Light Emitting Diode Fluorescent Microscopy and GeneXpert MTB/RIF Assay for the Diagnosis of Tuberculosis against LJ Culture among Patients attending Ambo Hospital, West-Central Ethiopia

By

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List of abbreviations/Acronyms

AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
BSC	Biological safety cabinet
CI	Confidence Interval
cZN	concentrated Ziehl-Neelsen
dLED-FM	direct light emitting diode Florescent Microscopy
DST	Drug sensitivity test
EPHI	Ethiopian Public Health Institute
EPTB	Extra-pulmonary TB
FM	Florescent microscopy
FMoH	Federal Ministry of Health
GLI	Global Laboratory Initiative
HIV	Human Immunodeficiency Virus
INH	Isoniazid
LJ	Lowenstein-Jensen
LM	Light microscopy
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NALC	N-acetyl-L-cysteine
NTM	Nontuberculous Mycobacteria
NPV	Negative predicative value
NTRL	National TB Reference Laboratory
PPV	Positive predicative value
PTB	Pulmonary Tuberculosis
RIF	Rifampicin
TB	Tuberculosis
WHO	World Health Organization
ZN	Ziehl-Neelsen

Abstract

One of the most important reasons for the high number of tuberculosis (TB)-related deaths in low-income countries is due to the challenge of diagnosis. The simple and cheaper light-emitting diode fluorescent microscopy (LED-FM) having long life-span, not producing ultraviolet (UV) light, minimal power requirements and better performance is recommended by the World Health Organization (WHO), in 2009 to replace the conventional microscopy in both high- and low-volume laboratories. The WHO has also endorsed (in 2010) one more technique, *GeneXpert MTB/RIF assay (Xpert)*, for better TB diagnosis particularly among human immunodeficiency virus (HIV)-infected cases. However, the relative performance of both of these tools differs from setting to setting in reference to culture as a gold standard. This study was, thus, aimed at evaluating these tools for TB detection in individuals visiting Ambo Hospital, west-central Ethiopia. Cross-sectional early-morning sputum samples were collected from presumptive TB patients attending Ambo Hospital between January and August 2015. Socio-demographic data were captured using a semi-structured questionnaire. Clinical information was gathered from patients' medical records. The sputum samples were diagnosed using LED-FM, *Xpert*, concentrated Ziehl-Neelsen (cZN) staining and Lowenstein-Jensen (LJ) medium culture. Drug sensitivity test (DST) was also conducted. Totally, 362 sputum samples were collected and processed. Of these, 36(9.9%) samples were positive by LED-FM, 42(11.6%) by cZN and 50(13.8%) by *Xpert*. All 362 samples were cultured but 22 were contaminated and 8 nontuberculous Mycobacteria (NTM) leaving only 332 with a definite culture status. From these 332, 45(13.6%) had culture-confirmed TB with 11(24.4%) being HIV co-infected. Two samples were rifampicin resistant by both DST and *Xpert*. The sensitivity, specificity, positive and negative predictive values of LED-FM and *Xpert* were 77.8, 100, 100 and 96; and 93.3, 98, 97.5 and 98.9% respectively. Xpert demonstrated 100 and 71.4% sensitivity in smear positive-culture positive and smear negative-culture positive sputum samples in detecting MTB respectively and increased MTB detection rate by 28% compared to dLED-FM.

Keywords: Sensitivity, Specificity, Xpert MTB/RIF assay, LED FM

1 Introduction

Tuberculosis (TB) is an infectious disease caused by the species of *Mycobacterium tuberculosis* (MTB) complex. Among the species of *M. tuberculosis* complex, *M. tuberculosis* is the dominant cause of tuberculosis. Occasionally, *M. bovis*, *M. africanum* can also cause TB. In immune competent individuals mycobacterial infection is mostly asymptomatic but it becomes activated and life-threatening when the immune system is compromised. Although mycobacteria infect the lungs and cause pulmonary TB (PTB), organs other than the lung can also be infected by the bacilli and cause extra-pulmonary TB (EPTB).

MTB is the most common opportunistic infection in human immunodeficiency virus (HIV)-infected individuals and cause of HIV-related deaths. In 2014, TB killed 1.5 million people (1.1 million HIV-negative and 0.4 million HIV-positive). Globally, 12% of the 9.6 million new TB cases in 2014 were HIV-positive (WHO 2015). TB is responsible for about 26% of AIDS-related deaths, with 99% occurring in developing countries (Roshanaei 2014). This makes it the leading killer particularly among HIV-seropositives. At least one in four deaths among people living with HIV can be attributed to TB (Gao et al. 2013). Conversely, the risk for TB is 20-37 times greater among persons infected with HIV, and in some countries in sub-Saharan Africa, up to 80% of patients with TB have HIV infection (Granich et al. 2010). Among estimated 1.2 million people living with HIV who developed TB in 2014 globally, 32% of TB cases were estimated to be co-infected with HIV in Africa, which accounted for 74% of TB cases among people living with HIV worldwide (WHO 2015). According to this WHO TB report, Ethiopia is one of the high TB burden countries. The estimated country TB prevalence and incidence is to be 200 and 207 per 100 000 population, respectively. The country is among the 22 high TB-burden countries and 27 high multidrug-resistant TB (MDR-TB) burden countries in the world (WHO 2014, 2015). Moreover, the country is among high TB/HIV burden countries with approximately 10% co-infection rate and TB case detection rate is 60%, which is below the target (72%) (WHO 2015). This indicates that Ethiopia should do very well to maximize TB case finding.

The most challenge in TB control and reasons for high number of TB-related deaths in low-income countries are the difficulty in diagnosis. Despite its requirement for sophisticated laboratory facilities and delayed results, making it difficult for early clinical management of severe cases, MTB culture remains the gold standard for TB diagnosis (Nyendak et al. 2009). Conventional light microscopy (LM) screening of Ziehl-Neelsen (ZN) stained sputum smears is still the mainstay of PTB diagnosis in rural settings. But many studies indicate the lower sensitivity of conventional LM especially in HIV-positive patients, EPTB cases, children and patients with low bacterial load (MaHTAS 2012, Suhasini and Mamilla 2015).

In general, these conventional MTB detection methods are labor-intensive and time-consuming. Apart from severely affecting individual patients, failure to detect TB early and rapidly is the main source of MTB dissemination in a community. Among people living with HIV, diagnosis of TB is more difficult compared to HIV negatives, and mortality rates are higher (FMoH/EPHI 2014).

