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Extrapulmonary Tuberculoses and Rifampicin Resistance in St.Luke Catholic Hospital and Tullu Bollo Hospital, Oromia, Ethiopia.

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Table of Contents

Table of Contents.....	II
List of tables	V
List of figure	VI
Abbreviations.....	VII
Abstract.....	VIII
1 Introduction	1
1.1 Background	1
1.2 Statement of the problem	3
1.3 Significance of the study	5
2 Literature Review	6
3 Objectives.....	9
3.1 General objectives	9
3.2 Specific objectives.....	9
3.3 Hypothesis.....	9
4 Materials and methods.....	10
4.1 Study area.....	10
4.2 Study period	10
4.3 Study design	10
4.4 Population.....	10
4.4.1 Source population	10
4.4.2 Study population	10
4.5 Inclusion and Exclusion criteria.....	11
4.5.1 Inclusion criteria	11
4.5.2 Exclusion criteria	11
4.6 Study variables	11
4.6.1 Dependent variables.....	11
4.6.2 Independent variables	11
4.7 Measurement and data collection.....	11
4.7.1 Sample size determination	11

4.8	Data collection instrument and procedure.....	12
4.8.1	Data collection instrument	12
4.8.2	Sample collection and analysis procedure	12
4.8.3	GeneXpert principle and procedure	12
4.8.4	Ziehl-Nelson smears.....	13
4.8.5	Fluorescent staining	13
4.8.6	White blood cell and differential count in body fluids	14
4.8.7	Gram staining.....	15
4.9	Data quality assurance.....	15
4.10	Data analysis and interpretation	15
4.11	Ethical consideration	16
4.12	Dissemination of the result.....	16
4.13	Operational definitions.....	16
5	Results	17
5.1	Socio-demographic characteristics of study subjects.....	17
5.2	Physical and staining characteristics of body fluids.....	19
5.3	Magnitude of extra pulmonary tuberculosis.....	20
5.4	Extrapulmonary tuberculosis cases in relation to cell count and staining status	20
5.5	Yield of extra pulmonary tuberculosis using different laboratory methods.....	22
5.6	Rifampicin resistance of EPTB.....	22
6.	Discussion.....	25
6	Limitation of the study	29
7	Conclusion and Recommendations	29
7.1.	Conclusion.....	29
7.2.	Recommendation.....	29
8	Reference.....	30
9	Annexes	34
9.1	Annex1: Participant Information sheet	34
9.2	Annex II: Informed consent form (English version).....	36
9.3	Annex III Informed consent form (Afan Oromo).	37
9.4	Annex IV: Data collection sheet	38

9.5	Annex V: Data collection sheet (Afan Oromo).....	40
9.6	Annex VI: GeneXpert result collection sheet	42
9.7	Annex VII Principles and procedure of GeneXpert rif assay, ZN and FM.....	43
	Ziehle-Nelson smears	44
	Fluorescent staining.....	44
9.8	Annex VIII: Declaration.....	45

List of tables

Table 1: Socio- demographic characteristics of study participants, St.Luke& Tullu Bollo Hospital, 2017.....	18
Table 2: physical and staining characteristics of body fluids of study participants in St.Luke and Tullu Bollo Hospital, 2017.....	19
Table 3: Extrapulmonary cases in relation to cell count and staining status of study participants in St.Luke and Tullu Bollo Hospital, 2018.....	21
Table 4: Yield of Extrapulmonary TB using different laboratory methods of study participants in St.Luke and Tullu Bollo Hospital, 2017.....	22
Table 5: Associated risk factors of Extrapulmonary among study participants in St.Luke and Tullu Bollo Hospital, 2017.....	24

List of figure

Figure 1: Extrapulmonary TB and RIF resistance of study participants St. Luke and Tullu Bollo Hospital, 2017.....	22
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Abbreviations

AOR: Adjusted odds ratio

CDC: Center for Disease Control and prevention

CSF: Cerebrospinal fluid

DST: Drug susceptibility test

EPTB: Extra pulmonary tuberculosis

HBCs: High-burden countries

HIV: Human immune deficiency virus

LJ: Lowenson Jeensen

MDR-TB: Multi drug resistance TB

MTB: Mycobacterium tuberculosis

MTBC: Mycobacterium tuberculosis complex

NAAT: Nucleic acid amplification test

COR: Crude odds ratio

PCR: Polymerase chain reaction

RIF: Rifampicin resistance

SOPs: Standard Operational Procedures

SPSS: Statistical Package for Social Sciences

TB: Tuberculosis

TBL: Tuberculosis lymphadenopathy

XDR-TB: Extensively drug resistant TB

WHO: World health organization

ZN: Zeihl-Neelsen

Abstract

Background: Tuberculosis affects millions of peoples in the world which involves any organ system of the body. The extrapulmonary tuberculosis (EPTB) problem is increasing mainly due to drug resistant *M. tuberculosis* especially in developing countries. For the diagnosis of EPTB, GeneXpert has recently been developed for rapid detection of *M.tuberculosis* and rifampicin resistance. Assessing the prevalence of extrapulmonary tuberculosis provide effective patients management and help policy makers.

Objectives: To assess the magnitude of extrapulmonary tuberculosis and rifampicin resistance at St.Luke Catholic Hospital and Tullu Bollo Hospital, Oromia, Ethiopia.

Methods: A cross sectional study was conducted from March to August, 2017. A total of 310 body fluid specimens were collected and analyzed by gene Xpert. Ziehlle-Nelson and Florescent microscopic examination were done for each specimen. Clinical history of all patients was reviewed for HIV. Binary and logistic regression was performed to assess the strength of associations between independent and dependent variables. Data entry and analysis was done using SPSS statistical software version 20.

Results: Out of 310 body fluid specimens, 16.5% (n=51/310) were positive for MTB of which 3.9 % (n=2/51) specimens also exhibited resistance to RIF. About 3.2% (n=10/310) cases of GeneXpert MTB/RIF assay positive were ZN positive, 13.2% (n=41/310) cases of GeneXpert MTB/RIF assay positive were ZN negative and 6.8%(n=21/310) cases of GeneXpert MTB/RIF assay positive were FM positive, 9.7 % (n=30/310) cases of GeneXpert MTB/RIF assay positive were FM negative. A significant proportion of EPTB cases were also co-infected with HIV.

Conclusion: The prevalence of GeneXpert confirmed extra pulmonary tuberculosis infection was lower. Regular initiation and awareness for clinician about EPTB and its diagnostic tool is expected from health offices and stake holders. Further studies are required to clarify operational difficulty, challenges and limitations in rule-out GeneXpert MTB/RIF in current TB control/treatment algorithms in the country.

Keywords: Extrapulmonary tuberculosis, Rifampicin resistance Oromia, Ethiopia

1 Introduction

1.1 Background

Tuberculosis (TB) is a disease caused by a bacterium called *M. tuberculosis*(1). Among infectious diseases, tuberculosis (TB) is one of the most frequent causes of death in the world, with more than 2 million TB-related deaths reported each year (2).WHO estimated a total of 9.27 new cases worldwide in 2007. The same report showed 13.7 prevalence and 1.3 million death with more than 90% in developing countries in 2009 (3).

In 2012, an estimated 8.6 million people developed TB and 1.3 million died from the disease (4). TB treatment averted 49 million deaths globally between 2000 and 2015, but important diagnostic and treatment gaps persist. In 2015, 6.1 million new TB cases were notified to national authorities and reported to WHO (5).

The crisis of multi-drug resistance detection and treatment continues. In 2015, of the estimated 580 000 people newly eligible for multi-drug resistance treatment, only 20% were enrolled (5,6).

Tuberculosis (TB) can involve any organ system in the body. The term EPTB describes isolated occurrence of TB at body sites other than the lung (7). EPTB involve organs such as pleura, lymph nodes, abdomen, genito-urinary tract, skin, joints, bones, tubercular meningitis, tuberculoma of the brain, etc. The problem of EPTB is still high, both in developing and developed countries. In India, EPTB forms 10 to 15 percent of all types of TB, in comparison to 25 percent in France and 50 percent in Canada, partly due to the dual infection of TB with human immunodeficiency virus (HIV) (8).Worldwide, extrapulmonary tuberculosis accounts for 25% of all TB cases, and even higher percentages in HIV-infected individuals and children (9).

