunosuppression (36, 39, 52) and positive Determine TB LAM test is as a predictor for mortality(26, 52).

The overall sensitivity of the combination of Xpert and Determine TB LAM test to diagnose EPTB irrespective of HIV status was higher than either test alone in this study, which was 61.1% and it is comparable to LJ culture (62.2%). This finding suggests that it is better to use the combination of Xpert and TB LAM test than each test alone or culture alone when diagnosing EPTB cases, since both Xpert and TB LAM test has short turnaround time than culture techniques which resulted in early diagnosis and treatment of tuberculosis patients, which is one of the main pillars and components of WHO end TB strategies (4). Using the combination of Xpert and Determine TB LAM test is relatively more expensive than using each test alone or culture alone, however it provides important contribution for the diagnosis of EPTB especially when there is a need of very sensitive test and short time to treatment initiation as in the case of Miliary TB, disseminated TB, TB meningitis and TB in HIV infected patients, however a negative result of the combination of Xpert and Determine TB LAM test does not exclude the diagnosis of EPTB. Patients whose sign and symptom, strongly suggests EPTB or patients whose ultrasound and radiological findings strongly suggests EPTB, should start ATT despite negative Xpert and LAM results.

In this study the sensitivity and specificity of Determine TB LAM test as compared with CRS, in HIV negative EPTB cases were 25.9% and 95.7% respectively, which is comparable to studies done in Thailand, by G. Suwanpimolkul et al, who reports a sensitivity of 20% and a specificity of 100 % (52). This might be due to very low concentration of LAM in urine of non-HIV infected TB patients, since lam concentration in patient's urine is directly proportional to disease burden and immunosuppression (53).

The specificity of Determine TB LAM test in the present study was low, 91.3% and 93.3% as compared to culture and CRS respectively. It is also 86.7% and 95.7% in HIV positive and HIV negative TB cases respectively. This is actually below the value of optimal requirements for the diagnostic specificity of a rapid biomarker-based, non-sputum-based test for detecting or diagnosis of extra-pulmonary TB in adults and children (98%) which was set by WHO(54). There are two possible explanations for this. Since specificity is inversely proportional to false positivity, a decreased specificity indicates that there is high number of false positive cases which might be due to NTM co-infection of study participants. Lipoarabinomannan (LAM) is a glycolipid present in the cell wall of all species within the Mycobacterium genus which may results cross-reactivity between MTBC and NTMS and it is supported by a study in South Africa (55).

The second possible explanation is that in our study we use grade 1 as a cut off-point and all cases with equal or greater than grade 1were classified as Determine TB LAM test positive cases. Using grade 1 as a cut point increases the positive results as well as false positive cases and according to studies switching from a grade 1 to grade 2 cut-point increases specificity (56). However, Determine TB LAM test in this study has high sensitivity for HIV positive participants and in combination with other diagnostic tests.

Limitation of the study

The relatively small sample size in our study may be considered as a limitation; as a result it was difficult to check the association between Determine TB LAM tests with HIV stages. Due to shortage of Determine TB LAM test kit the urine sample was stored. Despite these limitations, our study provides information on the diagnostic performances of Determine TB LAM test, Xpert MTB/RIF test and their combinations. In addition we have used CRS reference that could improve the specificity of new tests.

Chapter 7: Conclusion

This study set out to evaluate the diagnostic performance of Determine TB LAM test and Xpert MTB/RIF assay for the diagnosis of EPTB cases, which is the most difficult form of TB to diagnose, most sever and highly lethal form of TB. Xpert test had high sensitivity for abscess specimen and low sensitivity for body fluid specimens. The sensitivity of combination of Determine TB LAM test and Xpert MTB/RIF test was superior to either test alone and equivalent to culture to diagnose EPTB. The evidence from this study suggests that the combination of these tests can solve diagnostic challenges associated with EPTB diagnosis, especially for patients infected with HIV.

Chapter 8: Recommendations

Having this finding we recommend that incorporating TB LAM test in EPTB diagnostic algorithm in addition to Xpert MTB/RIF test will result for the detection of more TB cases; especially for countries with high EPTB and TB/HIV cases like Ethiopia. However further researches and efforts are needed to improve its sensitivity and specificity.

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Annex

1. **Questionnaire**

Information sheet

Title: - Evaluation of Xpert MTB/RIF assay and Determine TB LAM test for the diagnosis of extra-pulmonary tuberculosis at Jimma University medical center, Southwest Ethiopia.

Principal investigator: - Asnake Simieneh (MSc candidate)

Address: - Jimma University Institute of Health Sciences, Department of Medical Laboratory sciences.

Mobile phone: - +251923536275 E-mail:- asnakesimieneh@gmail.com

Introduction

The main aim of this research is to evaluate the clinical performance of rapid diagnostic tools for disseminated tuberculosis from presumptive EPTB patient's at Jimma University medical center. The study will be undertaken by **Asnake Simieneh**, candidate master of medical microbiology, school of medical laboratory, institute of health science at Jimma University. This consent form contains all the information you will need to know about the study to be undertaken before you decide to consent to take part in the above-mentioned study.