Light-emitting diode fluorescent microscopy (LED-FM) is simpler, cheaper having long lifespan. It does not produce ultraviolet (UV) light, has minimal power requirements and better performances, on average 10% more sensitive than conventional ZN LM. It uses the auramine-stained smear techniques (MaHTAS 2012, Minion et al. 2009, 2011, Steingart et al. 2006, WHO 2011). Additional advantages of this technology is lower magnification can be used, enabling rapid screening over a wider area of the smear to be seen and resulting in up to 4 times faster examination of smear, lower maintenance requirements and able to run on batteries (Rodrigues 2012, Shenai 2015). This technology is more beneficial in high-burden and resource-limited settings. LED-FM is recommended by the WHO (in 2009) to replace LM in both high- and low-volume laboratories.

In fact some studies recorded lower specificity of LED-FM compared to conventional LM (Cattamanchi et al. 2009, Cuevas et al. 2011, Shenai et al. 2011). It was suggested that this comparatively lower LED-FM might be attributed to the WHO advocated (WHO 2007) low threshold for positivity of FM (≥1 acid-fast bacillus/smear). LED-FM performance can possibly be impacted by sputum processing and only limited studies addressed this factor (Whitelaw et al. 2011, Bonnet et al. 2011). Therefore, further studies are warranted to evaluate the optimal technical conditions to fully implement this technology under different endemic settings and patient conditions including HIV-infected patients and MDR-TB cases (Van Deun et al. 2002, 2004, Gilpin et al. 2007).

As part of the continued search for innovative technologies for accurate and reliable diagnosis of TB, the WHO has endorsed another tool, Xpert MTB/RIF assay in December 2010. The development of the Xpert MTB/RIF assay which is an automated molecular diagnostic test with simple, rapid and highly sensitive is to be deployed close to the point of patient care in the fight against TB.

High sensitivity and specificity of Xpert MTB/RIF assay for detecting MTB is indicated in almost all literatures. Additional advantage of Xpert MTB/RIF test is providing a result within two hours, ability to diagnose drug susceptibility and has minimal bio-safety requirements and training. The determination of drug susceptibility is particularly relevant because MTB becomes increasingly resistant to two of the major anti-TB drugs, isoniazid (INH) and rifampicin (RIF), leading to rapid global emergence MDR-TB. When it detected MTB simultaneously it identifies mutations in the *rpoB* gene, which are associated with rifampicin resistance. The use of the rapid test Xpert MTB/RIF assay has expanded substantially since 2010, when WHO first recommended its use it is highly effective for diagnosing MDR-TB (WHO 2014, 2015).

2 Significance of the study

Difficulty in diagnosing TB is one of the challenges for TB control. Culture is the gold standard for TB diagnosis but, it requires expensive and sophisticated laboratory facilities and not able to provide a rapid result for the clinical management of severe cases (WHO 2011). The recent TB diagnosing techniques like Xpert MTB/RIF assay and LED-FM has improved the speeds, sensitivities and specificities due to its short turnaround time and automation of the procedure.

Evaluating these TB diagnosing technologies is one method for maximizing TB detection rate in the country. Ambo Hospital is one of the Hospitals in west-central Ethiopia has been reporting high TB cases for the regional TB control program and recently uses the above two technologies for TB diagnosing methods according to the national guideline. This study was aimed at evaluating the performance of the two technologies against the gold standard LJ culture.

Therefore, the generated new data may have implications for policy makers in scaling-up implementation of both technologies in the national algorisms. Governmental and non-governmental organizations working in the area of TB control and other TB researchers may utilize the information generated to accelerate and improve detection and treatment rates for TB.

3 Objectives of the study

3.1 General objective

The main aim of the study was to evaluate the diagnostic performance of LED-FM and Xpert MTB/RIF assay for diagnosis of TB.

3.2 Specific objectives

- To determine the sensitivity, specificity, PPV and NPV of LED-FM and Xpert MTB/RIF assay against the solid LJ medium for detection of MTB.
- To compare the performance sensitivity and specificity PPV and NPV of LED-FM against Xpert MTB/RIF assay for the detection of TB.
- To see the drug resistance positive of *M. tuberculosis* in the area.
- To compare diagnostic performance of the two technologies in HIV-positive and HIV-negative individuals.

4 Material and Methods

4.1 Study site

The study was conducted at Ambo Hospital in Ambo town from January through August 2015. Ambo is located in west central Ethiopia, 112 kms from Addis Ababa covering 4470.5 hectare. The town has latitude and longitude of 8°59′N 37°51′E/8.983°N 37.850°E and an elevation of 2101 meters above sea level. The mean annual temperature and annual rain fall of the area is 18^{0C} and 800-1200 mm respectively. According to information from statistic agency of the area (Ambo), the population of the town in 2013 was 61900 (male 31655 and female 30245).

The information from Ambo Hospital administration office indicates that, the Hospital gives service to 70,000 to 82,000 patients per year. One year data (July 2013 to June 2014) indicated that from the total patients attended the hospital, 1760 PTB presumptive cases were diagnosed by LED-FRM and 188(10.7%) were smear positive. The Hospital used Xpert MTB/RIF assay according to the national algorithm for diagnosing TB starting from November 2014.

4.2 Study design

The study was a health-facility-based cross-sectional survey. All patients who were visiting Ambo Hospital during the study period formed the source population. Of which presumptive active TB patients from outpatients departments (OPDs), wards (inpatients) and/or antiretroviral therapy (ART) clinic were the targets of the study. The sample size was determined using the formula for estimating a single population proportion method (Daniel 1999). Considering the sensitivity of LED-FM and Xpert MTB/RIF assay and 95% confidence interval (CI), a total sample size for the current study was 384.

That means, $$n = \frac{(Z\,\alpha/2)^2\, p\,(1-p)}{d^2};$$

Where: n is the required sample size (TB presumptive cases), the value of Z is the standard normal distribution that corresponds to α-level 0.05, p is assuming proportion of LED-FM and Xpert MTB/RIF performances for diagnosis of TB patients (P=50%), d the margin of error (precision) =5%. Thus n is computed to be 384.

4.3 Inclusion criteria

All TB suspects, both new and having previous history of self-reported TB medication, including HIV-infected and non-infected cases were eligible for inclusion in the study. Participants who aged ≥5 years and could provide sufficient sputum sample (4ml) were included in the study.

4.4 Exclusion criteria

Follow-up patients (or patients who were already on anti-TB medication), patients who could not provide sufficient sample, under-5 children and those with severe illness were excluded. Patients were recruited prospectively in a consecutive manner until the estimated sample size was attained as per the inclusion criteria following their arrival (for able patients) at the microbiology laboratory of the Hospital.

4.5 Sample collection and analysis

A semi-structured questionnaire with slight modification from (WHO stop TB partnerships 2008) was administered to capture socio-demographic and clinical data for consenting/assenting eligible patients before sputum sample collection. Each patient was provided a clean, dry, sterile wide-neck, leak-proof container (falcon tube) and instructed how s/he could collect productive sputum sample and submit it on the recruitment day. Patients were also advised to fetch adequate fresh early morning sputum samples in a new container for the next working day. After the patient brought the early morning sputum sample, a third container was given for a second-spot sample. The first- and second-spot samples were not processed in the study as per the WHO recommendation.