Tuberculosis (TB) lymphadenitis is the most common presentation and has been shown in about 35% of EPTB cases. TB meningitis is the most devastating form of meningitis and occurs in 7–12% of TB patients in developing country. Osteo articular TB accounts for about 1–3% of all TB cases and is the major cause of Osteomyelitis. Any bone, joint or bursa can be infected but the spine; hip and knee are the preferred sites of infection. Genitourinary TB comprising of genital and renal TB is the second most common EPTB and contributes up to 46% cases of EPTB while abdominal TB contributes up to 10–12% of EPTB cases, and much increase in this disease is because of HIV pandemic(10,11).

Existing tests for the diagnosis of EPTB are limited in accuracy and time to diagnosis, and often require invasive procedures and special expertise (12).

The absence of some typical TB symptoms hinders the clinical diagnosis of EPTB and may mislead physicians to suspect other diseases. Extrapulmonary specimens have a very low bacterial load, so the sensitivity of direct microscopy examination for detecting MTBC is low. This is particularly true of pleural TB, for which *M. tuberculosis* complex detection is achieved by smear microscopy in fewer than 5% of cases (13).

The diagnosis of pulmonary tuberculosis is usually established by examination of three Zeihl-Neelsen stained smears and Auramine O staining by fluorescent microscopy for both pulmonary and extrapulmonary tuberculosis in most of health facilities but in body fluids, smear microscopy mostly shows negative results, which do not preclude extrapulmonary TB. In line with these limitations more rapid and reliable methods are needed. In December 2010, WHO endorsed GeneXpert MTB/RIF for use in TB laboratory. It can be used by operators with minimal technical expertise, enabling the diagnosis of TB and simultaneous detection of rifampicin resistance within 2 hours (14). Recently a number of studies were done to evaluate this assay using non-respiratory clinical samples from patients suspected of having EPTB. In 2014, WHO has recommended GeneXpert over the conventional tests for testing specific non-respiratory specimens from presumptive EPTB patients. However, this was a conditional recommendation due to very low-quality evidence available. More studies are therefore needed particularly in setting with high EPTB prevalence.

1.2 Statement of the problem

Mycobacterium tuberculosis is a serious public health problem worldwide. It is the leading cause of morbidity and mortality. In 2012, an estimated 8.6 million people developed TB and 1.3 million died from the disease(14).

Ethiopia is one of the 22 high-burden countries that account for about 80% of the world's TB cases. Global TB report 2013 showed an estimated 247 per 100,000 populations' incident cases of TB and 16,000 deaths (18 per 100,000) due to TB, excluding HIV related deaths in Ethiopia in 2012. The rates for extrapulmonary TB are as high as those for smear positive and smear negative TB. The proportion of extra-pulmonary TB cases is 35-40%. Such low case detection rate reflect the critical deficiency in diagnostic laboratory capacity. In Ethiopia the MDR-TB prevalence based on the 2005 nationwide survey was 1.6% among new cases. Rifampicin resistance was lower than 2% in new cases(15).

Inability to rapidly diagnose and treat the affected patients leads to increased morbidity and mortality, development of secondary resistance (including extensively drug-resistant tuberculosis) and ongoing transmission of the disease. In this situation, not only rapid TB case detection, but also the early determination of MDR TB status is important. Conventionally the diagnosis of pulmonary tuberculosis has been based on clinical history, smear microscopy for acid fast bacillus, or bacterial isolation by culture (15,16).

In developing countries, out of all the laboratory investigations, diagnosis still relies heavily on the use of smear microscopy, which has a low sensitivity and specificity as compared to the culture. The microbiological identification of *M.tuberculosis* by culture remains the gold standard. However, the conventional culture technique for *mycobacterium* does not provide a rapid diagnosis, is a cumbersome procedure and requires sophisticated laboratory facilities of biological safety laboratory level II/III that cannot be afforded in most of resource limited settings(16).

The diagnosis of EPTB is often difficult to establish, considering that number of bacteria in specimens is often very low, a collection often requires invasive procedures, and it is not easy to obtain multiple samples. It is also not given priority in TB control programs in developing countries as the proportion is low and less infectious than PTB(16,17). There is also a major problem of drug resistance in EPTB individuals and particularly in those individuals co infected

with HIV. RIF resistance is used as a surrogate marker for uncovering MDR TB as more than 90% RIF resistant isolates are also isoniazid resistance(17,18).

The recent molecular diagnostic techniques are increasingly being promoted owing to their rapid turnaround time and high sensitivity and specificity. The World Health Organization (WHO) has endorsed the implementation of GeneXpert MTB/ RIF assay for national tuberculosis programs in developing countries. The GeneXpert MTB/RIF is an automated, user friendly and rapid test based on nested real-time PCR assay and molecular technology for MTB detection and RIF resistance. The results are obtained within a short period of 2 hours. Further on, the technique is not prone to cross-contamination, requires minimal biosafety facilities and has a high sensitivity in smear-negative extra pulmonary TB(18,19).

In Ethiopia, data on the burden of extrapulmonary tuberculosis are limited. Yet, few studies have been conducted so far from extrapulmonary samples using GeneXpert but no data is available on the yield of Zeihl-Neelsen and fluorescent staining on extrapulmonary tuberculosis. Available data were also from few body fluid specimens which cannot tell more about the issue. Moreover, data on rifampicin resistance *M.tuberculosis* from EPTB cases is very limited. Hence, this study aimed to detect extrapulmonary tuberculosis and rifampicin resistance by GeneXpert and to compare the detection rate of fluorescence and light microscopy against GeneXpert in St.Luke Catholic Hospital and Tullu Bollo Hospital, Oromia, Ethiopia.

1.3 Significance of the study

This study provided current information on the burden of EPTB. In addition, it generated information on the magnitude of MDR EPTB. This study was also help health care workers to strength the evaluation of the presumptive TB patients other than pulmonary and increase the utilization of GeneXpert assay for diagnosis of TB that involves different organs.

It is also helpful for policy makers in establishing guidelines and re-evaluation of criteria for GeneXpert request and enhance detection rate of TB from body fluid samples. It helps public health workers to strength prevention strategy and reduces the TB case mortality and morbidity.

Moreover, the study can be used as an initial data for future researchers in evaluation of GeneXpert, comparative study among different diagnostic instrument for EPTB, prevalence of EPTB and rifampicin resistance TB strains and help them to do community based research.

2 Literature Review

Deferent studies were carried out in detection of *M.tuberculosis* and RIF resistance in extrapulmonary specimens. Mostly GeneXpert assay has been evaluated against existing reference standards, microscopy and culture for TB testing and phenotypic DST for rifampicin testing.

Meta –analysis on 18 studies that included 4461 samples were done from different body fluid samples. On these studies EPTB prevalence ranged from 0% to 81%. Eight (44%) studies were conducted in low/middle-income countries. Six studies did not include any HIV-positive patients and for two studies, HIV status was unknown. One study only included HIV-positive patients. The percentages of HIV-positive patients included in remaining studies ranged from 1% to 87% of the study population. 10 studies included children, with percentages ranging from 2% to 34%. The median number of samples per study was 137. Seven studies included only one sample type (pleural fluid only). The remainder of the studies included different sample types in varying percentages(20).

Study conducted in Pakistan by Ahmad *et al*, on total of 100 clinically suspected cases of extra pulmonary tuberculosis showed that maximum positivity rate by GeneXpert. Out of 100 cases, 17 cases of GeneXpert assay positive were LJ culture positive, 20 cases of GeneXpert assay positive were LJ culture negative, 63 cases of both GeneXpert assay and ZN smear were negative. Out of 100 cases, 12 cases of GeneXpert assay positive were ZN smear positive, 25 cases of GeneXpert assay positive were ZN smear negative, 63 cases of both GeneXpert assay and ZN smear were negative. It is noted that none of the ZN smear positive and LJ culture positive samples gave negative results by GeneXpert (21).

Similar study was conducted in German on 521 specimens from May 2009 and August 2010. Among the 245 tissue samples, the majorities were lymph node specimens. Overall, 62 (11.9%) of the 521 specimens tested were positive for *mycobacterium* by culture. Out of these 62 positive cultures, 6 (9.7%) were also smear positive for acid-fast bacilli. 8, 5, and 2 *M. tuberculosis* strains were isolated from gastric fluid, urine, and stool specimens, respectively. 29 *M. tuberculosis* isolates were tested for rifampicin resistance by conventional drug susceptibility testing. All strains were found to be susceptible to rifampicin. Of the isolates positive by GeneXpert tests, 3 of 29 (10.3%) had an indeterminate rifampicin resistance result. For the remaining 26 samples, 25 were found to be susceptible and 1 was found to be resistant(22).