Participation

What is expected from you is to respond some question which take about five minutes and give necessary specimens.

Risks

While you are participating there is no risk.

Benefits

Your sample will be diagnosed with different rapid techniques in addition to the routine diagnostic tests and that might increase the diagnostic accuracy.

Confidentiality

Any information that we will collect about you during this study will be kept confidential. Only the principal investigators will be able to link your identity with the code number, if this becomes necessary to assist you in any way.

Right to refuse

Since participation in this study is entirely voluntarily, you can refuse to participate in this study at any time. Your refusal will not affect your taking health care.

Participant consent

I have read the above information and the purpose of the study explained to me. I had the chance to ask questions about the study and all questions have been answered to my understanding. I have been informed and have understood that participation is entirely voluntary and that I can withdraw my consent at any time if I wish so. I consent so that voluntarily to participate in this study as a respondent.

Name of participant----------

Signature----------

Date ----------

Participant Assent form for children aged 10 - 14 years

I have read the information above, or it has been read to me. I have been given the opportunity to ask questions and my questions have been answered to my satisfaction. I voluntarily assent that I would participate in this study provided my parents/guardians give their consent**.** To give my body fluids and urine samples to inform my permission on providing the specimens and be a participant in this study and understand that I have the right to withdraw from the study at any time.

Name of participant----------

Signature----------

Date ----------

1.1 Questionnaire in English form

JIMMA UNIVERSITY

INISTITUTE OF HEALTH SCIENCES

SCHOOL OF MEDICAL LABORATORY SCIENCES

Thesis title: - Evaluation of new approaches and rapid diagnostic tools for disseminated tuberculosis at Jimma University Medical Center, Southwest Ethiopia.

Date ---------- unique ID number----------

Dear respondents,

This project is proposal thesis for the partial fulfillment of master of degree in Medical Microbiology. Please give your responses by accordingly. Your concise and clear responses would facilitate smooth data analysis. All information provided will be treated as confidential.

PART 1: Socio demographic information

S.no	Questions	Respondent answers	Skip to	Remark
01	Sex	1. Male 2. Female		
02	Age	____years		
03	Address	1.urban 2 rural		

PART 2: Clinical information

S.no	Questions	Respondent answers	Skip to	Remark
		1. Yes (how long……)		

04	Do you have Cough????	2. No		
05	Do you have Fever????	1. Yes (how long…….)		
		2. No		
06	Do you have Night sweating???	1. yes (how long……)		
		2. No		
07	Do you have weight loss????	1. Yes(how long…….....)		
		2. No		
08	Have you lost appetite?????	1. Yes(how long……..)		
		2. No		
09	Do you feel Fatigue/ less active?	1. Yes(how long……..)		
		2. No		
10	Difficulty of breathing	1. Yes(howlong……..)		
		2. No		
11	Other clinical features (specify……..)	For how long…….		
Clinical information from card review				
12	HIV status of the patient	1. Positive 2. Negative 3. Unknown		
13	If positive for HIV, WHO category ?????	1. Stage 1 2. Stage 2 3. Stage 3 4. Stage 4 5. Un known		
14	If positive for HIV, ART started???	1. Yes 2. No		
15	Co morbid other than HIV	1. Yes (specify…….) 2. No		
16	Site of infection	…….		

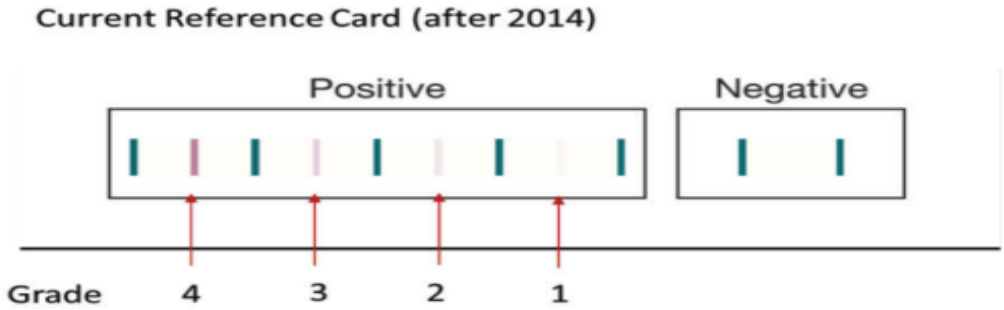
17	Type of specimen diagnosed	1. Cerebro spinal fluid (CSF) 2. Pericardial fluid 3. Peritoneal fluid 4. Pleural fluid 5. Pus		

PART 3: Laboratory investigation Results

Date processed	Lab code	Primary smear	Primary media	Growth detection weeks								BHI	Media smear	Capilla	Culture	Date report	Initials
				1	2	3	4	5	6	7	8						
			LJ														
			MGIT														

Lab Serial no	Sample	MRN	HIV status	Patient registration group (N,R,D,F)	Xpert result	Urine sample code

TB LAM test result



Observer 1

1. Positive (grade ……)
2. Negative

Observer 2

1. Positive (grade ….)
2. Negative

Thank you for your participation!