Before analysis, each morning sputum sample was carefully homogenized and partitioned into two equal parts. One of the splits was used for detection with LED-FM and Xpert MTB/RIF methods. The other half was stored at 2-8 $^{\circ C}$ and after a maximum of 7 days transported, in cold chain, to the NTRL, EPHI for MTB culture. All laboratory experiments were done following GLI mycobacteriology laboratory manual (GLI 2014).

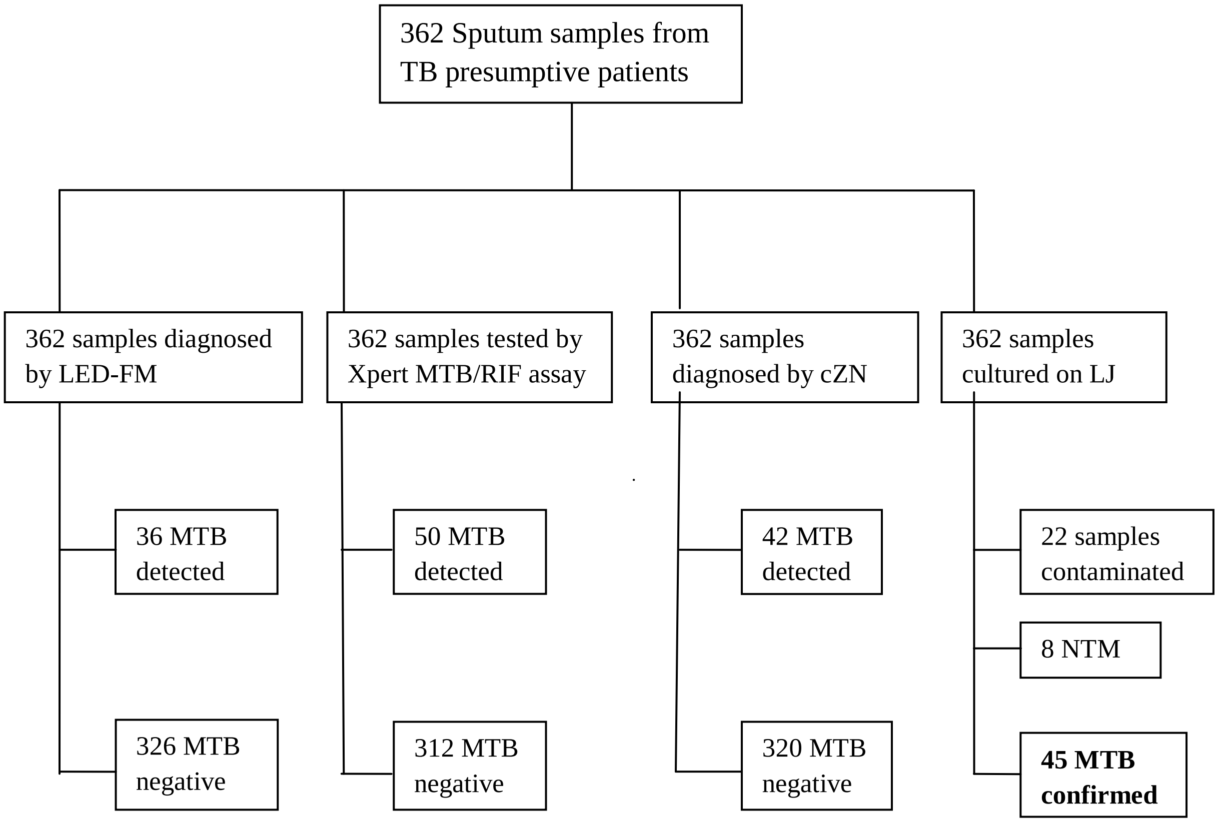

Figure 1: Flow chart showing series of diagnosis in the recruitment procedures and results. The same samples were diagnosed by different methods

4.5.1 LED-FM

The smear was prepared on new and clean frosted slides from the sputum sample and stained with the staining reagents, Auramine O stain (0.1% auramine O, 0.5% acid alcohol and 0.5% potassium permanganate) and examined by the LED-FM (Primo Star iLED, Carl Zeiss Micro imaging, Göttingen, Germany).

4.5.2 Xpert MTB/RIF assay

The Xpert MTB/RIF machine (GeneXpert®Dx System version 4.4a, Cepheid Company, 904 Caribbean Drive, CA 940889, USA) was used for this study. The sputum samples were treated with sample reagent (SR) containing NaOH and isopropanol that is provided with kit as per the manufacturer's instruction (Cepheid, Xpert MTB/RIF assay manual 2012). The SR is added using a 2:1 ratio of the sputum sample, homogenized and incubated for 15 minutes at room temperature. Then from the treated samples 2 ml were transferred into multi-chambered plastic cartridge preloaded with the liquid buffers and lyophilized reagent beads necessary for sample processing, DNA extraction, and hemi-nested RT-PCR. The cartridge is loaded into the Xpert MTB/RIF instrument, and an automatic process completes the remaining assay steps. The results could be visualized and printable in the view results window.

4.5.3 Concentrated ZN smear

Immediately after arrival at the NTRL, the samples were processed for concentrated ZN (cZN) smear test and MTB culture on the same day. A concentrated sputum sample ready for culture was examined by the cZN smear technique using conventional LM. From specimens that were processed for culture by decontaminating using NALC-NaOH (4%) and concentration by centrifugation at 3000 x g for 15 minutes, after decanting the supernatants smears were prepared from the sediment on new and clean forested slide. After the smears were well dried, ZN staining (1% carbol fucshin, 3% acid alcohol and 0.1% methylene blue) was performed and examined.

4.5.4 MTB culture on LJ medium

As the sample arrived at NTRL, the sputum samples were decontaminated using N-acetyl-L-cysteine (NALC)-sodium hydroxide NaOH (4%). After concentration by centrifugation at 3000 x g for 15 minutes, the sediment was re-suspended with 2ml of 0.5M phosphate buffer (pH 6.8) and inoculated into LJ medium and incubated at 37°C for up to 8 weeks to declare culture-negative. Each LJ culture positive isolates were stored in to NALC-tube labeled with study ID containing the storage media of MTB and stored in a deep freeze (-70^{oC}) refrigerator.

When the stored isolates were needed for DST testing, the stored isolates were removed out from the deep freezer and MTBC and NTM were identified by using One *Step Identification of MTBC by RAPID Test* using TB Ag MPT 64 Rapid (SD Bio Line, Kyonggi-do, Korea) and inoculating the isolates on blood agar media for 48 hour incubation. DST procedures were carried on confirmed MTB culture-positive samples.