Study conducted in India on total of 547 patients were showed, 150/547 (27%) were culture-positive “confirmed TB” cases (58/547 (11%) being smear negative and 92/547 (17%) being smear positive. Out of 547 patients, 16 patients (3%) were found to be HIV positive. The median age of the patients was 37 years (range, 8 months to 94 years). The male-to-female ratio was 0.85(23).

Other similar study conducted in India on 378 body fluid samples that consisted of 164 pleural fluids, 148 CSF, 59 ascitic fluid and 7 other body fluid (synovial, pericardial, peritoneal) specimens. Of these, 32(8.5%) specimens were positive for MTB with a positivity rate of 10.1%, 8.8% and 9.3% respectively for pleural fluids, CSF and ascitic fluid. Rifampicin resistance was detected in 4 of the pleural fluid and one of the ascitic fluid samples. Collectively, 19.2% of the MTB positives in our study were multidrug resistant (MDR) TB (24).

Another study conducted in Pakistan on the total 245 samples (205 pulmonary TB, 40 EPTB). *Mycobacterium tuberculosis* was detected by GeneXpert assay 9/40 (22.5%) presumptive EPTB. EPTB samples where GeneXpert detected MTB in 9 (22.5%) out of 40 cases, whereas only 4 (10%) and 03 (7.5%) cases were positive on LJ culture and ZN smear respectively. It was observed that GeneXpert could detect 12.5% and 15% additional positive cases as compared to LJ culture and ZN microscopy respectively in EPTB. There is a significance difference found between ZN and LJ culture for EPTB ($P<0.05$) (25).

Study conducted in Jimma University specialized Hospital on total of 143 patients with clinical presumptive TB presenting with lymphadenopathy were shows, 18.9% (27/143) were positive for TBL on smear microscopy, 60.1% (86/143) on GeneXpert and 61.5% (88/143) on culture. Overall, 64.3% (92/143) of tested cases were positive for TBL by culture and/or smear microscopy (23 smear/culture-positive, 65 culture-positive/smear-negative, 3 smear-positive/culture-negative and 1 smear-positive/culture contaminated). The Xpert result was invalid for 1.4% (2/143) of tests performed. Smear microscopy detected AFB in 26% (23/88) of culture-positive and 6% (3/49) of culture-negative cases. Of five contaminated samples on culture, one was positive on smear microscopy. Culture was positive in 74% (71/96) of cases with suggestive cytomorphology of TB and in 36.2% (17/47) of non-TBL suggestive cases. *M. tuberculosis* DNA was detected by GeneXpert in 3 out of 5 samples with contaminated cultures(26).

An institution based cross-sectional study was conducted among EPTB suspected patients at the Gondar University Hospital. A total of 141 extrapulmonary suspected patients were enrolled in this study. The overall Prevalence of culture confirmed extrapulmonary tuberculosis infection was 29.8%, but the GeneXpert result showed a 26.2% prevalence of *M. tuberculosis* complex infection. The 78.4% prevalence of extrapulmonary tuberculosis infection was found to be higher among the adult population. The prevalence of HIV infection among EPTB suspected patients was 14.1%, while it was 32.4% among GeneXpert-confirmed extrapulmonary TB cases (12/37). Tuberculosis lymphadenitis was the predominant (78.4%) type of EPTB infection followed by tuberculosis cold abscess (10.7%). Adult hood, previous history of contact with known pulmonary tuberculosis patients, and HIV co-infection showed a statistically significant association with extrapulmonary tuberculosis infection ($P<0.013$)(27).

A cross-sectional study was conducted in patients with presumptive TB from 1st April 2015 to 30th August 2016 in Gambo Hospital, Ethiopia on 309 unique patients; 197 (63.8%) were less than 14 years old, and 165 (53.4%) were male. The most commonly analyzed sample was gastric aspiration (n=144, 46.6%) followed by sputum (n=92, 29.8%). Gastric aspiration was performed mainly in children (98.6%, 142/144; $p<0.001$), while peritoneal effusion (94.4%, 17/18; $p<0.001$), pleural effusion (80.8%, 21/26; $p<0.001$), lymph node (63.6%, 14/22; $p=0.01$), and sputum (56/92, 60.9%; $p<0.001$) were performed mainly in adults. The results of GeneXpert were positive in 76.2% (16/21) of the lymph node samples ($p<0.001$), 22.3% of the gastric aspiration samples, 1.5% (1/17) of the ascitic fluid samples, and 0.0% (0/25) of the pleural effusions ($p=0.002$) (28).

Study conducted in Addis Ababa University on bacterial isolate from different body fluid samples such as CSF 264(68.8%), pleural fluids 76(19.8%),peritoneal fluids 34(8.9%),synovial fluids 10(2.6%) and gram stain from body fluid samples showed 41/384 (10.7%) bacteria were seen from total body fluid samples. Among these majority of body fluids 173(44.1%) had abnormal white cell count (WBC). Out of them 91(21.1%) had polymorphic features (29).

3 Objectives

3.1 General objectives

To assess the magnitude of extrapulmonary tuberculosis and rifampicin resistance by GeneXpert and compare the yields with smear examined by fluorescence and light microscopy in St. Luke Catholic Hospital and Tullu Bollo Hospital, Oromia, Ethiopia.

3.2 Specific objectives

- To determine the burden of extrapulmonary *M. tuberculosis*
- To determine the proportion of rifampicin resistance Using Xpert assay
- To compare the yield of Zeihl-Neelsen and, fluorescent staining techniques against GeneXpert assay method.
- To assess associated risks factors for EPTB

3.3 Hypothesis

The burden of EPTB in this research is not similar with a research done in University of Gonder Specialized Hospital, 2016.

4 Materials and methods

4.1 Study area

The study was conducted in St. Luke Catholic Hospital and Tullu Bollo Hospital, South West Shoa, Oromia. St. Luke Catholic Hospital is the only NGO Hospital found in South West Shoa Zone with bed capacity of 350. Geographically, it is located in Woliso Town 114 km south west of Addis Ababa. Tullu Bollo Hospital is also found in south West Shoa zone in Tullu Bollo town 78 km southwest of Addis Ababa. Both Hospitals provides services for approximately 12000 inpatient and 110,000 outpatient clients a year with a catchment population of about 4 million people. Data from Hospital TB registration book showed a total of 2200 tuberculosis patients sought medical care at St. Luke Catholic and Tullu Bollo Hospital per year. From these data only in 2016, a total of 450 TB patients were requested for GeneXpert from body fluid samples.

4.2 Study period

The study was conducted from March to August 2017 at St. Luke Catholic Hospital and Tullu Bollo Hospital, South West Shoa, Oromia, Ethiopia.

4.3 Study design

A cross sectional study was conducted over the study period of March 2017 to August 2017 among all presumptive EPTB and requested for GeneXpert at St. Luke Catholic Hospital and Tullu Bollo Hospital who fulfill the inclusion criteria.

4.4 Population

4.4.1 Source population

All presumptive TB patients who visited St. Luke Catholic Hospital and Tullu Bollo Hospital during the study period were considered as source population.

4.4.2 Study population

All presumptive EPTB patients who requested for GeneXpert at St. Luke Catholic Hospital and Tullu Bollo Hospital during the study period and fulfill the inclusion criteria were eligible for enrolment in the study.

4.5 Inclusion and Exclusion criteria

4.5.1 Inclusion criteria

Presumptive extrapulmonary tuberculosis patients willing to participate in the study were included.

4.5.2 Exclusion criteria

Previously confirmed pulmonary tuberculosis (PTB) cases and known extrapulmonary tuberculosis patients who were on anti-TB treatment were excluded from the study.

4.6 Study variables

4.6.1 Dependent variables

- The yield of EPTB by GeneXpert, ZN and FM
- Prevalence of EPTB
- Proportion of rifampicin resistance

4.6.2 Independent variables

- Age, sex, Socio-demographic & related factors
- Physical appearance of the body fluid samples
- WBC count

4.7 Measurement and data collection

4.7.1 Sample size determination

The sample size was determined using the following single population proportion formula:

$N = z^2 p (1-p) / w^2$, where N = the number of EPTB suspected patients; Z = Standard normal Distribution value at 95% CI which is 1.96; P = the prevalence of extrapulmonary tuberculosis infection = 26.2% (27); W = the margin of error taken as 5% with 10% contingency sample i.e. $297 \times 0.1 = 29.7$. Accordingly, the sample size was 326. A total of 310 study participants were recruited using consecutive convenient sampling techniques sampling methods.

Samples were collected from presumptive extrapulmonary tuberculosis patients who came to St. Luke Catholic Hospital and Tullu Bollo Hospital consecutively until the required number of patients offered the specimens.