Name of data collector ---------- **Signature** ----------**Date**----------

1.2 መጠይቅ በአማርኛ

<u>አርዕስት</u>- በደቡብ ምዕራብ ኢትዮጵያ የጂማ ዩኒቨርሲቲ ሕክምና ማዕከል ከሳንባ ውጭ የሳንባ ነቀርሳ በሽታን ለመመርመር የ ‹Xpert MTB / RIF’s assay› እና <Determine TB LAM> ምርመራን መገምገም ።።

ዋና ተመራማሪ - <u>አስናቀ ስሜነህ</u>

አድራሻ:-ጅማ ዩኒቨርሲቲ የጤና ሳይንስ ኢንስቲትዩት ፣ የህክምና ላብራቶሪ ሳይንስ ክፍል ።።

ሞባይል ስልክ: - +251923536275

ኢሜል: - asnakesimieneh@gmail.com

<u>መግቢያ</u>

የዚህ ምርምር ዋና ዓላማ በጅማ ዩኒቨርሲቲ የህክምና ማዕከል የሳንባ ነቀርሳ በሽታ ህመም ለታመመ ህመምተኞች ፈጣን የምርመራ መሳሪያዎችን ክሊኒካዊ አፈፃፀም መገምገም ነው ።። ጥናቱ የሚከናወነው በጅማ ዩኒቨርሲቲ የጤና ሳይንስ ኢንስቲትዩት የሁለተኛ ዓመት የህክምና ማይክሮባዮሎጂ ማስተርስ ተማሪ በሆኑት በ አስናቀ ስሜነህ ነው ።። ይህ የስምምነት ቅጽ ከዚህ በላይ በተጠቀሰው ጥናት ለመሳተፍ እና ለመስማማት ከመወሰንዎ በፊት ስለሚደረገው ጥናት ማወቅ ያለብዎትን ሁሉንም መረጃዎች ይይዛል

<u>ተሳትፎ</u>

ከአንተ የሚጠበቀው ነገር ለአምስት ደቂቃ ያህል የሚወስድ ጥያቄ መመለስ እና አስፈላጊ ናሙናዎችን መስጠት ነው።።

<u>አደጋ/</u>

እርስዎ በሚሳተፉበት ጊዜ ምንም አደጋ የለም።።

<u>ጥቅሞች</u>

ከተለመደው ምርመራ በተጨማሪ የናሙናዎ የምርመራው ትክክለኛነት እንዲጨምር ሊያደርግ በሚችሉ በተለያዩ ፈጣን ቴክኒኮች ይመረመራል ።።

<u>ምስጢራዊነት</u>

በዚህ ጥናት ወቅት ስለ እርስዎ የምንሰበስበው ማንኛውም መረጃ በምስጢር ይጠበቃል ።። በማንኛውም ሁኔታ እርስዎን ለማገዝ አስፈላጊ ከሆነ ዋና ማንነትዎን ከኮዱ ቁጥሩ ጋር ማገናኘት የሚችሉት ዋና መርማሪዎቹ ብቻ ናቸው ።።

እምቢየማለትመብት

በዚህ ጥናት ውስጥ ተሳትፎ ሙሉ በሙሉ በፍላጎት ስለሆነ በዚህ ጥናት ውስጥ በማንኛውም ጊዜ ለመሳተፍ እምቢ ማለት ይችላሉ ። እምቢታዎ የጤና እንክብካቤዎን አይጎዳውም ።

የተሳታፊ ስምምነት

ከዚህ በላይ ያለውን መረጃ አንብቤያለሁ እናም የጥናቱ ዓላማ ለእኔ ተብራርቷል። ስለ ጥናቱ ጥያቄዎች የመጠየቅ እድል ነበረኝ እናም ሁሉንም ጥያቄዎች ተገንዝቤያለሁ እናም ተሳትፎየ ሙሉ በሙሉ በፍቃደኝነት መሆኑን እና ከፈለኩ በማንኛውም ጊዜ ፈቃዴን መሰረዝ እንደምችል ተረድቻለሁ። በዚህ ምርምር በፈቃደኝነት ለመሳተፍ እስማማለሁ።

የተሳታፊ ስም ----------

ፊርማ ---------- -----

ቀን ---------- ----------

ከ 10 እስከ 14 ዓመት ለሆኑ ሕፃናት የአሳታፊነት ማረጋገጫ ቅጽ

ከላይ ያለውን መረጃ አንብቤያለሁ ወይም አንብበውልኛል ። ጥያቄዎችን የመጠየቅ እድል ተሰጥቶኛል እና ጥያቄዎቼ ተመልሰዋል ። ወላጆቼ / አሳዳጊዎቼ ፈቃዳቸውን ከሰጡ በዚህ ጥናት ውስጥ እሳተፋለሁ ብዬ በፈቃደኝነት አረጋግጫለሁ ። ተሳትፎየ ሙሉ በሙሉ በፍቃደኝነት መሆኑን እና ከፈለኩ በማንኛውም ጊዜ ፈቃዴን መሰረዝ እንደምችል ተረድቻለሁ። በዚህ ምርምር በፈቃደኝነት ለመሳተፍ እስማማለሁ።