4.5.5 DST testing for LJ culture positive

The stored isolate was cultured again to MIGIT tube to get new (fresh) isolates and first-line anti-TB drugs INH and RIF were tested on positive MGIT cultures by the phenotypic DST method. Lyophilized INH and RIF drug vials in MIGIT 960 Kit were identified by identification methods as far the manufacturer’s manual. Three 7ml MIGIT tubes per sample were labeled for each test isolate and with one of each of the following GC (Growth Control), INH and RIF. By placing the tubes in the GC, INH and RIF sequence. Aseptically 0.8 ml of BACTEC MGIT SIRE Supplement (provided in the SIRE kit) was added to each tube. Hundred microliters of the reconstituted INH and RIF drugs was added to the labeled tubes for INH and RIF aseptically. The final concentration of INH and RIF drugs in the test tubes were 0.1μg/ml and 1.0μg/ml respectively and MTB suspension were inoculated and then incubated. All procedures were carried out inside a biological safety cabinet (BSC) by using full personal protective equipment.

4.6 Quality assurance

Samples and clinical/questionnaire data were collected following established procedures by the assistance of trained laboratory personnel/clinicians. All reagents and chemicals were of analytical grade from renowned companies. BSC was used during culture inoculation and DST setting to ensure safety and contamination of samples. All laboratory tests were performed under strict follow-up of experienced professional TB laboratory personnel. Standard operation procedure of the NTRL was solely used to ensure the reliability and validity of test results.

4.7 Data analysis

Socio-demographic, clinical and laboratory data were recorded on a registration sheet developed by the principal investigator, entered into SPSS version 20 (IBM SPSS) and analyzed. Sensitivity, specificity, positive predicative value (PPV) and negative predicative value (NPV) at 95% CI of dLED-FM, cZN and Xpert MTB/RIF were calculated and compared against the gold standard LJ culture. The chi-squared test was used to test statistical significance for differences between values. All statistical tests were considered significant if the two sided p-value (p) was <0.05.

4.8 Ethical considerations

Ethical approval was obtained from College of Natural Sciences Institutional Review Board (IRB), Addis Ababa University, and Scientific and Ethical Review Office of EPHI. A support letter was obtained from Ambo Hospital administrative office. Consent and assent were also obtained from the study participants. TB positive test results were communicated to the responsible physicians immediately for proper patient management.

5 Results

5.1 Socio-demographic and clinical characteristics

A total of 362 presumptive TB patients were enrolled during the study period. Majority (56.6%) of the participants were males resulting in a male to female ratio of 1.2:1.0. The age of the participants ranged from 5-80 years with mean of 35.3. Most (90.4%) knew their HIV status.

Among these, 31.9% were HIV-infected. Most (46.7%) were farmers and the least daily laborer (5.3%). Over 77.7% were able to read and write with most having knowledge about TB transmission. One hundred thirty one (39.5%) had self-reported history of previous TB medication (table 8).

5.2 Detection rates

All of the samples were sputum samples except for pleural fluids samples from two patients. MTB was not detected from these two pleural fluids samples by any of the three detection methods. The plan to investigate EPTB cases unfortunately could not materialize. EPTB presumptive cases were not encountered, during the study period. While of the sputum samples collected (360) and the two pleural fluids total samples (362) were successfully analyzed by three methods (LED-FM, cZN and Xpert) only 340 were successfully cultured on LJ medium and declared positive or negative for MTB, the rest 22 were found contaminated and discarded and 8 from 340 were NTM.

In overall, 53 samples were found to be positive for MTB by at least one of the detection methods used and 35 were positive by all four method including culture. Specifically, 36(9.9%) samples were MTB-positive by LED-FM, 42(11.6%) by cZN, 50(13.8%) by *Xpert*. *Xpert* displays a semi-quantitative result as 'very low', 'low', 'medium', and 'high' for its positive cases. Accordingly, 7(14.0%) samples had a 'high' semi-quantitative result, 20(40.0%) 'Medium', 16(32.0%) 'Low', and 7(14.0%) 'Very low'. Of the total 53 growth on LJ culture media, 45(13.2%) were MTB whereas 8 of them were NTM by identification test. Some samples were positive by one method and not by the other(s) and vice-versa. The number of samples positive by the respective methods singly and in combination is indicated in table 1.

Regarding HIV infected and non-infected patients, LED-FM 6/11(54.5), Xpert MTB/RIF 10/11(90.9), cZN 7/11(63.6) and culture 11/11(100%) detected MTB cases in HIV-positive individuals and 26/32(81.3), 28/38(73.7), 26/33(78.8) and 30/41(73.2%), respectively detected MTB cases in HIV negatives individuals.

5.2.1 dLED-FM and culture

Out of 36 dLED-FM-positive samples 35 were culture-confirmed, 1 was contaminated. Proportionally there was 100% agreement between LED-FM and culture concerning the former's specificity. However, 10 culture-confirmed TB cases were missed by the dLED-FM. Thus, the sensitivity, PPV and NPV of dLED-FM in comparison with the gold standard LJ culture method were respectively 77.8, 100 and 96.6% (table 2).

Table1. Positivity of sputum samples analyzed by dLED-FM, cZN, Xpert and LJ culture singly and in combination

		dLED-FM (n=362)		**Xpert (n=362)**		**cZN (n=362)**		**LJ culture(n=332)**	
		positive	negative	positive	negative	positive	negative	positive	negative
dLED-FM	positive (36)			36	0	36	0	35	0
	negative			14	312	6	320	10	287
Xpert	Positive(50)	36	14			42	8	42	6
	negative	0	312			0	312	3	281
cZN	positive (42)	36	6	42	0			37	3
	negative	0	320	8	312			8	284
LJ culture	positive (45)	35	10	42	3	37	8		
	negative	0	287	6	281	3	284		

* Of 362 sputum samples only 340 were successfully cultured on LJ medium and declared positive or negative for MTB, the rest 22 were found contaminated and disregarded. Eight from 340 were NTM (n=332).

Table 2: Performance of dLED-FM for detection of TB against the LJ medium culture (n=332)

Characteristics	**Value (%)**	**95% CI**
Sensitivity	77.8	62.9 - 88.8%
Specificity	100	98.7 - 100%
PPV	100	90.0 - 100%
NPV	96.6	93.9 - 98.4%

PPV: positive predictive value, NPV: negative predictive value

5.2.2 dLED-FM and cZN

All the 36 dLED-FM-positive samples were positive by the cZN as well showing 100% agreement. But 6 samples that were MTB negative by the dLED-FM were positive by cZN smear. Thus the sensitivity of dLED-FM compared to the conventional cZN smear is 85.7%. Thus sensitivity, specificity, PPV and NPV were 85.7, 100, 100 and 98.2% respectively (table 3). The reading agreement between cZN and dLED-FM was kappa value (K) = 0.9

Table 3: Comparison of cZN against dLED-FM for diagnosis of TB (n=362)

Characteristics	**Value (%)**	**95% CI**
Sensitivity	85.7	71.5-94.6%
Specificity	100	98.9-100%
PPV	100	90.3-100%
NPV	98.2	96.0-99.3%

PPV: positive predictive value, NPV: negative predictive value

5.2.3 cZN and culture

When the cZN smear was compared against LJ culture, 3 MTB detected by cZN were culture negative. But cZN missed 8 MTB cases that were culture-positive. Sensitivity and specificity of the cZN method were, thus, 82.2, 98.9% respectively (table 4).