4.8 Data collection instrument and procedure

4.8.1 Data collection instrument

A tested structured questionnaire was used to collect socio demographic and risk factors data.

4.8.2 Sample collection and analysis procedure

All body fluids were collected by physicians using aseptic procedure from the patients who visited St. Luke Catholic Hospital and Tullu Bollo Hospital during the study period then sent to laboratories for GeneXpert, gram stains, AFB stains, WBC, and differential count.

4.8.3 GeneXpert principle and procedure

The assay utilizes single-use plastic cartridges with multiple chambers that are preloaded with liquid buffers and lyophilized reagent beads necessary for sample processing, DNA extraction and hemi nested RT-PCR. Clinical body fluid samples are treated with a sodium hydroxide and isopropanol-containing sample reagent (SR). The SR is added to the sample 2:1 ratio and incubated at room temperature for 15 min. This step is designed to reduce the viability of *M. tuberculosis* in the sample at least 10⁶-fold to reduce biohazard risk. The treated sample is then manually transferred to the cartridge which is loaded into the GeneXpert instrument. Subsequent processing is fully automated (30).

The cartridge incorporates a syringe drive, a rotary drive and a filter upon which *M. tuberculosis* bacilli were deposited after being liberated from the clinical material. The test platform employs a sonic horn that inserts into the cartridge base to cause ultrasonic lyses of the bacilli and release of the genetic material. The assay then amplifies a 192 bp segment of the *rpoB* gene using a hemi-nested RT-PCR reaction. The assay also contains lyophilized *Bacillus globigii* spores which serve as an internal sample processing and PCR control. The *B. globigii* PCR assay is multiplexed with the *M. tuberculosis* assay(30).

Mycobacterium tuberculosis is detected by the five overlapping molecular probes (probes A–E) that collectively are complementary to the entire 81 bp *rpoB* core region. *M. tuberculosis* is identified when at least two of the five probes give positive signals with a cycle threshold (C_T) of ≤ 38 cycles and that differ by no more than a pre specified number of cycles. The *B. globigii* internal control is positive when the single *B. globigii*-specific probe produces a C_T of ≤ 38 cycles. The standard user interface indicates the presence or absence of *M. tuberculosis* and the

presence or absence of rifampicin resistance and a semi-quantitative estimate of the concentration of bacilli as defined by the C_T range (high, <16; medium, 16–22; low, 22–28; very low, >28). Assays that are negative for *M. tuberculosis* and for the *B. globigii* internal control are reported as invalid assays. The basis for detection of rifampicin resistance is the difference between the first (early C_T) and the last (late C_T) *M. tuberculosis*-specific beacon (ΔC_T). The system was originally configured such that resistance was reported when ΔC_T was >3.5 cycles and sensitive if ≤ 3.5 cycles. Since the assay terminates after 38 cycles, the assay was deemed indeterminate for rifampicin resistance if the first probe C_T is >34.5 cycles and the last probe has a C_T of >38 cycles. The result was presented in text format by using computer soft(30).

4.8.4 Ziehlle-Nelson smears

After processing the specimens, smears were prepared from all samples and were examined at St. Luke Catholic Hospital and Tullu Bollo Hospital laboratory for the presence of AFB. After labeling centrifuged body fluids were smeared, dried and passed over the flame for fixation. Then 1% carbon fuschin solution was added and heated until vapor rises. After 3 minute it was washed off by 3% acid alcohol and flooded with 0.1% methylene blue as counter stain. There was washing procedure in between and were examined by light microscope. Finally reported as no AFB seen after at least 100 fields examined, 1-9 /100 fields actual number, 10-99/100 fields is +1, 1-10/ fields is +2 and more than 10 AFB per field in at least 20 fields is +3 (31).

4.8.5 Fluorescent staining

After processing the specimens, smears were prepared from all samples and were examined at St. Luke Catholic Hospital and Tullu Bollo Hospital laboratory for the presence of AFB. After labeling centrifuged body fluids were smeared, dried and passed over the flame for fixation. Then 0.1% auramine o solution was added. After 20 minute it was washed off by 0.5% acid alcohol and flooded with 0.5% potassium permanganate as counter stain. There was washing procedure in between and was examined by fluorescent microscopy. Finally reported as no AFB seen after at least 40 fields examined, 1-19 /40 fields actual number, 20-199/40 fields is +1, 5-49/20 fields is +2 and more than 50 AFB per field in at least 8 fields is +3(32).

4.8.6 White blood cell and differential count in body fluids

Body fluid analysis covers several analytical disciplines. This includes counting and differentiating cells and other particles. Counting and differentiating cells in a range of different body fluids, such as cerebrospinal fluid, serous fluids, and synovial fluid is possible with analyzers. Cell counts and differentiation in body fluids is one important aspect in the process of finding the right diagnosis. Automating these processes for body fluids has several advantages compared to manual methods using a traditional counting chamber. It is fast and convenient. Its quality does not depend on subjective skills, so it is an appropriate way of standardizing the procedure. And the number of time-consuming manual chamber counts can be reduced. However some body fluids manual counting is mandatory since unclear samples are not good to process by analyzer(33).

In hematology analyzer the body fluids was mixed well and aspirated by aspirating needle of the analyzer. This analyzer uses electrical impedance to count and size blood cells. It is based on the measurement of change in electrical resistance produced by particle suspended in conductive diluents as it passes through an aperture of known dimensions which make pulses. The number of each pulses generated indicates the number of particles that pass through the aperture. The amplitude of each pulse is essentially proportional to the particle volume which is specifically related to differential cell counts. For unclear samples manual cell and differential count is mandatory. Well mixed body fluid was filled in Thoma pipette to 0.5 marks with capillary action. Then pipette was filled WBC to the top mark on the pipette and place the pipette on the pipette shaker for at least two minutes. Place cover slip over the cleaned chamber and add gently the well mixed sample to hemocytometer chamber. Allow to settle for 3 minute and count cell on specified area and finally multiply with diluting factors. Manual differential count is also mandatory if not possible with analyzer. Place small to medium drop sample on the slide and hold the spreader and completely back to the drop to run along the rear edge of the side edges of the spread slide. Move the spreader in opposite with even motion. Allow the smear to dry and stain with Wright stain. Examine systematically with 100x objectives until 100 cells are counted and differentiate them in to different cells based on their shape, size and granularity(33).

4.8.7 Gram staining

This is the most extensively used differential stain that divides bacteria into two major groups. Those which retain crystal violet dye after treatment with iodine and alcohol appear purple or bluish purple and are designated as Gram positive. Those bacteria which lose the crystal violet show the color of the counter stain employed. The commonly-used counter stain is safranin which gives a pink/red color to bacteria and these organisms are labeled as Gram negative (34).

Make a thin smear on a clean glass slide, dry it in air and fix by passing through flame of a burner. Then cover the smear with crystal violet, keep for one minute. Wash the slide with water, then cover with Gram iodine and let it stand for one minute. Wash the slide with water. Decolorize with acetone/alcohol, rocking the slide gently for 10-15 seconds till the violet color comes off the slide. Wash with water immediately. Counter stain with safranin. Let the counter stain stand for 30 seconds. Wash with water, blot dry and examine under the oil immersion lens microscope(34).

4.9 Data quality assurance

Data quality was ensured through use of standardized data collection materials, pretesting of the questionnaires, proper training before the start of data collection and intensive supervision during data collection by the principal investigator. For laboratory analysis Pre-analytical, analytical and post-analytical stages of quality assurances that were incorporated in Standard operating procedures (SOPs) of the microbiology laboratory of St. Luke Catholic Hospital and Tullu Bollo Hospital was strictly followed. Internal quality control was done for each examination along with study samples and passed. In addition, well-trained and experienced laboratory professionals were participated in the laboratory analysis procedure.

4.10 Data analysis and interpretation

Data was entered into epi info and exported to the Statistical package for Social Science (SPSS) version 20.0 for analysis. Descriptive analysis was used to determine demographic and other characteristic. Logistic regression was done to show the association between dependent and independent variables.

4.11 Ethical consideration

Ethical clearance was collected from research and ethics review committee of the Department of Medical Laboratory Sciences, School of Allied Health Science, College of Public Health Sciences, Addis Ababa University. The participants after reading the consent form if agreed were involved on the study. Concerning the confidentiality of the result, since all clients who were participated on the study have unique ID number confidentiality was kept throughout the study.