የተሳታፊ ስም----------
ፊርማ----------
ቀን ---------- ----------

ጅማ ዩኒቨርሲቲ
የጤና ሳይንስ ተቋም
የህክምና ላብራቶሪ ሳይንስ ትምህርት ቤት

<u>አርዕስት</u>- በደቡብ ምዕራብ ኢትዮጵያ የጂማ ዩኒቨርሲቲ ሕክምና ማዕከል ከሳንባ ውጭ የሳንባ ነቀርሳ በሽታን ለመመርመር የ ‹Xpert MTB / RIF’s assay› እና <Determine TB LAM> ምርመራን መገምገም ::

ቀን ---------- ልዩ መታወቂያ ቁጥር ------- ----------

ውድ ምላሽ ሰጪዎች
ይህ ኘሮጀክት በሕክምና ማይክሮባዮሎጂ የሁለተኛ ዲግሪ ከፊል ማጠናቀሪያ ፕሮፖዛል ነው :: እባክዎ ምላሾችዎን በዚሁ መሠረት ይስጡ :: የእርስዎ አጭር እና ግልጽ ምላሾች የመረጃ ትንተናውን ያመቻቻል :: የተሰጠው መረጃ ሁሉ በሚስጥር ይያዛል ::
<u>ክፍል 1:የስነ ሕዝብ አወቃቀር መረጃ</u>

መለያ ቁጥር	ጥያቄዎች	የመልስ ሰጭ መልሶች	ዝለል ወደ	ምልክት ያድርጉ
01	ፆታ	1. ወንድ 2. ሴት		
02	ዕድሜ	____ዓመት		
03	አድራሻ	1. ከተማ 2. ገጠር		

ክፍል 2: ክሊኒካል መረጃ

መለያ ቁጥር	ጥያቄዎች	የመልስ ሰጭ መልሶች	ዝለል ወደ	ምልክት ያድርጉ
04	ሳል አለብህ ????	1. አዎ (ለምን ያህል ጊዜ .....)		
		2. የለም		
05	ትኩሳት አለህ ????	1. አዎ (ለምን ያህል ጊዜ .....)		
		2. የለም		

06	ክብደት መቀነስ አለብዎት ????	1. አዎ (ለምን ያህል ጊዜ .....)		
		2. የለም		
07	በሌሊት ጊዜ ላብ ላብ ይልዎታል ???	1. አዎ (ለምን ያህል ጊዜ .....)		
		2. አይለኝም		
08	የምግብ ፍላጎት መቀነስ አለ??	1. አዎ (ለምን ያህል ጊዜ .....)		
		2. የለም		
09	ድካም ይሰማዎታል????	1. አዎ (ለምን ያህል ጊዜ .....)		
		2. የለም		
10	የመተንፈስ ችግር አለብዎት ???	1. አዎ (ለምን ያህል ጊዜ .....)		
		2. የለብኝም		
11	ሌሎች ክሊኒካዊ ባህሪዎች (ይግለጹ ....)	ለምን ያህል ጊዜ		

1.3 Questionnaire in afaan oromoo version

Mata duree: Dhukkuba TB Kan sombarraa gara qaama biraati fafaca'u yaaluf Madaalli teknikaa Xpert MTB/RIF fi TB lam jiddu gala yaalaa jimma unibarsiititi hojjachuf, Kibba Itoophiyaa.

Qorannoo Kan gaggeessu: Asnaaqa Simeenah

Tessoo: jimma Unibarsiititi inistitiwuti fayyaa muummee medikaal laboratorii

Lakk Bilbilaa;-+251923536275

E-mail: -asnakesimieneh@gmail.com

Seensa: Kayyoo qorannoo kana barbachisumma teknikaa akka salphaatti saffisan fafaca'insa dhukkuba sombaa Kan sombaan alaa dhukkubsatoota jiddu gala mana yaala unibarsiiti jimmarrati madaaluf. Qorannichi Kan gageeffamu barata Asnaaqa Simeenah kan jedhamun jimma unibarsitiitti inistitiwuti fayyaatti muummee medical laboratory keessaa digrii lammaffaa medikal mayikrobiyologidhan kan ta'eni. Guchni waligaltee Kun oddeeffannoo wa'ee qorannichaaf si barbachisuu hundaa waan qabuuf osoo waligaltee kana hin xumurin hubadhu

HirmaannaaIsin irraaa Kan eegamu daqiiqa 5nif gaafilee gafatamtanif deebi lachuu fi namuuna barbachisu kennu qofa

Miidhaa Yeroo qorannoo kanarratti hirmaatu Miidhaa muraasa wayitu isin irra hin ga'u

Fayiidaa: Namuuna keessan malleen yeroo kanin hojjatamanirraatti dabalatan teknikaa akka salphaatti dafee waan yaaluf haqummaa yaali keessani dabala.