Table 4: Sensitivity, specificity, PPV and NPV of cZN against gold standard LJ culture (n=332)

Characteristics	**Value (%)**	**95% CI**
Sensitivity	82.2	67.9-92%
Specificity	98.9	97-99.8%
PPV	92.5	76.6-98.4%
NPV	97.3	94.7-98.8%

PPV: positive predictive value, NPV: negative predictive value

5.2.4 Xpert and culture

While 3 culture-confirmed mycobacterium cases were missed by *Xpert*, 6 of 7 *Xpert*-positive cases were culture-negative. Two *Xpert*-positive cases were indeterminate by culture because of contamination. The sensitivity and specificity of the *Xpert* assay in comparison with LJ culture method were 93.3 and 98%, respectively (table 5).

5.2.5 Xpert and dLED-FM

Fourteen Xpert-positive cases were not detected by dLED-FM. But all the 36 (100%) MTB-positives by dLED-FM were also positive by Xpert. Thus, the sensitivity and specificity of Xpert versus dLED-FM was 100 and 72%, respectively (table 6). Comparison of diagnosing performance of dLED-FM, cZN and *Xpert* between previous study and current result is indicated in table 7.

Table 5: Performance of *Xpert* assay for detection of TB against LJ culture (n=332)

Characteristics	**Value (%)**	**95% CI**
Sensitivity	93.3	81.7-98.6%
Specificity	98.0	95.6-99.2%
PPV	87.5	74.8-95.3%
NPV	98.9	97.0-99.8%

PPV: positive predictive value, NPV: negative predictive value

Table 6: Performance of Xpert against dLED-FM for detection of TB (n=362)

Characteristics	**Value (%)**	**95%CI**
Sensitivity	100	98.9-100%
Specificity	72	57.7-83.8%
PPV	100	90.3-100%
NPV	95.9	93.2-97.77%

PPV: positive predictive value, NPV: negative predictive value.

Xpert detected 2 RIF-resistant (MDR-TB) cases which were confirmed by the phenotypic DST test. There was no statistically significant difference in MTB case detection between dLED-FM, *Xpert* and culture among HIV-infected and non-infected TB patients.

5.3 Socio-demography and TB positivity

Most of the cases (64.4%, 29/45) belonged to the most productive age group (15-34 years). Occupation wise, the proportion of infected daily laborers (27.8%) was the highest (table 8). Among HIV positive TB presumptive (106) patients 10.4% were TB confirmed cases but from HIV negative TB presumptive individuals (194) about 15.5% were infected with TB. No patient had TB among individuals who refused to declare their HIV status. In all cases there was no statistically significant difference in TB prevalence with respect to the different socio-demographic variables.

Table 7: Comparison of diagnostic performance of dLED-FM, cZN and *Xpert* between previous study and current result.

Method	Secondary data					Current result (n=362)		
	Sensitivity (%)	Specificity (%)	p-value (<)	n	Reference	Sensitivity (%)	Specificity (%)	P-value
Xpert	80.2 90.6 97.14 98.4 65.5 57.4 74.7	99.0 94.3 100.0 95.9 96.3 ND ND	0.05 0.05 0.05 0.05 0.05 0.05 0.05	648 80 40 504 227 812 124	Ngabonziza 2014 Ioannidis et al. 2011 Darwish et al. 2013 Oluwasen et al. 2015 Geleta et al. 2015 Balcha et al. 2014 Alvarez-Uria et al. 2012	93.3	98.0	<0.05
dLED-FM	75.5 97.9 51.5 45.8 63.9	90.0 95.2 99.8 100.0 ND	0.01 0.00 0.01 0.10 0.00	404 150 648 178 106	Chaidir et al. 2013 Rahman et al. 2011 Ngabonziza 2014 Getachew et al. 2015 Alvarez-Uria et al. 2012	77.8	100	<0.05
cZN	72.7 45.8	97.8 100.0	0.01 0.01	404 178	Chaidir et al. 2013 Getachew et al. 2015	82.2	98.9	<0.05

*The p-value is stand for sensitivity and specificity, ND= not done

Table 8: Culture-confirmed TB cases in different groups of the study population (n=332).

Variable		Tested (no (%)	Culture-confirmed (no (%)	p-value
Age (years)	≤14	44(13.3)	4(9.1)	
	15-24	47(14.2)	12(25.5)	0.767
	25-34	75(22.6)	17(22.7)	
	35-44	74(22.3)	8(10.8)	
	45-54	51(15.4)	4(7.8)	
	55-64	32(9.6)	0(0.0)	
	≥65	17(5.1)	0(0.0)	
Sex	Male	188(56.6)	23(12.2)	
	Female	152(45.8)	22(14.5)	0.695
Occupation	Employed	25(7.5)	6(24.0)	
	Farmer	155(46.7)	17(10.9)	
	Merchant	57(17.2)	9(15.8)	0.630
	Daily laborer	18(5.3)	5(27.8)	
	Student	48(14.1)	4(8.3)	
	Housewife	18(5.1)	2(11.1)	
	Others	19(5.7)	2(10.5)	
Education	Primary	193(58.1)	26 (13.5)	
	Secondary	48(14.5)	8(16.7)	0.099
	Tertiary	17(5.1)	5(29.4)	
	Uneducated	82(24.7)	6(7.3)	
HIV/AIDS status	Positive	106 (31.9)	11(10.4)	
	Negative	194(58.4)	30(15.5)	
	Unwilling to respond	7 (2.1)	0(0.0)	0.327
	Never tested	33 (9.9)	4 (12.1)	
Previous TB treatment	Yes	131 (39.5)	12 (9.2)	
	No	209 (62.9)	33 (15.8)	0.920

6 Discussion

The 13.6% TB culture-positivity rate found in this study is comparable to previous reports from various settings of Ethiopia 13.5% (Getachew et al. 2015). But lower compared to other reports, 16.9% (Geleta et al. 2015) and 25.5% (Balcha et al. 2014). Even though it is difficult to compare prevalence rate of a single setting with WHO country TB estimation rate, it is higher than WHO 2015 estimates for Ethiopia (200 per 100 000 population). TB is a common public health problem in Ethiopia. Its prevalence is expected to be higher than normally reported. This is mainly because of paucity of nationwide representative incidence and prevalence data due to diagnostic challenges in most rural areas of the country. But, various prevalence data emerging from different settings are hard to compare for reasons related to the variability of such studies in their design.