4.12 Dissemination of the result

This study on completion can serve as a reference material for any health professionals, researchers, experts and policy makers for intervention. To reach these bodies the finalized paper would be submitted to Addis Ababa University, College of Health Sciences, School of Allied Health Sciences, and Department of Laboratory Sciences. So it can serve as a reference in the library. Additional effort will also be made to present on conferences to reach the medical/scientific community and publish the article on reputable Journals after the final reports.

4.13 Operational definitions

MDR.TB: is defined as when *M. tuberculosis* is resistance to rifampicin.

EPTB: isolated occurrence of TB from body fluids other than the lung.

5 Results

5.1 Socio-demographic characteristics of study subjects

A total of 310 presumptive EPTB patients age ranged from 1 day to 70years (mean =21.06 and 19.707 +/- SD) were included in this study and about 41 %(n=127/310) were children less than five years of ages. About 41.6 %(n=151/310) of the study participants were adults. Participants 55.5 % (n=172/310) were male. About 68.4 %(n=212/310) the study participants were living in rural area. More than half (53.2%) (n=165/310) of the study participants had no formal education. Only 8.1 %(n=25/310) of study participants were college and above. Marital status data showed that more than half (52.9 %(n=164/310)) were single. A total of 310 presumptive EPTB samples were collected from different body sites of patients. From these samples, 40.6 %(n=126/310) samples were from gastric aspirates which is the dominant samples and ascitic fluids (1/310). Majority presumptive EPTB patients were new and few of them were presumptive MDRTB (1%). Of 310 samples, 17.4% (n=54/310) were positive for HIV status” (Table 1).

Table 1. Socio- demographic characteristics of study participants in St.Luke& Tullu Bollo Hospital, March to August 2017.

Variables		Frequency	Percentage (%)
Age	1 day -5 year	127	41.0
	6 year- 10 year	9	2.9
	11-20 year	23	7.4
	21- 30 year	54	17.4
	31- 40 year	44	14.2
	41- 50 year	22	7.1
	>50 year	31	10.0
Gender	Male	172	55.5
	Female	138	44.5
Residence	Urban	98	31.6
	Rural	212	68.4
Educational status	No formal education	165	53.2
	Primary	78	25.2
	Secondary	42	13.5
	College and above	25	8.1
Marital status	Single	164	52.9
	Married	132	42.6
	Divorced	7	2.3
	Widowed	7	2.3
Site	Peritoneal	63	20.3
	Gastric aspirate	126	40.6
	Lumbar	16	5.2
	Pleural	60	19.4
	Lymph node	1	.3
	Abscess site	23	7.4
	Synovial	17	5.5
	Urine &asciatic	4	1.3
	New	309	99.7
	MDR-TB contact	1	.3
TB DIAGNOSIS	MDR-TB contact	1	.3
	HIV Status		
	Positive	54	17.4
	Negative	256	82.6

5.2 Physical and staining characteristics of body fluids

Physical assessment of all body fluids showed that 36.8 % (114/310) were clear and 22.9 % (71/310) were turbid. Gram staining of 184 samples showed that 13.6% were gram positive and 2.2% were gram negative (Table 2).

Table 2: Physical and staining characteristics of body fluids of study participants in St.Luke and Tullu Bollo Hospital, March to August 2017.

Parameters		Frequency	Percentage (%)
Sample appearance	Clear	114	36.8
	Turbid	71	22.9
	Bloody	2	.6
	Mucoid	123	39.7
Gram stain	No organism seen	155	84.2
	Gram Positive	25	13.6
	Gram Negative	4	2.2
ZN stain	Positive	10	3.2
	Negative	300	96.8
FM stain	Positive	21	6.8
	Negative	289	93.2

ZN= Zeihl-Neelsen

FM=fluorescent microscopy

5.3 Magnitude of extra pulmonary tuberculosis

Total of 310 different body fluid samples were analyzed by GeneXpert and about 16.5 % (n=51/310) of these samples were positive for extrapulmonary tuberculosis. The magnitude of extrapulmonary tuberculosis was higher in 21-30 age groups with positivity rate of 29.6% (n=16/54) and lower in 6-10 age groups (3%). Prevalence of extrapulmonary tuberculosis infection was almost the same among male 16.8 % (n= 29/172) and females 16 % (n= 22/138).

5.4 Extrapulmonary tuberculosis cases in relation to cell count and staining status

Due to small volume and sample variety of body fluids cell count were done only for 157 samples. About 52.9 % (n=83/157) of body fluids had abnormal white cell count and 15.7% (n=13/83) were xpert positive.

Out of 154 body fluid analyzed for Neutrophil and lymphocyte differential count 29.9% (n=46/154) samples had normal Neutrophil and 30.4 % (14/46) had extrapulmonary tuberculosis as measured by GeneXpert. Similarly, 24% (n=37/154) of body fluids had abnormal lymphocyte count and 32.4% ((n=12/37) of them had EPTB ($p<0.001$).

About 39.7% (n=123/310) and 22.9 % (n=71/310) of body fluids were Muroid and turbid and 14.6% (18/123) and 36.6 % (n=26/71) were positive for EPTB respectively ($p<0.001$) (Table 3).

Table 3: Extra pulmonary tuberculosis cases in relation to cell count and staining status of study participants in St.Luke and Tullu Bollo Hospital, March to August 2017.

Sample cell count		Gene Xpert detection		Total	P value
		Detected (n (%))	Not detected (n (%))		
Total WBC(n=157)	Normal	7(9.5%)	67(90.5%)	74	0.178
	Abnormal	13(15.7%)	70(84.3%)	83	
	Total	20(12.7%)	137(87.3%)	157	
Neutrophil(n=154)	Normal	14(30.4%)	32(69.6%)	46	<0.001
	Abnormal	5(4.6%)	103(95.4%)	108	
	Total	19(12.3%)	135(87.7%)	154	
Lymphocytes(n=154)	Normal	7(6%)	110(94%)	117	<0.001
	Abnormal	12(32.4%)	25(67.6%)	37	
	Total	19(12.3%)	135(87.7%)	154	
Cell appearance (n=310)	Clear	7(6%)	107(94%)	114	<0.001
	Turbid	26(36.6%)	45(63.4%)	71	
	bloody	0(0%)	2(100%)	2	
	Mucoid	18(14.6%)	105((85.4%)	123	
	Total	51(16.5%)	259(83.5%)	310	
Gram stain(n=184)	No organism	28(18%)	127((82%)	155	0.884
	gram pos	4(16%)	21(84%)	25	
	gram neg	1(25%)	3(75%)	4	
Sample site	Peritoneal	9(14.3%)	54(85.7%)	63	<0.001
	Gastric aspirate	18(14.3%)	108(85.7%)	126	
	Lumbar	1(6.25%)	15(93.75%)	16	
	Pleural	7(11.7%)	53(88.3%)	60	
	Lymph node	1(-)	0(-)	1	
	Abscess site	12((52.2%)	11(47.8%)	23	
	Synovial	3(17.6%)	14(82.6%)	17	
	Urine	0	3	3	
	Asciatic	0	1	1	

5.5 Yield of extra pulmonary tuberculosis using different laboratory methods

We have compared the yield of EPTB using gene xpert which is our “gold standard” and ZN and FM. About 16.5% (n=51/310) of body fluids were positive by Gene xpert with 2 high, 9 medium, 14 low and 26 very low bacterial loads while ZN detected 3.23 % (n=10/310) EPTB and FM detected 6.8 % (n=21/310) (table 6.4).

Table 4: Yield of extra pulmonary tuberculosis using different laboratory methods of study participants in St.Luke and Tullu Bollo Hospital, March to August, 2017.

GeneXpert detection	ZN		Total	FM		Total
	Positive	Negative		Positive	Negative	
Positive	10	41	51	21	30	51
Negative	0	259	259	0	259	259
Total	10	300	310	21	289	310

5.6 Rifampicin resistance of EPTB

We have the existence of rifampicin resistance TB using GeneXpert methods and only 3.9 % (n=2/51) of EPTB cases had rifampicin. However, five EPTB isolates were found to be indeterminate (Figure 1).