Iccitii: Odeeffannoon wa'ee kee walitti qabame hundi bifa dhokatan koodin ittii waan gadhamuf Kan qorannicha gaggeessuu qofatu wa'ee odeeffannoo sirra gurame beeka malee namni bira tokko iyyuu hin beeku.

Mirga diduu: Hirmannan kee guutuma guututti fedhiidhan waan ta'ef yeroo barbadetti dhiisu Ni dandeessa. Qorannicha adda kuutun kee yaali argachu keerratti dhiibba tokkoolee hin qabu

2. Consent/Assent Form in afaan oromoo version

Kayyoo qorannoon kun gaggeefamuf fi odeeffannoo barbaachisan dubbiseen jira, Carraa gaffii wa'ee qorannichaa gaafatan gafatamun hanga hubannoo kooti deebi debiseen jira Odeffannoo fi hubannaa wa'ee qoranichan walqabatan feedhi koo guutudhaan yeroon barbadu hirmachudhan yeroon hin barbanne dhiisudhan fedhi horadhen jira. Kanaaf waligaltee kana fedhi Koo gutuudhan akka hirmata qarannichati waligalerra

Mallattoo hirmaata/ dhukubsachiisa ----------

Guyyaa----------

Assent Form in afaan oromoo version

Kayyoo qorannoon Kun gaggeefamuf fi odeeffannoo barbaachisan dubbiseen jira, Carraa gaffii wa'ee qorannichaa gaafatan gafatamun hanga hubannoo kooti deebi debiseen jira, Odeffannoo fi hubannaa wa'ee qoranichan walqabatan feedhi Koo guutudhaan yeroon barbadu hirmachudhan yeroon hin barbanne dhiisudhan fedhi horadhen jira Kanaaf waligaltee kana fedhi Koo gutuudhan akka hirmata qarannichati akka maatin koo itti wali galanitti waligalerra. Kanaaf fedhi kootin namuuna dhangala'a qaama fi namuuna fincaani keenuf eeyamamera

Maqaa hirmaata----------

Malattoo----------

Guyyaa ----------

YUNIBARSIITI JIMMA

DHAABBATA SAAYINSI FAYYAA

KUTAA BARUMSA MEDICAL LABORATORII

Mata duree: Dhukkuba TB kan sombaan alaa yaaluf Madaalli teknikaa Xpert MTB/RIF fi TB lam jiddu gala yaalaa jimma unibarsiititi hojjachuf, Kibba Itoophiyaa

Guyyaa ---------- Lakk. Icciiti ----------

Deebin isiin nuuf kennatun gaaffii gaafatamtanif gabaaba fi ifa yoo ta'e qorranno keenyaa dhugaan bu'ureessa waan ta'ef maloo hangaa dandeessanitti akka nuu gargartan irraa debinee isiin yaadachifna

PART 1: Socio demographic information

Lakk.	Gaaffii	Deebi hirmatota	gara---Darbii	Yaada
01	Saala	1. Dhiira 2. Dubara		
02	Umrii	____Waggaadhan		
03	Teessoo	1.magaala 2 baadiyyaa		

PART 2: Clinical information

Lakk.	Gaffii	Deebi gaafatamaa	Gara--darbi	yaada
04	Qufaa qabduu????	1. Eeyyeen (hangamiif……) 2. Lakkii		
05	Ho'iinsa qaama qabduu????	1. Eeyyeen (hangamiif……) 2. Lakkii		
06	Halkaan isin dafqisiisa???	1. Eeyyeen (hangamiif……) 2. Lakkii		
07	Ulfatinni keessan hir'ate jiraa????	1. Eeyyeen (hangamiif……) 2. Lakkii		
08	FEddhii nyaata keessan hir'ate jiraa?????	1. Eeyyeen (hangamiif……) 2. Lakkii		
09	Dadhabinni qaama isiinitti dhaga'aame?	1. Eeyyeen (hangamiif……) 2. Lakkii		
10	Hargaansun isinitti cimaa	1. Eeyyeen (hangamiif……) 2. Lakkii		
11	Mallattoon biraa jira (adda baasa himaa……..)	Hangaamif…….		

2. Sops for performed tests

2.1 Xpert MTB RIF test

Principle

This diagnostic test (also known as the Xpert *M.tb*/RIF) test is based on a semi-quantitative, nested real-time PCR for the detection of *M. tuberculosis* complex DNA in sputum samples and the detection of rifampicin (RIF) resistance associated mutations of the *rpoB* gene in samples from patients at risk for RIF resistance. The *M.tb*/RIF test is intended for use with specimens from untreated patients for whom there is clinical suspicion of tuberculosis, so that the results can be provided within 2 hours.