Among all TB suspects, majority (56.6%) were males. But the proportion of confirmed TB cases was higher among females (14.5%) than males (12.2%) although the difference was not statistically significant. May be males have better treatment-seeking behavior and/or financial ability to travel to health-facilities. But the higher proportion of female TB patients is similar to studies elsewhere in Ethiopia (Bezabih et al. 2002, Biadglegne et al. 2013). Similar findings were reported from other countries as well (Ersöz et al. 1998, Chan-Yeung et al. 2002, Ong et al. 2004, Pahwa et al. 2005). Although reasons for such sex-based difference are not yet clear, differences in hormonal influences and production of some cytokines like tumor necrosis factors and interleukin-10 is suggested as biological factors that could enhance susceptibility of females to TB (Click et al. 2012, Polesky et al. 2005, Chan-Yeung et al. 2002). In other settings, a male preponderance has also been reported (Gomes et al. 2013, Birhanu et al. 2014).

Twenty nine (23.8%) culture-positive cases aged 15-34.9 years, and these constituted 64.4% of all culture-confirmed cases. This shows the impact of the disease on this particular group. Similarly, this age category had the highest burden in previous reports from Ethiopia (Birhanu et al. 2014, Seyoum 2007, Bruchfeld et al. 2002) and beyond (Khandkar et al. 2015, Viegas et al. 2015).

Of the patients with known HIV status, about one-third was HIV-infected showing the sustained public health impact of the virus. Among these, 11(10.4%) had culture-confirmed active TB. But, this is lower compared to the proportion of TB cases (15.5%) among HIV-negatives. The lower proportion of TB cases among HIV-infected patients may be explained by the fact that this

subpopulation has better health-oriented education and awareness. Probably these patients take their ART drugs effectively and get better feeding.
Of 362 sputum samples tested 36(9.9%), 42(11.6%), 50(13.8%) by dLED-FM, cZN, Xpert respectively showing comparable performance of the methods used. From culture-confirmed TB cases, 11(24.4%) were HIV co-infected. Of these 11 samples, only 6 were positive by dLED-FM. But Xpert has detected all of the samples from HIV-positive cases except one. This demonstrates the diagnostic accuracy of Xpert for detection of TB among HIV-coinfected cases. Over one-third (38.5%) of the patients had history of previous treatment for TB indicating the recurring burden of the disease in the population. This study confirmed TB among 12(9.2%) of these patients. This lower finding compared to that among patients without history of previous TB treatment (15.8%) may show recent new infections rather than reactivation of past cases.

The semi-quantitative results of Xpert assay indicated the assay's ability to detect a low mycobacterial load very well. Nevertheless, from 7 very-low Xpert semi-quantitative results, 6 were culture negative. These might be the dead bacteria or due to very low bacteria in the sample, it was possibly missed during inoculation on LJ culture tube. MTB were scantly detected by cZN stain in 3 of 6 culture-negatives samples although all 7 very-low Xpert semi-quantitative results were negative by dLED-FM. It shows that Xpert assay is highly sensitive and specific.

Various authors from different settings evaluated the diagnostic performance of both dLED-FM and Xpert MTB/RIF. Chaidir et al. 2013, from Indonesia, reported the sensitivity and specificity of dLED-FM to be 75.5 and 90.0% and that of cZN smear 72.7 and 97.8% respectively. This result is lower than ours where the respective values are 77.8, 100 and 82.2, 98.9%. The disparity might be due to differences in the status of the two study populations including the quantity of sputum samples provided. A similar study from Bangladesh (Rahman et al. 2011) reported LED-FM sensitivity, specificity, PPV and NPV 97.9, 95.2, 97.9 and 76.9% respectively. The 97.9% sensitivity of that study was greater compared to the present finding although all other values were lower. The explanation why the sensitivity was greater and others lower might be because of the smaller sample size (150) the authors used, quality of sputum and diagnosing techniques. The current findings demonstrate lower sensitivity and higher specificity of LED-FM compared to the 84% and 98%, respectively, in a recent comprehensive review (Shenai 2015). The lower sensitivity may be attributed to sample quality and clinical condition of the patient. A recent study in Ethiopia also indicated the sensitivity, specificity, PPV and NPV of LED-FM to be 62.5, 100, 100, and 94.5%, respectively (Getachew et al. 2015) sensitivity and NPV lower compared to the 77.8, and 96.6%, respectively, in present study. This might also be because of quality of sputum sample analyzed or clinical condition of the patients.

The sensitivity, specificity, PPV and NPV of Xpert MTB/RIF assay were found to be 80.2, 99, 96.6 and 97% respectively (Ngabonziza 2014). Among smear positive-culture positive and smear negative-culture positive cases the sensitivities were 91.8 and 60% respectively, according to the same report. The findings of our study which are 93.3, 98, 87.5 and 98.9% respectively are higher. For smear and culture positive and smear-negative culture positive cases our Xpert performed superior. The above authors analyzed sputum samples that were collected from eight health facilities and transported to the national referral laboratory for culture. This might have influenced the culture yield.

Another study from Greece found the sensitivity, specificity, and PPV and NPV of Xpert MTB/RIF as 90.6, 94.3, 93.5, and 91.7% (Ioannjdis et al. 2011) which was also lower compared to ours of 93.3, 98, 87.5 and 98.9% respectively. The reason might be because of small samples (80) although specimens were selected from strong clinical indications for TB patients.

The evaluation of *Xpert* in comparison with LJ culture in Egypt revealed sensitivity, specificity, PPV and NPV of 97.14, 100, 100 and 85.6% respectively (Darwish et al. 2013) which were higher than the results of this study. This might be due to the small sample size (40), strongly presumptive patients and 33-58 age category used by the Egyptian investigators. A recent study is lower compared to study in Nigeria recorded sensitivity, specificity, PPV and NPV of 98.4, 95.9, 98.8 and 94.0%, respectively, for Xpert assay (Oluwaseun et al. 2015) except for specificity and NPV of the present result. The higher sensitivity of Xpert MTB/RIF assay might be attributed to the greater sample size (504) and systematic sampling method the authors used. The authors recruited every third person from a long list of TB suspect as well as the clinical status of the patients.

The 2 RIF-resistant cases detected by Xpert and phenotypic DST test were not from HIV-infected individuals showing that RIF-resistance is not related to TB-HIV infection. Both of the cases were from TB patients with previous history of treatment suggesting reactivation of previous latent case rather than a new infection.

7 Conclusion and recommendation

Xpert MTB/RIF assay demonstrated 100 and 71.4% sensitivity in smear positive-culture positive and smear negative-culture positive sputum samples, in detecting MTB respectively. The assay increased MTB detection rate by 28% compared to LED-FM and ability to detected very low bacterial load in the sputum sample. This study generated additional data showing the added value of Xpert assay over LED-FM in detecting MTB for organizations working in the area of TB control and TB researchers.

