

Conventional and Molecular Methods for the Diagnosis of Smear Negative Pulmonary TB and Concordance of Empirical TB Diagnosis with Culture Assay

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Abstract

Background: Ethiopia is among high TB burden countries. The proportion of smear negative patients among pulmonary TB cases was 51%. Nevertheless, microscopy is still a primary tool for TB diagnosis. In the absence of sensitive diagnostic methods, clinicians often make the diagnosis of smear-negative pulmonary TB with information obtained through the clinical history, physical examination and chest X-ray. The treatment of smear negative TB patients with those criteria largely depends on the treating clinician. There is limited data on the utilization of clinical criteria commonly used to initiate TB treatment empirically when culture is used as the reference method. Beside this, there are a number of sensitive diagnostic methods that have a substantial contribution for the diagnosis of smear negative TB patients, but only few studies were conducted in Ethiopia to address this issue. Thus, the present study is designed to assess the contribution of conventional and molecular methods for smear-negative patients and describe the concordance rate of empiric TB treatment with culture assay.

Methods: A cross-sectional study was designed on smear negative TB presumptive patients referred to St. Peter's TB Specialized Hospital from December 2014 to June 2015. Consecutive smear negative TB presumptive patients, aged greater or equal to 15 and willing to participate were included in the study. Socio-demographic and clinical data were collected using the data collection form designed for the study purpose. The data collection form was designed to capture patient demographic data, signs and symptoms, chest X-ray findings and empirical TB treatment initiations. Spot-Morning-spot sputum was collected using sterile falcon tubes for routine diagnostic purpose. Direct ZN examination was done on Spot-Morning-spot sputum at St. Peter's TB Specialized Hospital. The morning sputum was used for culture (LJ and MGIT), TB-LAMP, Xpert MTB/RIF assay and fluorescent microscopy examination. Data were captured and analyzed using SPSS.

Results: This study enrolled 459 smear negative presumptive TB patients. Most (57%) of the study participants were female; with median age of 40 IQR (28–55) years. HIV test results were available for 41% of the study participants and the prevalence of HIV among the study participants was 30%. Three hundred eighty three cases were having both treatment and lab results. Forty six cases were treated empirically. The sensitivity and specificity of empiric TB treatment when compared to culture were 45.8% and 90% respectively. The overall culture positivity rate was 6.8% (30/439), of which 6.6% (26/391) was by MGIT and 5.3% (23/436) was by LJ method. Direct and concentrated fluorescent microscopy adds 0.9% and 1.3% detection rate compared to the direct ZN. The overall sensitivity and specificity of TB-LAMP was 61.5% (16/26) and 96.6% respectively. The overall sensitivity and specificity of Xpert MTB/RIF was 70.8% (17/24) and 97.2% respectively.

Conclusion: TB-LAMP and Xpert MTB/RIF assay can provide confirmatory results for at least two third of TB cases.

Key words: TB-LAMP, Xpert, Smear negative TB

Introduction

Globally, 9.6 million people are estimated to have fallen sick with tuberculosis (TB). In 2014, 6 million new TB cases were reported to the World Health Organization (WHO) and 5.2 million of them were pulmonary TB. Among the 5.2 million pulmonary TB patients, 42% of patients were diagnosed based on the information obtained from clinical evidences (WHO 2015). In Ethiopia, TB is a major public health problem and WHO categorizes Ethiopia as a high TB and multi-drug resistant TB burden country. According to the WHO annual report, the proportion of smear negative patients among pulmonary TB

cases was 51% (WHO 2015). Even if majority of the patients are smear negative TB cases, microscopy is still the primary tool for TB diagnosis; the method has low sensitivity and can only detect 36 % of tuberculosis cases (Dagnaw et al. 2014). Clinicians often make the diagnosis of smear-negative pulmonary TB with information obtained through the clinical history, physical examination and chest X-ray (Siddiqi et al. 2003, Soto et al. 2013). The treatment of smear negative TB patients with the criteria according to the national guideline largely depend on the treating clinician. Even if half of the pulmonary TB patients are diagnosed on clinical evidences, there

is limited data on the utilization of clinical criteria commonly used by clinicians to initiate empirical TB treatment

when cultures are used as the reference method. Currently, there are a number of sensitive diagnostic methods that have a substantial contribution for the diagnosis of smear negative TB patients. Fluorescence microscopy improves the diagnostic yield, around 10% increase in sensitivity than conventional ZN microscopy. Xpert MTB/RIF assay has 67% pooled sensitivity for smear negative TB patients (Steingart et al. 2014). TB-loop mediated isothermal amplification (LAMP) assay adds on test for smear negative TB patient has 48.8% to 55.6% sensitivity (Boehme et al. 2007, Mitarai et al. 2011, Ou et al. 2014).