Test Procedure

- Using a sterile transfer pipette, transfer at least 0.5 mL of re-suspended pellet to a conical screw-capped tube.
- Add 1.5 mL of Xpert *M.tb*/RIF Sample Reagent (SR) to the 0.5 mL of re-suspended sediment using a sterile transfer pipette and shake vigorously 10 – 20 times, (a single shake is one back-and-forth movement).
- Incubate the specimen for 15 minutes at room temperature. Between 5 and 10 minutes of incubation, shake the specimen vigorously again 10 – 20 times. Samples should be liquefied with no visible clumps of sputum.
- Label each Xpert *M.tb*/RIF cartridge with the lab accession number by writing on the sides of the cartridge or attach ID label. Note: do not put the label on the lid or obstruct the existing 2D barcode on the cartridge.
- Using the sterile transfer pipette provided with the kit, aspirate the liquefied sample into the transfer pipette until the meniscus is above the minimum mark. **Do not process the sample further if there is insufficient volume.**
- Open the cartridge lid and transfer sample into the open port of the Xpert *M.tb*/RIF cartridge. Dispense sample slowly to minimize risk of aerosol formation.
- Close the cartridge lid and make sure the lid snaps firmly into place. Remaining liquefied sample may be kept for up to 12 hours at 2 – 8 ºC should repeat testing be required.
- Be sure to load the cartridge into the GeneXpert Dx
- Instrument and start the test within 30 minutes of preparing the cartridge.
- In the GeneXpert Dx System window, click **Create Test**. The Scan Cartridge Barcode dialog box appears. Scan the 2D barcode located on the Xpert *M.tb*/RIF cartridge. The Create Test window appears. The software will automatically fill the boxes for the following fields: Select Assay, Reagent Lot ID, Cartridge SN, and Expiration Date based on the barcode information.

- In the **Sample ID** box, scan or type the sample lab accession number. Cross-check to ensure it is typed or scanned correctly. The sample ID/lab accession number is associated with the test results in the "**View Results**" window and all generated reports.
- Click **Start Test**. In the dialog box that appears, type your password.
- Open the instrument module door with the flashing green light and load the cartridge.
- Close the door. The green light will stop flashing and become steady once the test starts. When the test is finished, the green light will turn off and the system will release the door lock.
- Once the system releases the door lock at the end of the run, open the module door and remove the cartridge.

Interpretation of Results

The results are interpreted by the GeneXpert DX System from measured fluorescent signals and embedded calculation algorithms and will be displayed in the "**View Results**" window of the GeneXpert machine.

M.tb Detected

M.tb target DNA is detected.

- *M.tb* Detected - The *M.tb* result will be displayed as High, Medium, Low or Very Low depending on the Ct value of the *M.tb* target present in the sample.
- Rif Resistance DETECTED, Rif Resistance NOT DETECTED, or Rif Resistance INDETERMINATE will be displayed only in *M.tb* DETECTED results and will be on a separate line from the *M.tb* DETECTED result.
- Rif Resistance DETECTED; a mutation in the *rpoB* gene has been detected that falls within the valid delta Ct setting.
- Rif Resistance INDETERMINATE; the *M.tb* concentration was very low and resistance could not be determined.
- Rif Resistance NOT DETECTED; no mutation in the *rpoB* gene has been detected.
- SPC— NA (not applicable); SPC signal is not required since *M.tb* amplification may compete with this control.
- Probe Check—PASS; all probe check results pass.

M.tb Not Detected

M.tb target DNA is not detected, SPC meets acceptance criteria.

- *M.tb* NOT DETECTED—*M.tb* target DNA is not detected.
- SPC— Pass; SPC has a Ct valid range and endpoint above the endpoint minimum setting.
- Probe Check—PASS; all probe check results pass.

2.2 LJ culture

Principle

Many different solid media are available for cultivating mycobacteria. Most are variations of egg-potato base or albumin-agar base media. There is no general consensus on which medium is best for routine isolation. The advantages of egg-based media such as LJ are:

1) It is easy and economical to prepare, 2) it is associated with lower contamination rates, and 3) isolated colonies with characteristic colony morphology for MTB can be observed.

Disadvantages are: 1) when contamination occurs, it often involves total surface of medium, 2) If contamination is slight, it is not evident when mycobacterial growth is confluent, and 3) drug susceptibility tests are more difficult to perform using egg-based media because some drugs must be adjusted to account for their loss by heating or by interaction with certain components of the egg. As with all media preparation, attention must be given to purity of chemical components, including quality of eggs; preparing and sterilizing medium and glassware; exposure of final product to excessive heat or sunlight; and method and length of storage. All lab-prepared media must be tested for sterility and performance characteristics before being used.

Procedure

Inoculation of slants must be done inside the biosafety cabinet, using full PPE.

1. Label LJ tube, using the study-specific labels that contain identifying information
2. Remove any excess water in the slant using a sterile transfer pipette.
3. Inoculate the tube with 200 µL of the sample (either well-mixed, processed sputum) Spread inoculum evenly over entire surface of medium.
4. Replace cap and ensure there are no droplets around the rim of the tube. Wipe off the outside of the tube with a paper towel soaked in tuberculocidal disinfectant.
5. Leave tube in slanted position with cap loosened until inoculum is absorbed (about a week), then tighten cap securely and incubate in upright position at 37°C (±1°C). Alternatively, the tube can be incubated immediately in an upright position with cap loose for the first week of incubation.
6. Examine and record results for the cultures weekly, for 8 intervals. Cultures can be read on the bench, as long as the caps are NOT loosened.
7. To observe fine growth, a strong direct light from the angle poise lamp must be shone onto the slant surface. *M. tuberculosis* usually grows as a buff-colored, dry colony, which is very distinctive.