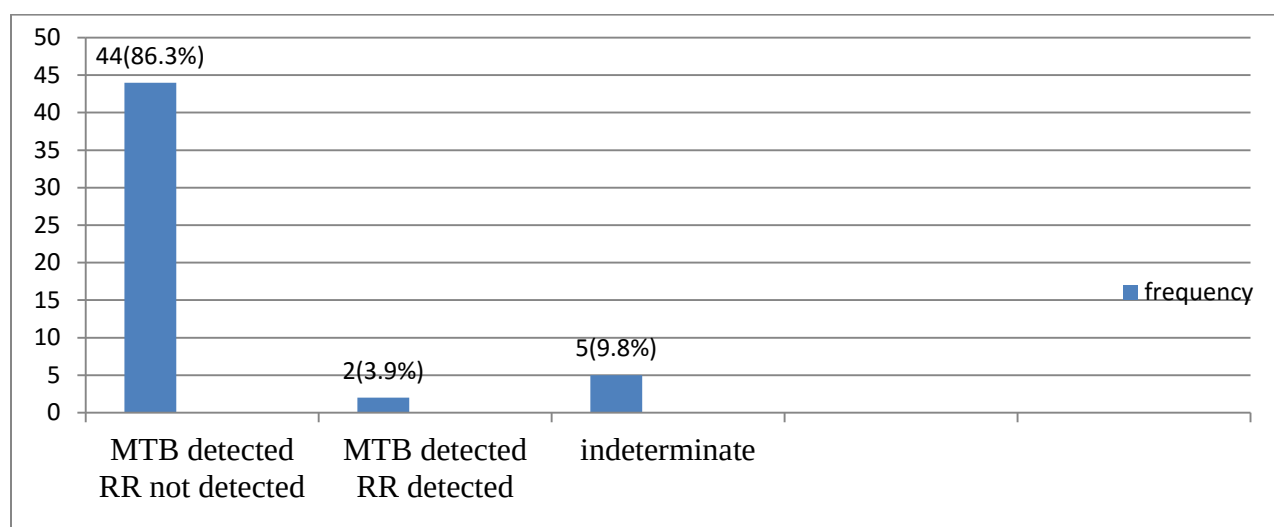


Figure 1: EPTB and RIF resistance in St. Luke and Tullu Bollo Hospital, March to August, 2017.

5.7. Associated risk factors to EPTB

Socio-demographic characteristics such as sex, residence, and educational status were not significantly associated with extra pulmonary tuberculosis infection. Age group of 21-30 years had less likely to develop EPTB than younger age groups. (COR =95%CI 0.392 (0.182, 0.845, P 0.017). This is statistically significant. On the other hand, HIV positive patients had two times more likely to develop EPTB than HIV negative one (CRO=95%CI 2.072(1.027, 4.18) P 0.042).

Table 5: Associated risk factors of EPTB among study participants in St.Luke and Tullu Bollo Hospital, March to August 2017.

Variables		GeneXpert result		COR (95 % CI)	p-value	AOR(95%CI)	P- Value
		Detected (n (%))	Not detected (n (%))				
Age	1 day -5 year	18(14.2%)	109(85.8%)	.724(.140,3.379)	0.700		
	6 year- 10 year	1(3%)	8(11.1%)	1.5(0.156, 15.171)	0.712		
	11-20 years	0(0%)	23(100%)	0.884(.145,12.12)			
	21- 30 year	16(29.6%)	38(70.4%)	0.392(0.182,0.845)	0.017	.354(.104,1.208)	0.097
	31- 40 year	7(16%)	37(84%)	0.873(0.338,2.256)	0.78		
	41- 50 year	4(18.2%)	18(81.8%)	0.743 (0.225, 2.45)	0.626		
	>50 year	5(16%)	26(84%)	1	-	-	-
Gender	Female	22(16%)	116(84%)	1.00	-	-	-
	Male	29(16.8%)	143(83.3%)	0.935 (.510, 1.714)	0.828		
Residence	Urban	16(16.3%)	82(83.7%)	1.00	-	-	-
	Rural	35(16.5%)	177(83.5%)	0.987 (0.517, 1.88)	0.968		
Educational status	No formal education	24(14.5%)	141(85.5%)	1.47 (0.503, 4.28)	0.482		
	Primary	15(19.2%)	63(80.8%)	1.05(0.339, 3.25)	0.933		
	Secondary	7(16.7%)	35(83.3%)	1.25(0.350, 4.46)	0.731		
	College and above	5(20%)	20(80%)	1.00	-		
Marital status	Single	19(11.6%)	145(88.4%)	1.00	-	-	-
	Married	30(22.7%)	102(77.3%)	0.446(0.238,0.835)	0.012	.825(.359,1.897)	.650
	Divorced	1(14.3%)	6(85.7%)	0.786(0.090, 6.88)	0.828		
	Widowed	1(14.3%)	6(85.7%)	0.786(0.090, 6.88)	0.828		
IType TB (clinical)	Presumptive TB	50(16.3%)	257(83.7%)	2.57(0.229, 28.88)	0.444		
	Presumptive MDR-TB	1(33.3%)	2(66.7%)	1.00	-	-	-
Sero-status	Positive	14(26%)	40(74%)	2.072(1.027,4.18)	.0042	.486(.230,1.027)	0.059
	Negative	37(14.5%).	219(85.5%)	1	-	-	-

6. Discussion

The Xpert MTB/RIF assay is an automated nucleic acid amplification test for clinical specimens that can identify MTB and resistance to RIF with minimal labor. When tested in areas with high TB incidence, the Xpert assay was highly accurate, with a sensitivity of 88% and specificity of 98% (5). It can rapidly detect the presence of *M. tuberculosis* and identify the mutations most frequently associated with rifampin resistance directly from smear-negative and smear-positive clinical samples (5).

Worldwide, extra pulmonary tuberculosis (EPTB) accounts for 25% of all TB cases, and even higher percentages in HIV-infected individuals (9). In India, EPTB forms 10 to 15% of all types of TB, in comparison to 25% in France and 50% in Canada, partly due to the dual infection of TB with human immunodeficiency virus (HIV) (8). Existing tests for the diagnosis of EPTB are limited in accuracy and time to diagnosis, and often require invasive procedures and special expertise. Extra pulmonary specimens have a very low bacterial load, so the sensitivity of direct microscopy examination for detecting MTBC is low and culture is time consuming. Gene Xpert (Cepheid, Sunnyvale, CA) is an automated, integrated, real-time PCR system which has recently been developed for rapid detection of MTBC and rifampicin (RIF) resistance. The current study was conducted to determine the burden of *Mycobacterium tuberculosis* complex isolates from different body sites among presumptive EPTB cases.

In the current study, out of 310 body fluid specimens, 51 (16.5%) were positive for EPTB of which 2(3.9%) specimens also exhibited resistance to RIF. The result for body fluids is comparable to the study done in India by Pravin K Nair in (2016) (24); Where the positivity rate in pleural fluids and CSF was 10.1% and 8.8% against 11.7% and 6.25% respectively in this study. In a study from Pakistan (21), the positivity rate with GeneXpert assay was 51.7% for pus samples which is comparable with present study (52.2%). Another study from University of Gondar have reported 33.3%, 33.3%, 33%, 0%, and 10.7% respectively, of pus, lymph node, genitourinary, peritoneal and synovial fluid samples to be positive (27).

The current study showed higher positivity rate in synovial fluids (17.6%) and peritoneal fluids (14.3%). Study in southern Ethiopia (28) by GeneXpert were positive in 76.2% (16/21) of the lymph node samples, 22.3% of the gastric aspiration samples, 1.5% (1/17) of the ascitic fluid samples, and 0.0% (0/25) of the pleural effusions against current study with 14.3% (18/126)

gastric aspirates and 11.7%(7/60) pleural fluids. This difference may be due to variety of the sample in each study, sample size difference, study area and period and the dynamics of EPTB epidemiology specific to geographic location.

Compared to previous reports (27) in University Of Gondar (26.2%), the current prevalence of *Mycobacterium tuberculosis* complex caused EPTB (16.5%) is lower, however higher (8.5%) as compared to study in India (24). Understanding the reasons for the difference EPTB infection prevalence could be difficult although several reports showed that HIV infection is the common risk factor for EPTB infection. Moreover, EPTB prevalence variation was reported depend on sex, age group, and HIV status. The current study also showed that the prevalence of extra pulmonary tuberculosis infection was almost the same among male (16.8%) and females (16%). This result was different from study reported in the University of Gonder which documented a 27% prevalence of EPTB infection among females compared with a 25.4% among males (27). Extra pulmonary tuberculosis infection was more prevalent (16.7%) among adult patients than pediatric age groups in current study which correlate with study done in Gonder University (30.4%) (27), however, previous reports documented that EPTB infection was more common at younger ages (< 14 years) (23.2%) (27). These differences may be due to samples variation in each study and number of samples included.

In this study, unfortunately only one tuberculosis lymphadenitis was tested and positive. Previously, the proportion of TB lymphadenitis cases was reported as (33.3%) University of Gonder (27).According to literatures, a proportion of as high as 76.2% TB lymphadenitis was reported in Southern Ethiopia (28). A report from Jimma University 87.8% showed TB lymphadenitis (26).