Few studies were conducted in Ethiopia on the contribution of fluorescence microscopy and Xpert MTB/RIF assay for the diagnosis of smear negative TB patients. Two studies conducted in Addis Ababa indicated the detection rate of fluorescence microscopy 17.7% from all referred TB presumptive cases and 8.4% from HIV positive individuals (Getachew et al. 2015 and Solomon, et al. 2015). The values of Xpert MTB/RIF assay were evaluated at Karamara Hospital on consecutively recruited TB presumptive patients and at five Health Centers around Adama on HIV-positive adults. The sensitivity of Xpert MTB/RIF assay for smear negative and culture positive was 48.6% for TB presumptive patients and 57.4% for HIV-positive adults (Geleta et al. 2015; Balcha et al. 2014). There is no data on the diagnostic yield of TB-LAMP. Therefore, the present study was designed to assess the contribution of TB-LAMP as well as Xpert MTB/RIF and fluorescence microscopy for diagnosis of smear negative TB and describe the concordance rate of empiric TB treatment with culture.

Materials and methods

A cross-sectional study was designed to evaluate the contribution of conventional and molecular methods for the diagnosis of smear negative pulmonary TB patients and assess the concordance rate of empirical TB treatment with culture results. The study was conducted on smear negative TB presumptive patients referred to St. Peter's TB Specialized Hospital (SPH) from December 2014 to June 2015. Smear negative TB presumptive patients, age greater or equal to 15, referred to SPH during the study period were included in the study. All TB presumptive patients referred during the intake period and those who were willing to participate and provide informed consent were enrolled for the study. Smear negative and TB patients on anti-TB treatment were excluded from the study. A total of 459 TB presumptive patients were enrolled during the study period. Socio-demographic and clinical data were

collected using the data collection form designed for purposes of the study. All missing information was collected from patient medical record. The data collection form captures patient demographic data, signs and symptoms, chest X-ray findings and empirical TB treatment initiation.

Spot-Morning-spot sputum was collected for diagnostic purposes using sterile falcon tubes then direct ZN examination was done at St. Peter's TB Specialized Hospital. Morning sputum this found three times smear negative was stored at 2-8°C for further testing and used for culture assay, TB-LAMP, Xpert MTB/RIF assay and fluorescent microscopy examination. Culture assay was done using Löwenstein-Jensen (LJ) and Mycobacterium Growth Indicator Tube (MGIT). The quality of morning sputum was evaluated and graded as M₁ (mucoid with no suspicion of pus), M₂ (predominantly mucoid, suspicion of pus), P₁ (1/3 purulent 2/3 mucoid), P₂ (2/3 purulent 1/3 mucoid), and P₃ (greater than 2/3 purulent) (Mitarai et al. 2011).

Loop-mediated isothermal amplification: TB LAMP was done on morning sputum at SPH and Ethiopian Public Health Institute (EPHI). The LAMP reaction was performed with a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tochigi, Japan). DNA was extracted using the Loopamp extraction kit. Sixty micro liter of sputum was added into Extraction kit tube and incubated for 5 minute at 90°C. Thirty micro liter of DNA extract was then dispensed into a reaction tube and to reconstitute the dried reagent. The reaction tube was inverted several times, then the tube was incubated at 67°C for 40 min and the reaction was terminated by heating at 95°C for 2 min. The result was viewed for green color production in the presence of a LAMP amplicon, and the absence of amplification is shown by reaction tubes retaining the orange color (Kohan et al. 2011). TB-LAMP was taken as positive if either of the study sites was reported as positive.

Xpert MTB/RIF assay: Xpert MTB/RIF assay was done on morning sputum samples. At least 2ml were taken from morning sputum sample for Xpert MTB/RIF assay and the remaining sample was used for culture. Xpert MTB/RIF assay was performed in a self-contained cartridge using Xpert MTB/RIF amplification kit (Cepheid, France). Reagent was added into sputum sample in 2:1 ratio. The mixture was incubated with continuous shaking for 15 minutes at room temperature. Two milliliters of diluted sample was added into cartridge. The cartridge was inserted in the machine. The result was interpreted by GeneXpert DX System (WHO 2013). When the result was error/invalid, retesting was done

on the leftover sample. If the test remains error/invalid for the repeated GeneXpert testing, the result was taken as error/invalid.