2.3 p-nitrobenzoic acid (PNB) inhibition test

1. **Purpose:** PNB test is used for the presumptive differentiation of MTBc from NTM

2. **Principle:** The LJ containing PNB is used to confirm that strains belong to MTBc. It contains a pre-defined concentration of PNB added before coagulation. The addition of the PNB is strict, well-adjusted and controlled. The concentration of PNB incorporated in the LJ media is as **500 mg/L**.

3. **Preparation of L-J media containing PNB**
   - **I.** Weigh out 0.5 gm PNB and dissolve in the minimum amount of dimethylformamide (~15ml).
   - **II.** Add to 1 liter of L-J fluid, distribute and inspissate once for 50 minutes at 85 degrees. The final concentration of PNB should be 500 µg/ml
   - **III.** Store in cold room (2-8^{o}c).

4. **Preparation of bacterial suspension and inoculation**
   1. Prepare inoculum by scraping freshly grown colonies from the surface of the LJ medium.
   2. A few colonies are emulsified in one flask containing glass beads and 2 ml of sterile distilled water to obtain turbidity greater than McFarland 1 standard.
   3. Homoginze the content of the flask, allowed to stand for 5min to allow larger clumps to settle,
   4. Transfer 1 ml of the supernatant suspension to another tube, where the turbidity is adjusted to McFarland 1 standard adding sterile distilled water. This bacterial suspension is used as the work suspension.
   5. Inoculate two LJ media (one containing PNB and the other PNB free-control) with 100 µl of the work suspension and incubate at 37^{0}c
   6. Read on 28th day. PNB should not be kept for reading at 42 day.

5. **Results and interpretation**

M. tuberculosis complex (MTBc) strain does not grow on PNB medium.

All other mycobacteria (Non-tuberculous mycobacteria-NTM) are resistant to PNB.

- If No growth was seen on PNB media----------MTBc
- If growth was seen on PNB media----------NTM

Note: There should be a growth on drug free L-J media

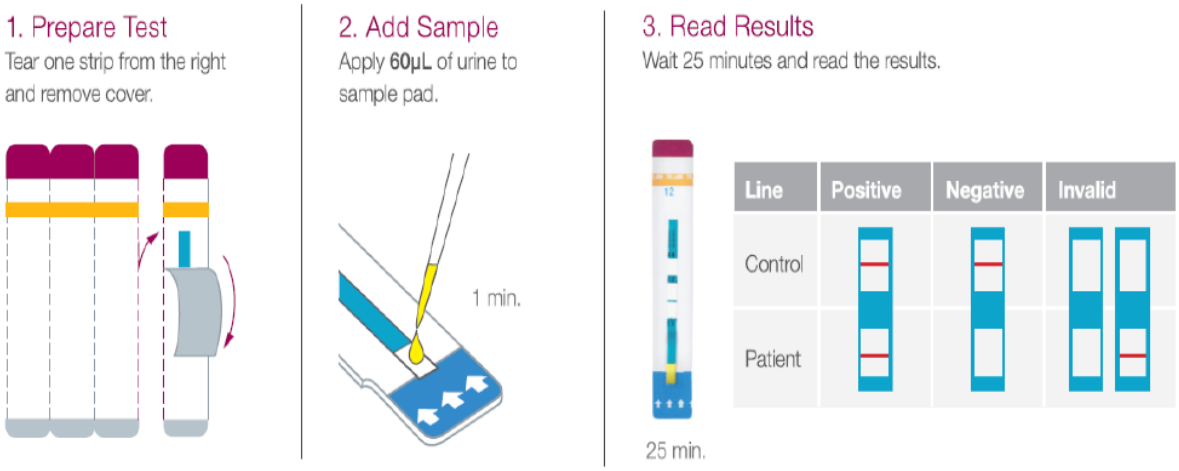
2.4 Determine TB LAM test

Principle

Alere Determine TB LAM Ag is an immunochromatographic test for the qualitative detection of lipoarabinomannan (LAM) antigen of Mycobacteria in human urine. Alere Determine TB LAM Ag employs highly purified antibodies specific to the major polysaccharide antigen of the genus Mycobacterium: lipoarabinomannan (LAM). These antibodies are used for both the capture and the detection tracer. The capture antibodies are adsorbed onto the nitrocellulose membrane of the test strip. The detection antibody is labeled by conjugation to colloidal gold particles. After a urine specimen is added to the sample pad, the colloidal gold conjugated antibodies attach to the LAM antigen and are released by the specimen from the conjugate pad. This immunological complex is then captured by anti LAM antibodies immobilized on the nitrocellulose membrane and made visible due to the presence of the colloidal gold label. A positive result (a visible purple/gray line) indicates that LAM antigen of Mycobacteria is present in the sample at or above the detection limit of the test; whereas a negative result (no visible purple/gray line) indicates it is not present or below detection limit. To ensure assay validity, a procedural control bar is incorporated in the assay device.