Health facilities in the country that reported high TB and RIF-resistance cases should be implemented with this Xpert MTB/RIF assay to increase the MTB case detection rate.

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9. Annexes

Annex I: Consent and assent forms

1. Informed consent and assent forms (English version)

1.1. Informed consent form for adults

Name of study participant: ___________________________ Age ______ Sex _____

I have very well understood that the aim of this study will to assess the Performances of LED FM and Xpert MTB/RIF Assay for the Diagnosis of Tuberculosis among HIV- infected and Non-infected Patients at Ambo Hospital, West-central Ethiopia. I will be asked to provide information that I know about TB and samples such as sputum or other body fluids for laboratory examination of this study. I have asked some questions and clarification has been given to me. I have been informed that there is no direct benefit provided to me except receiving Laboratory result for my medication only. I have given my consent freely to participate in this study. I have been given enough time to think over before I signed this informed consent.

Participant's signature _______________ Date______/____/_______

Researcher Name _________________________ signature _____Date____/____/_____

1.2. Informed Assent Form for children less than 18 years old

Name of Participant Child ______________________________ Age ______ Sex ______________

Parent Name (Participant Child parent) __________________________Age ______Sex _____

I have very well understood that the aim of this study will to assess the Performances of LED FM and Xpert MTB/RIF Assay for the Diagnosis of Tuberculosis among HIV- infected and Non-infected Patients at Ambo Hospital, West-central Ethiopia. I will be asked to provide information that I know about TB and my child his/her name mentioned above provide samples such as sputum or other body fluids for laboratory examination of this study. I have asked some questions that is not clear to me and clarification has been given to me. I have been informed that there is no direct benefit provided regarding this study to me except receiving Laboratory result for my child medication purpose only.

Based on this, by getting full understanding of the situation, I have agreed and consent freely to participate on this study with my child. I have been given enough time to think over before I signed this informed consent.

Signature of parent (Participant Child parent) ____________________Date ___/___/____

Name of researcher ________________________ sign ____________ Date ____/___/______

2. Informed consent and assent form (Afaan Oromoo)

2.1. Consent form (Waliigaltee)

Maqaa hirmaattichaa _______________________ Umurii _______ Saala __________

Kaayyoo qo'anna kanaa sirriitti hubadhee, hospitaala Ambootti wa'ee sakatta'a danddeettii meeshota dhukkuba sombaa mul'isan jechuunis "LED fluorescent Mikroscopy" fii "GeneXpert MTB/RIF" qorachuudhaaf akkata'e sirriitti hubadhee mirkaneeffadheera. Kanaafis immoo Akkitaa koo ykn dhangala'aa biro kan qaama keessa jiru qo'annichaaf kanta'u akkan kennuu fii hubannaan ani dhukkuba sombaatiif qabu akkan himu gaafatameera. Anis waan naaf hingalle gaafadhee sirriitti naafdeebi'eera. Qo'annoo kana irraa frii Laaboraatory dhibeekoo akkan ittiin yalamuf naaf kenna mamalee fayidaa biraa kallattiidhaan akkan hinarganne natti himameera. Waliigaltee kanas otoon hinmallatteessin yeroon bal'aa naaf kennamee sirriitti itti yaadeera.

Kanaafis dirqama tokko malee qo'annoo kana irratti hirmaachuuf dhaaf waliigalee mallatteesseera.

Mallattoo hirmaattichaa _______________________ Guyyaa ____/_____/________

Mallattoo qo'ataa ____________________________ Guyyaa ____/____/_________

2.2. Assent form (Waliigaltee maatiii joollee Umuriin waggaa 18 gadii)

Maqaa mucaa (Daa'imaa) _______________________ Umurii _______ Saala _________

Maqaa maatii mucaa (Daa'imaa) _________________________Umurii ______ Saala _______

Kaayyoo qo'annaa kanaa sirriitti hubadhee, hospitaala Ambootti wa'eesakatta'a danddeettii meeshota dhukkuba sombaa mul'isan jechuunis "LED fluorescent Mikroscopy" fii "GeneXpert MTB/RIF" qorachuudhaaf akka ta'e sirriitti hubadhee mirkaneeffadheera.

Kanaafis immoo mucaan koo kan maqaan isaa/ishee armaan olittii bsame/ibsamte Akkitaa ykn dhangala'aa biro kan qaama keessa jiru qo'annichaaf kanta'u akka kennuu/kennituu fii hubannaan ani dhukkuba sombaatiif qabu akkan himu gaafatameera. Anis waan naaf hingalle gaafadhee sirriitti naaf deebi'eera. Qo'annoo kana irraa frii ykn bu'aa Laaboratory dhibee mucaa koof akkan ittiin yaalladhuuf naaf kennama malee kallattiidhaan waan biraa akkan hinarganne nattihimameera. Waliigaltee kanas otoon hin mallatteessin dura yeroon bal'aa naaf kennamee sirriitti ittiyaadeera. Kanaafis dirqama tokko malee qo'annoo kana irratti mucaan koo akka hirmaatu/ttu anibakka bu'ee waliigaltee kana mallatteesseera.

Maqaa Maatii Mucaa (Daa'imaa) ____________________ Mallatoo______ Guyyaa___/__/____

Maqaa qo'ataa ________________________ Mallatoo ______ Guyyaa ___/___/________

3. **የጥናቱ ምንነትና ስምምነት በአማርኛ**

የጥናቱ ታሳታፍ ስም ____________________________ዕድሜ__________________________ፆታ__________

ስለጥናቱ አስፈላጊ የሆኑትን ማስረጃዎች አንብቤ ወይም ተናቦልኝ አለማውም በአምቦ ሆስቲታል የኤል ኢ ዲ ፍሎረሰንት ማይክሮስኮፐ (LED FM) እና የጂን ኤክስፐርት (Xpert MTB/RIF) የቲቢ በሽታ መመርመርያ መሣሪያን የመመርመር አቅም ለማጥናት መሆኑን ተረድቻለሁ ፡፡ ለዚህም ጥናት ስለ ቲቢ ያለኝን አስፈላጊ መረጃዎች እና ለዝህ ጥናት እንድዉል የአክታ ናሙና ወይም ከሰዉነቴ ዉስጥ የሚቀዳ ፈሳሽ ነገር እንዲሰጥ ታጠይቃለሁ፡፡ ከዚህም ጥናት ለበሽታዬ መድሃንት እንዲሰጠኝ የ LED FM, Xpert MTB/RIF እና የ DST ውጤት በሽታዬን እንድታካም ይሰጠኛል እንጂ ሌላ የተለዬ ጥቅም እንደማለገኝ ተገልፆልኝ ወይም ተረድቼ እንድሁም ስምምነቱን ከመፈረሜ በፊት በቂ ጊዜ አግንቼ አስቤበት በጥነቱ ለመሳታፍ ፍቃደኛ መሆኔን በፍርማዬ አረጋግጣለሁ ፡፡