Culture and identification: The sample was processed using NALC-NaOH method. Equal amount of NALC-NaOH was added into the sputum samples. Then the mixture was vortexed and kept for 15 minutes at room temperature. Phosphate buffer (pH =6.8) was added up to 45 ml to neutralize the NaOH and then centrifuged for 15 minutes at 3000g. After decanting the supernatant, a 2ml amount of phosphate buffer was added to reconstitute the sediment. An aliquot of 50-100µl of the sample was then inoculated into two bottles of LJ medium and 500ml to MGIT. The inoculated media were then incubated at 37°C. Growth is monitored for contamination and fast growers in the first week. Growth is followed weekly until the 8th week for LJ and 6th week for MGIT. LJ culture was considered negative when no colony was seen after 8 weeks of incubation and MGIT culture was considered negative after 6th week incubation and flagged as negative by the machine (Kantor et al. 1998). Identification of MTB complex was done by Capilia TB test on positive cultures. Testing and interpretation was done as per the manufacturer's recommendation (Abe et al. 1999). The culture result was reported as positive if *Mycobacterium* TB Complex grows on at least either of the two culture methods (MGIT and LJ). The result was taken as negative if no *Mycobacterium* TB Complex grows on both culture methods/ either of the two culture methods was negative and the other is contaminated.

Data management and Statistical analysis: Data was captured and analyzed using SPSS (IBM, version 20). Chi-square test compared the characteristics of the study population. For the predictors of empiric TB

treatment initiation, Odds ratio (OR) with 95% confidence interval was used. Sensitivity, specificity, negative and positive predictive values were defined for smear microscopy, molecular detection and empirical TB treatment using the culture result as gold standard. P-value less than 0.05 were taken as statistically significant while comparing the diagnostic performance of the detection methods.

The study was performed on the left over samples that were collected for diagnostic purposes. Before the patient gives the sample, he/she was informed to use the left over sample and written consent was obtained. The smear microscopy, Xpert and culture results were reported back to the physician on time for treatment purpose. Ethical clearances were obtained from EPHI and St. Peter's TB Specialized Hospital.

Results

Study participant characteristic: Four hundred fifty nine smear negative TB presumptive patients were enrolled in this study. Most (57%) of the study participant were female; the median age was 40 IQR (28–55) years. HIV test results were available for 41% (190/459) of the study participant and the prevalence of HIV among those individuals were 30%. Anti-TB treatment was known for 383 study participants and 46 of them were on anti-TB treatment empirically based on site smear result and other findings. Majority (68%) of the study participants were submitted good quality specimen. The background information of the study participants is summarized in Table 1.

Table 1: Background information of the study participant

		%	N
Sex	Female	43	197
	Male	57	262
HIV status	Positive	30	57
	Negative	70	133
TB in close contact	Yes	9	33
	No	91	359
X-ray	Yes	83	340
	No	17	72
Treated for TB	Yes	12	46
	No	88	337
Rx for smear negatives`	Clinical only	16	7
	Clinical and X-ray	84	36
Sputum quality	M1&M2	32	146
	P1 to P3	68	312

N-Total number of participants; HIV-Human Immunodeficiency Virus; TB-Tuberculosis

Association of empirical treatment with confirmatory results and patient symptoms: Forty six smear-negative patients using direct ZN were treated empirically. The sensitivity and specificity of

empirically treated when compared with culture assay was 45.8% and 90% (Table 2). Patients who have HIV positive results [OR=4.62, 95% CI (2.30-9.31), $p<0.001$], chest pain [OR=2.27, 95% CI (1.32-

3.90), $p=0.003$] and hemoptysis [OR=2.79, 95% CI (1.40-5.54), $p=0.005$] were significantly associated

with empiric TB treatment initiation.

Table 2: Empirical treatment of the patient and its culture result

	Treated for Anti-TB	Not treated for Anti-TB	Unknown treatment status	Total
Culture Positive	11	13	6	30
MGIT	10	10	6	26
LJ	10	9	4	23
Culture Negative	34	306	67	407

Diagnostic contribution of FM microscopy and culture over direct ZN: The overall culture positivity rate was 6.8% (30/439), of which 6.6% (26/391) was by MGIT and 5.3% (23/436) was by LJ method. Direct and concentrated fluorescent microscopy adds 0.9% and 1.3% detection rate compared to direct ZN.