In present study of 157 eligible samples GeneXpert TB detection was seen in 15.7% of total white count was above the normal value and 32.4% of TB detected had abnormal lymphocytes count which correlates with EPTB($p < 0.001$).

Gross body fluids was described as turbid in 21.9%, Muroid in 39.7% ,clear in 36.8% and blood stained in 0.6% of the cases and 52.9%(83/157) WBC and 23.6%(37/154) lymphocytes were above normal value which is comparable in total cell count with study done in Addis Ababa(44.1%)(29). The number of GeneXpert positivity rate was highest in body fluids with turbid appearance (29.6%), and lowest in blood stained aspirates (0%). Gram stain was done and 13.6% was gram positive and 2.2% gram negative which were slightly higher than study in

Addis Ababa University (29). This may be due to variety of samples and differences in number of samples included.

Out of 310 cases, 10/310(3.2%) cases of GeneXpert assay positive (with 8 high and 2 medium bacterial load) were ZN positive, 41/310(13.2%) cases of GeneXpert assay positive were ZN negative. This is against study in Pakistan (21), of which 12% cases of Gene Xpert MTB/RIF assay positive were ZN smear positive, 25% of the cases of Gene Xpert MTB/RIF assay positive were Zn smear negative. This difference may be due to variety of body fluid samples (only pus, CSF, Pleural fluids, and Asiatic fluids analyzed in previous study) and epidemiology of study population.

On the other hand bacterial loads were not identified in previous study as low and very low bacterial load were ZN negative in present study. Study in German(22), India (23), Jimma University Hospital(only on lymph node aspirate) (26), Pakistan (25) showed 9.7% ,17%,18.9% and 7.5% smear positive respectively were higher than present study(3.2%). There were discordant results among each reports and present study showed sample types, study area and sample size may contribute to these differences. In current study 259/310(83.5%) cases of both GeneXpert assay and ZN smear negative and 21/310(6.8%) cases of GeneXpert assay positive (with 2, 9, 8 as high, medium and low bacterial load) were FM positive, 30/310(9.7%) cases of GeneXpert assay positive were FM negative, 259 cases were both GeneXpert assay and FM smear negative. It is noted that none of the ZN smear positive and FM positive samples gave negative results by GeneXpert indicating GeneXpert assay is highly sensitive and specific technique. On the other hand all ZN smear positive was positive in FM showed FM was more sensitive than ZN. Current study showed of 51 GeneXpert positive samples 3.9% were resistance to rifampicin which was higher than previous reports; 0.3% in Gambo Hospital (28), 2.4% in Gondar Hospital (27), and lower (19.2%) of study in India (24) and 9.8% were indeterminate for rifampicin in present study. The main differences may be due to types of presumptive EPTB included in the previous study with higher value and study area and sample types.

Socio-demographic characteristics such as sex, residence, and educational status were not significantly associated with extra pulmonary tuberculosis infection which is similar with study in University of Gondar (27). This may be due to single sample from gastric aspirate was positive in these age category. Age group of 21-30 years had less likely to develop EPTB than

younger age groups. (COR = 0.392, 95%CI 10.182, 0.845, P 0.017) which is statistically significant. On the other hand, HIV positive patients had two times more likely to develop EPTB than HIV negative one (2.072(1.027, 4.18), P 0.042).

6 Limitation of the study

- Unable to culture and DST for all Xpert MTB/RIF tested specimen and to confirm rifampicin resistance case as MDRTB for Xpert positive.
- Operational and logistic difficulties to increase the size and to link Xpert MTB/RIF result with clinical outcomes of the study participants.
- Unable to retest indeterminate Rifampicin resistance isolates.

7 Conclusion and Recommendations

7.1. Conclusion

The burden of Gene Xpert confirmed extra pulmonary tuberculosis infection was low. The most prevalent type of extra pulmonary tuberculosis infection was TB from cold abscess followed by TB from synovial fluids. A significant proportion of EPTB cases were also co-infected with HIV. There was a significant difference between smear microscopy (FM and ZN) results compared with GeneXpert results. A more accurate test would contribute to improve EPTB case detection, and thus reducing misdiagnosis, the morbidity and mortality.

7.2. Recommendation

Regular initiation and awareness for clinician about EPTB and its diagnostic tool is expected from health offices and stake holders on EPTB and enhancing Gene Xpert utilization for body fluid samples.

Further studies are required to clarify operational difficulty, challenges and limitations in rule-out Xpert MTB/RIF in current TB control/treatment algorithms in the country.

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9 Annexes

9.1 Annex1: Participant Information sheet

Date -----

Department of Medical Laboratory Science, Collage of Allied Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia.

Principal Investigator: Alemayehu Feyissa

First of all I would like to thank you in advance for your cooperation and consent in participation in this study. Please read or listen when it is read for you about the general information of the study. If you have any question regarding the study please ask freely.

Title: detection of EPTB and rif resistance by gene Xpert from body fluid samples in St. Luke catholic hospital and Tullu Bollo hospital.

Background: Tuberculosis is the opportunistic infections which increases the mortality in human immunodeficiency virus infected individual. The diagnosis of EPTB is usually established by examination of hisopathological samples, radiology, ZN stained smears and culture. However, rapid and sensitive diagnostic tool should be used since the mentioned diagnostic tests are not sensitive, time consuming and rif resistance detection is not possible. In order to overcome this problem other more sensitive diagnostic instrument that replaces the conventional method should be addressed. Since tubercle bacilli or their nucleic acids are also expected to be found in body fluid samples, it was interesting to assess all body fluids for diagnosing E PTB.

Objective of the study: The aim of this study is detection of presumptive EPTB by gene Xpert machine and compares the findings with smear examined by fluorescence microscopy and ZN in St. Luke catholic hospital and Tullu Bollo Hospital, Oromia, Ethiopia.

Participation: The procedure was carried out after getting your willingness to participate. All volunteer patients with EPTB, fulfilling inclusion criteria, were included.

Benefit: You benefited from the study; because it was part of your diagnosis and might be a key to your current and/or your future problem if it came up with positive result.

Compensation: You/your child were receiving your result through your physician. You got treatment for free if you became positive for EPTB.

Risks and complication

There are no anticipated risks to your participation. As routine laboratory procedure body fluid sample will be taken once from your different organ. During sample collection you may feel some discomfort but this does not produce serious problem.

Confidentiality

There is no sensitive issue that you asked related with your social desirability but any information that was obtained in connection with this study and that can be identified with you remained confidential. Participants were not prohibited to stop or withdraw at any time from the study. Only interested participants can retrieve their own lab result using their code number. The information collected about you was coded using numbers. No personal information was disclosed to third party or was not appeared in any report from this study except your physician.

Approval: This research project has got ethical clearance from the Departmental Research and Ethics Review Committee (DRERC) of Addis Ababa University, College of Health Sciences School of Allied Health Sciences, Department of Medical Laboratory Science.

Whom to contact: If you have any question or description about this study, you can communicate on the following address:

1. Addis Ababa University, College of Health Sciences, School of Allied Health Sciences,
Department of Medical Laboratory Sciences

Tel: +251-112-75-51-70

Fax: +251-112-75-46-69

E-mail: SMLT@ethionet.et

P.o.Box: 1176, Addis Ababa, Ethiopia

2. Principal Investigator: Alemayehu Feyissa (Bsc)

Tel. +251-913852208

E-mail: lammifeye96@gmail.com

9.2 Annex II: Informed consent form (English version)

Dear sir /Madam

My name is Alemayehu Feyissa. I am currently studied in Addis Ababa University, department of medical laboratory science and undertaking a master's degree (Msc) in Diagnostic and Public health microbiology on detect ion TB from body fluid samples from presumptive EPTB by gene Xpert machine and compare the findings with smear examined by fluorescence and light microscopy.

I like to ask all presumptive EPTB patients requested for gene Xpert to participate in responding the questioners.

Signature of person taking the consent_____

Date _____D/M/Y

I read the information or have been read to me. I have had the opportunity to answer the question on the questioner. I consent voluntary give permission for my/my child sample to be used in the current research project: detection TB from body fluid samples from presumptive EPTB by gene xpert machine and compare the findings with sputum smear examined by fluorescence and light microscopy.