Test Procedure

- Remove the protective foil cover from each test and label the test kit. The assay should be initiated within 2 hours after removing the protective foil cover from each test.
- Apply 60 µL of sample (or 2drops of urine) to the sample pad (White pad marked by the arrow symbol).
- Result should be interpreted between 25 and 35 minutes after sample application. Visualize the strip under standard indoor lighting conditions or in the shade. Do not visualize the strip under direct sun light. Do not read beyond 35 minutes.



Interpretation of test results

The interpretation of the result must be made using the reference scale card (provided in the kit) by holding it alongside the patient window in a well-lit environment.

LAM Antigen POSITIVE (Two Bars - Control and Patient Bars)

The test result is positive even if the patient line appears lighter or darker than the control line, **as long as the "Patient" line is equal to or stronger than any of the colored lines in the "POSITIVE" range** on the Reference Scale Card

NEGATIVE (One Bar)

One purple/gray bar appears in the control window of the strip (labeled "Control") and no purple/gray bar appears in the Patient window of the strip (labeled "Patient").

Faint line (lighter than grade 1) should be considered as indefinite/equivocal (check section).

2.5 LED FM Microscopy (Auramine staining)

The property of acid-fastness is based on the presence of mycolic acids in the mycobacterial cell wall. Primary stain (Auramine) binds cell-wall mycolic acids. Intense decolourization (strong acids, alcohol) does not release primary stain from the cell wall and the mycobacteria retain the fluorescent bright yellow colour of auramine. Potassium permanganate is used to quench fluorescence in the background; however, it provides little contrast for focusing and stains are therefore sometimes preferred, of which blue ink may be the best.

Preparation of smears and staining

Smear is prepared after specimen decontamination and the concentrated material must be transferred to the slide with a sterilized loop to avoid splashing. Let the smears air-dry at room temperature; do not use heat to speed the drying. When dry, hold the slides in forceps and fix them by passing three times slowly through the flame of a spirit lamp or quickly through that of a Bunsen burner, smear upwards; do not overheat or AFB staining will be poor. Place the slides, smear upwards, on the staining rack over a sink, about 1 cm apart.

Place a new filter paper in a small funnel, keep it over the first slide and fill it up with auramine staining solution. Let the solution filter through the paper, covering each slide completely. Do not heat. Leave for 20 minutes. Using forceps tilt each slide to drain off the stain solution. Rinse the slides well with distilled water or clean tap water from a beaker (not directly from the tap). Pour the acid solution over the smears, covering them completely, and allow acting for 3 minutes. Using forceps tilt each slide to drain off the acid-alcohol solution. Gently rinse each slide again with distilled water or clean tap water from a beaker (not directly from the tap). Flood smears with potassium permanganate solution for 1 minute. Time is critical because counterstaining for longer may quench the AFB fluorescence. Using forceps tilt each slide to drain off the counterstain solution.

Gently rinse each slide again with distilled water or clean tap water from a beaker (not directly from the tap).Using forceps, take each slide from the rack and let the water drain off. Stand the slide on edge on the drying rack and allow air-drying.

Reading and reporting

- Keep stained smears in the dark (in a box or folder) and read as soon as possible – fluorescence fades quickly when exposed to light.
- Switch on fluorescent lamp 5 minutes before use; leave the lower ordinary lamp off.
- Rotate the nosepiece so that the 20x (or 25x) objective is in the light path.

- Load the positive control slide on the stage and move the stage to position the slide under the objective.
- Check that bright yellow fluorescent AFB are clearly seen. If not, adjust the lamp and/or the mirror position.
- Using the 20x (or 25x) objective, scan the stained smear systematically from one side to the other and back again – at least one length must be scanned before reporting a negative. At 200x magnification, this corresponds to three lengths or 300 high-power fields (HPF) using the oil-immersion 100x objective; at 400x it equals two lengths or 200 HPF with the oil-immersion objective.
- Use the 40x objective for confirmation of AFB

IUATLD/WHO scale (1000x field =HPF) Result	Bright-field (1000x magnification: 1 length = 2 cm = 100 HPF	Fluorescence (200–250x magnification: 1 length = 30 fields = 300 HPF)	Fluorescence (400x magnification: 1 length = 40 fields = 200 HPF)
Negative	Zero AFB / 1 length	Zero AFB / 1 length	Zero AFB/ 1length
Scanty	1–9 AFB / 1 length or 100 HPF	Zero AFB / 1 length	1–19 AFB / 1 length
1+	10–99 AFB / 1 length or 100 HPF	1–29 AFB / 1 length	20–199 AFB / 1 length
2+	1–10 AFB / 1 HPF on average	30–299 AFB / 1 length	5–50 AFB / 1 field on average
3+	>10 AFB / 1 HPF on average	10–100 AFB / 1 field on average	>50 AFB / 1 field on average