Performance of TB-LAMP and Xpert MTB/RIF: Participants whose cultures were contaminated ($n=20$), NTM ($n=2$), or error/invalid on Xpert MTB/RIF assay ($n=21$) were excluded from

performance analysis. The sensitivity, specificity, PPV and NPV of TB-LAMP were compared to culture and presented in Table 3. Intra-test performance of TB-LAMP between testing sites varies significantly ($p<0.001$). The performance of Xpert MTB/RIF assay was also analyzed using sensitivity, specificity, PPV and NPV of TB-LAMP and shown in Table 4. The agreement of TB-LAMP and Xpert MTB/RIF assay varied significantly (kappa value 0.628).

Table 3: The sensitivity, specificity, PPV and NPV of TB-LAMP

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy
All (HIV+, HIV- & unknown)	61.5	96.6	53.3	97.5	94.5
HIV positive	100	95.9	71.4	100	96.3
Testing sites					
SPH	50	97.5	56.5	96.8	94.6
EPHI	50	97.9	61.9	96.7	95

Table 4: The sensitivity, specificity, PPV and NPV of Xpert MTB/RIF

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy
All (HIV+, HIV- & unknown)	70.8	97.2	60.7	98.2	95.6
HIV positive	100	97.9	83.3	100	98.1
Before retesting	69.5	97.1	61.5	97.9	95.4

Discussion

In resource limited countries, smear microscopy is the frontline diagnostic tool for smear negative TB patients. Clinicians often make decisions to initiate TB treatment based on clinical and radiological findings (Siddiqi et al. 2003). In this study, the sensitivity of empirical TB treatment compared to culture was 46% which means 54% of culture confirmed TB patients will be left untreated unless sensitive diagnostic tools are used. The present findings agree with the report from Uganda (sensitivity=38.1%) and Tanzania (sensitivity=37%) (Swai et al. 2011; Davis et al. 2010). The low sensitivity in the present finding emphasizes the need

for sensitive diagnostic tools at point of care level. Conventionally, isolation of mycobacterium using culture is the most sensitive diagnostic tool. Culture can detect as few as 10–100 bacilli/ml of specimen (Parsons et al. 2011). In the present study, the diagnostic contribution of culture was 6.8% which was much lower than the findings reported by Swai et al. (2011) and Soto et al. (2013), the contribution of culture was 27% in both studies. The lower contribution of culture may be because having patients that are not TB presumptive are being examined rather than incipient TB cases. This indicates the need for strengthening TB presumptive selection. Several algorithms and clinical scoring

have been used to predict smear-negative tuberculosis based on local epidemiology. The algorithms they use provide reasonable approach for the diagnosis of smear negative TB (Swai et al. 2011; Soto et al. 2013).

Those algorithms allow the efficient use of laboratory resources for patient management particularly for cost intensive diagnostic tools.

Molecular diagnostic assay have great advantages in terms of speed and sensitivity which is key for early identification of TB (Boehme et al. 2007). Previous studies reported that the sensitivity of TB LAMP for smear negative and culture positive was 48.8-55.6% (Boehme et al. 2007; Mitarai et al. 2011; Ou et al. 2014). Xpert MTB/RIF assay as an add-on test following a smear negative has 67% pooled sensitivity (95% CI 60% to 74%) (Steingart et al. 2014). In the present finding, the sensitivity of TB LAMP and Xpert MTB/RIF assay was 61.5% and 70.8% respectively. Both molecular detection methods have sensitivity close to the upper limit of the previous report. Our finding indicates that TB LAMP and Xpert MTB/RIF assay can provide a confirmatory result for at least two third of TB cases which prompt initiation of timely therapy to reduce TB-associated morbidity and mortality as well as transmission.

The yield of TB diagnostic test performance can be increased by improving the quality of sputum, moreover, proper selection of purulent part for testing is also equally important (Ho et al. 2015). Here, the performance of TB-LAMP between individuals varies significantly. This might be due to the selection of purulent part of the sputum. TB-LAMP was tested by taking 60µl from the sputum. Those diagnostic tests which take small portion of sputum might be affected by improper selection of purulent part for testing.

Conclusions

The diagnostic contribution of culture and concentrated smear was low. The low positivity rate of laboratory diagnostic methods suggests the need to strengthening better TB presumptive selection. TB-LAMP and Xpert MTB/RIF assay can provide confirmatory results for at least two third of TB cases.

Acknowledgment

The authors thank FIND and EPHI for the financial support and St Peter's Hospital for the lab work and collection of clinical data.

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