የታሳታፊው ፊርማ__________________________________ ቀን______________________________

የተማራማሪው ፊርማ___________________________________ ቀን__________________________

የጥናቱ ታሳታፍ ልጅ ስም ________________________ዕድሜ_________________ፆታ____________

የልጁ ወላጅ ስም __________________________________ ዕድሜ _________ ፆታ ______________

ስለጥናቱ አስፈላጊ የሆኑትን ማስረጃዎች አንብቤ ወይም ተናቦልኝ አለማውም በአምቦ ሆስቲታል የኤል ኢ ዲ ፍሎረሰንት ማይክሮስኮፐ (LED FM) እና የጂን ኤክስፐርት (Xpert MTB/RIF) የቲቢ በሽታ ማመርመርያ መሣሪያን የመመርመር አቅም ለማጥናት መሆኑን ተረድቻለሁ ፡፡ ለዚህም ጥናት ከዚህ በለይ ስሙ የተገለጼዉ ልጄ እንዲሳተፍበት ተጠይቄ እኔም ጥናቱ ጠቃሚ መሆኑን ስላመንኩበት ስለ ቲቢ ያለኝን አስፈላጊ መረጃዎች እና የልጄን የአክታ ናሙና ወይም ከሰዉነቱ ዉስጥ የተቀደዉን ፈሳሽ ነገር ለዝህ ጥናት የሚዉል እንዲሰጥ ታጠይቃለሁ፡፡ ከዚህም ጥናት ልጄ ለበሽታዉ መድሃንት እንዲታዘዝለት እና እድታከምበት የ LED FM, Xpert MTB/RIF እና የ DST ውጤት ይሰጠኛል እንጂ ሌላ የተለዬ ጥቅም እንደማለገኝ ተገልፆልኝ ወይም ተረድቼ እንድሁም ስምምነቱን ከመፈረሜ በፊት በቂ ጊዜ አግንቼ አስቤበት በጥነቱ ለመሳታፍ ፍቃደኛ መሆኔን በፍርማዬ አረጋግጣለሁ ፡፡

የታሳታፊው ፊርማ_______________ ቀን_____/___/______የተማራማሪው ፊርማ__________ ቀን___

Annex II: Questionnaire

Participants will be asked the following questions in order to collect demographic and clinical information of the study subjects.

Study site _______________ Participants code no ________________

Name of responsible health worker ___________________ Signature _______ Date ___/___/___

1. Address of the participant: Zone __________________
   Woreda ________________
   Kebele _________________
   Card No ________________
   Tele. ___________________
2. Age of the participant _________________
3. Sex: A. ☐ Male
   B. ☐ Female
4. Ethnicity: A. ☐ Oromo D. ☐ Guragie
   B. ☐ Amhara E. ☐ Sidama
   C. ☐ Tigrie F. ☐ Others (please specify) ________
5. Religion: A. ☐ Protestant D. ☐ Wakefata
   B. ☐ Muslim E. Other (please specify) ________
   C. ☐ Orthodox
6. Marital status: A. ☐ Single C. ☐ Widowed
   B. ☐ Married D. ☐ Divorced E. ☐ No respond
7. If widowed**:** A. ☐ Due to tuberculosis
   B. ☐ Other cause (please specify) ______________________
8. If Divorced: A. ☐ Before contracting TB
   B. ☐ After contracting TB
9. How many family members do you have in the house you live? ______________
10. Educational level of the patient:
    A. ☐ Primary school
    B. ☐ Secondary school

C. ☐ Higher education

D. ☐ None educated

11. Occupation of the participant: A. ☐ Employed D. ☐ Daily laborer

B. ☐ Farmer E. ☐ Private Worker

C. ☐ Merchant F. ☐ Pensioned

G. Others (please specify) ________________

12. for how much time you cough? A. ☐ One week

B. ☐ Two week

C. ☐ Greater than two week

13. Are you smoking Cigarettes? A. ☐ Yes C. ☐ I stopped it before (when?)

B. ☐ No ↳ specify it _______________

13.1. If your answer is 'yes' for No 13 above, how many cigarettes per week do you smoke?______.

13.2. If your answer is 'C' for No 13 above, when was the last time you smoked? _______.

13.3. How long have you smoked? ______________________

14. Are you take any drugs for your disease before? A. ☐ Yes B. ☐ No

14.1. If your answer for No 14 is 'yes', what type of drug you taken?

A. ☐ antibiotic

B. ☐ ant pain C. ☐ both D. ☐ other (please specify)_____________

15. Are you treated for TB before? A. ☐ Yes B. ☐ No

15.1. Is there any in your family member treated for TB before? A. ☐ Yes

B. ☐ No

15.2. Is there any TB patient treated in your community before? A. ☐ Yes

B. ☐ No

15.3. If your answer is **yes** for No 15 to15.2 above, when? (Specify) _______________

16. How far do you live from the nearest health clinic or hospital?

A. ☐ 0–10 kilometers C. ☐ 21–30 kilometers

B. ☐ 11–20 kilometers D. ☐ More than 30 kilometers

17. What is your average monthly income?

A. ☐ No Income? C. ☐ 501-1000 Birr

B. ☐ < 500 Birr D. ☐ 1001-2000 Birr

E. ☐ > 2000 Birr F. ☐ Not willing to respond

18. Do you have (Television) or Radio? A. ☐ Yes B. ☐ No

19. Do you listen health education about TB through radio Or Television?

A. ☐ Yes B. ☐ No

20. Do you screen your blood for HIV/AIDS before? A. ☐ Yes B. ☐ No

20.1. If your answer is 'yes' for No 20 above, what is your status?

A. ☐ Positive B. ☐ Negative C. ☐ Not response

D. ☐ I did not screen my blood for HIV before.

20.2. If your answer for No 20 is 'yes' When did you screened? ______________

20.3. If your answer for No 20 above is 'No' why? (Specify) _________________

21. How can a person get TB from the following?

A. ☐ Through handshakes

B. ☐ Through the air when a person with TB coughs or sneezes

C. ☐ Through sharing dishes

D. ☐ Through eating from the same plate

E. ☐ Through touching items in public places (doorknobs, handles in transportation, etc.)

F. ☐ I do not know any G. ☐ Other (please explain):____________________

10. Declaration

I, the undersigned, declare that this is my original work and has never been presented for a Degree before and all source materials used are duly acknowledged.

Name: Alemu Gadissa (BSc)

Signature __________________ Date ________________________

11. Statement of the supervisor(s)

This thesis has been approved for submission to the Department of Microbial, Cellular and Molecular Biology for public defense.

Name: Hassen Mamo (PhD)

Signature ____________________ Date __________________