Name of participant _____signature_____

Date _____ D/M/Y

9.3 Annex III Informed consent form (Afan Oromo).

Maqaan koo Alamaayyoo Fayyisaa jedhama. Ammaan kana Yuunivasiitii Finfinneetti Muummee Yaala Fayyaa Namaa, dippaartimentii laaboraatoorii fayyaa namaatti digrii lammaffaa wal'ansaa fi fayyaa namaa lubbu qabeeyyii ijaan hin mul'anne (Diagnostic and Public health microbiology) irratti qorannoo dhukkuba TB sombaan alaa irratti meeshaa gene xpert jedhamuun gaggeessaan jira.

Kanaafuu namootni dhukkuba TB sombaan alaatiif yaalii laboratoorii argachuuf dhuftan yoo fedhii qabaattan gaafii afaanii akkanaaf guuttan kabajaan isin gaafadha.

Maqaa nama hirmaatee_____ mallattoo_____

Guyyaa_____

Waliigaltee kana dubbiseera ykn naaf dubbifameera. Aniscarraa kana argadhee akkan deebisuun eeyyamamaa ta'ee qorannoo ogeessi fayyaa yaalakootiif ajaje irratti yaalii TB sombaan alaa fi qoricha wajjin wal bare kan jedhu irratti akka gaggeessatan fedhii koo ta'uu mirkaneesseera.

Maqaa hirmaataa _____ mallattoo_____

Guyyaa _____

9.4 Annex IV: Data collection sheet

Data collection sheet

I. patients identification

1. Code No.: _____

2. Sample ID: _____

3. Date: _____

4. Age _____ (years)

5. Sex

☐ Male

☐ Female

6. Residence

☐ Rural

☐ Urban

7. Educational status

☐ No formal education

☐ Primary

☐ Secondary

☐ College and above

8. Marital status

☐ Single

☐ Married

☐ Divorced

☐ Widowed

II. TB disease and treatment history

1. Site: extra pulmonary (specify). _____

2. Registration group:

☐ New

☐ Relapse

☐ Treatment after loss to follow up

- ☐ After failure to first treatment
- ☐ MDRTB Contact
- ☐ Other

3. Request for testing

- ☐ Presumptive TB
- ☐ Presumptive MDRTB

4. Co-infections

- ☐ Yes (specify)_____
- ☐ No

5. Specimen appearance

- ☐ Clear
- ☐ Turbid
- ☐ Bloody
- ☐ Mucoid

6. Data collector: Name_____

Date_____

Signature_____

9.5 Annex V: Data collection sheet (Afan Oromo)

I .dhukkubsataa kan ittiin adda baasan

1. lakkoofsa koodii: _____

2.lakkoofsa addaa _____

3. guyyaa: _____

4. umurii _____

5. Saala

☐ dhiira

☐ dhalaa

6. bakka jireenyaa

☐ baadiyyaa

☐ magaalaa

7. sadarkaa barnootaa

☐ Barnoota idilee kan hin qabne

☐ Barnoota sadarkaa tokkoffaa

☐ Barnoota sadarkaa lammaffaa

☐ Kolleejjii fiikansanaa olii

8. haalagaa'ilaa

☐ Hinfuune/eerumne

☐ Fuudhe/eerumte

☐ Wal hike/hiikte

☐ Abbaanmanaakanirraadu'e

II.dhukkuba daranyoo sombaa fi seenaa isaa

3. bakka: dhukkuba daranyoo sombaan alaa (maqaa dhayi)._____

4. garee galmee:

☐ haaraa

☐ kanitti deebi'e

☐ Yaalii erga hordoffii addan kute

☐ Yaalii jalqabaa hojjachuu kan dide

☐ Dhukkuba daranyoo sombaan amma qabu waliin walitti dhufeen yakan qabu

☐ Kan biroo

3. qorannoof gaafii

- ☐ Shakkamaa dhukkuba daranyoo sombaa
- ☐ Shakkamaa dhukkuba daranyoo sombaa qoricha waliin wal bare

4. dhukkuba dabalataa jiraachuu

- ☐ eeyyeen (maqaadhayi)_____
- ☐ lakki

5 .bifa dhangala'ichaa

- ☐ qulqulluu
- ☐ kan booraye
- ☐ dhiiga kan qabu
- ☐ akkeekan qabu

6. Gama Raga Funaane: Maqaa_____

Guyyaa _____

Mallattoo _____

9.6 Annex VI: GeneXpert result collection sheet

[illegible]

9.7 Annex VII Principles and procedure of GeneXpert rif assay, ZN and FM

Gene xpert machine is a single use multi-chamber plastic cartridge with lyophilized reagents and buffers for sample processing, amplification, and detection. Clinical samples will be manually transferred to the cartridge which will be loaded into the Gene Xpert instrument and subsequently processed automatically. The samples will be processed by diluting it in a 2:1 ratio with a sample reagent (SR) buffer. The result will be presented in text format by using computer software.

The cartridge incorporates a syringe drive, a rotary drive and a filter upon which *M. tuberculosis* bacilli are deposited after being liberated from the clinical material. The test platform employs a sonic horn that inserts into the cartridge base to cause ultrasonic lysis of the bacilli and release of the genetic material. The assay then amplifies a 192 bp segment of the *rpoB* gene using a hemi-nested rt-PCR reaction. The assay also contains lyophilized *Bacillus globigii* spores which serve as an internal sample processing and PCR control. The *B. globigii* PCR assay is multiplexed with the *M. tuberculosis* assay.

Mycobacterium tuberculosis is detected by the five overlapping molecular probes (probes A–E) that collectively are complementary to the entire 81 bp *rpoB* core region. *M. tuberculosis* is identified when at least two of the five probes give positive signals with a cycle threshold (C_T) of ≤ 38 cycles and that differ by no more than a pre specified number of cycles. The *B. globigii* internal control is positive when the single *B. globigii*-specific probe produces a C_T of ≤ 38 cycles. The standard user interface indicates the presence or absence of *M. tuberculosis* and the presence or absence of rifampicin resistance, and a semi-quantitative estimate of the concentration of bacilli as defined by the C_T range (high, <16 ; medium, $16–22$; low, $22–28$; very low, >28). Assays that are negative for *M. tuberculosis* and for the *B. globigii* internal control are reported as invalid assays. The basis for detection of rifampicin resistance is the difference between the first (early C_T) and the last (late C_T) *M. tuberculosis*-specific beacon (ΔC_T). The system was originally configured such that resistance was reported when ΔC_T was >3.5 cycles and sensitive if ≤ 3.5 cycles. Since the assay terminates after 38 cycles, the assay was deemed indeterminate for rifampicin resistance if the first probe C_T is >34.5 cycles and the last probe has a C_T of >38 cycles.

Ziehl-Nelson smears

After processing the specimens, smears were prepared from all samples and was examined at the at St.Luke Catholic Hospital and Tullu Bollo Hospital laboratory for the presence of AFB. After labeling centrifuged body fluids were smeared, dried and passed over the flame for fixation. Then 1% carbon fuschin solution was added and heated until vapor rises. After 3 minute it was washed off by 3% acid alcohol and flooded with 0.1% methylene blue as counter stain. There was washing procedure in between and were examined by light microscope. Finally reported as no AFB seen after at least 100 fields examined, 1-9 /100 fields actual number,10-99/100 fields is +1,1-10/ fields is +2 and more than 10 AFB per field in at least 20 fields is +3 (31).

Fluorescent staining

After processing the specimens, smears were prepared from all samples and were examined at St.Luke Catholic Hospital and Tullu Bollo Hospital laboratory for the presence of AFB. After labeling centrifuged body fluids were smeared, dried and passed over the flame for fixation. Then 0.1% auramine o solution was added. After 20 minute it was washed off by 0.5% acid alcohol and flooded with 0.5% potassium permanganate as counter stain. There was washing procedure in between and was examined by fluorescent microscopy. Finally reported as no AFB seen after at least 40 fields examined, 1-19 /40 fields actual number,20-199/40 fields is +1,5-49/20 fields is +2 and more than 50 AFB per field in at least 8 fields is +3

9.8 Annex VIII: Declaration

I, the undersigned, declare that this MSc thesis is my original work, has not been presented for a degree in Addis Ababa University or any other universities. I also declare that all sources of materials used for the thesis have been duly acknowledged.

Name of the candidate: Alemayehu Feyissa (BSc)

Signature_____

Place: Addis Ababa University, School of Allied Health Science, Department of Medical laboratory Science, Ethiopia

Date of submission_____/_____/_____

This proposal has been submitted with my approval as university advisor.

Name of advisors: Mr. KassuDesta(MSc, PhD fellow) Signature _____

Mr.MeleseHailu (MSc, PhD fellow) Signature _____

Place: Addis Ababa University, Department of Medical Laboratory Sciences, Ethiopia

Date of submission_____/_____/_____

